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The identification and characterization of the upstream sequences regulating the expression of the maltose fermentation genes of *Saccharomyces*

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THE IDENTIFICATION AND CHARACTERIZATION OF THE UPSTREAM SEQUENCES REGULATING THE EXPRESSION OF THE MALTOSE FERMENTATION GENES OF SACCHAROMYCES

by

Joel Levine

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Abstract

THE IDENTIFICATION AND CHARACTERIZATION OF THE UPSTREAM SEQUENCES REGULATING THE EXPRESSION OF THE MALTOSE FERMENTATION GENES OF SACCHAROMYCES

by

Joel Levine

Adviser: Professor Corinne A. Michels

Maltose fermentation in Saccharomyces yeasts requires information from one of five unlinked MAL loci: MAL1, 2, 3, 4, or 6. Each locus consists of three genes encoding three proteins: maltose permease, maltase and a trans-activator protein, the MAL activator. At the MAL6 locus, the three genes are called MAL61, MAL62 and MAL63, respectively. Transcription of the MAL61 and MAL62 genes is coordinately induced by maltose and repressed by glucose; gene expression is regulated by sequences within the MAL61-MAL62 intergenic region acting in conjunction with the MAL activator.

By deletion analysis of the MAL61-MAL62 intergenic region, we show that an 81 basepair region, from basepairs -502 to -582 upstream of the MAL61 gene start codon, contains sequences necessary for the maltose induced expression of the MAL61 and MAL62 genes, the UAS_{MAL}. This sequence contains two copies of an 11 basepair dyad which may be the active elements of the UAS_{MAL}. Using heterologous gene plasmid constructs, we

demonstrate that the sequences of the UAS_{MAL} are sufficient for maltose inducibility of the MAL62 gene and that this regulated expression requires a functional MAL activator.

Our results suggest that the MAL61-MAL62 intergenic region contains additional distinct elements which function to precisely regulate MAL61 and/or MAL62 gene expression. Among these are repressing sequences including a glucose-responsive element, located between basepairs -583 and -638, which is partially responsible for mediating glucose repression of MAL62 gene expression.

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INTRODUCTION

One of the most fundamental problems under investigation by molecular biologists is the mechanism of the regulation of eukaryotic gene expression. During the past decade great strides have been made in this area particularly regarding regulation at the transcriptional level. The initial breakthrough came with the identification of an "enhancer" of transcription in the DNA sequences upstream of the SV40 early genes (Benoist and Chambon, 1981). The enhancer is required for high rates of transcription. Since this initial finding many enhancer-like sequences have been identified in many regulated systems and their ability to activate transcription has been shown to be mediated by trans-acting factors, that is, proteins which bind to these enhancer sequences (Scholer and Gruss, 1984; Sassone-Corsi and Borelli, 1986). This combination of enhancer and activation factor(s) allows gene expression to positively respond to various physiological changes, such as variation in the intracellular levels of metabolites, by altering the transcriptional activity of RNA polymerase II. The goal of my investigation is to identify the "enhancer-like" sequences involved in the regulation of inducible expression of the structural genes encoding the maltose fermentative enzymes in Saccharomyces yeasts.

Cis-acting regulatory elements in eukaryotic systems. A typical eukaryotic RNA polymerase II promoter is shown in

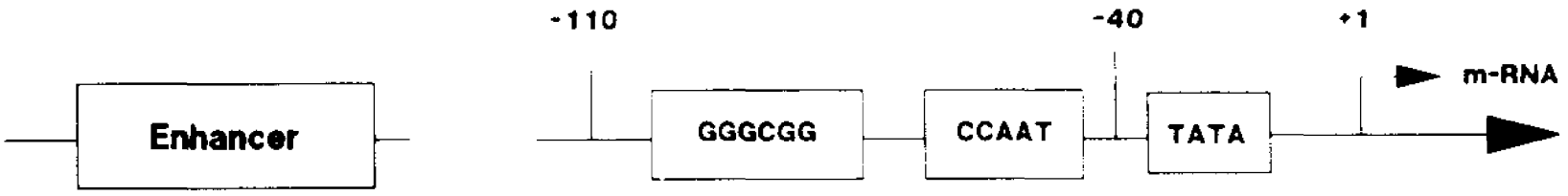
Figure 1 (Sassone-Corsi and Borelli, 1986). It contains several elements located at a distance upstream or 5' to the m-RNA start site. The elements are composed of functionally important sequences. Approximately 25-30 basepairs upstream of the transcriptional initiation site is the TATA box. A mammalian factor, TFIID, has been shown to interact with this sequence (Sawadago and Roeder, 1985). The TATA box is required for efficient initiation of transcription in many but not all promoters (Dynan, 1986). The sequences surrounding the transcriptional initiation site are involved in transcriptional initiation (Struhl, 1987; Chen and Struhl, 1988). Cordon et al. (1980) showed that a weak consensus sequence 5'-PyPyCAPyPyPyPyPy-3', is necessary to allow transcription to start at the adenine within this sequence in several mammalian genes containing TATA boxes. The transcriptional start sites could be changed by several mutations within this region. Recently, Smale and Baltimore (1989) found a 17 basepair element surrounding the transcriptional initiation site in the mouse TdT (terminal deoxynucleotidyl transferase) gene which they called the "initiator". Its function is to direct transcriptional initiation from a single site. When the initiator is inserted into a heterologous promoter along with a TATA or more distal promoter element, it is capable of activating transcription. Genes that do not contain TATA elements can be divided into two classes: housekeeping and developmentally regulated genes

Figure 1: Typical higher eukaryotic and yeast RNA polymerase II promoter regions.

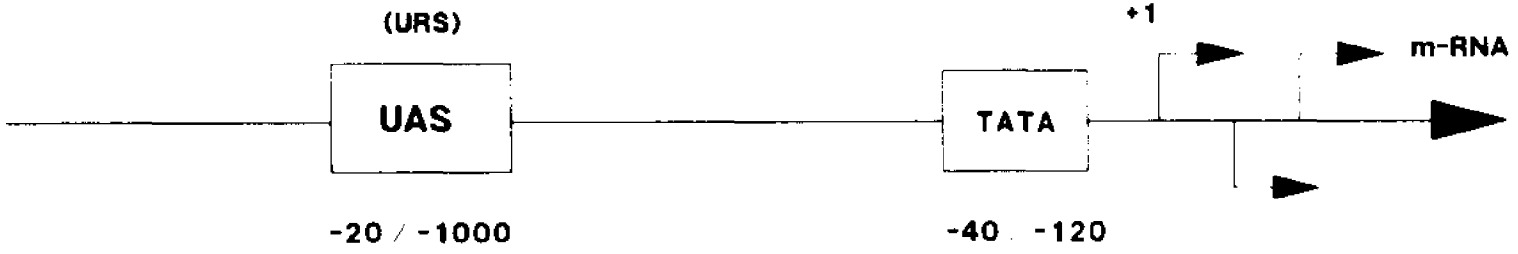
Higher Eukaryotic Promoter: The numbers represent the approximate locations of the elements contained in the promoter region of a typical gene. +1 represents the location of the invariant transcriptional initiation site and the arrow points in the direction of transcription. The approximate locations of the TATA box, upstream elements (consensus sequences) and enhancer are indicated.

Yeast Promoter: The typical yeast promoter contains several alternative transcriptional initiation sites. +1 represents the site of the one located furthest upstream and the arrows point in the direction of transcription. The approximate locations of the variably located TATA box and Upstream Activation Sequences (UAS) or Repression Sequences (URS) are indicated.

Higher Eukaryotic Promoter



Yeast Promoter



(Smale and Baltimore, 1989). Housekeeping genes are usually expressed constitutively throughout the cell cycle. They have promoters rich in GC nucleotides and usually contain several transcriptional start sites distributed over a fairly large region (Dynan and Tijan, 1983). The second class of TATA-less promoters are not as GC rich and possess one or a few start sites placed near each other. The Drosophilia homeotic and mammalian immunodifferentiation genes, including TdT, are examples of the second class (Smale and Baltimore, 1989).

Additional upstream elements are commonly found between 40 to 110 basepairs upstream of the m-RNA start site. The consensus sequences of two well studied examples are shown in Figure 1 (Sassone-Corsi and Borelli, 1986). They are involved in directing efficient transcription from certain mammalian promoters. They also have been shown to be binding sites for transcriptional activators. A transcriptional activator protein, Sp1, has been shown to bind to the hexanucleotide sequence GGGCGG (Jones et al., 1985). A CCAAT-binding protein has also been identified. These factors play a role in the activation of transcriptional initiation by RNA polymerase II (Kadonaga et al., 1986).

Enhancer elements are sometimes found further upstream. Enhancers are defined as cis-acting transcriptional activation sequences which can increase transcription rates of nearby genes by as much as 1000-fold (Khoury and Gruss, 1983; Sassone-Corsi and Borelli, 1986; reviewed in Atchison, 1988).

Enhancers are approximately 100-200 basepairs in length, can act in either orientation, can stimulate transcription in heterologous promoters, and can influence gene expression over variable distances, sometimes as much as several kilobasepairs. In at least one case, the immunoglobulin genes, the enhancer is located downstream of the gene (Queen and Baltimore, 1983). Enhancers have been found in many eukaryotic viral and cellular genes. Some are cell or tissue specific, others function more generally (reviewed in Voss et al., 1986; Atchison, 1988). Some enhancers function only in restricted stages of development. Most are positive regulatory sequences but a few have been found to be negative regulators (Scholer and Gruss, 1984; reviewed in Atchison, 1988). Trans-acting proteins have been proposed to bind to short repeated sequences within the enhancer and mediate its function by interacting with the transcription machinery (Gidoni et al., 1984; Peterson et al., 1986).

A higher eukaryotic system whose regulation has been studied in detail is that involving glucocorticoid induction. Glucocorticoids have been shown to be important in the regulation of growth, differentiation and development of higher eukaryotes (reviewed in Yamamoto, 1985; Beato, 1989; Burnstein and Cidlowski, 1989). After diffusing into a target cell, the glucocorticoid hormones bind to intracellular receptors. The binding, in turn, alters the receptor's three-dimensional conformation to increase the binding of the

glucocorticoid receptor complex to DNA sequences called glucocorticoid response elements (GRE's). If binding results in transcriptional activation, the GRE acts as an enhancer. GRE's have been identified within or adjacent to genes encoding mouse mammary tumor virus (MMTV), human metallothionein IIA, human growth hormone, chicken lysozyme and the tyrosine aminotransferase gene (TAT) (Yamamoto, 1985). The TAT gene's system is illustrative of the developmental specificity of glucocorticoid regulation (Jantzen et al., 1987). It is transcribed exclusively in the parenchymal cells of the liver and only after parturition.

Responsive elements to glucocorticoids and other steroid hormones have been identified by various methods. Among these are deletion analysis; chimeric GRE (or other RE)-promoter constructions; in vitro mutagenesis; retention of labelled DNA fragments on nitrocellulose filters of partially purified receptor preparations; DNAseI footprinting analysis; and the identification of sequences that are protected from methylation by dimethyl sulfate (reviewed in Yamamoto, 1985; Wynshaw-Boris et al., 1986; Jantzen et al., 1987; Beato, 1989).

The sequences of several GRE's have been identified and a 15-mer consensus sequence has been determined: 5'-GGTACAnnnTGTTCT-3' (reviewed in Beato, 1989). Interestingly, induction by several other steroid hormones, progesterone, androgens and mineralocorticoids, is regulated

by the same motif. Note that the sequence is partially rotationally symmetrical suggesting that binding of the glucocorticoid receptor may involve two binding domains possibly formed by dimerization of the receptor molecule (Jantzen et al., 1987). The 15-mers are usually located within several hundred basepairs upstream of the transcriptional start sites but can be active at larger distances. For example, in the TAT gene's regulatory region, the most distal GRE is located 2.5 kilobasepairs upstream (Jantzen et al., 1987). If a distantly located GRE is moved closer to the gene, its regulatory activity increases. In general, GRE's exert their effects in an orientation independent manner and can vary in length and separation from each other. A GRE has also been found downstream of the promoter (Slater et al., 1985).

Several copies of the GRE are often found in the regulatory region of glucocorticoid responsive genes. In the TAT gene system four copies are present (Jantzen et al., 1987). Three of these have been shown to be binding sites of the glucocorticoid receptor by footprinting studies. Deletion analysis has demonstrated that, of the three, two are functional and act cooperatively to activate transcription. One of these, GRE-III, is not functional by itself. This may be explained by the fact that it possesses less symmetry compared to the consensus sequence. Seven copies of the glucocorticoid receptor binding sites were found in the

upstream region of the prolactin gene (Sakai et al., 1988). By mutagenesis experiments, three were found to be important in the negative regulation of prolactin gene expression. Tsai et al. (1989) showed that multiple GRE/PRE's (an element that is regulated by glucocorticoids and progesterone) act synergistically. In contrast, mutational analysis of the MMTV hormonal responsive elements demonstrated that in this system, at least, they work independently (Buetti and Kuhnel, 1986).

In most systems studied, the effect of glucocorticoid addition results in transcriptional activation, but in at least three, the response is transcriptional repression. They are the genes encoding proopiomelanocortin, prolactin and the α-subunit of the glycoprotein hormone (Camper et al., 1985; Charron and Drouin, 1986; Akerblom et al., 1988; Sakai et al., 1988). A comparison of the responsive elements yields an ambiguous consensus sequence that possesses partial homology to the positive GRE (reviewed in Beato, 1989). It has been suggested that binding of the glucocorticoid receptor complex to the negative GRE does not cause repression by itself but acts to sterically inhibit the binding of additional factors to nearby sites (Akerblom et al., 1988). Alternatively, the negative GRE could alter the conformation of the bound glucocorticoid receptor to change it into a repressor (Sakai et al., 1988).

The upstream sequences of yeast genes are basically similar to that seen in higher eukaryotes in that they contain

a TATA box and, for regulated genes, one or more cis-acting elements, referred to as the upstream activating (UAS) or upstream repressing sequences (URS) (see Figure 1) (reviewed in Struhl, 1987; Guarente, 1988; Struhl, 1989). The yeast UAS/URS sequences possess properties similar to the upstream regulatory elements of higher eukaryotes. They can act in either orientation, at variable distances and in a heterologous promoter, but none has been shown to function in a downstream position. Recently, research on yeast and other eukaryotic promoters indicates that multiple, different UAS and/or URS elements are present in a promoter thereby enabling the regulated gene to respond to a variety of physiological controls (West et al., 1987; Pfeifer et al., 1987a; reviewed in Guarente, 1987; Struhl, 1989).

Yeast genes differ from those of higher eukaryotes in several ways. Whereas in higher eukaryotes the distance between the TATA and the initiation site is a fixed 25-30 nucleotides, in yeast the distance varies from 40-120 (reviewed in Guarente, 1987; Struhl, 1989). Yeast genes often contain several transcriptional initiation sites whereas higher eukaryotic genes contain one.

To identify a UAS/URS element in a regulatory region of a gene a series of plasmids containing deletions of the region is constructed. Each is transformed into yeast and tested separately for its effect on transcriptional regulation. Several common methods are used to assay the deletion

containing plasmids for activity. The most direct is to measure specific protein concentrations, enzymic activity, m-RNA levels or other phenotypic effects. Alternatively, one can fuse the upstream region of the gene to a reporter gene. One used in many studies of this type is the E. coli gene lacz, encoding β-galactosidase, an enzyme not normally synthesized in Saccharomyces (Rose et al., 1981). The lacz gene has been cloned into several series of yeast/E. coli shuttle vectors which contain multiple cloning regions adjacent to the 8th codon of lacz thereby allowing the upstream region and amino terminus of the structural gene of interest to be fused to lacz (Hill, J.E. et al., 1986; Myers et al., 1986). The fusion plasmids are then transformed into yeast. Transformants containing the deletion plasmids are assayed for β-galactosidase whose activity should be regulated by the gene's physiological control signals and trans-acting factors. Results will usually yield a candidate or putative regulatory sequence(s) which when deleted alters the pattern of expression of the gene. By this method sequences are identified that are **necessary** for activation or repression of transcriptional expression.

To determine if a putative sequence is **sufficient** as well as necessary for proper gene expression, the approach is to insert the sequence into the upstream region of a heterologous gene whose upstream regulatory sequences have been deleted (Guarente et al., 1982; Guarente, 1984). A

plasmid often used for these studies is plasmid pLG312 which contains a *CYC1-lacz* fusion gene and a yeast origin of replication (Guarente and Mason, 1983; Guarente et al., 1984). Fully functional TATA and initiator elements are located upstream of the *CYC1-lacz* fusion. Further upstream is the *CYC1* UAS region which possesses strategically placed restriction sites enabling it to be easily removed and replaced by the suspected activation sequence (UAS). The plasmid construction is transformed into a yeast host and assayed for β-galactosidase synthesis under different growth conditions. If *CYC1* expression, as measured by levels of β-galactosidase, is now regulated in a similar fashion as is the gene from which the sequence was derived, the sequence being studied is both **necessary** and **sufficient** for transcriptional control. The same plasmid can be used to identify repression sequences (Guarente, 1984; West et al., 1987). A putative repression sequence is inserted between the *CYC1-lacz* fusion and its UAS, transformed into an appropriate host yeast strain, and tested for its ability to repress β-galactosidase synthesis.

Several other techniques have been used to identify a UAS. Examples are footprinting analysis, alteration of methylation protection patterns, gel retardation and mutational analysis. Footprinting analysis utilizes the principle that trans-activation or repression factors bind to the regulatory elements thereby protecting the sequences from

DNAseI digestion (Galas and Schmitz, 1978; Bram and Kornberg, 1985). Similarly, the binding of regulatory factors will interfere with the methylation of nucleotides by dimethyl sulfate (Giniger et al., 1985). Studies utilizing gel retardation or the decrease in gel migrational velocity of specific DNA sequences by purified or crude extracts of trans-regulatory proteins has also identified regulatory sequences (Hope and Struhl, 1985; Olesen et al., 1987; Pfeifer et al., 1987b; Shore and Nasmyth, 1987). Caution in the interpretation of binding studies should be used because in vitro experiments use concentrations of regulatory factors far above physiological levels leading to the identification of binding sequences which may have no physiological significance (Burnstein and Cidlowski, 1989). Mutational analyses may be used to locate exact nucleotides necessary for upstream regulation. The putative region is mutagenized and each mutation is assayed for its effect on regulation (Lalonde et al., 1986; Fosburg and Guarente, 1988). Those that show significant alterations are sequenced and the individual nucleotides identified.

When characterizing a UAS/URS by deletion analysis, care must be taken in attributing activity to precise sequences. First, active sequences may not work independently but in conjunction with other sequences or require a secondary structural environment created by other sequences (West et al., 1984; Andrews and Herskowitz, 1989). Thus, deletion of

an inactive sequence may result in loss of regulation due its effect on another active region. An example is found in the experiments conducted by Takahashi and coworkers (1986) with the SV40 enhancer. Sequences between the enhancer and the TATA box were deleted. Deletions of multiples of five basepairs resulted in inhibition of transcriptional activation whereas multiples of ten had no effect. The former removed a sufficient number of nucleotides so that the enhancer was now located on the opposite side of the DNA helix whereas the latter deletions resulted in the realignment of the enhancer on the original side. Therefore, although deleted sequences by themselves lack regulatory activity, they can affect others by altering the secondary structure of DNA. Other secondary effects can be envisioned. Similar results have been observed in E. coli although none have been reported so far with yeast genes (Ptashne, 1986; Giniger and Ptashne, 1988).

A second precaution which should be observed is that a deletion may result in the joining of non-adjacent sequences thereby creating an artifact with regulatory activity. Included here are the effects of the linker sequences which when cojoined to deletion termini could possibly produce an active region. Subcloning the putative sequence into a heterologous promoter and demonstrating appropriate regulation can be used to overcome this ambiguity although intact secondary structural requirements may be important here, too. When inserted upstream of heterologous genes, suspected UAS

sequences have been shown to function but not always with wild type activity (Sarokin and Carlson, 1986; Struhl, 1989). Point mutations within a suspected UAS or URS, rather than deletions, can also be used.

A negative result involving heterologous genes does not necessarily indicate lack of activity. Putative regions of activity may not function independently of other regions (Andrews and Herskowitz, 1989). An active motif may confer regulation only when inserted in several or a minimum number of copies. Three examples illustrate this. The pentanucleotide ACCGA has been identified to be an important activation motif in region B of UAS1 of the CYC1 gene (Lalonde et al., 1986). A fragment containing eleven or twelve tandem repeats of it functions properly when inserted into a heterologous promoter whereas one with five copies (and presumably less) does not. Similar results have been obtained with a 7 basepair motif upstream of SUC2 which has been shown to be necessary for derepression when cells are switched from glucose to an alternative carbon source (Sarokin and Carlson, 1986). Insertion of multiple copies confers derepression more readily to a heterologous gene than does a single copy. A possible explanation of these phenomena will be explored below. A third example is the CCB element of URS2 of the HO gene (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). It is a 7 basepair motif found repeated ten times. When inserted into a heterologous promoter in ten tandem

copies it functions properly, but interestingly, the whole URS2 region does not. Obviously, the URS2 contains other modifying sequences essential for wild type control.

An examination of the characterized UAS's of yeast genes to date shows that each is comprised of functional units of unique short sequence motifs. Specificity is expected since each gene's expression is regulated differently with respect to cell type, timing in the cell cycle, stage of development and response to endogenous regulatory substances and environmental cues. Homology of UAS sequences in different genes indicates coordinate regulation. For example, the GCN4 gene product functions as the trans-activator of 30-50 genes encoding amino acid biosynthetic enzymes which are coordinately activated during amino acid starvation (Hope and Struhl, 1985). Upstream regions of each gene contain a strongly homologous short sequence motif. But each gene's motif varies from the other's and from the sequence which promotes optimum binding of GCN4 protein and possesses maximum inducing activity suggesting that regulation of the various genes is similar but also retains a degree of specifity (Hill, D.E. et al., 1986). Not as well understood is the situation in which different UAS's are partly homologous. The defined UAS sequences of the H2A-H2B and HO promoters are different but somewhat related (Osley et al., 1986; Andrews and Herskowitz, 1989). Both systems are cell cycle controlled. Partial homology here may suggest regulation by a common or

similar trans-regulator which is involved in cell cycle control but the similarity also may only be coincidental. Often, the motifs identified cannot function independently. As discussed above other nearby sequences and the proper secondary structural background may influence a motif's activity (West et al., 1984; Andrews and Herskowitz, 1989).

Yeast UAS/URS's fall into three categories with respect to the active motifs which have been identified: dyad repeats, direct repeats and multiple subsites. The dyad repeat elements consist of short sequences of nucleotides that are usually but not always found in multiple copies. Often the copies are not exactly identical but possess strong homology to a consensus sequence. The activating effects of the individual copies act cooperatively (Giniger and Ptashne, 1988). As in the *E. coli* lambda repressor system, binding of a regulatory factor may facilitate the binding of a second factor and thus impart fine tuned control to it (Schleif, 1987). In some cases the interior nucleotides within the rotationally symmetrical sequences of a dyad are not important. They can be deleted, altered or lengthened without affecting regulation (Struhl, 1989). The presence of a dyad suggests binding by dimers of identical subunits with features similar to the lambda repressor. Dyad repeat elements have been found in the upstream regions of the following genes: *GAL1* and *GAL10*, GCN4 regulated genes, and several a-specific genes repressed by *MATα2* (Giniger et al., 1985; Hill, D.E. et

al., 1986; reviewed in Guarente, 1989). Binding of a dimer of GCN4 monomers has been experimentally demonstrated (Hope and Struhl, 1987).

A genetic system whose UAS is composed of dyad repeats and whose regulation has been studied in detail is that of galactose fermentation. Transcription of four genes, GAL1, 2, 7 and 10, which make products necessary for growth on galactose, are coordinately induced by galactose and repressed by glucose (Guarente et al., 1982). GAL10 and GAL1 are transcribed bidirectionally and are separated by an intergenic region of 680 basepairs which contains sequences that are involved in their regulation. The products of two other genes, GAL4 and GAL80, are positive and negative regulators, respectively (Johnston and Davis, 1984; West et al., 1987). Analysis of a series of deletions within the GAL1-GAL10 intergenic region produced by linker scanning showed that the UAS for both GAL1 and GAL10 was confined to a 125 basepair region about midway between the two genes (West et al., 1984). Internal deletions which removed this region prevented induction by galactose. The region contains four, 17 basepair dyads which have a related sequence (Giniger et al., 1985). By DNA footprinting analysis, it was shown that the product of GAL4, the positive activator, can bind to all four. A similar sequence is found upstream of the GAL7 gene. Additionally, West et al. (1987) have identified several negative control regions in the intergenic region between GAL1 and GAL10 by

fusing various fragments of the GAL1-GAL10 intergenic region to CYC1 promoters. Several of the sequences prevented derepression of CYC1 expression by the UAS_{CYC} when the cells were transferred from glycerol to galactose containing growth media. The repression sequences are adjacent to or overlap the dyads comprising the activation regions. Thus, binding of GAL4 protein may inhibit repression by steric hindrance.

In a few cases, evidence exists which suggests that the activator binding to the dyad is a dimer of non-identical monomers. The STE3 gene is an example. Upstream deletion analysis of the STE3 gene by Jarvis et al. (1988) has identified two sequences necessary for regulation: a P box possessing a 16 basepair imperfect palindrome and an adjacent 10 basepair Q box. A P box inserted upstream of a UAS-less CYC1-lacz gene lacked activity but a synthetic perfect consensus was functional. Presumably, a perfect palindrome can bind two identical molecules or domains of the transcription factor whereas the Q and P boxes of the wild type UAS can bind a complex composed of different subunits.

UAS's containing direct repeats of a short sequence motif fall into a second category. Upstream regulatory regions of the following genes are examples: SUC2, H2A-H2B, PHO5, and the HAP1 protein binding site of CYC7 (Bergman et al., 1986; Osley et al., 1986; Sarokin and Carlson, 1986; Pfeifer et al., 1987a). The last is representative. Transcription of iso-2-cytochrome c, the minor isozyme of

cytochrome c and the product encoded by the CYC7 gene, is mediated through the HAP1 activator (Prezant et al., 1987; Pfeifer et al., 1987b). HAP1 protein binds to a short sequence upstream of CYC7 that contains two adjacent copies of an asymmetrical 9 basepair sequence, 5'-ATTATCGCT-3'. The arrangement suggests binding by dimers of identical subunits. Interestingly, HAP1 protein also binds to two sites in the CYC1 upstream region: subsites A and B of UAS1 (Pfeifer et al., 1987a; Kim et al., 1990). The binding sites lack homology, suggesting that HAP1 protein either can recognize and interact with several entirely different sequences or that some hidden common secondary structure exists in the HAP1 binding regions (Guarente, 1989).

The SUC2 UAS is a another example of the second category of UAS sequences. The SUC2 gene produces two transcripts encoding the cytoplasmic and secreted forms of invertase, an enzyme necessary for the extracellular hydrolysis of sucrose (Carlson and Botstein, 1982). The larger transcript, which encodes the secreted form of invertase, is regulated only by glucose repression. Sarokin and Carlson (1985) have identified a 32 basepair sequence in the upstream region that, when inserted in tandem copies into a heterologous promoter, confers partial control by glucose repression. Within the 32 basepair region is a 7 basepair element, (A/C)(A/G)GAAT, which is found in five places in the upstream region (Sarokin and Carlson, 1986). In contrast to

the CYC7 upstream element, the active SUC2 elements are separated by other sequences.

A complex unit, composed of several active regions, makes up a third class of UAS. Some genes have upstream regulatory elements consisting of two or more adjacent or separated subsites. Several activator or repressor substances can bind to the various subsites thereby allowing the gene to respond very precisely to a variety of physiological signals. Sometimes the individual subsites have no obvious symmetry, direct or palindromic, and therefore, could possibly be regulated by the binding of dimers consisting of nonidentical subunits which could bind to the two subsites (Guarente, 1989). Several promoters of this category contain negative control elements or repression sequences in addition to activation motifs. The upstream regions of CYC1, HO and several MATα2-responsive genes fall into this category (Guarente et al., 1984; Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989; reviewed in Guarente, 1989). It is likely that all genes which respond to more than one physiological signal mediated through different regulatory proteins will have multi-element upstream regions (Guarente et al., 1984).

The CYC1 upstream region is illustrative of the third category of UAS's. Transcription of iso-1-cytochrome c, the product encoded by the CYC1 gene, is regulated by two adjacent but separated upstream sites, UAS1 and UAS2, centered approximately 220 and 270 basepairs, respectively, upstream of

the region of transcriptional initiation (Guarente et al., 1984). Each is about 40 to 50 nucleotides in length. They were identified by deletion analysis of the CYC1 upstream sequences (Guarente and Mason, 1983). A short block of homology exists between the two UAS's but each interacts with different trans-activating proteins (Olesen et al., 1987; Pfeifer et al., 1987a). UAS1 consists of two short nonhomologous subsites, regions A and B, separated by about 20 nucleotides, which may work independently to mediate UAS1 function, that is, induction by heme and catabolite repression (Lalonde et al., 1986). Two proteins, the HAP1 gene product and RC2 factor, bind to region B whereas another protein factor, called RAF, may bind to region A (Pfeifer et al., 1987a). A recent report states that region A of UAS1 may bind HAP1 protein and not RAF as previously thought (Kim et al., 1990). UAS2 is responsible for transcriptional induction in nonfermentable carbon sources (Guarente et al., 1984). Induction requires the products of the HAP2, HAP3 and HAP4 gene products whose binding to UAS2 is interdependent (Olesen et al., 1987; Forsburg and Guarente, 1989). As in UAS1, two subsites exist in UAS2 (Forsburg and Guarente, 1988). The upstream region 1 mediates induction whereas region 2 functions to augment region 1's activity. Region 1 contains a sequence which bears a striking similarity to the CCAAT box of promoters of higher eukaryotic genes (Forsburg and Guarente, 1988). Taken together, the complexity of the

multiple subsites and trans-activational factors of the UAS of CYC1 allows a varied expression in response to a variety of physiological states. (Forsburg and Guarente, 1988; Hahn and Guarente, 1988)

The HO system is an example of a gene whose upstream region is unusually large and complex, involving the interplay of several positive and negative elements, allowing its expression to be temporally and cell-specifically regulated (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). HO is the structural gene encoding a site specific endonuclease which initiates the process of mating type interconversion between **a** and α cells of yeast (Nasmyth and Shore, 1987). HO gene transcription is activated during a short period in late G1, only in mother cells, and is repressed in diploid cells (Andrews and Herskowitz, 1989). The 1400 basepair upstream region contains two essential sequences: URS1 and URS2. URS1, encompassing about 500 basepairs, is responsible for expression in mother cells. The more proximal region, URS2, is the responsive element of cell cycle temporal specificity. In contrast to URS1, URS2 is not essential for transcription and cannot function alone in a heterologous promoter. But within URS2 there are 10 repeats of a hexanucleotide, 5'-CACGAAA-3', referred to as the CCB (cell-cycle box element). Tandemly repeated CCB's lacking URS2 intervening sequences can confer cell cycle control to a heterologous promoter, but interestingly, the intact URS2 cannot. As

previously discussed, the native URS2 must contain modifying sequences which influence CCB activity. In contrast, URS1 can function properly in a heterologous promoter. Several activator and repressor proteins have been shown to bind to and affect either URS1 and URS2, thus enabling the expression of HO to be regulated precisely. The core sequence of the H2A-H2B upstream activation element, 5'-GCGAAA-3', bears a striking resemblance to the CCB. The significance of this similarity was discussed above.

Many upstream regions of yeast structural genes contain repression sequences or regions of negative control (Guarente, 1984; Brent, 1985; reviewed in Guarente, 1989; Struhl, 1989). In prokaryotes, the negative control sequences, called operators, are regions to which repressor proteins bind (Ptashne, 1988). RNA polymerase binding is blocked and interference of transcription is the result. Yeast promoter regions contain sequences which act similarly to prokaryotic repressors, but not all exert their effects by the same mechanism. For example, the MATα2 protein binding site is a negative control site which mediates repression in a-specific genes such as BAR1 and STE2 (Miller et al., 1985). The MATα2 protein binding site of BAR1 overlaps the UAS (Kronstad et al., 1987). Therefore, it may act similarly to a prokaryotic repressor binding site by interfering with the binding of some essential transcriptional activator (Guarente, 1989). But this may not be the mechanism of action because a synthetic 32

basepair MATα2 protein binding site can repress transcription when inserted into a *CYC1-lacZ* promoter between the UAS and TATA or when placed upstream of the UAS (Brent, 1985). In the latter case, transcriptional repression is not as effective.

A yeast negative control element may act by inhibiting the interaction between the UAS and trans-activator protein. RC2 competes with HAP1 for the same binding site in region B of UAS1 of *CYC1* (Pfeifer et al., 1987a). It has been postulated that RC2 could be a negative regulator which together with HAP1 protein could fine tune the expression of UAS1.

There may be repression sequences that are non-specific and which are found in the upstream region of many genes. Sumrada and Cooper (1987) found a 12 basepair motif which mediates repression in *CAR1*, the gene encoding arginase. It possesses a core sequence, 5'-CCGCC-3', which is found upstream of several yeast, eukaryotic and prokaryotic genes. It may be the binding site of a global repressor substance or a sequence which mediates repression in several genes by a non-specific mechanism.

Other examples of negative regulatory elements occur in yeast. The "silencer" found at the mating type loci is an example of a complex negative control element involving several gene products (Nasmyth and Shore, 1987). It possesses an ARS and may act by altering the properties of chromatin to repress transcription (Brent, 1985). Another example is the

phenomenon of glucose or catabolite repression. Expression of many yeast genes is repressed when glucose is present in the growth medium (reviewed in Carlson, 1987). Transcription of glucose controlled genes is lowered below constitutive levels. The mechanism of glucose repression is unknown. While many reports indicate that modifications of regulatory proteins, such as phosphorylation, which has the potential of altering their functional activity, is occurring in the glucose-repressed state, sequences in upstream regions may be in part responsible (Bemis and Denis, 1988; Mylin et al., 1989). Methylation protection studies have shown that GAL4 binding to its promoter region does not occur under glucose repressed conditions (Giniger et al., 1985). West et al. (1984) found that glucose repression of GAL1 promoter activity was reduced from 150-fold to 5 or 10-fold when sufficient sequences were deleted between the UAS_{GAL} and the GAL1 TATA box. They postulated that the reduction of glucose repression could be a result of either the abridged distance between the TATA and the UAS_{GAL} or the deletion of inhibitory elements.

In addition to activation and repression control elements, there may be sequences within yeast regulatory regions which mediate other effects. Struhl (1985) demonstrated that poly dAT tracts act as constitutive expression elements in at least three different genes: DED1, HIS3 and PET56. Increased tract length was associated with increased expression. Poly dAT tracts were shown by Nelson et

al. (1987) to prevent formation of nucleosomes, thereby implicating them in the mediation of constitutive expression.

The mechanism by which any UAS can mediate transcriptional activation is unknown. Future research will probably reveal several to be operative. Any proposed mechanism must include the role of the trans-activator proteins (reviewed in Guarente, 1989; Johnson and McKnight, 1989). Much work has been focused on these and a common characteristic has been observed. They are composed of modular units in which their separate functions appear to reside in distinct independently acting domains (reviewed in Struhl, 1987; Guarente, 1988; Ptashne, 1988; Struhl, 1989). The separate functions include DNA binding, transcriptional activation, nuclear localization, ligand binding and dimer (or heteromer) formation. The modular arrangement of the trans-activator proteins suggests that the mechanism by which a UAS mediates transcriptional activation in response to an environmental or endogenous signal is to provide a specific binding site for a dimeric activator molecule's binding domain to allow its activation domain to articulate with one or more components of the transcriptional machinery. RNA polymerase II, TATA-binding factors or some constituent of chromatin are probable candidates for this interaction (Guarente, 1988; Struhl, 1989). Ptashne (1986) has proposed a model in which two separated regions of the DNA are brought into juxtaposition by looping. The binding domain of one or

both subunits of the dimer binds to the UAS. Then its activation domain makes contact with another protein, perhaps TFIID (the yeast TATA-binding factor), or some other component of the transcriptional apparatus which, in turn, seeks out a distant target sequence on the DNA. Recent evidence has shown that other factors, called coactivators, are involved in the formation of the complex (reviewed in Lewin, 1990; Ptashne and Gann, 1990). The result is the formation of a loop in the DNA. The complex could then presumably bind to RNA polymerase II to initiate transcription. Conceivably, RNA polymerase II, the activator protein and TFIID could bind in a complex in some other way with or without the participation of other factors. Support for Ptashne's model comes from an experiment performed by Horikishi et al. (1988) who showed that the activation domain of GAL4 protein shifted the DNA binding contacts of mammalian TFIID. Most importantly, the model explains how a UAS or other cis-acting element can exert its effects over large and/or variable distances, in opposite orientations and in heterologous genes.

Scleif (1987) has explained why looping of DNA would be a viable mechanism controlling gene expression by regulatory proteins. First, a short stretch of DNA has a limit to its surface available to accommodate the binding of more than just a few regulatory factors. Looping allows gene expression to be controlled by a multitude of factors which have separate DNA binding regions and a common activation mechanism.

Second, looping permits cooperativity in the binding of two proteins to sites on the DNA. Suppose that to regulate a gene's expression, two proteins must bind in a complex to two separated sites on the DNA. After the first factor binds its non-DNA binding region is now closer to the other site. Thus, the second factor can bind more easily to the first protein and to its DNA binding site. Effectively, looping increases the local concentration of the second binding factor thereby allowing for a reduced rate of synthesis and overall concentration of these factors. In a cell with thousands of regulatory proteins controlling the expression of a large set of genes this effect would be advantageous because the resulting reduction in required concentrations of regulatory factors would result in the concomitant reduction of energy expended to synthesize them.

Ptashne (1986) supports the DNA looping model as the mechanism of action of UAS sequences, but other models have been proposed. He has categorized them with dramatic titles such as "twisters", "sliders" and "oozers". Nasmyth (1986) has defined four: "supercoiling", "chromatin structure", "bind and slide" and "direct contact". Each has a modicum of substantiation. One which has much experimental support proposes that a UAS or other cis-activating elements (i.e. enhancers) can activate (or repress) transcription by adjusting the structure of chromatin. As discussed above, Struhl (1989) has suggested that constitutive expression by

poly dAT tracts can be explained by their inhibition of nucleosome formation. Enhancers have been shown to alter the pattern of DNAseI hypersensitive sites in linked DNA (reviewed in Atchison, 1988). The E site of the "silencer" of mating type in yeast affects DNAseI hypersensitive sites, too (Nasmyth, 1982; Guarente, 1987). Electron microscopy has shown that the SV40 enhancer alters nucleosome structure (Jacobovitz et al., 1980). Nacheva et al. (1989) demonstrated that histone binding patterns and nucleosomal structure are changed during transcriptional activation. Taken together, the mechanism of UAS mediated transcriptional activation so far is mostly conjectural. Most probably, future investigations will reveal that specific gene systems are activated by one of several alternative mechanisms which need not be mutually exclusive.

Maltose fermentation in *Saccharomyces* yeasts. Since my investigation is concerned with the identification and characterization of the upstream regulatory sequences of the maltose fermentative genes, a discussion of the system is appropriate. Maltose fermentation in *Saccharomyces* yeasts requires two enzyme functions: maltase and maltose permease (Van Wijk et al., 1969; de Kroon and Koningsberger, 1970). Transcription of the genes encoding these enzymes is induced by maltose and repressed by glucose. There are five unlinked ***MAL*** loci identified in *Saccharomyces cerevisae*: ***MAL1***, *2*, *3*, *4* and *6* (Barnett, 1976). Each locus consists of three genes

(Cohen et al., 1984; Needleman et al., 1984; Charron et al., 1986). GENEs 1 and 2 code for the enzymes, maltose permease and maltase, respectively; GENE 3 encodes the trans-activator protein referred to as the MAL activator (Needleman et al., 1984; Dubin et al., 1985; Chang et al., 1988; Chang et al., 1989; Charron et al., 1989). At the MAL6 locus, the three genes are called MAL61, 62 and 63, respectively (Needleman et al., 1984). The complete MAL6 locus encoding all three genes is contained within a 9.0 kilobasepair fragment and is sufficient for maltose fermentation. Strains lacking any one of the three genes are unable to ferment.

Haploid fermenting yeast strains possess one fully functional MAL locus and may also possess one or more cryptic or partially functional loci (Michels and Needleman, 1983; Needleman and Michels, 1983). The cryptic loci that have been characterized lack, (as a result of naturally occurring deletions and/or mutations), one or more of the three GENEs discussed above (Charron, 1988; Charron and Michels, 1988). For example, 332-5A, a strain used in this study, possesses a complete MAL6 locus and a partially functional allele of the MAL1 locus in which only the MAL12 gene is active (Dubin et al., 1985). No other MAL loci are present in the strain. Southern blot analysis has shown that all five MAL loci have sequences that are significantly homologous (Michels and Needleman, 1983; Needleman and Michels, 1983; Michels and Needleman, 1984). Genes derived from different loci can

complement each other (Needleman et al., 1984; Chang et al., 1989; Charron et al., 1989). For example, a strain carrying MAL61 and MAL62 but lacking MAL63 can be transformed to a fermenter by the gene encoding the MAL activator from any of the other loci (i.e. MAL13, MAL23, etc.).

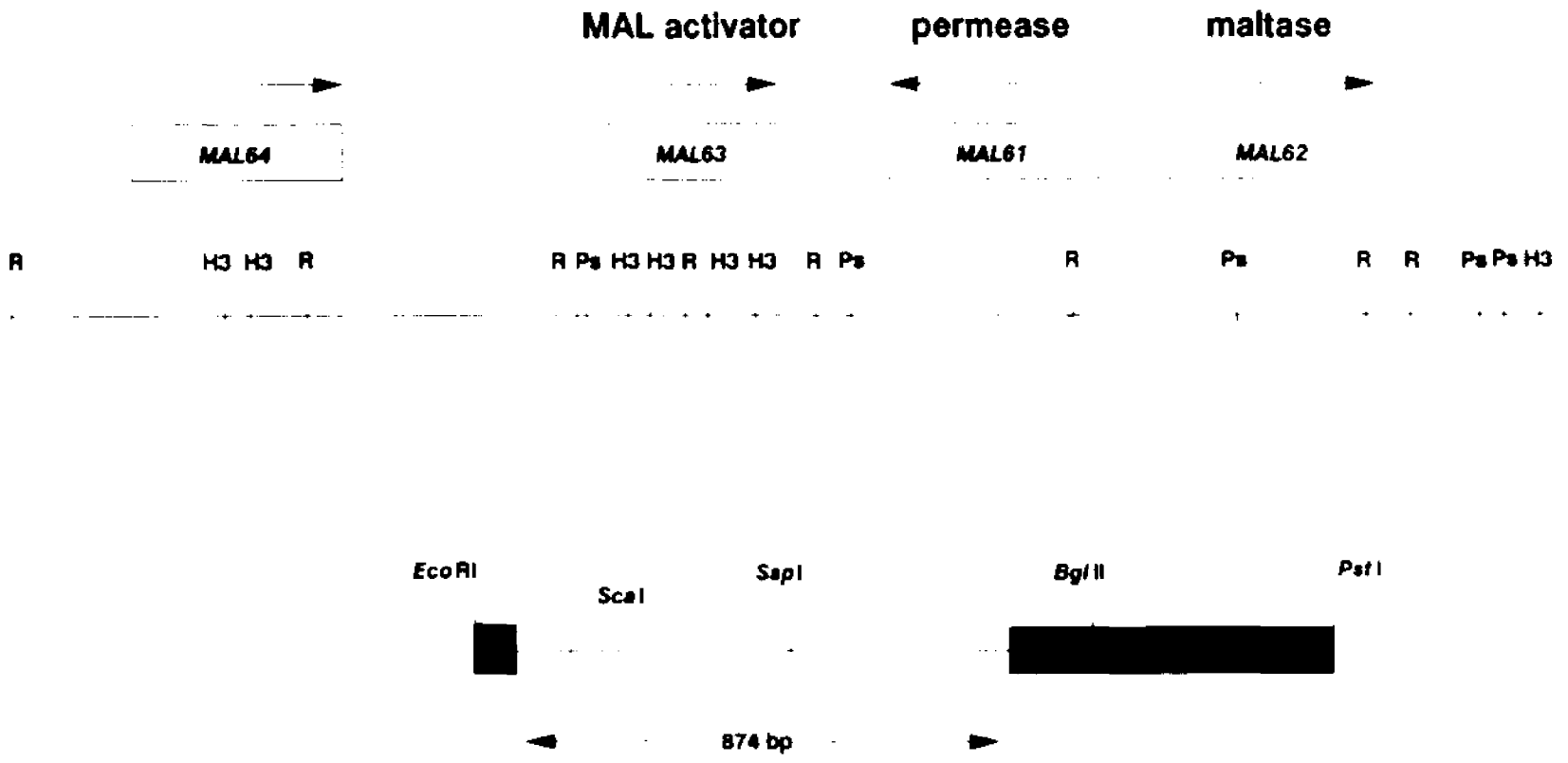
A map of the MAL6 locus is shown in Figure 2. The organization of the three GENEs required for maltose fermentation is indicated. Note that MAL61 and MAL62 are transcribed bidirectionally (Needleman et al., 1984). Their transcription is induced by maltose and repressed by glucose. The region separating MAL61 and MAL62 has been proposed to be the binding site of the MAL activator and thus would mediate the coordinate transcriptional regulation of the adjacent structural genes.

MAL63 and its homologues code for a positive trans-activator protein required for maltose inducibility (Chang et al., 1988). Its presence was implied from a study of the characteristics of several maltose nonfermenting strains which were isolated by ten Berge et al. (1973a) upon mutagenesis of a maltose fermenting strain. The mutations mapped at the MAL6 locus, were recessive and formed a single complementation group. The mutant strains could synthesize only basal levels of the maltose fermentative enzymes, maltase and maltose permease, and were uninducible by maltose. Collectively, these characteristics suggest that the strains carried mutations in a gene encoding a trans-acting positive

Figure 2: Restriction endonuclease map of the MAL6 locus.

The approximate location of the MAL64, MAL63, MAL61 and MAL62 genes are indicated above the map and the direction of transcription of each of the genes is shown by the arrows. The expanded region represents the restriction endonuclease map of the EcoRI-PstI fragment, containing the MAL61-MAL62 intergenic region, which was cloned into the EcoRI-PstI site of plasmid pBR325 to construct plasmid pIGR61,62, the starting plasmid for all deletion constructs (see Methods and Materials). The darkened rectangles represent the portions of the MAL61 and MAL62 genes contained in the EcoRI-PstI fragment.

Recognition sites for restriction endonucleases are abbreviated as follows: R, EcoRI; H3, HindIII; and Ps, PstI.


MAL activator
permease
maltase
MAL64
MAL63
MAL61
MAL62
R
H3 H3 R
R Ps H3 H3 R H3 H3 R Ps
R
Ps
R R
Ps Ps H3
EcoRI
ScaI
SapI
BglII
PstI
874 bp

regulator. Confirmation came from molecular studies. Chang et al. (1988) constructed a yeast strain, A9, containing a deletion/disruption of the MAL63 gene in strain 332-5A. When grown under maltose inducing conditions, A9 failed to synthesize either the MAL61 and MAL62 gene transcripts or the maltose fermentative enzymes. In addition, the several non-inducible mal6 mutations of ten Berge (1973a), as well as additional mutations, were shown to map within the MAL63 gene (Chang et al., 1988). Thus, collectively, the evidence confirms that MAL63 encodes a positive activator required for inducible expression of the maltose fermentative genes. Sequence analysis has shown that the MAL63 gene encodes a 470 residue protein containing a cysteine-rich region of significant homology to the DNA binding domain of GAL4 protein and several other yeast activators (Kim and Michels, 1988).

Recently, a second gene encoding a trans-acting activator product having homology to MAL63 was identified and designated MAL64 (Dubin et al., 1986). Its presence was demonstrated from analyses of maltose fermenting revertants of two mutant mal63 nonfermenting strains (ten Berge et al., 1973b; ten Berge et al., 1974). The revertant strains were constitutive for the synthesis of the maltose fermentative enzymes. The mutational alteration in the revertant strain, C2, was shown by ten Berge et al. (1973b) to be linked to MAL6. They incorrectly proposed that the reversion mutation was a second site suppressor mutation within the gene encoding

the activator. Subsequently, Dubin et al. (1986) showed that the site of the reversion was located outside of the MAL61, 62, 63 gene cluster and in a previously undefined gene located 2.3 centimorgans to the left of the MAL63 gene. They called this gene MAL64. The MAL64 gene was shown to possess positive regulatory function in the constitutive revertant strains. The wild type MAL64 gene and two constitutive alleles have been cloned (Dubin et al., 1988; Lori A. Young, personal communication). MAL64 encodes a protein that, like the MAL63 gene product, contains 470 amino acids (Lori A. Young, personal communication). The two are 85% homologous.

Two lines of evidence point to the view that MAL64 is a non-essential homolog of MAL63 (Dubin et al., 1988). First, deletion of MAL64 has no effect on maltose inducibility or maltose fermentation in inducible strains. Second, of the other MAL loci, only MAL3 possesses an analogous linked MAL64-homologous region (Charron et al., 1989). Dubin et al. (1988) proposed that MAL64 may have arisen by gene duplication of MAL63 and may function only as a reserve of genetic information.

We report here a deletional analysis of the MAL61-MAL62 intergenic region. Our results allow us to identify an 81 basepair sequence required for inducible expression of both MAL61 and MAL62. This sequence is also sufficient for conferring maltose inducibility on the heterologous CYC1-lacz fusion gene. Maltose inducible expression from this chimeric

gene requires a functional MAL activator indicating that this region contains the binding site of this protein, that is, it is the UAS_{MAL}.

METHODS AND MATERIALS

Yeast Strains. Table 1 lists the genotypes of the yeast strains used in this study. The full MAL genotype of each strain is given utilizing the nomenclature described in Needleman et al. (1984) and Charron and Michels (1988). Also listed is the previous designation of each MAL locus based on the nomenclature of G. Naumov (1971; 1972; 1976). The information presented below explains both nomenclatures.

At least one of five unlinked, dominant MAL loci (MAL1, MAL2, MAL3, MAL4, MAL6) is present in maltose fermenting Saccharomyces yeasts strains (Barnett, 1976). Each locus encodes three genes: GENEs 1 and 2 encode enzymes maltose permease and maltase, respectively; GENE3 encodes the positive trans-activator protein (Cohen et al., 1984; Needleman et al., 1984; Cohen et al., 1985; Dubin et al., 1985; Chang et al., 1988; Chang et al., 1989; Charron et al., 1989). A two digit numbering system is used to designate each of the GENEs at each of the MAL loci with the first digit indicating the locus and the second digit indicating the GENE (Needleman et al., 1984). Thus, the three genes located at the MAL6 locus are called MAL61, MAL62 and MAL63. MAL11, MAL12 and MAL13 refer to the genes at the MAL1 locus. Maltose fermentation requires the presence of at least one functional copy of each gene. The genes derived from the different MAL loci differ only slightly based on complementation analysis, restriction site polymorphisms and Southern analysis (Charron

Table 1: Yeast strains used in this study.

Strain	Genotype	MAL Locus	Source
332-5A	MATa MAL64 MAL63 MAL61 MAL62 mal13 mal11 MAL12 ura3-52 leu2-3,112 trp1 his	MAL6 $mal1^0$	Needleman et al.(1984)
A9	Isogenic to 332-5A except mal63Δ::URA3	mal6 $mal1^0$	Chang et al. (1988)
A9u	Isogenic to A9 except mal63Δ::ura3	mal6 $mal1^0$	Lori A. Young
332-5AΔF-1-5	Isogenic to 332-5A except mal64:LEU2	mal6 $mal1^0$	Dubin et al. (1988)
SW14	MATa MAL64 mal63-14 MAL61 MAL62 mal13 mal11 MAL12 ura3-52 leu2-3,112	mal6 $mal1^0$	Chang et al. (1988)
340-2A	MATα mal13Δ MAL11-2 MAL12 ura3-52 ade	MAL1g	R. Needleman
8-2BΔF-5-21	MATa mal64-C2:LEU2 mal63-13 MAL61 MAL62 mal13 mal11 MAL12 ura3-52 leu2-3,112 trp1 ade	mal6 $mal1^0$	Dubin et al. (1988)

et al., 1989). Based on his earlier genetic analysis, Naumov (1976) designated the MAL loci of several nonfermenting strains as MALp, MALg, or mal^0. Examples are MAL1p or MAL3g loci. A MALp locus was proposed to encode a functional MAL activator gene but lacks the genes encoding the maltose fermentative enzymes. A MALg locus lacked a functional MAL activator gene but was proposed to contain the wild type alleles of the structural gene(s) at the designated locus. Strains containing either a MALp or a MALg locus were thus unable to ferment maltose, but diploid strains heterozygous for these loci were fermenters. Thus, the MALp and MALg loci appeared to define two complementation groups. A third class of nonfermenting strains, complemented neither MALp nor MALg strains, and these were referred to as mal^0 containing strains. Naumov suggested that such strains lacked all functional genes required for maltose fermentation.

Charron and Michels (1988) have completely characterized the nature of the three GENEs of the different MAL1 alleles. The dominant MAL1 is a complete locus containing functional copies of the three genes, MAL13, MAL11, and MAL12. The MAL1p and MAL1g loci are partially functional and are capable of complementing each other. The former contains a fully functional MAL13 gene; a mutant mal11 gene inactivated by one or several point mutations; and lacks the MAL12 gene due to a deletion. The MAL1g locus lacks MAL13 due to a deletion but possesses functional genes encoding maltose

permease and maltase. Curiously, the maltose permease encoded by the MAL1g locus lacks sequence homology to the MAL11 gene of the dominant MAL1 locus; thus, the gene encoding maltose permease at the MAL1g locus is designated MAL11-2, in contrast to MAL11-1, its designation at the dominant MAL1 locus. Charron and Michels (1988) propose that the presence of this variant maltose permease encoding gene at the MAL1g locus is the result of a rearrangement which brought the variant gene under the control of maltose induction. Strains designated mal^0 by Naumov (1976) were found to contain MAL-homologous sequences mapping to the MAL1 locus and are referred to as mal1^0 (Michels and Needleman, 1983; 1984; Needleman and Michels, 1983). Dubin et al. (1985) showed that the mal1^0 locus contains a functional maltase gene. MAL11- and MAL13-homologous sequences are found at the mal1^0 locus but are inactive, most likely as a result of the accumulation of point mutations (Charron and Michels, 1988). Thus, the relevant MAL genotype of the mal1^0 locus is mal13 mal11 MAL12. To date, all laboratory strains investigated have been shown to carry one of the four alleles of the MAL1 locus.

The other MAL loci have fewer alleles than MAL1. The MAL3 locus has two: MAL3 and MAL3g (Michels and Needleman, 1983; 1984; Needleman and Michels, 1983). The MAL3 allele posseses all three genes and strains carrying it are inducible and maltose fermenters. The MAL3g allele contains genes encoding maltase and maltose permease but is devoid of

sequences homologous to any MAL activator gene. To date partially functional alleles of the other MAL loci, MAL2, MAL4 and MAL6, have not been found (Charron and Michels, 1988).

A detailed description of the strains used in the present study follows. Strain 332-5A, a MAL6 $mal1^0$ strain, is the strain used in this study to define and characterize the upstream activation region of the MAL6 locus (Dubin et al., 1985). Transcription of the structural genes is inducible and glucose-sensitive (Needleman et al., 1984). This strain has been used as the standard MAL6 strain in several previous studies (Dubin et al, 1985; 1986; 1988; Chang et al., 1988). Strain 332-5A was derived from strain CB11 (MAL6 MAL1g MAL3g) of the Berkeley Yeast Stock Center (Needleman and Michels, 1983). Its relevant MAL genotype is MAL64 MAL63 MAL61 MAL62 mal13 mal11 MAL12. Thus, there are two functional copies of the gene encoding maltase.

Strain A9u is a derivative of strain A9 which, in turn, is a derivative of strain 332-5A. Strain A9 contains a deletion/disruption of the MAL63 gene of strain 332-5A in which the HindIII fragments of MAL63 are replaced by a HindIII fragment containing the URA3 gene (Chang et al., 1988). Strain A9u is a ura3 mutant of strain A9 selected using 5-fluoroorotic acid according to the method of Boeke et al. (1984). Thus, strain A9u is isogenic to strain 332-5A except that it possesses a mutation in the URA3 gene used to disrupt the MAL63 gene. Strain A9u is a nonfermenter and is

uninducible by maltose. Thus, its MAL-related genotype is MAL64 mal63Δ::ura3 MAL61 MAL62 mal13 mal11 MAL12.

Strain 332-5AΔF-1-5 is a derivative of 332-5A. It contains an insertion/disruption of the MAL64 gene constructed by inserting a HindIII fragment carrying the LEU2 gene into the HindIII site in MAL64 (Dubin et al. 1988). It is a fermenter and is inducible by maltose. Its MAL-related genotype is mal64:LEU2 MAL63 MAL61 MAL62 mal13 mal11 MAL12.

The parent strain used to construct strain SW14 was 269-5B (genotype MAL64 MAL63 MAL61 MAL62 mal13 mal11 MAL12) (Chang et al.,1988). It was mutagenized with N-methyl-N'-nitrosoguanidine and several maltose nonfermenting mutants were isolated, one of which was SW14. The latter complemented MAL1p but not MAL1g strains and its mutation has been mapped to the MAL63 gene.

Strain 8-2BΔF-5-21 is mutant for both MAL activator genes of the MAL6 locus. Its derivation is complex. ten Berge et al. (1973a) mutagenized an inducible MAL6 strain to produce a nonfermenting, uninducible strain, 6-13. Chang et al. (1988) show that the MAL6-linked mutation in strain 6-13 maps to MAL63. Using strain 6-13, ten Berge et al. (1973b) isolated C2, a maltose fermenting revertant of strain 6-13, which synthesized the maltose fermentative enzymes constitutively. The revertant was shown to be linked to the MAL6 locus and later was localized to the MAL64 gene (Dubin et al., 1986). Strain C2 was subsequently crossed to $mal1^0$

strains to remove the MALg loci in the background resulting in strain 8-2B. The genotype of 8-2B is MAL64-C2 mal63-13 MAL61 MAL62 mal13 mal11 MAL12 and it was used to construct 8-2BΔF-5-21. The latter was derived from 8-2B by inserting the LEU2 gene into the HindIII site of the MAL64-C2 gene (Dubin et al., 1988). Thus, 8-2BΔF-5-21 lacks functional products encoded by both the MAL64 and MAL63 genes. It is a nonfermenter and is uninducible. Its MAL-related genotype is mal64-C2:LEU2 mal63-13 MAL61 MAL62 mal13 mal11 MAL12.

Strain 340-2A is another yeast strain lacking functional MAL regulators. It carries only the MAL1g allele of the MAL1 locus and no other MAL loci. Thus, it lacks all functional MAL activator genes and its MAL-related genotype is mal13Δ MAL11-2 MAL12.

Growth Conditions. Yeast strains were grown on either complete media, referred to as YP media (1% yeast extract, 2% peptone, 2% glucose or substituted sugar) or minimal media, referred to as SD (0.67% yeast nitrogen base without amino acids, 2% glucose or substituted sugar, and the appropriate amino acids minus uracil) at 30°C.

Fermentation of yeast strains was determined by the evolution of CO_2 and substantial growth in 1-3 days on 5 ml of YP media supplemented with 2% (wt/volume) of the specified sugar, as previously described (Needleman and Michels, 1983).

E. coli strain RR1 was used for routine cloning procedures of all plasmids. Bacteria were grown on LB media

(1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with the appropriate antibiotic for selection and maintenance of the plasmid.

Preparation and Analysis of DNA. Large scale plasmid DNA's were prepared by the procedure described by Maniatis et al. (1982). Strains were grown in M9 media containing 5 g casamino acids/liter and 1 mM $MgCl_2$. Where applicable, final concentrations of the following antibiotics were used for amplification of the plasmids: ampicillin, 100 μg/ml, tetracycline, 25 μg/ml, chloramphenicol, 300 mg/liter, or spectinomycin, 300 mg/liter. Small scale plasmid DNA preparations were performed by the method of Holmes and Quigley as described in Maniatis et al. (1982). Yeast DNA's were isolated by the method of Cryer et al. (1975) with modifications as described by Michels and Needleman (1983). Standard methods were used for gel electrophoresis, restriction enzyme analysis, DNA subcloning and DNA purifications. GeneClean was used for the purification of several DNA fragments following the procedure described by the manufacturer (BIO 101 Inc., La Jolla, CA). GeneClean utilizes the selective adsorption of DNA fragments from an excised agarose gel slice to a silica matrix (GLASSMILK™). Southern DNA analysis was done as described (Michels and Needleman, 1983). Four-fold concentrated SSC and 0.5% SDS were used for hybridizations followed by washes in two-fold concentrated SSC and 0.1% SDS. SSC contains 0.15 M NaCl and 0.015 M sodium

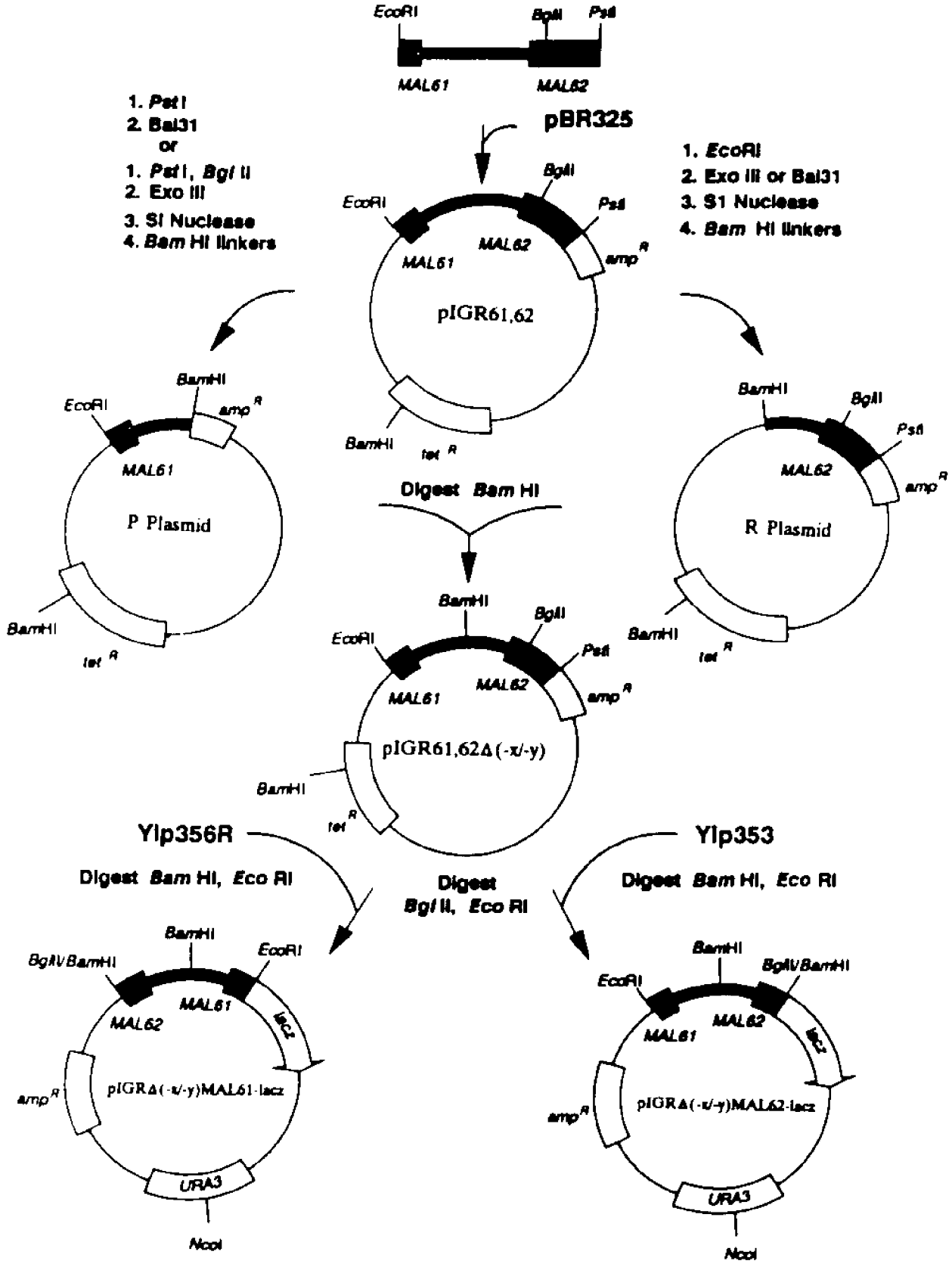
citrate. Colony filter hybridizations were performed by standard methods (Grunstein and Wallis, 1979). Nick translations were performed using ^{32}P-dCTP (Amersham) as described by Rigby et al. (1977).

Construction of lacz Fusions to Internal Deletions Within the MAL61-MAL62 Intergenic Region. Plasmid pIGR61,62 was used as the starting plasmid for the construction of all deletions as diagramed in Figure 3. It was obtained by subcloning the EcoRI-PstI fragment containing the MAL61-MAL62 intergenic region and extending from the MAL61 coding region to the MAL62 coding region into plasmid pBR325 (see Figure 3). This fragment was obtained from plasmid YEpMAL which is described in Federoff et al. (1982) and Needleman et al. (1984). External deletions into this yeast insert from the EcoRI site (the R plasmid series) or from the PstI site (the P plasmid series) were obtained. Individual members of the two sets of deletion plasmids were paired to create internal deletions within the yeast insert. The procedure is called linker scanning (McKnight and Kingsbury, 1982; West et al., 1984; Forsburg and Guarente, 1988).

The set of R plasmids was prepared by cleaving plasmid pIGR61,62 at the EcoRI site and then digesting the linearized DNA with Bal31 or ExoIII for various incubation times resulting in the deletion of both insert and plasmid sequences starting from the EcoRI site (Henikoff, 1984). S1 nuclease was used to digest the single stranded termini leaving blunt

Figure 3: Cloning strategy used for the construction of plasmids containing deletions of the MAL61-MAL62 intergenic region fused to lacz.

Plasmid maps containing pertinent restriction endonuclease sites, truncated portions of the MAL61 and MAL62 genes and strategic marker genes are indicated. Darkened segments of the plasmid maps represent the MAL61-MAL62 intergenic regions with or without deletions. Plasmid nomenclature and cloning strategy are as described in the Methods and Materials.


EcoRI
BglI
PstI
MAL61
MAL62
pBR325
1. PstI
2. Bal31
or
1. PstI, Bgl II
2. Exo III
3. S1 Nuclease
4. Bam HI linkers
1. EcoRI
2. Exo III or Bal31
3. S1 Nuclease
4. Bam HI linkers
pIGR61,62
BamHI
ampR
tetR
P Plasmid
R Plasmid
Digest Bam HI
pIGR61,62Δ(-x/-y)
Ylp356R
Ylp353
Digest Bam HI, Eco RI
Digest Bgl II, Eco RI
BglIVBamHI
lacZ
pIGRΔ(-x/-y)MAL61-lacz
pIGRΔ(-x/-y)MAL62-lacz
URA3
NcoI

ends to which BamHI linkers were ligated and then joined by T4 ligase. Plasmids were transformed into E. coli strain RR1 and screened by digestion with restriction enzymes to estimate the extent of the deletion within the yeast insert. The exact endpoint of each deletion was determined from the BamHI linker using dideoxy sequencing in M13 as described in Sanger et al. (1977). Thus, a nested set of R plasmids was constructed, each possessing the 5' end of the MAL62 coding sequence (from the PstI site) and different sized fragments of the intergenic region upstream of MAL62 extending to the EcoRI site.

The P plasmids were prepared similarly except that plasmid pIGR61,62 was first linearized by digestion with PstI followed by treatment with Bal31 exonuclease. Alternatively, the plasmid pIGR61,62 was cleaved with BglII and PstI and incubated with ExoIII. This strategy is preferred because ExoIII cannot attack the 3' single stranded termini formed by PstI, and therefore, the ExoIII digestion occurs unidirectionally from the BglII site into the MAL61-MAL62 intergenic region without affecting the plasmid sequences adjacent to the PstI site (Henikoff, 1984). The yeast insert of each P plasmid was sequenced from the BamHI linker to determine the exact deletion endpoints using the dideoxy method of Sanger et al. (1977). To construct an internal deletion of the MAL61-MAL62 intergenic region, an R and a P plasmid were paired as shown in Figure 3. Each was first digested with BamHI, then the larger BamHI fragment from the

R plasmid (containing some MAL61-MAL62 intergenic region, 5' MAL62 coding sequence and some vector sequences) was ligated to the smaller BamHI fragment from the P plasmid (containing some MAL61-MAL62 intergenic region, 5' MAL61 coding sequence and some vector sequences). Note that all pBR325 sequences between the EcoRI and PstI sites are regenerated including the gene for tetracycline resistance. The correct plasmid construction was isolated by transformation into RR1 selecting for tetracycline resistance. The plasmids were screened by restriction analysis to demonstrate the presence of the desired EcoRI-PstI fragment containing an internal deletion marked by the presence of a BamHI site. By judicious pairing of various R and P plasmids, a series of plasmids containing differently sized and placed internal deletions was constructed (Figure 3). This series is referred to as pIGR61,62Δ(-x/-y) where x and y indicate the endpoints of the deletion. The bases are numbered starting at the first base upstream of the translational start site of the MAL61 coding region.

To measure the ability of the MAL61-MAL62 intergenic region deletions to activate transcription in yeast, each was fused to a reporter gene, the *E. coli lacz* gene encoding β-galactosidase, an enzyme not normally synthesized by Saccharomyces yeasts (Rose et al., 1981). These fusions were constructed using a series of yeast/bacteria shuttle vectors developed by Myers et al. (1986). Each contains a truncated

copy of *lacz* lacking all sequences upstream of the 8th codon but containing a multiple cloning site (MCS) at this position (Hill J.E. et al., 1986). The MCS is available in three reading frames and in both orientations. In addition, these shuttle vectors provide the gene for ampicillin resistance (for selection in bacteria), either the *LEU2* or the *URA3* genes (for selection in yeast), and are constructed either with or without the yeast 2 micron circle replication origin. Of this series we chose the vectors YIp353 and YIp356R because these enabled us to create an in-frame fusion to *lacz* using the *Bgl*II site of the *MAL62* coding region and the *Eco*RI site in the *MAL61* coding region, respectively. Each of the *MAL61*-*MAL62* intergenic deletions was subcloned into both YIp353 and YIp356R and transformed into the *MAL6* *mal1*^{0} strain 332-5A directing integration of the plasmid to the *ura3-52* gene of the host as described below.

Bacterial and Yeast Transformations. Bacterial transformations of strain RR1 were performed by standard methods using 0.1 M $CaCl_2$ as described by Maniatis et al. (1982).

Yeast transformations were done by the method of Ito et al. (1983) using lithium acetate. Site directed integrations of the *MAL61*-*lacz* and *MAL62*-*lacz* fusion plasmids were targetted to the genomic *ura3-52* gene by linearizing the plasmid DNA with *Nco*I which makes a single cut in the plasmid *URA3* gene (Orr-Weaver et al., 1983). Stability of the Ura+

phenotype was determined after growth overnight at 30°C in YP media containing 2% (wt/volume) glucose. Stable Ura+ colonies were screened for the presence of the appropriate nutritional markers including fermentation in maltose. The stable Ura+ colonies were then subjected to colony filter hybridization using ^{32}P-labeled pBR322 to determine those colonies having integrated MAL61-lacz or MAL62-lacz fusion plasmids. Total genomic DNA was prepared from each integrative transformant and Southern analysis performed digesting the DNA with either EcoRI or NcoI and hybridizing with ^{32}P-labeled pBR322 to detect the integrative plasmid. The presence of a single, uniformly sized, hybridizing EcoRI fragment, larger in size than the linearized plasmid, indicated the presence of a single copy insertion of the deletion plasmid. Any integrative transformants possessing additional hybridizing fragments identical in size to the linearized plasmid were discarded since this result is indicative of multiple tandem insertion of the transforming plasmid. Hybridizing NcoI fragments having the same size as the linearized transforming plasmids confirmed the presence of multiple integrations. Only single copy integrations were used in our analysis.

Episomal plasmids were transformed by the method of Ito et al. (1983) using lithium acetate. Instability of the Ura+ phenotype was determined after growth overnight at 30°C in YP media containing 2% (wt/volume) glucose. Unstable Ura+ transformants were screened for the presence of the

appropriate nutritional markers and then assayed for β-galactosidase activity.

Construction of Heterologous Promoters Using the Putative UAS_{MAL}. DNA fragments containing the putative UAS_{MAL} were subcloned into the heterologous expression vector pLG312, a yeast/*E. coli* shuttle vector (Guarente and Mason, 1983). This vector contains 385 basepairs of the upstream regulatory region of the yeast *CYC1* gene and four basepairs of the 5' end of the *CYC1* coding region fused to *lacz* (Smith et al., 1979; Guarente and Ptashne, 1981; Guarente and Mason, 1983). The UAS_{CYC} can be deleted from pLG312 by digestion with *Sma*I and *Sal*I and the foreign DNA fragment to be tested for UAS activity may be inserted at this site. Two fragments containing the putative UAS_{MAL} were obtained from plasmids pIGR61,62Δ(-583/-643) and pIGR61,62Δ(-639/-769) as follows. The *Eco*RI-*Bam*HI fragment of each plasmid was subcloned into the multiple cloning region of pUC18. These were then digested with *Ssp*I, whose cleavage site is located just 5' to the putative UAS_{MAL} and *Sal*I, of the MCS, and this DNA fragment then was cloned into the *Sma*I-*Sal*I sites of pLG312. These plasmids are referred to as $pUAS_{MAL}$(-490/-582) and $pUAS_{MAL}$(-490/-638). The plasmids were transformed into *ura3-52* yeast strains having various *MAL*-related genotypes. Ura+ transformants were selected and shown to contain the plasmids as episomes (pLG312 contains the yeast 2 micron origin of replication).

Beta-galactosidase Assay. Yeast strains were grown at 30°C with vigorous shaking until an optical density at 600 nm of about 1.0 was reached. Stable, integrative transformants were cultured in YP media supplemented with 2% (wt/volume) of the appropriate sugar (maltose, glucose and galactose for induced, repressed and uninduced cultures, respectively). Transformants carrying episomal plasmids were cultured in SD media to which the appropriate sugar was added (maltose and galactose, glucose, and galactose for induced, repressed and uninduced cultures, respectively). It was necessary to add galactose to the induced cultures because strains carrying mutations of the MAL63 regulatory gene are nonfermenters and unable to grow appreciably on maltose alone.

Two 4ml samples of culture were removed, centrifuged at 3000 RPM in a Sorvall, washed once with 0.1 M phosphate buffer (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4, pH 7.0), resuspended in 0.2 ml of 0.1 M phosphate buffer. An equal volume of glass beads (425-600 microns, Sigma) was added. Cells were disrupted by vortexing 5 times at maximum speed for 15 seconds each of which was followed by 15 seconds on ice. 0.4 ml of 0.1 M phosphate buffer was added, the tubes briefly vortexed and centrifuged. The supernatant was removed with a Pasteur pipette and kept on ice until assayed or placed at 4°C and assayed the following day. β-galactosidase activity was determined as described by Miller (1972) except that the calculation of units was normalized to the protein

concentration of the extracts. Protein concentration was determined by the method of Lowry (1951) using bovine serum albumin as the standard.

Each cell extract sample was assayed in duplicate as follows. Tubes containing Z buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot 4H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, 0.05 M β-mercaptoethanol, pH 7.0) and cell extract to a final volume of 1 ml were pre-incubated at 28°C to temperature equilibrate. Incubation times were measured beginning with the addition of 0.2 ml of freshly prepared o-nitrophenyl-β-D-galactoside (4 mg/ml) until a pale yellow color was observed. To stop the reaction the pH was adjusted to alkalinity by the addition of 0.5 ml of 0.5 M Na_2CO_3. Tubes were centrifuged briefly at 3000 rpm in a Sorvall and the OD at 420 nm was measured. Activity was measured as the number of mμmoles of o-nitrophenol released per minute per mg protein at 28°C and pH 7 (Miller, 1972). To calculate the number of mμmoles of o-nitrophenyl per ml in the assay mixture, the OD_{420} was divided by 0.0045, which is the extinction coefficient of 1 mμmole per ml. For ten determinations, each performed in duplicate, the average activities of the induced (maltose grown) 332-5A strains integrated with single copies of the plasmids, pIGR61,62-MAL61-lacz or pIGR61,62-MAL62-lacz were 149.0 and 218.1 mμmoles/min/mg protein, respectively.

For reasons which remain unexplained, the absolute activities varied considerably from day to day, but the

relative activities of induced, repressed and uninduced cultures remained relatively constant. Therefore, for each series of assays, a standard strain was selected and always included in each days set of strains to be assayed. All activities were normalized to the daily activity of this strain. For the series of strains carrying a single integrated copy of a plasmid with an internal deletion of the MAL61-MAL62 intergenic region, the β-galactosidase activity was normalized to that of the 332-5A transformant carrying plasmid pIGR61,62-MAL61-lacz or pIGR61,62-MAL62-lacz containing the undeleted MAl61-MAL62 intergenic region grown under induced conditions (2% wt/vol maltose). Either the 332-5A integrative transformant carrying plasmid pIGR61,62-MAL61-lacz or pIGR61,62-MAL62-lacz was used depending upon which was appropriate for the deletion series assayed. For the series of transformants carrying the pLG312 derived episomal plasmids, the activities were normalized to that of plasmid $pUAS_{MAL}$(-490/-582) in yeast strain 332-5A grown under induced conditions (2% wt/vol maltose and 2% wt/vol galactose). Values are the average of assays performed on single transformants in duplicate measured on either two or three days. The average of seven determinations each performed in duplicate for induced cultures of strain 332-5A transformed with the episomal plasmid $pUAS_{MAL}$(-490/-582) was 340.7 mμmoles/min/mg protein.

RESULTS

The MAL61 and MAL62 genes are divergently transcribed (Needleman et al., 1984). Other similar structural organizations of coordinately regulated genes are seen in yeast; for example, the GAL1 and GAL10 genes and the histone genes H2A-H2B and H3-H4 (West et al., 1984; Osley et al., 1986; Cross and Smith, 1988). In these systems, a common upstream activation sequence (UAS) has been identified which regulates expression from both genes of the coordinately controlled pair. This report describes experiments which define the UAS_{MAL} and show that this sequence is responsible for the coordinate maltose inducible expression of MAL61 and MAL62 and partially responsible for the glucose-sensitivity of MAL62 expression.

Nucleotide sequence of the MAL61-MAL62 intergenic region. The sequence of the region extending from the EcoRI site within MAL61 to the PstI site within MAL62 (see Figure 4) was determined by constructing two series of nested deletions as described in Methods and Materials. Deletions into the region from the EcoRI site are referred to as the R plasmid series and from the PstI site as the P plasmid series. A BamHI linker was placed at each deletion endpoint and the EcoRI-BamHI or PstI-BamHI fragment was sequenced using the method of Sanger et al. (1977). The sequence of the non-coding intergenic region is given in Figure 4. The endpoints of each deletion series are indicated below the sequence in

Figure 4: The nucleotide sequence of the MAL61-MAL62 intergenic region.

Numbers represent the basepairs of the MAL61-MAL62 intergenic region upstream of the MAL61 gene. Basepairs -1 and -874 are adjacent to the translational start codons of MAL61 and MAL62 genes, respectively. The TATA boxes of the respective genes are indicated by a series of asterisks below the nucleotides comprising them. The transcriptional start sites of the MAL61 and MAL62 genes are indicated by open triangles (Hong and Marmur, 1986). Pertinent restriction endonuclease sites are indicated.

The endpoints of the deleted sequences removed from the series of P and R plasmids are indicated by arrows below the sequence, the arrows pointing in the direction of the deleted sequences (see Methods and Materials).

The putative UAS_{MAL} region, from basepair -502 to -582 is contained within the dotted lines. The two copies of the 11 bp element possessing partial dyad symmetry which we suggest may be active elements of the UAS_{MAL} are contained within solid rectangles. Another partially dyad symmetric element, which is located within a region possessing possible maltose inducible activity, is indicated by double darkened lines below the sequence.

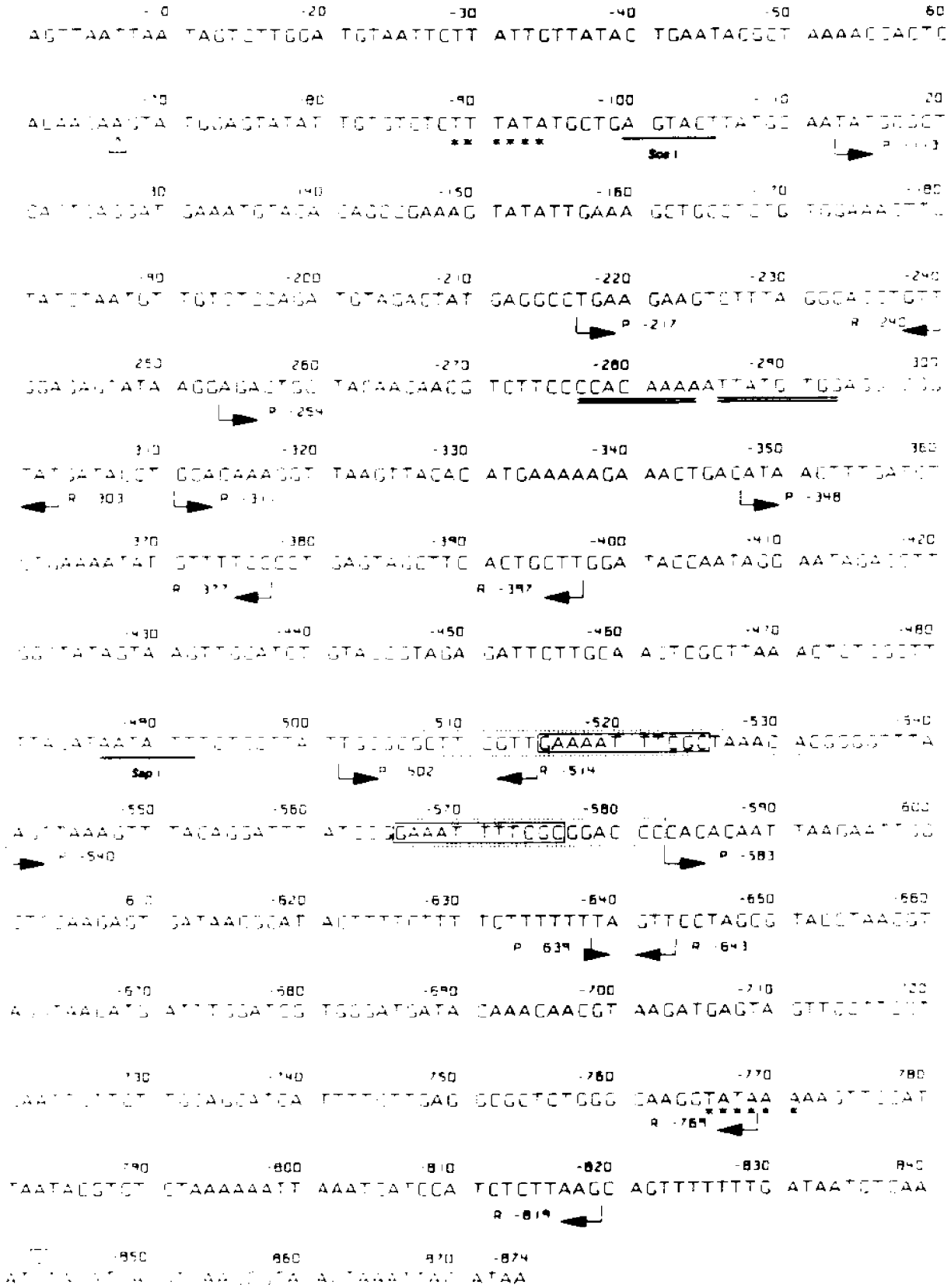


Figure 4, with an arrow pointing in the direction of the deleted region. This sequence has also been reported by Hong and Marmur (1986); the two differ by several nucleotide additions and deletions which are scattered throughout. Portions of the sequence reported here have been confirmed (Ni and Needleman, 1990). Bases -1 and -874 designate the first basepairs upstream of the MAL61 and MAL62 translational start sites, respectively. Hong and Marmur (1986) have demonstrated that the A at basepair -67 and the G at basepair -843 are the transcriptional initiation sites for the MAL61 and MAL62 genes, respectively. The proposed TATA elements of both genes are marked by asterisks below the sequence.

Localization of the UAS_{MAL}. To determine the sequences necessary for upstream regulation of the MAL61 and MAL62 genes, a series of overlapping internal deletions of the MAL61-MAL62 intergenic region was constructed by linker scanning procedures as described in Methods and Materials. For this, an external deletion from the P plasmid series was paired with one from the R plasmid series using the BamHI site at each deletion endpoint, as described in Figure 3 and the Methods and Materials, producing an internal deletion. The EcoRI-BglII fragments containing these deletions and spanning the MAL61-MAL62 intergenic region from basepair +93 of MAL61 to basepair +152 of MAL62 were fused to the *E. coli lacz* gene utilizing the yeast/*E. coli* shuttle vectors, YIp353 and YIp356R (Myers et al., 1986). These vectors differ in the

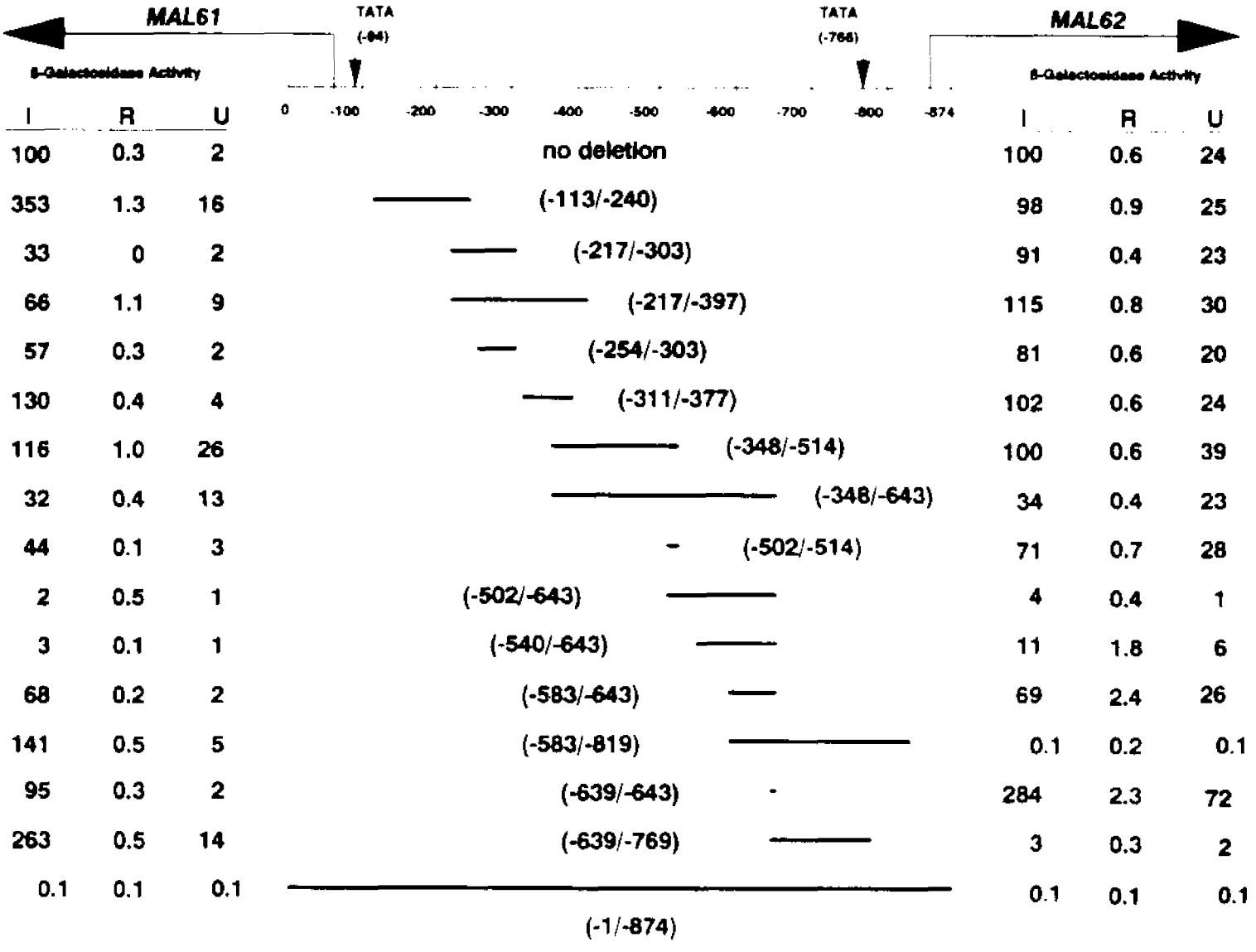
orientation and reading frame of their multiple cloning regions allowing in-frame fusion to *lacz* at the *Bgl*II site of *MAL62* in YIp353 and the *Eco*RI site of *MAL61* in YIp356R. The plasmids containing the *MAL61*-*lacz* and *MAL62*-*lacz* gene fusions are referred to as pIGRΔ(-x/-y)*MAL61*-*lacz* and pIGRΔ(-x/-y)*MAL62*-*lacz*, respectively where -x and -y indicate the deletion endpoints. Each fusion plasmid was integrated, in single copy, at the *ura3-52* gene of strain 332-5A, a *MAL6* *mal1*^{0} fermenting strain. The constructions containing the complete *Eco*RI-*Bgl*II fragment without any deletions are referred to as pIGR61,62-*MAL61*-*lacz* and pIGR61,62-*MAL62*-*lacz*. Expression of the reporter *lacz* gene was measured under induced (2% maltose), repressed (2% glucose) and uninduced (2% galactose) conditions. The results are shown in Figure 5.

The results obtained with plasmids pIGR61,62-*MAL61*-*lacz* and pIGR61,62-*MAL62*-*lacz* clearly indicate that the wild type *MAL61*-*MAL62* intergenic region sequence is able to activate transcription of the *lacz* reporter gene bidirectionally, that is, when fused to either the *MAL61* or *MAL62* coding regions. Thus, this intergenic region contains the UAS_{MAL}. In addition, activation appears to be normally regulated in that expression of both genes is maltose inducible and glucose repressible. In plasmid pIGR61,62-*MAL61*-*lacz*, expression is induced approximately 50-fold which is consistent with the reported induction ratios seen for maltose permease, the product of the MAL61 gene (Dubin et al., 1989). Only 4-fold induction of

Figure 5: β-galactosidase activities of yeast strain 332-5A transformed with plasmids containing internal deletions of the MAL61-MAL62 intergenic region adjacent to the MAL61-lacz or MAL62-lacz genes.

The numbered line represents the nucleotide sequence of the MAL61-MAL62 intergenic region. TATA boxes and transcriptional initiation sites are indicated. The direction of transcription of the MAL61 and MAL62 genes are shown by arrows. Darkened lines below the MAL61-MAL62 intergenic region represent the deleted sequences in the deletion plasmids (see Methods and Materials). Their endpoints are indicated by numbers in parentheses.

β-galactosidase activities are measured in mμmoles of o-nitrophenyl released per minute per mg protein (see Methods and Materials). Each activity is the average from duplicate assays of single transformants on two separate occasions. The abbreviations I, R and U represent β-galactosidase activities in induced (2% wt/vol maltose), repressed (2% wt/vol glucose) and uninduced (2% wt/vol galactose) cultures, respectively. β-galactosidase activities were normalized to that of the 332-5A transformant carrying plasmid pIGR61,62-MAL61-lacz or pIGR61,62-MAL62-lacz containing the undeleted MAl61-MAL62 intergenic region grown under induced conditions (2% wt/vol maltose).


MAL61
TATA
(-94)
TATA
(-766)
MAL62
β-Galactosidase Activity
β-Galactosidase Activity
I R U
0 -100 -200 -300 -400 -500 -600 -700 -800 -874
I R U
100 0.3 2 no deletion 100 0.6 24
353 1.3 16 (-113/-240) 98 0.9 25
33 0 2 (-217/-303) 91 0.4 23
66 1.1 9 (-217/-397) 115 0.8 30
57 0.3 2 (-254/-303) 81 0.6 20
130 0.4 4 (-311/-377) 102 0.6 24
116 1.0 26 (-348/-514) 100 0.6 39
32 0.4 13 (-348/-643) 34 0.4 23
44 0.1 3 (-502/-514) 71 0.7 28
2 0.5 1 (-502/-643) 4 0.4 1
3 0.1 1 (-540/-643) 11 1.8 6
68 0.2 2 (-583/-643) 69 2.4 26
141 0.5 5 (-583/-819) 0.1 0.2 0.1
95 0.3 2 (-639/-643) 284 2.3 72
263 0.5 14 (-639/-769) 3 0.3 2
0.1 0.1 0.1 0.1 0.1 0.1
(-1/-874)

β-galactosidase synthesis is seen from plasmid pIGR61,62-*MAL62*-*lacz*. Hong and Marmur (1987) found a 30-fold induction ratio for maltase activity in cells grown in the nonrepressing sugar raffinose. In their analysis of the regulation of expression of maltase and maltose permease by the *MAL63* and *MAL64* encoded MAL activators, Dubin et al. (1986) reported induction ratios varying from ten to sixty-fold when measuring the enzymic activity of maltase in strain 332-5A. The lower induction ratios reported here appear to be a consequence of an elevated rate of uninduced expression of the *MAL62* gene in these constructions.

To test if the high uninduced expression of the *MAL62* gene found here is a consequence of the site of integration of the plasmid, in other words a position effect, the *MAL62*-*lacz* fusion plasmid containing the complete *MAL61*-*MAL62* intergenic region was integrated at the *MAL6* locus, instead of the *ura3-52* gene, by transforming strain 332-5A with uncleaved plasmid pIGR61,62-*MAL62*-*lacz* and selecting for stable Ura$^+$ transformants which retained pBR322 homologous sequences. Southern analysis utilizing several restriction enzymes confirmed that the plasmid had integrated at the *MAL6* locus in single copy. Comparison of the level of expression of the *MAL62*-*lacz* gene fusion from the two different integration sites indicate a similar high basal level of constitutive expression suggesting that it is not the result of integration at *ura3-52* (unpublished results). The basis of this elevated

uninduced expression of MAL62 in this construction is not clear. Perhaps the MAL62 gene is under some form of positional regulation at the wild type locus which is absent in the conditions used in these experiments.

The results reported in Figure 5 also allow us to localize the UAS_{MAL} within the MAL61-MAL62 intergenic region. Deletion of the sequences from -502 to -643 and from -540 to -643 significantly decrease the level of β-galactosidase synthesis under induced and uninduced conditions and this effect is seen for fusions to both the MAL61 and MAL62 genes. Deletion of the sequences from -502 to -514 and from -583 to -643 result in a slight but significant decrease of maltose inducible expression. Thus, the sequences between -502 and -643 contain the putative UAS_{MAL}. This conclusion is supported by experiments to be described below, which demonstrate that the sequence from -490 to -582 is capable of conferring maltose regulated expression in a heterologous system and which allow us to further limit the UAS_{MAL} to the region -502 to -583. Paradoxically, the larger deletion removing basepairs -348 to -643 does not result in as dramatic a reduction of expression as did the shorter deletions containing the UAS_{MAL}. It is likely that this result is an artifact of the construction since deletion of the sequences from -348 to -514 has little or no effect on expression on either the MAL61-lacZ or MAL62-lacZ fusion genes. Perhaps,

the deletion plus the insertion of the BamHI linker fortuitously created a maltose-responsive sequence.

Certain internal deletions appear to have unilateral effects on expression of either the MAL61 or MAL62 genes. A glucose responsive element controlling MAL62 expression may be located between bases -583 and -643. Transformants containing pIGRΔ(-583/-643)MAL62-lacz exhibit only a 10-fold reduction in repressed vs. uninduced expression as opposed to the 25-50 fold reduction in expression seen for transformants carrying plasmids containing this sequence and the UAS_{MAL}. Based on the results reported in Figure 5, the effect is not obvious but the presence of this element will be supported by experiments to be described below.

Three deletions, removing basepairs -217 to -303, -254 to -303 and -217 to -397, partially lowered transcriptional activation of the MAL61 gene alone. Note that the three deletions remove a sequence in common, from -254 to -303, which possesses the dyad palindrome element, 5'-CCACAAAAATTATGTGG-3', from sequences -277 to -293. Note that it is symmetrical for 7 out of 8 basepairs on either side of the centrally located A nucleotide (-285). We have not extended our investigation of the role of this sequence or the dyad.

The deletions removing basepairs from -113 to -240 or from -639 to -769 resulted in an increase in expression of the MAL61 gene suggesting that these deletions may have removed a

repressing element. Without characterization in a heterologous gene system, it is not possible to draw any firm conclusions.

Expression of MAL62 alone was increased in strains transformed with plasmids lacking sequences -639 to -643 from the MAL61-MAL62 intergenic region. This deletion is actually an insertion because addition of the BamHI linkers necessary for ligation actually resulted in the insertion of more nucleotides than were removed. The deletions which removed basepairs -583 to -819 and -639 to -769 resulted in a dramatic reduction of expression of the MAL62 gene supporting the view that the TATA sequence at -766 is functional.

The putative UAS_{MAL} confers maltose regulated expression to a promoterless CYC1-lacz fusion. To determine whether the sequence containing the putative UAS_{MAL} is sufficient to confer maltose regulated expression, two fragments of different sizes containing the putative UAS_{MAL} were separately placed upstream of a heterologous gene and the regulation of this gene by maltose and the MAL activator determined. Plasmid pLG312 (from L. Guarente) is a yeast episomal plasmid containing 385 basepairs of the CYC1 upstream region and four basepairs of the amino terminus of CYC1 fused in frame to lacz so as to place expression of the lacz gene under the control of the CYC1 upstream region (Smith et al., 1979; Guarente and Ptashne, 1981; Guarente and Mason, 1983). We deleted the UAS_{CYC} from pLG312 and replaced it with a

fragment containing basepairs -490 to -638 (called $pUAS_{MAL}$(-490/-638)) or a fragment containing basepairs -490 to -582 (called $pUAS_{MAL}$(-490/-582)) from the MAL61-MAL62 intergenic region. The orientation of the fragments is the same as that upstream of the MAL62. Plasmids $pUAS_{MAL}$(-490/-582) and $pUAS_{MAL}$(-490/-638) were transformed into strain 332-5A and into other yeast strains containing various mutations in the MAL activator genes.

The results (see Table 2) show that both plasmids confer maltose regulated expression to the CYC1-lacz gene fusion in the MAL6 strain 332-5A. The ratio of induced to uninduced levels is similar to that seen for the MAL62-lacz fusion containing the undeleted MAL61-MAL62 intergenic region. Approximately 4-fold induction is exhibited both in strains containing the MAL62-lacz fusion plasmid (plasmid pIGR61,62-MAL62-lacz) and in strains containing the CYC1-lacz plasmids containing either of the putative UAS_{MAL} containing fragments (plasmids $pUAS_{MAL}$(-490/-582) or $pUAS_{MAL}$(-490/-638)). Based on these results and on those of the deletion analysis described above, we conclude that the UAS_{MAL} is located within the sequence from -502 to -582. Expression of the heterologous genes in both plasmids is repressed by glucose although to a lesser extent than that seen in strain 332-5A containing plasmid pIGR61,62-MAL62-lacz. Additionally, the glucose-repressed level of expression in strain 332-5A transformed with $pUAS_{MAL}$(-490/-582) is three times higher than

Table 2: Beta-galactosidase activities of the UAS_{MAL}-*CYC1*-*lacz* fusions.

β-galactosidase activities are the averages from duplicate assays of single transformants on at least two separate occasions. Units of activity were calculated as described by Miller (1972) (see Methods and Materials). The abbreviations I, R and U represent β-galactosidase activities in induced (2% wt/vol maltose and 2% wt/vol galactose), repressed (2% wt/vol glucose) and uninduced (2% wt/vol galactose) media, respectively. Activities were normalized to the activities of strain 332-5A transformed with plasmid $pUAS_{MAL}$(-490/-582) in induced medium.

Strain	Activator Genotype		Plasmid	Beta-Galactosidase Activity		
	MAL64	MAL63		I	R	U
332-5A	MAL64	MAL63	$pUAS_{MAL}$(-490/-582)	100.0	5.7	24.5
			$pUAS_{MAL}$(-490/-638)	81.3	1.8	18.1
			pLG178	0.8	1.3	0.7
			none	0.6	0.7	0.6
SW14	MAL64	mal63-14	$pUAS_{MAL}$(-490/-582)	17.7	3.0	18.1
			$pUAS_{MAL}$(-490/-638)	15.6	1.7	14.9
			pLG178	1.4	2.1	1.3
			none	0.8	0.6	0.8
A9u	MAL64	mal63Δ::ura3	$pUAS_{MAL}$(-490/-582)	22.1	3.9	21.9
			$pUAS_{MAL}$(-490/-638)	16.5	2.3	16.7
			pLG178	0.9	0.5	0.8
			none	0.6	0.4	0.6
340-2A	--	--	$pUAS_{MAL}$(-490/-582)	3.9	2.4	3.5
			$pUAS_{MAL}$(-490/-638)	2.1	1.6	1.4
			pLG178	0.6	0.8	0.5
			none	0.4	0.7	0.4
332-5AΔF-1-5	mal64:LEU2	MAL63	$pUAS_{MAL}$(-490/-582)	58.8	3.5	9.7
			$pUAS_{MAL}$(-490/-638)	40.0	1.4	4.1
			pLG178	0.7	0.6	0.9
			none	0.1	0.4	0.0
8-2BΔF-5-21	mal64-C2:LEU2	mal63-13	$pUAS_{MAL}$(-490/-582)	8.6	5.4	7.9
			$pUAS_{MAL}$(-490/-638)	5.0	1.9	5.5
			pLG178	0.6	0.8	0.7
			none	0.2	0.2	0.2

in strain 332-5A transformed with $pUAS_{MAL}$(-490/-638) indicating, as described above, that the region from -583 to -638 contains a sequence which is partially responsible for the glucose sensitivity of *MAL62* expression. A sequence of similar function has been identified upstream of *GAL1* (West et al., 1984). β-galactosidase activities of the untransformed strain 332-5A and of the 332-5A transformants containing the plasmid pLG178, a promoterless *CYC1-lacz* fusion, were minimal and uninducible.

In order to demonstrate dependence of expression of β-galactosidase on the MAL activator, the two UAS_{MAL}-*CYC1-lacz* gene fusion plasmids, $pUAS_{MAL}$(-490/-582) and $pUAS_{MAL}$(-490/-638), were transformed into *Saccharomyces* strains containing various MAL activator gene mutations and assayed for β-galactosidase expression (Table 2). The results show that the maltose inducible expression of the *CYC1-lacz* gene fused to either UAS_{MAL} fragment is dependent upon the presence of a functional MAL activator. No induction over uninduced levels is seen in transformants of strains A9u, SW14 and 340-2A containing either $pUAS_{MAL}$(-490/-582) or $pUAS_{MAL}$(-490/-638). Strain A9u carries a deletional disruption of the *MAL63* gene and strain SW14 contains a point mutation in *MAL63* (Chang et al., 1989; J. Kim, unpublished results). The *MAL1g* allele of strain 340-2A is a naturally occurring variant of *MAL1* and contains the *MAL11-2* gene (encoding maltose permease) and *MAL12* gene (encoding maltase) but lacks the gene encoding the MAL

activator, and thus, the strain is uninducible (Charron and Michels, 1988).

The results in Table 2 suggest that the product of the MAL64 gene may be responsible for the high uninduced levels of β-galactosidase expression in strains transformed with either UAS_{MAL}-CYC1-lacz containing plasmid. This is surprising because the MAL64 gene product does not appear to play a significant role in maltose fermentation in wild type fermenting strains. Uninduced β-galactosidase expression in transformants of host strains lacking the MAL64 gene (332-5AΔF-1-5, 340-2A, and 8-2BΔF-5-21) is significantly lower compared to strains carrying the MAL64 gene. Induction in strain 332-5AΔF-1-5 transformed with $pUAS_{MAL}$(-490/-582) and $pUAS_{MAL}$(-490/-638), respectively, resulted in a six and ten-fold increase in β-galactosidase activity, and differed slightly from the four-fold increase observed in transformants of strain 332-5A. Strain 332-5AΔF-1-5 is a derivative of strain 332-5A in which the MAL64 gene has been disrupted by the integration of LEU2. In addition, strains 8-2BΔF-5-21 and 340-2A (described above), which lack any functional MAL activator, exhibit only low levels of expression and are uninducible. Strain 8-2BΔF-5-21 contains a LEU2 integrative disruption of MAL64-C2 (a constitutive allele of MAL64) and the mal63-13 mutant allele (a substitution mutation) (Dubin et al., 1989; J. Kim, unpublished results). Previous studies of the MAL64 gene showed that this gene is an apparently

nonfunctional homolog of *MAL63* which can be activated by mutation to encode a constitutive MAL activator (Dubin et al., 1989). Disruption of the *MAL64* gene in inducible *MAL6* strains does not affect the ability to ferment maltose or reduce the induced or uninduced levels of maltase or maltose permease synthesized by strain 332-5A (Dubin et al., 1989). Thus, it is likely that the high uninduced expression of β-galactosidase in the $pUAS_{MAL}$ plasmids (as was seen in plasmid pIGR61,62-*MAL62*-*lacz*) is a function of the construction.

DISCUSSION

In this study we have sought to identify and characterize the upstream sequences which control the expression of the maltose fermentative enzymes at the MAL6 locus of Saccharomyces. Using deletion analysis of the MAL61-MAL62 intergenic region, we have localized the sequences responsible for the maltose inducible regulation of both the MAL61 and MAL62 genes to a 81 basepair region, from bases -502 to -582 upstream of the MAL61 gene start codon, which we designate the UAS_{MAL}. Our results indicate that the UAS_{MAL} is necessary for maltose induction and that this regulated expression requires the MAL activator. Deletions removing the UAS_{MAL} significantly reduce the maltose inducible β-galactosidase expression from MAL61-lacz and MAL62-lacz gene fusions (see Figure 5). Since a deletion of the UAS_{MAL} inhibits induction of both MAL61 and MAL62, these results also support our previous conclusions that the expression of both genes is under positive control (Chang et al., 1988). The UAS_{MAL} is located at different sites relative to the initiation of the regulated genes. It lies 502 and 292 nucleotides upstream of the translational start codons of the MAL61 and MAL62 genes, respectively. A similar bidirectionally functioning element, the UAS_{GAL}, has been found in the intergenic region between the GAL1 and GAL10 genes (West et al., 1984).

Our results also demonstrate that the UAS_{MAL} is sufficient for maltose inducible expression. Two fragments of the MAL61-MAL62 intergenic region containing bases -490 to -582 and -490 to -638, which span and contain the UAS_{MAL}, were inserted into plasmid pLG312 upstream of the CYC1-lacz fusion replacing the UAS_{CYC}. Expression of the UAS_{MAL}-CYC1-lacz fusion genes in the MAL6 host strain 332-5A is maltose inducible. The results show that the UAS_{MAL} acts in conjunction with the MAL activator to regulate expression by maltose. In strains transformed with either of the UAS_{MAL}-CYC1-lacz gene fusions, $pUAS_{MAL}$(-490/-582) or $pUAS_{MAL}$(-490/-638), the presence of the wild type allele of MAL63 is required to confer maltose inducibility to the heterologous gene. Induction does not occur in strains lacking a functional MAL activator gene. These include a strain carrying a point mutation in MAL63 (SW14), a strain carrying a deletion/disruption of MAL63 (A9u), and a strain carrying a naturally occurring partially functional MAL locus lacking a MAL activator gene (340-2A).

The 81 basepair UAS_{MAL} contains two copies of an 11 basepair element, 5'-GAAA(A/T)TTTCGC-3', located at -515 to -525 (copy I) and -566 to -576 (copy II) possessing almost complete dyad symmetry (see Figure 4). We suggest that the 11 basepair dyads could be active elements in the regulation of maltose induction of the MAL61 and MAL62 genes. This hypothesis is based on the following. Multiple copies of short sequences having dyad symmetry have been identified as

regulatory elements in the upstream regions of several yeast genes including, in particular, the UAS_{GAL} (West et al., 1984; Giniger et al., 1985; Hill, D.E. et al., 1986; reviewed in Guarente, 1989; Struhl, 1989). The yeast GAL1-GAL10 intergenic region contains four 17 basepair dyad elements which have been shown to be binding sites for the GAL4 activator protein (West et al., 1984; Giniger and Ptashne, 1988). If the 11 basepair element of the MAL61-MAL62 intergenic region identified in this report is, in fact, the MAL activator binding site, our results suggest that either only copy II is functional or the two 11 basepair dyad elements act synergistically to activate transcription. Removal of one dyad repeat (copy II in deletion -540/-643) or both dyads (copies I and II in deletion -502/-643) reduces expression of both the MAL61 and MAL62 genes to similar low levels under induced or uninduced growth conditions. Since our results do not include a deletion of only copy I, we are unable to distinguish between these possibilities. Recently, Ni and Needleman (1990) have shown by DNAse I footprinting that MAL63 protein, synthesized in E. coli, binds to a region between basepairs -556 and -579 (our numbers). This region contains copy II of the 11 basepair dyad. Therefore, it is possible that copy II of the dyad may be acting alone to mediate transcriptional activation. In this respect, it resembles an artificially constructed consensus sequence of the four GAL4 binding sites of the GAL1-GAL10 promoter which

by itself is capable of activating transcription of a GAL1 gene fusion (Giniger and Ptashne, 1988). Alternatively, if both copies I and II acting synergistically are necessary for maltose inducible gene expression of the MAL61 and MAL62 genes, the situation resembles the naturally occurring GAL1-GAL10 promoter. In the latter, evidence exists that two (or more) of the naturally occurring GAL4 binding sites act synergistically, and not additively to activate transcription. Removal of either site 2 or site 3 is sufficient to reduce activation by galactose to low levels (West et al, 1984). Sites 3 or site 4 alone, when present upstream of a GAL1-lacz gene fusion, cannot activate transcription appreciably, but when present together they can, and the level of activation is not additive but synergistic (Giniger and Ptashne, 1988). The naturally occurring GAL4 binding sites bind activator with low affinity. Synergism could be a manifestation of the effect of cooperative binding of GAL4 molecules to low affinity binding sites. Whether the suggested dyad copy II of the MAL61-MAL62 intergenic region acts alone or synergistically with copy I, transcriptional activation can be explained in a model proposed by Ptashne (1988). Two or more activators bound on the DNA at low affinity binding sites or one at a high affinity site are needed to interact with and precisely position a common target protein enabling the latter to interact with some other component of the transcriptional apparatus, perhaps TFIID or RNA polymerase II.

In a previous study, Hong and Marmur (1987) purport that the inducible expression of the MAL62 gene requires sequences between -497 and -544 (our numbers) of the MAL61-MAL62 intergenic region. This conclusion is based upon a deletion analysis, similar to ours, of the MAL61-MAL62 intergenic region but in which only MAL62 expression is measured. One unrecognized discrepancy in their interpretation of their results is that a deletion which removes basepairs -497 to -534, and therefore almost entirely deletes their proposed UAS_{MAL}, does not affect maltose induction of MAL62. By our interpretation, the results of Hong and Marmur (1987) suggest that two equally active sites exist in the UAS_{MAL}, one in the -497 to -557 region and a second between -557 and -612, since deletion of either region alone has no effect but deletion of both leads to the loss of inducibility. This is in contrast to our findings in which deletion of the -540 to -583 sequence has a very dramatic effect on expression of both MAL61 and MAL62 indicating that either the UAS_{MAL} is completely contained within this region or that this region acts synergistically with additional binding sites in the -502 to -539 region. While our results do not allow us to distinguish these two possibilities, we prefer the latter based on the finding that the -502 to -514 deletion has a partial but significant effect on expression of both MAL61 and MAL62. Taken together, the results of Hong and Marmur (1987), Ni and Needleman (1990) and the deletion analysis of

the present study strongly suggest that the UAS_{MAL} is contained within the region from basepair -502 to -582. Our results, along with those of Ni and Needleman (1990), perhaps imply that the UAS_{MAL} is entirely contained within basepairs -540 to -583. Ni and Needleman (1990) also find a second MAL63 protein binding site centered around basepair -480. This site lies outside of the region defined here as the UAS_{MAL} and appears only at higher protein concentrations, and therefore may not be functionally significant. We suggest that different methods, such as point mutation analysis, will be needed to further define the structure of the UAS_{MAL}.

It is interesting to note that the sequence between basepairs -556 and -565, part of the MAL activator binding site found by Ni and Needleman (1990), possesses homology to the Y factor consensus binding site of several yeast genes (Chasman et al., 1990). Factor Y binding is inactive by itself but acts synergistically to stimulate transcription in conjunction with factor(s) binding to a nearby pyrimidine-rich sequence. Since the absence of the pyrimidine-rich sequence between basepairs -622 and -639 of the MAL61-MAL62 intergenic region does not affect transcriptional activation in the strains transformed with the UAS_{MAL}-CYC1-lacZ fusions, the Y factor binding site may not be significant for maltose regulated expression; however, it is possible that information contained in the CYC1 upstream sequences of plasmid pLG312 may be providing the function of the pyrimidine-rich sequence.

Two *MAL61* upstream deletions, pIGRΔ(-113/-240) *MAL61*-*lacz* and pIGRΔ(-639/-769)*MAL61*-*lacz*, exhibit elevated maltose induced levels of β-galactosidase expression suggesting the presence of repressing elements. Repression elements have been found upstream of several other yeast genes (reviewed in Guarente, 1987; Struhl, 1989). To confirm the presence of an upstream repressing sequence (or URS), such sequences have to be tested in a heterologous promoter system such as the *CYC1*-*lacz* fusion plasmid pLG312 used in this study.

Our results suggest that the region between basepairs -583 and -638, located adjacent to the UAS_{MAL} and between it and the *MAL62* gene, contains sequences which may partially mediate glucose repression of the *MAL62*. This conclusion is based on a comparison of the results shown in Table 2 obtained with plasmids $pUAS_{MAL}$(-490/-582) and $pUAS_{MAL}$(-490/-638) and are supported by the results with plasmids pIGR61,62-*MAL62*-*lacz* and pIGRΔ(-583/-643)*MAL62*-*lacz* shown in Figure 5. In glucose repressing growth conditions, *MAL62*-*lacz* fusion expression is repressed approximately 40-fold below the uninduced galactose grown levels in strain 332-5A transformed with plasmid pIGR61,62-*MAL62*-*lacz*. Deletion of the sequences from basepairs -583 to -643 partially relieves this repression. Only 10-fold glucose repression is seen and no effect is evident on expression of the *MAL61* gene. Glucose repression has an approximately 10-fold effect on *MAL61*-*lacz* expression.

In the heterologous $pUAS_{MAL}$ plasmids, where expression of the *CYC1-lacz* fusion is regulated by the proposed UAS_{MAL}, the plasmid containing the -490 to -638 UAS_{MAL} sequence was three times as sensitive to glucose repression as the plasmid containing the sequences -490 to -582. Similar results were found in the transformants of the MAL activator mutant strains. Thus, the region containing sequences -583 to -638 appears to be involved in the glucose repression mechanism. The work of Hong and Marmur (1987) delineates approximately the same region as a glucose-responsive element.

Glucose repression of *GAL1* expression is regulated by two independent regions within the *GAL1* promoter: one within the region containing the GAL4 protein binding sites, the UAS_{GAL}, and a second involving sequences between the UAS_{GAL} and the *GAL1* TATA box, the URS_{GAL} (Flick and Johnston, 1990). In glucose grown cells, the binding of GAL4 protein to its binding site(s) on DNA, the UAS_{GAL}, is inhibited *in vitro* implying that one possible mechanism of glucose repression is by the inhibition of binding of a trans-activator protein to its UAS (Giniger et al, 1985). Since repression by glucose is accompanied by dephosphorylation of the GAL4 protein, the mechanism may involve the conversion of the active trans-activator to an inactive, dephosphorylated form (Mylin et al., 1989).

The second GAL4-independent sequence involved in glucose repression is located in the region between the UAS_{GAL}

and the GAL1 TATA box and is designated the URS_{GAL} (Flick and Johnston, 1990). West et al. (1984) found that glucose repression of GAL1 expression was partially relieved by the deletion of a sufficient amount of nucleotides between the UAS_{GAL} and the TATA box of the GAL1 gene. Elevated expression under glucose repressing conditions did not depend on a functional GAL4 gene product. Similarly, our results show that relief from glucose repression of MAL62 expression by the deletion of the sequences between -583 and -638 in the MAL61-MAL62 intergenic region does not require the MAL activator (Table 2). Flick and Johnston (1990) showed that the URS_{GAL} is contained within the 89 basepair sequence from basepair -126 to -214 upstream of the GAL1 transcriptional start site. The region can confer glucose regulated repression to a heterologous gene fusion. By deletion analysis they demonstrated that the region contains two or three glucose repression sensitive elements. Analysis of the URS_{GAL} sequence elements reveals that several short repeated (or inverted) motifs are found in two of various combinations of the elements. Our proposed glucose-sensitive sequence contains homology to two of these repeated motifs. One is the sequence between -580 and -588 of the MAL61-MAL62 intergenic region, 5'-CCCCACACA-3', which is homologous to 8 of 9 nucleotides of the URS_{GAL} repeat motif, 5'-CCCCACAAA-3'. The second URS_{GAL} sequence motif found in our region from basepairs -612 to -618, 5'-ATAACGC-3', is identical for 6 out of 7 nucleotides

to the one contained in the URS_{GAL}. Additional homology exists beyond this second motif in both directions in our sequence and theirs although these sequences are not part of the repeat motif found in the URS_{GAL} glucose-sensitive elements. It is possible that glucose repression of MAL62 gene expression involving the region between basepairs -583 to -638 of the MAL61-MAL62 intergenic region may share a common mechanism with the URS_{GAL} and that they both are mediated by similar sequence motifs.

As in the GAL1 promoter, glucose repression of maltose fermentative gene expression appears to be under the control of several components of which the glucose-responsive element between -583 and -638 is only one. The repression of β-galactosidase activity by glucose in strain 332-5A transformed with $pUAS_{MAL}$(-490/-583) is still evident, although to a diminished extent. In addition, MAL61 expression is clearly sensitive to glucose repression but is unaffected by the deletion of this element. Based on studies of the constitutive, glucose-repression insensitive allele of MAL43, the MAL activator may be a second important component of the glucose-responsive effect (Charron and Michels, 1987).

In this study we have identified and characterized the UAS_{MAL}, the sequences of the MAL61-MAL62 intergenic region which are responsible for the maltose inducibility of the transcription of MAL61 and MAL62, the genes encoding the maltose fermentative enzymes at the MAL6 locus. Like several

other yeast UAS's, the UAS_{MAL} contains short dyad symmetric sequences which we suggest may be the active elements within the region (Giniger et al., 1985; Hill, D.E. et al., 1986; reviewed in Guarente, 1989; Struhl, 1989). We have also identified additional sequences which play a role in transcriptional expression including an apparent glucose-responsive element which is partially responsible for mediating glucose repression. In this respect, the MAL61-MAL62 intergenic region resembles the upstream regions of other yeast genes, in that it is composed of several distinct elements which act in concert with trans-activating factors to precisely regulate gene expression (reviewed in Guarente, 1989; Struhl, 1989). Further experiments, including binding studies and the systematic mutagenesis of the suspected regulatory sequences, are planned to more completely identify the sequences involved and to more fully clarify their role in the regulation of maltose fermentation.

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