Characterization of activation and inhibitory regions in the ADR1 transcriptional regulator

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Characterization of activation and inhibitory regions in the ADR1 transcriptional regulator

**Abstract**

The regulation of the ADH2 gene from the yeast Saccharomyces cerevisiae is carbon source-dependent. Expression of ADH2 requires the activity of the transcriptional activator ADR1. This dissertation addresses three separate aspects of the regulation of ADR1 activity.

We have identified eleven additional ADR1\(\sp{c}\) mutations which allow ADH2 to partially escape glucose repression. These eleven new mutations cluster to one region of the ADR1 peptide between amino acids 227 and 239, previously identified by four other ADR1\(\sp{c}\) mutations. These are the only mutations in ADR1 which have been isolated by virtue of their ability to activate ADH2 transcription under repressed conditions. These mutations have previously been shown not to affect ADR1 mRNA or protein levels suggesting that they likely affect the ability of ADR1 to interact with other proteins. This may entail relief of repression or an increased ability to interact with other transcription factors.

We have used an in vivo transcription assay to identify three distinct regions of the ADR1 protein which are capable of transactivation function. The location of these activation domains coincide with regions of the protein that were shown to be important for activation function by previous deletion analyses. We also identified two regions which display repressor function with this assay. One of these regions corresponds to the site of the ADR1\(\sp{c}\) mutations.

In a modified in vivo transcription assay, we show that the UAS2 element, located adjacent to the binding site for ADR1 in the ADH2 promoter, is able to repress the activity of LexA-ADR1 fusions containing various activation domains of ADR1 as well as diminishing the activity of other transcriptional activators. The extent of repression varied depending on the particular activation domain of ADR1 present. Under derepressed conditions, inclusion of the UAS2 element in a hybrid promoter containing the LexA binding site increased the activity of the promoter when coexpressed with LexA-ADR1 fusions. This increase in activity was not specific for ADR1 as we also observed an increase in activity when other activators were bound to the template. These results suggest that ADR1 transcriptional activity is the mediated by complex interactions between activation and inhibition domains located within the molecule and that the ability of ADR1 to function at the ADH2 locus is governed by other factors which bind to the promoter region at UAS2.

**Keywords**

Biology, Molecular

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Characterization of activation and inhibitory regions in the
ADR1 transcriptional regulator

Chase, Dan, Ph.D.
University of New Hampshire, 1993
CHARACTERIZATION OF ACTIVATION AND INHIBITORY REGIONS IN
THE ADRI TRANSCRIPTIONAL REGULATOR

by

Dan Chase

B.S., University of New Hampshire, 1987

DISSERTATION

Submitted to the University of New Hampshire in Partial
Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in

Biochemistry

December, 1993
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11/3/93
Date
This work is dedicated to my Mom and Dad

Dave and Joan Chase

I love you both
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I would like to thank my family, first and foremost, for all their love and support over the years.

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ABSTRACT
CHARACTERIZATION OF ACTIVATION AND INHIBITORY REGIONS IN
THE ADR1 TRANSCRIPTIONAL REGULATOR
by
Dan Chase
University of New Hampshire, December, 1993

The regulation of the \textit{ADH2} gene from the yeast
\textit{Saccharomyces cerevisiae} is carbon source-dependent.
Expression of \textit{ADH2} requires the activity of the
transcriptional activator ADR1. This dissertation addresses
three separate aspects of the regulation of ADR1 activity.

We have identified eleven additional \textit{ADR1i} mutations
which allow \textit{ADH2} to partially escape glucose repression. These
eleven new mutations cluster to one region of the ADR1 peptide
between amino acids 227 and 239, previously identified by four
other \textit{ADR1i} mutations. These are the only mutations in \textit{ADR1}
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mutations have previously been shown not to affect \textit{ADR1} mRNA
or protein levels suggesting that they likely affect the
ability of ADR1 to interact with other proteins. This may
entail relief of repression or an increased ability to
interact with other transcription factors.
We have used an in vivo transcription assay to identify three distinct regions of the ADR1 protein which are capable of transactivation function. The location of these activation domains coincide with regions of the protein that were shown to be important for activation function by previous deletion analyses. We also identified two regions which display repressor function with this assay. One of these regions corresponds to the site of the ADR1 mutations.

In a modified in vivo transcription assay, we show that the UAS2 element, located adjacent to the binding site for ADR1 in the ADH2 promoter, is able to repress the activity of LexA-ADR1 fusions containing various activation domains of ADR1 as well as diminishing the activity of other transcriptional activators. The extent of repression varied depending on the particular activation domain of ADR1 present. Under derepressed conditions, inclusion of the UAS2 element in a hybrid promoter containing the LexA binding site increased the activity of the promoter when coexpressed with LexA-ADR1 fusions. This increase in activity was not specific for ADR1 as we also observed an increase in activity when other activators were bound to the template. These results suggest that ADR1 transcriptional activity is the mediated by complex interactions between activation and inhibition domains located within the molecule and that the ability of ADR1 to function at the ADH2 locus is governed by other factors which bind to the promoter region at UAS2.
GENERAL INTRODUCTION

All cells must engage in some form of communication which provides the cell with the ability to detect and respond to environmental stimuli. The molecular details required for signal recognition and transduction processes are only recently being elucidated. It has become clear, however, that there are a variety of mechanisms for the transduction of signals across the outer membrane of the cell as well as diverse mechanisms by which the external signal is manifest in intracellular responses.

Some of these responses are effected by modifications of extant molecules within the cell. Perhaps the best studied example of this is the regulation of macromolecule activity through substrate phosphorylation-dephosphorylation. Another mechanism through which cells adapt to their changing environment is by the regulation of gene expression. Initiation of messenger RNA synthesis is a primary control point in the regulation of differential gene expression. Cells respond to intra- and extra-cellular cues by turning on or off and by modulating the extent of transcription of active genes. Although the mechanisms and biochemical pathways by which cells integrate physiological cues to bring about appropriate transcriptional changes are still largely unknown, it is clear that the frequency of initiation of mRNA synthesis depends
ultimately on factors that interact with specific elements in gene promoters.

An assortment of regulatory elements for RNA polymerase II-mediated transcription can be scattered both upstream and downstream of the RNA start site for a gene. Systematic mutational analyses have revealed that each gene in a eukaryotic cell has a particular combination of positive and negative regulatory cis elements that are uniquely arranged in number, type, and spatial array. This organization of elements within a single promoter was originally demonstrated in viral promoters (Herr and Clarke 1986) and has since been described for various eukaryotic promoters such as those of the CYC1 and CYC7 genes (Pfeifer et al. 1987). These elements are binding sites for sequence-specific transcription factors that either activate or repress transcription of the gene. Usually cis elements are located within several hundred base pairs of the initiation site, but some elements can exert control over much greater distances. Overlapping or superimposed binding sites for multiple factors can result in different positive and negative factors competing for sites; and in some cases, synergistic effects that are dependent on strict spacing between adjacent cis elements have been observed (Akerblom et al. 1988; Schule et al. 1988).

One of the most striking features of the sequence-specific factors at the molecular level, are their apparent functional modularity. These factors are generally capable of
four distinct functions. They contain a nuclear localization sequence so that they may enter the nucleus following translation in the cytoplasm. They are also able to recognize and bind to specific DNA elements in the promotor regions of genes from where they exert their effects. Often these factors are capable of dimerization in either a homo- or heteromeric fashion. This dimerization can be a requirement for activity of the factor and can also be a source of differential regulation particularly in the formation of multiple heterodimers (O’Shea et al. 1992). The factors also must present a surface capable of protein-protein interaction. It is this region, known as the activation region, which imparts to the molecule its trans-activation function. It has been shown recently through the construction of chimeric molecules that the functions of these trans-acting factors, now known as transcriptional activators, are distinct and separable (Review see Frankel and Kim 1991). While these domains are functionally interchangeable, the affected gene is dictated by the DNA binding domain present on the particular chimera. The DNA binding activities of several eukaryotic transcription factors have been characterized and tend to be defined by relatively small subregions consisting of 60-100 amino acids. These studies have demonstrated that a DNA binding domain is necessary but not sufficient for transcriptional activation. Transcriptional activators often have more than one activation domain and several apparently unrelated structural motifs have
been identified that confer this function. The first activation regions identified in eukaryotic transcription factors were in the yeast factors GAL4 and GCN4 (Ma and Ptashne 1987; Hope and Struhl 1988). The activation domains in these factors are of relatively short amino acid sequence and contain a high proportion of acidic residues. It was originally thought that these domains simply formed an acidic blob which could interact with components of the transcriptional machinery (Sigler 1988). Further studies suggested that these regions could form an amphipathic alpha helix (Ptashne 1988), with one face of the helix containing the acidic residues which would contact the transcriptional machinery and the other face containing hydrophobic residues which would serve to anchor the domain to the surface of the activator. More recent evidence suggests that these domains assume a $\beta$-sheet conformation (Van Hoy et al. 1993), while mutational analysis has revealed that the relative acidity of these domains has little effect on the domain’s ability to activate transcription (Leuther et al. 1993). These domains, when fused to a heterologous DNA binding domain, can activate transcription of reporter genes which contain a binding site for the heterologous factor in yeast as well as in a variety of higher organisms (Gill et al. 1990). Other activation domains have since been identified which show little homology to the yeast factors. Mammalian factors such as Spl contain activation domains which have a high glutamine content or
contain a preponderance of basic residues (Kadonaga et al. 1988). Another type of activation domain, originally identified in the mammalian CTF/NF1 factor, contain a large proportion of proline residues (Mermod et al. 1989). While these domains have been shown to be functionally interchangeable in higher organisms, many, like the glutamine-rich domains of Sp1, do not activate transcription in yeast (Kadonaga 1988).

Regardless of the particular type of activation domain present, it is likely that they function by contacting other proteins. A particularly good candidate for interaction with these transcriptional activators are the members of the general transcription complex. Most eukaryotic promoters contain an element located in the proximal promoter known as the TATA box. It is at this site that a number of proteins collectively known as the transcriptional machinery or the transcription complex bind. Binding of these proteins to the TATA box is apparently ordered and sequential, initiating with the binding of the TBP protein, (TATA binding protein), followed by TAFs, TFIIA, TFIIIB, and a host of other proteins including RNA polymerase II (review see Weinmann 1992). Clearly, recruitment of any of these factors through a physical interaction with a transcriptional activator would lead to an increase in the rate of transcription of the downstream gene. A number of transcriptional activators have been shown to be capable of interactions with various members
of the general transcription complex (Pugh and Tijan 1990; Goodrich et al. 1993). The developing model from these studies is that transcriptional activators function by stabilizing the formation of the pre-initiation complex, thereby increasing the rate of mRNA synthesis.

My research has focused on the characterization of the yeast transcriptional activator ADR1 which is responsible for regulation of the ADH2 gene in yeast. ADH2 encodes the isozyme alcohol dehydrogenase II (ADH II) which catalyzes the oxidation of ethanol to acetaldehyde. This is the initial step in the utilization of ethanol as a carbon and energy source under nonfermentative growth conditions (Figure 1). The ADH2 gene is repressed under conditions of fermentative growth as simple sugars such as glucose are metabolized by the organism with the concomitant production and secretion of ethanol. Once the glucose is depleted from the medium, ADH2 becomes derepressed effecting a 500-fold induction of ADH II activity (Ciriacy 1975). This derepression is linked to an ADR1 dependent increase in transcription of ADH2 (Ciriacy 1975). In addition to ADH2, ADR1 has been shown to affect other glucose-repressed genes including genes involved in glycerol metabolism (Bemis and Denis 1988), mitochondrial function (Cherry and Denis 1989), and peroxisomal genes such as CTA1 (Simon et al. 1991). The ADH2 locus has been mapped to chromosome XIII and contains cis elements typically found in eukaryotic promoters. Transcription of ADH2 begins
Figure 1. Alcohol dehydrogenases in yeast metabolic pathways.
predominantly from position -57 relative to the ATG translational start site and a consensus TATA box is located at position -160. Promotor sequences from base pair -453 to -176 have been shown to be required for regulation of ADH2 expression (Beier and Young 1982). Located within this promotor region are two activation sequences which appear to be directly responsible for this regulation. The first upstream activation sequence, UAS1, is comprised of a 22 base pair perfect inverted repeat which has been shown to bind two molecules of ADR1 (Thukral et al. 1991). The second element, UAS2, lies immediately upstream of UAS1 and is necessary for full derepression of ADH2 expression (Schuster et al. 1986). As yet, no factor has been identified which acts through this second element (Figure 2). A number of other trans-acting factors have been shown to affect ADH2 transcription including CCR4 (Denis 1984), REG1 (Dombek et al 1993), SNF1 (Ciriacy 1977), SWI3 (Peterson and Herskowitz 1992), SPT6 and SPT10 (Denis 1986). How these factors are involved is not clear.

ADR1 has been cloned (Denis and Young 1983) and sequenced (Hartshorne et al. 1986) and encodes a protein of 1323 amino acids with a molecular mass of 152 kD. Several functional domains have been identified in this transcriptional activator. Nuclear localization of ADR1 is dependent on amino acids 1-16 (Thukral et al. 1989). There are two DNA binding Cys_2-His_2 zinc fingers, homologous to those first identified in Xenopus transcription factor TFI1IA (Miller et al. 1984),
Figure 2 Regulatory regions in the ADH2 gene.
located between amino acids 99 and 155 (Yu et al. 1989; Taylor and Young 1990). Mutations within these zinc fingers results in an \texttt{adrl} phenotype indicating that this region is essential for ADR1 function (Blumberg et al. 1987; Cook et al. 1993). Previous deletion studies of ADR1 suggest there are multiple activation domains located throughout the protein and that no one domain is responsible for full activation function (Bemis and Denis 1988; Thukral et al. 1989). A domain which is capable of weak activation function is located within the N-terminal 220 amino acids since amino acids 76-172 and 1-220 by themselves are able to activate \texttt{ADH2} expression (Thukral et al. 1989; Bemis and Denis 1988). Further deletion analysis indicated that amino acids 220 through 642 were most critical for activation function, while amino acids 642 through 1323 were required for full activation function.

Mutant searches which were screened for the ability to bypass glucose control of \texttt{ADH2} expression revealed four separate mutations which were located within the \texttt{ADRI} coding sequence. The mutant alleles were sequenced and determined to each contain single base pair substitutions all of which caused single amino acid changes within the sequence between amino acids 227 and 231 (Denis and Gallo 1986; Cherry et al. 1989). This sequence, \texttt{RRASF}, conforms to a \texttt{cAMP} dependent protein kinase consensus sequence in which serine 230 is the phosphoacceptor. These results led to the suggestion that phosphorylation at serine 230 inactivated the ADR1 molecule
and caused transcriptional repression of the ADH2 gene. My research has centered on ADR1 and its role in the regulation of ADH2 expression. This research has included the study of mutant alleles of ADR1 which are capable of inducing ADH2 expression under normally repressive conditions. I have also been involved in the identification and characterization of the functional domains located throughout the ADR1 molecule. And finally, I have investigated the interaction of ADR1 with UAS2, a second regulatory site located in the promoter of ADH2.

In the first portion of this dissertation I describe our examination of mutant yeast strains which we believed were the result of aberrations within the coding sequence of ADR1. These strains were all able to escape glucose repression at ADH2 as evidenced by their ability to grow in the presence of glucose and the respiratory inhibitor Antimycin A. We discovered that in a number of these mutant strains this phenotype was the result of a mutation within the coding sequence of ADR1. The portion of the ADR1 sequence which contained the mutation was rescued from the different mutant strains and sequenced. Eighteen mutations were sequenced, from which we identified eleven new mutations in ADR1 which would permit escape from glucose repression. All of these mutations occur in a small region of the ADR1 between amino acids 227 and 239. Contained within this region is the sequence RRASF which conforms to the cAPK consensus sequence. There have been
no mutations isolated in any other region of ADR1 which allow this bypass of glucose repression. Therefore, we suggest that this region plays a critical role in the regulation of ADR1 function.

The second area of my research focuses on the identification of functional regions within the ADR1 molecule. These studies are based on the observation that there are multiple domains responsible for activation function within the molecule, as suggested by the deletion analysis of Bemis and Denis. We have complemented their efforts with the analysis of additional ADR1 C-terminal truncation alleles and by using a positive in vivo transcription assay which can further delineate the activation domains of ADR1. With these techniques we have identified the location within ADR1 of three activation domains as well as two domains which function to repress transcription.

In the third portion of this dissertation I discuss results which indicate that the UAS2 element found in the promotor region of ADH2 is capable of repressing ADR1-dependent transcription from UAS1 under fermentative growth conditions. Additionally, I show that the UAS2 element is capable of elevating the level of transcription from a heterologous promoter when juxtaposed to the binding site for ADR1. This increase in transcription occurs only in the absence of glucose and is seen when LexA-ADR1 hybrids are bound to either UAS1 or a LexA operator. This ethanol
responsiveness appears to be transcription factor dependent and does not function when the GAL4 activator is tethered to the adjacent site. This clearly indicates that the UAS2 element plays a critical role in the derepression of ADH2 expression under nonfermentative growth conditions.
CHAPTER 1
IDENTIFICATION AND CHARACTERIZATION OF ADR1\textsuperscript{C} MUTATIONS

Introduction

The yeast \textit{Saccharomyces cerevisiae} is a facultative anaerobe, capable of fermenting sugars as well as using non-fermentative carbon sources such as ethanol. Given a mixture of carbon sources, this yeast will prefer the utilization of glucose. This metabolic course is energetically most economical and is facilitated by the repression of genes not involved in glucose metabolism, a phenomenon known as catabolite repression (review see Gancedo 1992). Glucose repression is one mechanism developed in yeast which allow the organism to better adapt to changes encountered in their environment.

While the mechanisms of catabolite repression have been extensively characterized in bacteria (Ullman 1985; Saier 1989), little is known of how glucose exerts its repressive effects in yeast. The degree of repression seen in yeast varies according to strain and depends on the particular enzyme studied. Invertase activity is repressed as much as 700-fold in the presence of glucose (Estruch and Carlson 1990), whereas aconitase displays only a 5 to 10-fold repression (Polakiss and Bartley 1965). The decrease in
enzymatic activity caused by glucose is generally paralleled by a decrease in the concentration of the corresponding mRNA. This correlation could be the result of a glucose-dependent decrease in transcription rates, or on the decreased stability of the corresponding mRNA (Schleffer 1992), or on both. Direct measurements of transcription rates have been performed for the glucose repressed genes CYC1 and MAL65. In these studies, derepressed cells synthesized the corresponding mRNA 6 and 15 times faster, respectively, than repressed cells (Zitomer et al. 1979; Federoff et al. 1983).

While the mechanisms of glucose repression of metabolic enzymes in yeast appear to act primarily at the level of transcription, it is not likely that the signal produced by glucose binds to DNA. Regulation of transcription is accomplished by the interplay of transcription factors within the promotor regions of genes; these factors provide the best target for the glucose signal.

ADR1 is the key regulatory factor affecting ADH2 expression and its activity is controlled by the glucose signal. ADR1 controls a variety of other glucose repressed genes including genes involved in glycerol metabolism, mitochondrial function, and peroxisome function. ADH2 expression is fully repressed in the presence of glucose and displays a 500-fold increase in ADHII activity upon derepression. This derepression is strictly dependent upon ADR1 activity. In an effort to isolate mutations within the
ADR1 protein which allowed ADH2 expression to bypass glucose repression, cells lacking the fermentative isozyme ADH1 were grown in the presence of the respiratory inhibitor Antimycin A. This selection forces the yeast to ferment glucose by either increased expression of ADH2 or by increased ADHII activity. Mutants were isolated which bypassed glucose repression by increased expression of ADH2 and four of these mutations were linked to ADR1. These mutants showed a 60-fold increase in ADHII activity under repressed conditions, and were designated ADR1c for ADR1 constitutive (Ciriacy 1979). Sequencing of the mutant alleles revealed single base pair substitutions within the coding region of ADR1 which translated into single amino acid changes all of which clustered to one region of the protein (Cherry et al. 1989). This region encompassed amino acids 227-231 whose sequence, RRASF, conforms to a cAMP dependent protein kinase (cAPK) consensus sequence with serine 230 being the phosphoacceptor. The mutations had no effect on mRNA (Denis and Gallo 1986) or protein abundance (Vallari et al. 1992), suggesting that phosphorylation of ADR1 at serine 230 by cAPK inactivated ADR1 and that mutations in this region prohibited this phosphorylation and led to the constitutive phenotype. In support of this model, strains which harbor a disruption in the BCY1 locus, and therefore have unregulated cAPK activity, show diminished ADHII activity, whereas the ADR1c counterparts show less of an effect (Cherry et al. 1989). In addition,
studies to assess the phosphorylation state of ADR1 at serine 230 in wild-type and ADR1' strains show a reduction in phosphorylation of the ADR1' protein in vitro when compared to the wild-type ADR1 protein (Cherry et al. 1989). However, conflicting evidence demonstrates that strains lacking cAPK activity showed no increase in ADH2 expression under glucose growth conditions (Denis et al. 1992). This suggested that while phosphorylation had an ADR1-dependent effect on ADH2 expression, this effect may not occur through the 227-231 region.

The first part of this dissertation describes the characterization of twenty additional mutant strains which allow bypass of glucose control at ADH2. Of these twenty strains, seventeen define new mutations in the ADR1 protein. All of these mutations occur either within or immediately surrounding the serine 230 site, suggesting that this may be the only region within ADR1 which is regulated by the glucose signal.

Results and Discussion

In order to more extensively examine the effect of cAPK phosphorylation and ADR1' protein function, we characterized seventeen additional ADR1 mutant alleles which allow yeast strains to partially bypass the glucose repression normally exerted on ADH2 expression. My participation in this project involved rescue of one of the mutants from yeast as well as
performing tetrad analysis on four of the seventeen new mutations to determine linkage of the constitutive phenotype to ADR1. In addition, I performed the sequence analysis of twelve of these mutations which resulted in the identification of four of the eleven new ADR1<sup>c</sup> alleles. The results from my research and related studies have been described fully in a published manuscript (see Appendix A).

Evidence presented in Appendix A suggests that the model in which ADR1<sup>c</sup> mutations make ADR1 less sensitive to cAPK phosphorylation of Ser-230 may not be correct. Peptides which have been modelled on the 222-234 region of ADR1 were examined for their ability to serve as substrates for phosphorylation by cAPK. ADR1<sup>c</sup> alleles which allow comparable levels of ADH2 activity showed marked differences in their ability to serve as a substrate for cAPK. One of the peptides was actually phosphorylated with a lower K<sub>m</sub> than the peptide modelled after wild-type sequences. In conjunction with the observation that strains lacking cAPK activity do not have elevated ADH2 expression, our data suggests that while cAPK is in part responsible for ADR1-mediated regulation of ADH2 expression it probably is not working through the 230 site. The mutations in ADR1 which confer the constitutive phenotype may either disrupt an interaction with a repressor molecule, or may cause a change in the folding pattern of the molecule which exposes an activation domain normally masked in the wild-type protein.
CHAPTER TWO

IDENTIFICATION OF FUNCTIONAL REGIONS IN THE TRANSCRIPTIONAL ACTIVATOR ADR1

Introduction

In the yeast *Saccharomyces cerevisiae*, the transcriptional activator ADR1 is required for the regulated expression of several genes (Denis et al. 1981, Bemis and Denis 1988, and Simon et al. 1991). Included among these genes is ADH2 (Denis et al. 1981) which encodes the glucose-repressible isozyme of alcohol dehydrogenase, ADHII. ADR1 binds to an upstream activation sequence (UAS1) located between base pairs -271 and -291 upstream of the ADH2 translational start site (Eisen et al. 1988). UAS1 is comprised of a 22 base pair perfect inverted repeat and ADR1 binds to this sequence as two monomers (Thukral et al. 1991). Several functional regions have been identified within the transcriptional activator ADR1. The nuclear localization signal resides between amino acids 1 and 16 (Thukral et al. 1989). Two DNA binding zinc fingers analogous to those first identified in Xenopus transcription factor IIIA are located between residues 99 and 155 (Thukral et al. 1989), and there are at least two regions involved in transcriptional
activation located between amino acids 76 and 172 and between
amino acids 262 and 642 (Bemis and Denis 1988 and Thukral et
al. 1989). Additionally, the region encompassed by amino acids
227 and 239 plays a role in inhibiting ADR1 activation (Denis

This chapter of my dissertation details evidence which
localizes three distinct transcription activation domains
within the ADR1 protein as well as another domain, separate
from the 227-239 region which is capable of inhibiting ADR1
activity.

It has become clear over the last decade that the
regulation of gene expression is controlled by a variety of
sequence-specific DNA-binding proteins. These proteins bind
upstream of genes at sites which are usually less than 600
base pairs from the initiation of transcription (UAS elements
for upstream activation sequence) and either activate or
repress transcription of the downstream gene. More than 100
such proteins have now been identified mainly from yeasts,
Drosophila, rodents and man. Many of these factors have been
cloned and sequenced and the amino acid sequences of these
proteins suggest there is a limited number of families of
transcription factors. All of these factors have a number of
functional domains in common. Each contains a short region of
approximately 100 amino acids which functions in DNA-binding.
A number of binding motifs have been identified including
several types of zinc fingers (Miller et al. 1985; Klug and
Rhodes 1987; Freedman 1988), basic-leucine zipper (bZIP) (Landschultz et al. 1988), and homeodomain (Scott et al. 1989). These transcription factors also require a nuclear localization sequence as well as a region of the molecule capable of making protein-protein contacts and inducing transcription.

The regulatory proteins found in yeast were first identified by mutations that alter the transcription of a specific gene or set of genes. The binding of transcriptional activators to elements in the promoter region of these genes is necessary but not sufficient for activation of transcription. Deletion derivatives of the yeast factors GAL4 and GCN4 containing only their DNA-binding domains are not able to activate transcription but can actually repress transcription when their recognition sites are located in a heterologous promoter between an upstream activation sequence and a TATA box (Keegan et al. 1986, and Hope et al. 1986). Derivatives of these activators which lack their own DNA-binding domains can activate transcription of genes when fused to a heterologous DNA-binding domain such as that of the Escherichia coli LexA repressor (Hope et al. 1986, Brent and Ptashne 1985). These LexA fusions bind the hybrid promoter through a LexA operator site and activate transcription at a rate commensurate with the strength of their particular activation domain. These experiments provided the first evidence that the domains providing DNA-binding function and
transactivation function to transcriptional activators were separable and resided in distinct portions of the molecule.

Deletion analysis of these yeast factors localized the activation function to short regions of the molecule which contained a relatively high percentage of negatively charged amino acids (Hope et al. 1986; Ma and Ptashne 1987). The GCN4 molecule, which regulates a family of genes involved in amino acid biosynthesis, contains an activation domain encompassed by as few as 40 amino acids. This region, when fused directly to the DNA-binding domain is capable of wild-type levels of transcriptional activity (Hope et al. 1986). Interestingly, progressive deletions into this activation region led to progressive loss of activation function indicating that this domain does not have rigid structural requirements like those of active sites of catalytic proteins. The activation domains of GAL4 and GCN4, while similar with respect to charge, have no other striking similarities. Additional evidence suggests that these domains require few structural features. Different portions of these activation domains are capable of comparable activation function even though they show little interdomain homology (Hope et al. 1986; Hope et al. 1988). Further, single amino acid substitutions in the activation domain of GAL4 which increase or decrease activation potential also increased or decreased the net negative charge (Gill and Ptashne 1987).

The involvement of an alpha helical structure in the acidic activation domains of these transcription factors was
postulated by Giniger and Ptashne (1987). They designed a peptide that was predicted to form an amphipathic alpha helix with a net negative charge on one face and hydrophobic residues on the other. When this peptide was fused to the DNA-binding domain of GAL4 it was capable of inducing transcription. A second peptide containing identical residues but in scrambled order to disrupt the helical structure was not able to activate transcription. More recent evidence suggests, however, that the acidic domain of GAL4 actually adopts a β-sheet structure at low pH (Van Hoy et al 1993). This particular domain also contains a binding site for GAL80, a repressor molecule which prohibits GAL4 activation. Mutations in this domain which permit constitutive activation all lie on one face of the β-sheet structure presumably disrupting the interaction with GAL80. Further evidence against the alpha helical structure of these domains was shown by mutagenesis of the acidic activator VP16, in which two proline substitutions in the activation domain fail to affect activation function (Cress and Triesenberg 1991). Leuther et al (1993) have demonstrated that negative charge is not the sole determinant of activation in GAL4. Through various mutations they have eliminated the negative charge in this domain and saw only a modest reduction in activation function. It is possible that the only constraint on these acidic activator sequences is that the region have a high degree of freedom such that it can adapt its surface structure to that
of other molecules with which it interacts.

Berger et al. (1992) have proposed that there exist different classes of acidic activators. In an attempt to identify the target of acidic activators they isolated a mutant strain which blocked the activity of a hybrid GAL4-VP16 activator. Further, they showed that deletion of this gene (ADA2), severely reduced the ability of GCN4 and VP16 to activate transcription, but did not affect the activity of two other acidic transcription factors, HAP1 and HAP4. This suggests that not all acidic activators work by the same mechanism.

Analysis of the human transcription factor Sp1 has revealed the identity of another class of transcriptional activation domain. Sp1 binds to GC-rich recognition sequences (GC boxes) in the promoters of several viral and cellular genes. This protein contains three zinc fingers which recognize and bind DNA (Kadonaga et al 1987). Mutation analysis of Sp1 revealed four distinct regions other than the zinc finger region which are important for transcriptional activation(Kadonaga et al. 1988). Two of these domains map to regions in the N-terminal portion of the molecule that are rich in glutamine residues. Unlike the activation domains described for yeast activators, these domains contain almost no negatively charged residues. Furthermore, there appears to be no correlation between negative charge and activation function (Courey and Tjian 1988). However, like the domains of
GAL4 and GCN4, progressive deletion of the domain does not lead to an abrupt loss of function, suggesting there is no strict structural requirement for its interaction with target molecules. The third activation domain of Sp1 maps to a region of high charge density just N-terminal of the DNA-binding domain. There is no correlation in this domain between negative charge and activation function. The fourth activation domain of Sp1 shows no homology to other domain types and appears to require the presence of the other three domains for activation function.

The mammalian CTF/NF-1 proteins are typical of a third class of activation domain. These proteins activate transcription from a number of cellular and viral promoters and activate replication of adenovirus by binding to specific GCCAAT elements (Santoro et al. 1988). The CTF activation domain contains no net negative charge and instead contains approximately 25% proline residues which are incapable of forming alpha helical structures. These proline-rich domains also contain several stretches of serine and threonine residues similar to the glutamine-rich domains of Sp1. These residues may be sites of posttranslational modification such as O-linked glycosylation or phosphorylation not atypical of other activators. Modification of the proline-rich domains of CTF appear not to be essential for transcriptional activation but could regulate their activity.

It is interesting to note that while acidic, glutamine-
rich and proline-rich domains are the three dominant domains thus far identified for transcriptional activation, some transcription factors utilize activation domains composed of combinations of these motifs. The domains of c-Jun are proline-rich but also contain a high proportion of negative charges (Struhl 1987). Similarly, a region that is both proline-rich and glutamine-rich is present in the activation domain of the lymphoid-specific factor OTF-2 (Clerc et al 1988).

The ability of acidic activators such as GAL4 and GCN4 to activate transcription in mammalian systems as well as in yeast suggests that whatever interactions are required by these yeast-specific acidic activators are also available in mammalian systems. The glutamine-rich and proline-rich domains, however, which were identified in mammalian systems, cannot activate transcription in yeast. This would suggest that transcription factors in higher eukaryotes may function through special adaptor molecules that are not present in the yeast system. Such factors may include the TBP associated factors (TAFs) (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1992).

There exists a class of conditional transcription factors, the steroid hormone receptors, which are active only when bound by ligand. These factors activate transcription through the use of domains similar to those used by other transcription factors. Steroid hormones play important roles
in cell growth, differentiation, and development in higher eukaryotes. Upon entering the cell, steroid hormones bind to their high affinity receptors. Once bound by ligand these receptor-ligand complexes bind to cis-acting elements termed steroid response elements (STE) and stimulate transcription. All of these receptors contain the same structural design. They consist of a region required for activation in both their N-terminal and C-terminal domains, a cysteine-rich central region required for DNA-binding and a ligand binding domain in their C-terminus. Truncation of the ligand-binding domain of these receptor leads to constitutive activity with respect to both DNA-binding and activation function suggesting that this domain is responsible for suppressing the transactivation function of the receptor (Kumar et al. 1987). The activation domains of several of these receptors have been identified and in many cases activate transcription through domains that appear to be similar to domains already identified. Other receptors, like other types of transcription factors, appear to effect transcription from domains which exhibit no homology to any other domain previously identified. As more studies are conducted, it is likely that many more classes of activation domains will be identified.

It has been suggested that various activators are regulated by posttranslational modification. The cAMP responsive element-binding protein (CREB) a member of the basic-leucine zipper (bZIP) family of dimeric transcription
factors, which also include Jun, Fos, and NFIL-6, mediate transcriptional activation of cAMP inducible genes upon phosphorylation by cAMP dependent protein kinase (PKA) (Gonzalez et al. 1991). Most experiments rule out an effect of PKA on DNA-binding or dimerization of CREB in vitro (Yamamoto et al. 1988). The site of phosphorylation in this protein is serine 133 which lies outside the DNA-binding and dimerization domains and lies just downstream of its glutamine-rich activation domain (Gonzalez and Montminy 1989; Lee et al. 1990). Mutation of serine 133 to alanine abolishes the transactivation function of the protein, while fusion of the activation domain to a heterologous DNA-binding domain produces a chimera which is PKA inducible (Lee et al 1990). It appears, therefore, that the mode of regulation of CREB is phosphorylation of its activation domain. The mechanism by which phosphorylation of CREB causes activation is not known but phosphorylation has been shown to affect the conformation of this region (Gonzalez et al. 1991). c-Jun, a relative of CREB is subjected to similar regulation by phosphorylation.

Two basic mechanisms may account for the increased activity of activation domains by phosphorylation. In the first scenario, phosphorylation may increase the affinity of the transcription factor for its target. Certainly, phosphorylation of the domain will lead to an increased negative charge which has been shown to be an important characteristic of at least some activation domains. The second
mechanism involves negative regulation. Several transcriptional activators have been shown to bind repressor molecules. Phosphorylation of the transcription factor may effect dissociation of the repressor and thus activate the transcription factor. It should be noted that other transcription factors have been shown to be substrates for phosphorylation. In these cases phosphorylation occurs in and around the activation domain and leads to increased transcriptional activation potential.

The mechanisms employed by these different classes of transcriptional activators remains unclear. It is believed that these factors increase the rate of gene transcription by stabilizing the formation of the transcription initiation complex at the TATA box either by contacting various members of the transcriptional machinery directly, or indirectly through adaptor molecules (See discussion).

In the present study we have used deletion analysis coupled with the LexA in vivo transcription assay to localize the activation domains of ADR1. With this combination of techniques we have localized three transcription activation domains positioned between amino acids 76-172, 263-358, and between amino acids 397-505. In addition, we have localized two regions which inhibit activation function. One of these regions, positioned between amino acids 227-239, co-localized to the ADR1e region previously identified (Denis and Gallo 1986; Denis et al. 1992) and the second region is
located between amino acids 288 and 330.

**Results and Discussion:**

The results for the identification of ADR1 activation domains is presented in a manuscript accepted for publication (see Appendix B). My contributions to this project began with the idea of using the *in vivo* transcription system to identify and isolate the domains of ADR1 which are responsible for transactivation function as well as all the preliminary work required to verify the feasibility of this assay. I also constructed a number of the LexA-ADR1 fusions including those which localized TADII and TADIII.

Previous studies of the functional domains of ADR1 indicated that there were several regions of the protein which were required for full activation function (Bemis and Denis, 1988). Deletion of all regions C-terminal to the DNA-binding region resulted in a reduction in activity of ADR1. The activation function of these truncations roughly correlated with the length of the truncated molecule. There was not any region of ADR1 which when deleted abolished activity. These data indicate that there are multiple regions of the protein which are capable of transcriptional activity. Activity was abolished only after removal of portions of the DNA binding domain. After learning of the *in vivo* transcription assay devised by Brent and Ptashne (1985), in which they used fusion molecules containing the DNA-binding specificity of GAL4 to
identify E. coli genomic DNA fragments which could activate transcription, I decided to use this system to locate the activation domains of ADR1. This assay has the advantage of positive identification, whereby only fragments of ADR1 which could activate transcription would be identified. This technique, in combination with deletion analysis, would allow us to more precisely identify which regions of ADR1 were actually responsible for transcription activity. Initial constructs, which included the region of ADR1 between amino acids 147 and 359, established the viability of this technique. These initial constructs, however, gave rise to several problems. The GAL4 DNA binding domain used in these studies (amino acids 1-147) were capable by themselves of low levels of activation. Secondly, identification of hybrid molecules by Western blotting with GAL4 antibody was hindered by the high background caused by cross reactivity of the antibody.

With these factors in mind, we decided to switch from using the GAL4 DNA binding domain to using the DNA binding domain of the bacterial repressor LexA. The LexA protein by itself was not capable of activation function from our hybrid promoter. Using this system, we were able to localize three distinct activation domains in ADR1 as well as two domains which are capable of repressing ADR1 activity.

The first domain had previously been identified by Thukral et al (1989) and resides between amino acids 76 and
172. While we did not clone this particular fragment, we did show that amino acids 1-220 were capable of both activating transcription in our in vivo LexA system (Appendix B, Figure 5A) as well as functioning at the ADH2 locus.

The second domain, between amino acids 147 and 359, was originally identified in the GAL4-ADR1 constructs as a weak activation domain. Transfer of this domain to the LexA system confirmed this observation (Appendix B, Figure 5A) and allowed fine structure deletion analysis to better localize the activation function (Appendix B, Figure 5B). Deletion into either end of this domain reduced the activity of this region five-fold. Further removal of the N-terminal residues revealed the presence of a strong activation domain whose activity had been masked in the longer fusion. Thus the activation region in this domain was localized to amino acids 263 to 359. A repression domain which had previously been identified between amino acid 227 and 239 was present in the original 147-359 construct and is probably responsible for the reduced activity. The 263-359 region is rich in acidic residues with 13% of all residues being either glutamate or aspartate. This is typical of other transcriptional activators found in yeast. Besides its acidic character, this region shows no homology with other known activation domains.

Fusion of amino acids 1-642 displayed activity levels far in excess of either of these domains individually (Appendix B, Figure 5A) indicating that either these domains either
interact synergistically, a theory not borne out by internal deletion of full length ADR1 (Appendix B, Table 3), or there was another activation domain present. C-terminal deletion analysis of ADR1 suggested the presence of another domain between amino acids 397 and 642. In search of this third activation domain, we fused amino acids 359-740 to the LexA DNA binding domain. This fragment was capable of activation in our system (Appendix B, Figure 5A). C-terminal truncation analysis suggested that the activation function of this region of ADR1 probably resided between amino acids 397 and 571 (Appendix B, Figure 3). We therefore deleted the amino acids 397-505 from our 359-740 fragment and observed complete loss of function (Appendix B, Figure 5A). The implication that this region was responsible for the activity of the larger fusion is supported by internal deletion of both full length ADR1 (Appendix B, Table 3) and the 1-571 truncation (Appendix B, Table 4). Additionally, fusion of amino acids 642-1323 provided no activation function (Appendix B, Figure 5A). This domain, designated TADIII, also has a high acidic character typical of the activation domains identified in GAL4 and GCN4.

Deletion of residues 262-288 (shown to be critical for the function of TADII in Appendix B, Figure 5B) from full length ADR1 had no effect on activation function (Appendix B, Table 3). It is possible that since ADR1 regulates the transcription of several other genes, that this domain, while dispensable for ADH2 expression is required for transcription
of these other genes. Deletion of TADIII resulted in a derivative which was capable of 60% of the activity of the wild-type protein when assayed for ADHII activity (Appendix B, Table 3). Deletion of TADII in the 1-571 truncated derivative resulted in a 40% decrease in activity (Appendix B, Table 4), indicating that the activation domains identified are functionally redundant.

We have shown that ADR1 contains at least three separate activation domains (Appendix B, Figure 7). The human transcription factor Sp1 has been characterized as a transcriptional activator which contains multiple activation domains (Kadonaga et al 1988). Deletion analysis of Sp1 gave similar results to those that we have observed for ADR1. Deletion of either of the glutamine-rich activation domains in Sp1 resulted in a modest reduction in transactivation function. Deletion of individual activation domains in transcriptional activators which contain only two activation domains has a much more dramatic effect on activation potential(). We were not able to separate the activation domain TADI from the DNA-binding domain of ADR1, and therefore were unable to directly study the effect of deletion of this domain. However, the LexA-ADR1 fusion containing amino acids 220-1323 which includes TADII and TADIII but not TADI displayed activity comparable to that seen in the LexA-ADR1 fusion containing all three activation domains, suggesting that deletion of TADI would have at most a modest effect on
the transcriptional activity of the whole molecule.

The results of numerous studies suggest that transcriptional activators function by interacting through various members of the basal transcription complex to stabilize the formation of the preinitiation complex (Ha et al. 1993; Lin et al. 1991.) Some activators interact with this transcriptional machinery indirectly through contacts with intermediary proteins known as coactivators or adaptors (Dynlacht et al. 1991; Goodrich et al. 1993). Perhaps the best evidence for this interaction of activator proteins with essential, limiting factors involved in transcription was by Gill and Ptashne (1988) who showed that various GAL4 derivatives, when over-expressed, could inhibit the transcription of genes lacking GAL4 binding sites. The degree of inhibition directly correlated with the relative strength of the activation domain. VP16, a strong activator when expressed in yeast, actually lethal to the cell, supporting the notion that these activation domains titrate essential factors.

Several studies involving various transcription factors have identified a number of potential target molecules. The E1A activator from adenovirus has been shown by various techniques to bind to the TATA-binding protein (TBP) (Lee et al. 1991). Mutations within the activation of this protein which decrease the activation potential also diminish the interaction observed between E1A and TBP. The acidic activator
VP16 has been shown by chromatographic techniques to bind to TFIIB and to interact with one of the TBP associated factors (Goodrich et al. 1993). While these results appear to be conflicting, it is possible that activators are capable of interactions with different members of the transcriptional machinery thereby inducing synergistic activation. This is corroborated by evidence which suggests that there are few structural requirements imposed on transcriptional activators. When Zhou et al. (1993) compared the ability of a partially purified TFIID fraction and cloned TBP to support activation, they found that a number of transcriptional activators including GAL4-AH and Sp1 required factors present in the TFIID fraction to support activation. A separate study demonstrated a direct interaction between TAF110 and the activator Sp1 (Hoey et al. 1993).
CHAPTER 3

THE ROLE OF THE UAS2 ELEMENT ON THE REGULATION OF ADR1 ACTIVITY

Introduction

It is now well established that gene expression is regulated by the coordinated activities of multiple positive and negative sequence-specific trans-acting factors which bind to recognition elements in the promoter region of eukaryotic genes. The binding of two or more activators to the promoter region often leads to synergistic activation of transcription. Synergism can occur when two or more activators are bound simultaneously or when two or more activators act in the same pathway to stimulate transcription from a single template (review see Herschlag and Johnson). Synergistic activation may play a crucial role in coordinating responses from different intracellular signals and in allowing large responses from small changes in activator concentrations. Several mechanisms have been proposed to account for synergistic activation. Two activators can effect synergistic activation by binding to DNA in a cooperative fashion (Janson and Pettersson 1990). In this model, the binding of one activator increases the affinity for binding of the second factor through direct protein-protein
contacts. A second mechanism involves an indirect interaction in which two activators do not contact each other directly but simultaneously bind to different sites within a single transcription complex (Carey et al. 1990; Lin et al. 1990).

Wright and Guftafsson (1991) have demonstrated synergistic activation by the human glucocorticoid receptor from a promoter with two upstream response elements. They show that interactions involving the $r_1$ activation domain are solely responsible for this synergy and that the presence of this domain induces cooperative binding of the receptors to the template. This confirms earlier results which suggested that dimers of the glucocorticoid receptor bound to a double response element cooperatively (Schmid et al. 1989).

Kakidani and Ptashne (1988) have demonstrated cooperative activation between the rat glucocorticoid receptor and the yeast activator GAL4. In these experiments, DNA binding sites for each of the activators were located within a single gene. Transcription from this hybrid promoter was greater than twice the activity when either factor was expressed alone. In a later study (Lin et al. 1990) it was shown that a derivative of GAL4 and the mammalian factor ATF could cooperate to activate transcription synergistically. Carey et al (1990) showed similar results with singly and multiply bound GAL4 molecules. It is unlikely, in the experiments involving both yeast and mammalian factors, that there is a direct physical interaction between the activators to effect cooperative DNA-
binding. Further, synergy in these studies was demonstrated under conditions in which each activator was present in concentrations sufficiently high to ensure saturation of their binding sites. Under these conditions, it is postulated that synergy results from the activators simultaneously touching some part of the transcriptional machinery.

It has been suggested that activators in the proximal promoter region of eukaryotic genes interact with proteins bound to enhancers far upstream to effect synergistic activation. Li et al. (1991) have shown that the cellular transcription factor Sp1 and the BPV-1 enhancer protein E2 activate transcription synergistically from two papilloma viral promoters as well as from a series of synthetic promoter constructs. Additionally, they demonstrate that Sp1 can target E2 to a promoter in the absence of an E2 DNA-binding region. This clearly suggests that transcriptional activators, bound at the proximal promoter, are capable of recruiting other transcription factors to the site of initiation.

In addition to synergistic activities, promoters containing multiply bound proteins can also result in transcriptional repression. Transcriptional repression in prokaryotes is generally caused by the competition between DNA binding proteins and transcription factors. A repressor protein generally binds near the transcription start site and blocks the interaction of transcription factors with the promoter. Several additional models have been proposed for
transcriptional repression in eukaryotic systems (review see Renkawitz 1990). For one such mechanism, called quenching, the activator and repressor bind adjacent elements and protein-protein interactions between the repressor and activator prevent the activator from making the necessary contacts with the transcriptional machinery. In this model the repressor inhibits the activity of the activator without affecting its ability to bind to the template. Perhaps the best studied example of this sort of repression involves the regulation of a-specific gene expression by the yeast mating type locus (Johnson and Herskowitz 1985). MCM1, a sequence-specific DNA binding transcription factor, is responsible for the expression of several a-specific genes in yeast. The mating type protein α2 binds to a site adjacent to MCM1 and blocks MCM1-mediated activation of a-specific genes by modifying the activity of the activator.

In another model, the repressor molecule binds to sites distal to the binding site of the activator and interferes with the activity or formation of the transcription complex. This form of repression has been observed in the expression of the GAL genes of yeast, which are required for the catabolism of galactose. The expression of this family of genes is induced by the presence of galactose and repressed by the presence of glucose. GAL4, the transcription factor required for the expression of the GAL genes, binds to multiple sites in the promoter regions of these genes. Several cis-acting
elements (designated URS₉ for upstream repression sequences) located between the UAS₉ elements and the TATA box have been identified which repress the transcription of these genes (Flick and Johnston 1992). No factors have been identified which bind to these elements, however, oligonucleotides corresponding to these sequences have been shown to confer glucose-dependent repression upon a heterologous promoter (Flick and Johnston 1992). A third model involves a repressor which itself cannot bind DNA but represses the activity of the transcription factor by direct protein-protein interaction. In yeasts growing in the absence of galactose, the GAL4 transcriptional activator binds to its recognition sequence but is held in check by the binding of the repressor, GAL80 (Ma and Ptashne 1987). GAL80 binds to the C-terminal domain of GAL4 and prevents activation presumably by masking the activation domain.

Like GAL4, ADR1 appears to be bound to its upstream activation sequence under both repressed and derepressed growth conditions (Taylor and Young 1990). This would suggest the presence of a repressor function as the bound ADR1 is not able to activate transcription under repressed conditions. Two glucose responsive domains have been identified in ADR1 which may mediate this repression (Denis and Young 1983; Cook et al. 1994).

We have found that a LexA-ADR1 fusion containing the N-terminal 642 amino acids of ADR1 is able to functionally
substitute for the ADR1 allele and is able to cause derepression at the ADH2 locus in a strain containing a disruption of the wild-type ADR1 allele (adr1). This same fusion is unable to increase the activity of a heterologous promoter which contains only a LexA operator site when cells are grown under derepressed conditions (Table 1). This indicates that ADR1 is not capable of eliciting the derepressed activities observed at ADH2 alone and requires additional elements from the ADH2 promoter. Deletion analysis of the ADH2 promoter suggests that full derepression of ADH2 expression requires not only the presence of UAS1, but also the presence of a second element, UAS2, which is located immediately upstream of UAS1 (Yu et al. 1989). Deletion of UAS2 at the chromosomal locus virtually abolishes derepression of ADH2 expression (Schuster et al). Similarly, a strain which lacks a functional ADR1 allele (adr1-1) is not capable of derepressed activities near that found in a wild-type strain (Denis et al. 1981) These observations, taken together, suggest there may be an interaction between ADR1 and a second protein which binds the UAS2 element, which together provide synergistic activation of ADH2 expression under derepressed conditions. No factor has yet been shown to bind to this second element.

We have modified the in vivo transcription assay devised by Brent & Ptashne (1985) to allow the identification of promoter elements which regulate transcription factor
Table 1: LexA-ADR1-1-642 can functionally substitute for ADR1 at ADH2.

<table>
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<th>p1840</th>
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<th>ADH2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>B- Galactosidase Activity</td>
<td>U/mg</td>
<td>ADH II Activity</td>
<td>mU/mg</td>
</tr>
<tr>
<td>LexA Fusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADR1 1-642</td>
<td>8% Glucose</td>
<td>2% Ethanol</td>
<td>8% Glucose</td>
<td>2% Ethanol</td>
</tr>
<tr>
<td></td>
<td>1845</td>
<td>465</td>
<td>595</td>
<td>1500</td>
</tr>
</tbody>
</table>

Yeast strain 237-1b-10 containing a disruption in ADR1 (adr1::TRP1) was grown under repressed (8% glucose), or derepressed (2% ethanol) conditions. The standard errors of the means were less than 20%.
activity. In this study we examine the ability of the UAS2 element to regulate ADR1 transcriptional activity under both repressed and derepressed growth conditions. To accomplish this, we used a hybrid GAL1-lacZ gene which contains a LexA operator site as the upstream element in an otherwise UAS-less promoter. This is the site that is recognized by the LexA-ADR1 fusions and will serve to anchor ADR1 to the promoter. Immediately upstream of this operator, in a position analogous to that found at the ADH2 locus, we have inserted the UAS2 element (Figure 3). We examined the effect of the UAS2 element on ADR1-dependent transcription by comparing the ability of various LexA-ADR1 fusions to activate transcription from this promoter as compared to their ability to activate transcription from an identical promoter which lacks the UAS2 element. We found that UAS2 confers a glucose-dependent repression on ADR1 transactivation potential. This repression does not function through the glucose-responsive domains of ADR1 that have been previously identified (Cook et al. 1994) and is able to repress the activity of a variety of activators. We also show that this repression is relieved when the UAS2 element is distanced from the ADR1 binding site. This suggests that the repressor likely functions through either a quenching or an occlusion mechanism.

We also show that the UAS2 element is able to dramatically increase the ability of ADR1 to activate transcription under derepressed growth conditions. Further,
Figure 3  Heterologous promoters.

Schematic diagrams of the heterologous promoters used to determine the effect of UAS2 on the LexA-AD1 activity.

1B40

1840

LexA Operator

GAL1 Promoter

lacZ

LR2L

UAS2

LexA Operator

GAL1 Promoter

lacZ

LR21

UAS2

UAS1

GAL1 Promoter

lacZ

LR2-1

UAS2

UAS1

GAL1 Promoter

lacZ

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the UAS2 element is able to increase the transcriptional activity of the two strongest activation domains of ADR1 (TADII and TADIII) when expressed singly or in combination with each other or in combination with TADI. This increase in transcriptional activity in the absence of glucose is also observed when other activators are tethered adjacent to the UAS2 element. These results demonstrate the properties of both transcriptional repression and synergistic activation from a single promoter element.

Materials and Methods

Yeast Strain: Yeast strain 237-1b-10, MATα ura3, his3, trp1, leu2, adh1-11, adr1::TRP1 was used throughout this study.

Plasmid Constructions:
LexA-ADR1-1-397: ADR1 sequences were obtained from plasmid JS119 (Cherry et al. 1989). JS119 was digested with NcoI and EcoO109I. The EcoO109I site was filled in with the large subunit of E. coli DNA polymerase (Klenow) and the 1193 base pair fragment was isolated. This fragment was then inserted into plasmid pLexA202-5 (Cook et al. 1994) which had been digested with NcoI and XhoI. The XhoI site was filled in with Klenow. This fusion protein contains the intervening peptide ELIPGDPSN derived from the polylinker of pLexA202-5 between LexA and ADR1. The protein also contains at its C-terminus the peptide RVELQPS derived from the polylinker of pLexA202-5 and its termination sequence. All other LexA-ADR1 fusions have
been previously described (Cook et al. 1994). LexA202-B42 has already been described (Ruden et al. 1991). LexA202-Gal4-87-881 has already been described (Laurent et al. 1992). LexA-CCR4-1-345 was obtained as a nonsense mutation from plasmid MD33 which is described elsewhere (Draper et al. 1994). Plasmids ADCY4 and ADCY5 were provided by E.T. Young and have been described elsewhere (Yu et al. 1989).

**GAL1-lacZ promoter constructs:**

All promoter constructs are derivatives of LR1Δ1. LR1Δ1 is a derivative of plasmid RY131 which had been cleaved with XhoI and religated (Yokum et al. 1984). Cleavage of pRY131 with XhoI and religation removes the UASG from the GAL1 promoter. This leaves the XhoI site available for cloning UAS elements into the GAL1 promoter. Plasmid 1840 consists of a single LexA operator binding site at the XhoI site of pLR1Δ1 and has been described elsewhere (Cook et al. 1994). LR2L was made by annealing two oligonucleotides to create the LexA operator (Figure 4): the first oligonucleotide 5'–ccgggtactgtatgtacata cagtacgctgcagc–3' was annealed to the second oligonucleotide 5'aattcgtcgacgtactgtatgtacatacagtc–3' which created the double stranded oligonucleotide with a XmaI overhang on the 5' end and a sticky EcoR1 site on the 3' end. This was then inserted into plasmid AK29 that had been cut with XmaI and EcoR1 to create pAK29-L. Plasmid AK29-L was then digested with SalI and the small fragment was inserted into the XhoI site of LR1Δ1. pAK29 contains the sequence cccggttgagatgccggt
Figure 4  Construction of plasmid LR2L.

LexA operator

pAK29

pAK29-L

pLR2L

GAL10 promoter

GAL1

operator

Insert LexA oligonucleotide into Xmal/EcoRI
site of pAK29 to create pAK29-L.

Cleave pAK29-L with Sall and insert 70 bp Sall fragment
into pUL1 at Xmal site to create pLR2L.

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gttccggcagaggagatcagtctctgtgctgga between the SmaI and SalI site of pUC18. Plasmid LR2-1 was made by digesting pAK26 with Ssp1 and inserting an EcoR1 linker into this site to create plasmid E-5 (Figure 5). E-5 was then cut with EcoR1 and the resulting 640 base pair fragment was isolated and inserted into the EcoR1 site of plasmid AK29 to create plasmid AE-2. AE-2 was then cut with SalI and the resulting 100 base pair fragment was inserted into pLR1Δ1 digested with XhoI. Plasmid AK26 contains the sequence cccgggtctccacattataagttggagagtctgga between the SmaI and SalI sites of pUC18. Plasmid LR21 was created by digesting plasmid AK30 with SmaI and inserting a SalI linker to create plasmid S-14 (Figure 6). S-14 was then cut with SalI and the resulting 60 base pair fragment was inserted into pLR1Δ1 which had been cut with XhoI. Plasmid AK30 contains the sequence cccgggtctccacattataagttggagatgccccgggtgtttccggcagaggagatcagtctctgtgctgga between the SmaI and Sal sites of pUC18.

Activity Assays:
β-galactosidase assays were conducted as described (Brent and Ptashne 1985) except values were measured per milligram total protein. ADHII activities were conducted as described (Denis 1987) in the same yeast extracts as were used for the β-galactosidase assays. All assays values are the average of at least three separate determinations.

Yeast Transformations:
All yeast transformations were conducted using the lithium
Figure 5  Construction of plasmid LR2-1.

pAK26

EcoRI

UAS1

SmaI  Sall

Insert EcoRI linker into SspI site of pAK26 to create pE-5.

pE-5

EcoRI

UAS1

SmaI  Sall

Isolate 640 bp EcoRI fragment from pE-5 and insert into EcoRI site of pAK29 to create pAE-2.

pAE-2

EcoRI

UAS2

Sall  SmaI  SmaI  Sall

Isolate Sall fragment from pAE-2 and insert into the XhoI site of pLR2-1 to create pLR2-1.

pLR2-1

EcoRI

UAS2

Sall  SmaI

GAL1 promoter

UAS1

Sall

lacZ
Figure 6 Construction of plasmid LR21.

Insert Sall linker into the SmaI site of pAK30 to create pS-14.

Digest pS-14 with Sall and insert the 60 bp fragment into pUR1.1 to create pLR21.

GAL1 promoter
acetate method as described (Ito et al. 1983).

**Growth Conditions**

Growth conditions of yeast have been described (Cook et al. 1994; Denis and Young 1983).

**Results**

UAS2 confers synergistic activation upon ADR1 under derepressed conditions

Both UAS1 and UAS2 have been shown to be required for full derepression of ADH2 expression (Shuster et al. 1986; Yu et al. 1989). Deletion of either element from its chromosomal locus results in reduced ADHII enzyme activities. In the present study we determined the effect of the UAS2 element on the ability of ADR1 to activate transcription. LexA-ADR1 fusions which contain the DNA-binding function of the bacterial repressor LexA and the activation domains from ADR1 have been shown in a previous study to activate transcription from a hybrid promoter which contained a LexA operator in an otherwise UAS-less GAL1 promoter (Cook et al. 1994). Activation of lacZ expression from this plasmid (p1840) was dependent upon LexA-ADR1 expression. These LexA-ADR1 fusions are able to function at the ADH2 locus since expression of LexA-ADR1-1-642 allows ADH2 derepression in an adr1 strain. Similar levels of derepression were not seen when this fusion was bound to the GAL1-lacZ hybrid promoter containing only the
LexA operator (Table 1), suggesting that ADR1 alone was not sufficient to allow increased activity under derepressed conditions and that another factor was required.

We initially examined the ability of our LexA-ADR1 fusions to activate transcription of a CYC1-lacZ hybrid gene that contained ADH2 promoter sequences (Figure 7). The first reporter construct (pADCY4) contains sequences from the ADH2 promoter from base pair -220 to -321, relative to the translation start site, which includes both the UAS1 and the UAS2 elements. The second promoter (pADCY5) contains ADH2 sequences from -220 through -302 which includes sequences through the UAS1 element but does not include the UAS2 element. When these reporter plasmids were co-expressed with LexA-ADR1 fusions a three-fold increase in transcription from the promoter that contained both UAS1 and UAS2 elements was observed over that seen with the promoter that contained only UAS1 (Figure 7). This result confirmed that the UAS2 element is critical for derepressed activities.

In order to determine if ADR1 and the UAS2 element were sufficient for this derepression, we examined the ability of these LexA-ADR1 fusions to activate transcription from a set of simplified promoters. One of these promoters (pl840) contains only the LexA operator site which functions to anchor the LexA-ADR1 fusions to the template. To create the second promoter, we cloned an oligonucleotide consisting of the UAS2 and LexA operator elements into the promoter region of a GAL1-
Figure 7  UAS2 is required for derepression.

β-Galactosidase activity of hybrid genes containing ADH2 sequences. Plasmid constructions have been previously described (Yu et al. 1988). Fold increase was calculated as the ratio of the activity from pADCY4 divided by the activity from pADCY5.

Standard errors of the means are less than 10%. Strains were grown in the presence of 2% ethanol, 2% glycerol.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>8- Galactosidase Activity</th>
<th>U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LexA-ADR1 Fusion</td>
<td>ADCY4</td>
<td>ADCY5</td>
<td>Fold Increase</td>
<td></td>
</tr>
<tr>
<td>1-1323</td>
<td>450</td>
<td>160</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>1-642</td>
<td>550</td>
<td>180</td>
<td>3.1</td>
<td></td>
</tr>
</tbody>
</table>
lacZ fusion gene to create the reporter plasmid LR2L. This construction places the UAS2 element immediately upstream of the binding site for ADR1 when ADR1 is expressed as a LexA fusion, a situation which mimics the relative positions of the UAS1 and UAS2 elements found at the ADH2 locus (Figure 3). Except for the presence of the UAS2 element, these plasmids (LR2L and 1840) are identical. Direct comparisons of the in vivo transcription activities from these two promoter constructs in the presence of various LexA-ADR1 fusions allowed us to assess the effect of the UAS2 element upon ADR1 activation in the absence of all other ADH2 promoter sequences. By binding the template through the LexA operator site we were able to examine the effect of UAS2 on the individual activation domains carried on the LexA-ADR1 fusions independent of the DNA-binding domain of ADR1. The data from these experiments are shown in Table 2.

Under derepressed growth conditions, the LexA-ADR1 fusions were capable of increased activity in the presence of the UAS2 element when compared to the activity from the identical promoter which contained only the LexA operator. The transactivation ability of TADII was enhanced 1.5-fold in the presence of the UAS2 element. Likewise, TADIII activity was increased almost 3-fold. TADII and TADIII when expressed on the same fusion, showed a 2.4-fold increase in activity in the presence of UAS2. The most dramatic increases in activity were seen when TADI was present in the LexA-ADR1 fusion. An
Table 2 UAS2-dependent derepression of LexA-ADR1 activity.

<table>
<thead>
<tr>
<th>LexA Fusion</th>
<th>Amino Acids</th>
<th>8- Galactosidase Activity U/mg</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR1 TAD I,II,III</td>
<td>1-1323</td>
<td>1840</td>
<td>LR2L 2100</td>
</tr>
<tr>
<td>ADR1 TAD I,II,III</td>
<td>1-642</td>
<td>470</td>
<td>LR2L 2500</td>
</tr>
<tr>
<td>ADR1 TAD I*,II,III</td>
<td>1-642 E117A</td>
<td>490</td>
<td>LR2L 900</td>
</tr>
<tr>
<td>ADR1 TAD II</td>
<td>263-359</td>
<td>680</td>
<td>LR2L 1100</td>
</tr>
<tr>
<td>ADR1 TAD III</td>
<td>359-740</td>
<td>36</td>
<td>LR2L 100</td>
</tr>
<tr>
<td>ADR1 TAD I,II</td>
<td>1-397</td>
<td>24</td>
<td>LR2L 360</td>
</tr>
<tr>
<td>ADR1 TAD II,III</td>
<td>220-1323</td>
<td>700</td>
<td>LR2L 1000</td>
</tr>
</tbody>
</table>

Yeast strains were isogenic to 237-1b-10 and were grown in the presence of 2% ethanol, 2% glycerol. Fold increase was calculated as the ratio of the activity from the LR2L promoter divided by the activity from the 1840 promoter. Standard errors of the means are less than 20%.

* TAD1 contains the mutation E117A
increase in activity of 15-fold in the presence of UAS2 was observed when TADI and TADII were present in the same fusion. The activities of LexA-ADR1 fusions containing TADI as well as TADs II and III displayed increased activities greater than five-fold when tethered next to the UAS2 element. This suggests that TADI plays an important role in the derepression of this promoter. The presence of an ADR1' mutation in TADII allowed a 31-fold increase in transcription in the presence of UAS2 as compared to a 7-fold increase in the similar construct containing wild-type residues (Table 3).

This UAS2-dependent increase of expression was also observed with a LexA fusion which contained the bacterial activator B42, and the yeast activator CCR4 (Table 4). Little or no increase in activity was seen, however, for the GAL4 activation domains when they were expressed as LexA fusions. The UAS2 element, when present as the sole upstream element in a UAS-less CYC1 promoter, was able to activate transcription only slightly (Table 5). This suggests that in these studies the UAS2 element is capable of domain-independent synergism with all activators except GAL4.

We have shown that the UAS2 element is capable of augmenting the activity of LexA-ADR1 fusions when juxtaposed to the binding site for ADR1. We next addressed the possibility that this activity requires that the two elements be in close proximity to one another in order for the two binding proteins to successfully interact to achieve
Table 3 The ADR1° mutation causes dramatic increase in UAS2-dependent derepression

<table>
<thead>
<tr>
<th>LexA-ADR1 Fusion</th>
<th>8-Galactosidase Activity (U/mg)</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR1 147-359</td>
<td>4.0</td>
<td>31</td>
</tr>
<tr>
<td>ADR1-5° 147-359</td>
<td>20</td>
<td>600</td>
</tr>
</tbody>
</table>

Yeast strains were isogenic to 237-1b-10 and were grown in the presence of 2% ethanol and 2% glycerol. Fold increase was calculated as the ratio of the activity from the LR2L promoter divided by the activity from the 1840 promoter. Standard errors of the means are less than 20%.
Table 4  UAS2-dependent derepression of non-ADR1 activators.

<table>
<thead>
<tr>
<th>LexA Fusion</th>
<th>Amino Acids</th>
<th>1840</th>
<th>LR2L</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR4</td>
<td>1-345</td>
<td>110</td>
<td>350</td>
<td>3.2</td>
</tr>
<tr>
<td>GAL4</td>
<td>87-881</td>
<td>340</td>
<td>430</td>
<td>1.3</td>
</tr>
<tr>
<td>B42</td>
<td></td>
<td>540</td>
<td>1500</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Yeast strains were grown in the presence of 2% ethanol and 2% glycerol. Fold increase was calculated as described in Table 2. Standard errors of the means were less than 30%.

Table 5  Activity of UAS2 element at CYC1 promoter.

Promoter:

UAS2 ——— CYC1 ——— lacZ

<table>
<thead>
<tr>
<th>Strain</th>
<th>8% Glucose</th>
<th>2% Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>237-1b-10</td>
<td>2.0</td>
<td>15</td>
</tr>
</tbody>
</table>

Activities were determined as described in Table 2. Standard errors of the means were less than 20%.

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synergistic activation. To do this we constructed two additional promoters and inserted them in the upstream region of a GAL1-lacZ hybrid gene. One of these promoters (LR21) contains the UAS1 and UAS2 elements in direct contact with one another as they are found in the ADH2 promoter. The second construct (pLR2-1) contains an identical promoter, however, here the UAS elements are separated by 30 base pairs of pUC18 sequence. We found little or no difference in the transactivation ability of LexA-ADR1 fusions which contained the DNA binding domain of ADR1 from the these two reporters (Table 6). This suggests that the mechanism by which these factors interact to achieve synergistic activity is not dependent upon strict spacing between the two elements.

**UAS2 confers glucose-dependent repression on ADR1 activation**

The UAS2 element when present as the only upstream element in a CYC1-lacZ hybrid promoter was tightly glucose-repressed (Yu et al. 1989). To further examine this glucose repression and its effect on ADR1 activity, we compared the activities of the LexA-ADR1-1-1323 and LexA-ADR1-1-642 fusions from the ADCY4 and ADCY5 promoters. We saw little or no change in the activities of these fusions from these two promoters (Table 7). Comparison of these activities to those found at the ADH2 promoter for the same fusions (Table 1), suggests that the ADCY4 and ADCY5 promoters are not good candidates for studying the regulation of ADH2.
Table 6  Separation of UAS elements under derepressed growth conditions.

<table>
<thead>
<tr>
<th>LexA-ADR1 Fusion</th>
<th>LR2-1</th>
<th>LR21</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR1 1-1323</td>
<td>240</td>
<td>440</td>
<td>1.8</td>
</tr>
<tr>
<td>ADR1 1-642</td>
<td>270</td>
<td>330</td>
<td>1.2</td>
</tr>
<tr>
<td>ADR1-5° 1-642</td>
<td>780</td>
<td>610</td>
<td>0.8</td>
</tr>
<tr>
<td>ADR1 1-397</td>
<td>66</td>
<td>160</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Yeast strains are isogenic to 237-1b-10 and are grown under derepressed growth conditions (2% ethanol, 2% glycerol). Fold increase was calculated as the ratio of the activity from the LR21 promoter divided by the activity from the LR2-1 promoter. Standard errors of the means were less than 16%.
Table 7 LexA-ADR1 fusions are not repressed at complex promoter.

<table>
<thead>
<tr>
<th>LexA-ADR1 Fusion</th>
<th>ADCY4</th>
<th>ADCY5</th>
<th>Fold Repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1323</td>
<td>600</td>
<td>480</td>
<td>0.8</td>
</tr>
<tr>
<td>1-642</td>
<td>700</td>
<td>610</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Promoter constructs are described in Figure 3. Strains are grown in 8% glucose as the sole carbon source. Fold repersions are calculated as the ratio of the activity from the ADCY5 promoter divided by the activity from the ADCY4 promoter. Standard errors of the means are less than 10%.
To better study the effect of UAS2 on ADR1 activity, we examined the activities of LexA-ADR1 fusions from our simplified promoters. These promoters (p1840 and pLR2L) are shown in Figure 1. The ability of the UAS2 element to affect ADR1-dependent activation from these promoters when cells are grown under repressive conditions is shown in Table 8. UAS2 repressed the activity of the two strongest activation domains of ADR1 (TADII and TADIII) 47- and 11-fold respectively in this assay. Similarly, TADII and TADIII, when present together on the same LexA fusion were repressed almost eighteen-fold by UAS2. While the UAS2 element was still able to repress the activity of LexA-ADR1 fusions containing two activation domains, this ability was significantly diminished when TADI was part of the fusion molecule. We found that the activity of TADI and TADII, when present together as part of the LexA-ADR1 fusion, was repressed only three-fold by the presence of UAS2. In addition, LexA-ADR1 fusions that contained TADI along with TADs II and III showed little or no repression. A fusion which contained these three domains and also contained a point mutation in the first activation domain (TADI-E117A), however, was repressed twelve-fold by UAS2. Thus, it appears that TADI plays a critical role in the UAS2-dependent repression of ADR1 activity. It is possible that the identical LexA-ADR1 fusion containing ADR1 sequences 1-642 which was not repressed, escaped repression by virtue of binding DNA through the LexA domain thereby fully exposing a third domain (TADI) which is
Table 8 UAS2-dependent repression of LexA-ADR1 activity.

<table>
<thead>
<tr>
<th>LexA Fusion</th>
<th>Amino Acids</th>
<th>1840</th>
<th>LR2L</th>
<th>Fold Repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR1 TAD I,II,III</td>
<td>1-1323</td>
<td>2300</td>
<td>1800</td>
<td>1.3</td>
</tr>
<tr>
<td>ADR1 TAD I,II,III</td>
<td>1-642</td>
<td>1850</td>
<td>2000</td>
<td>0.9</td>
</tr>
<tr>
<td>ADR1 TAD I*,II,III</td>
<td>1-642 E117A</td>
<td>1500</td>
<td>130</td>
<td>12</td>
</tr>
<tr>
<td>ADR1 TAD II</td>
<td>263-359</td>
<td>1900</td>
<td>40</td>
<td>48</td>
</tr>
<tr>
<td>ADR1 TAD III</td>
<td>359-740</td>
<td>480</td>
<td>60</td>
<td>8.0</td>
</tr>
<tr>
<td>ADR1 TAD I,II</td>
<td>1-397</td>
<td>220</td>
<td>80</td>
<td>2.8</td>
</tr>
<tr>
<td>ADR1 TAD II,III</td>
<td>220-1323</td>
<td>2300</td>
<td>100</td>
<td>23</td>
</tr>
</tbody>
</table>

Yeast strains were grown in the presence of 8% glucose as the sole carbon source. Fold repression was calculated as the ratio of the activity from the 1840 promoter divided by the activity from the LR2L promoter. Standard errors of the means were less than 17%.

* TAD1 contains the mutation E117A.
normally involved in binding at the ADH2 locus. Mutations in TADI may disrupt this acquired activity and thus lead to repression by UAS2.

The identity of the TADI activation domain has not been firmly established because of its co-localization with the DNA-binding domain of ADR1. However, the region which displays transcriptional activity shows no homology to any other characterized activation domains. The other two activation domains of ADR1, TADII and TADIII, contain a disproportionate number of acidic residues and in this way are similar to other activation domains previously identified in yeast.

We next wanted to determine if the UAS2 element was specific for ADR1 or whether it could repress the activity of other transcriptional activators. For this study, we co-expressed the two reporter plasmids p1840 and pLR2L separately with a number of LexA fusions containing activation domains from other transcription factors. As shown in Table 9, the UAS2 element was capable of a 2.1-fold repression of the activation domains present in the yeast factor GAL4, and a 1.8-fold reduction in the activity of LexA-B42, which contains an E. coli derived transcriptional activator. Another hybrid activator contained a region of the yeast transcriptional activator CCR4 fused to LexA. This fragment of CCR4 contains two separate activation domains (M. Draper, personal comm.). One of these domains contains a high distribution of acidic residues and the second domain is rich in glutamine residues,
Table 9 UAS2-dependent repression of non-ADR1 activators.

<table>
<thead>
<tr>
<th>LexA Fusion</th>
<th>Amino Acids</th>
<th>1840</th>
<th>LR2L</th>
<th>Fold Repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR4</td>
<td>1-345</td>
<td>400</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>GAL4</td>
<td>87-881</td>
<td>960</td>
<td>500</td>
<td>1.9</td>
</tr>
<tr>
<td>B42</td>
<td></td>
<td>120</td>
<td>110</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Yeast strains were grown in the presence of 8% glucose as the sole carbon source. Fold repressions were determined as described in Table 8. Standard errors of the means were less than 30% except for GAL4-LR2L which was 34% and B42-1840 which was 35%.
a motif typical of mammalian activators (Kadonaga et al. 1988). This hybrid activator was repressed 15-fold by UAS2. It appears, therefore, that the UAS2 element is capable of repressing the activity of a number of transcriptional activators, although to much different extents. We have not examined the ability of the UAS2 element to repress the individual activation domains of either GAL4 or CCR4. It will be interesting to see if the two domains in these proteins are differentially repressed by UAS2. The bacterial activator B42 was originally isolated by Ma and Ptashne (1987) and contains sequences rich in acidic residues.

We extended these studies by examining the effect of UAS2 on the repression of ADR1 activity when UAS2 was physically separated from the site where ADR1 was binding. At the same time, we wanted to determine whether UAS2 could repress a fusion containing all three activation domains when the hybrid molecule binds UAS1. In this study we employed two different plasmids, one of which contained the UAS1 and UAS2 elements aligned next to each other as they appear at the ADH2 locus (pLR21). The other plasmid contains a promoter identical to the first except that the UAS1 and UAS2 elements are separated by 30 base pairs of pUC18 sequence (pLR2-1 see Figure 1). The remainder of the promoter regions for the two reporter plasmids are identical to that found on p1840 and pLR2L including the spacing between the UAS elements and the TATA box. Because the LexA fusions were now required to bind to the
UAS1 element instead of a LexA operator, this assay allowed us to study the repression of ADR1 when it bound DNA through its normal DNA-binding domain located within TADI. We found that the activation potential of our LexA-ADR1 fusions when bound to the UAS1 element was greater when UAS1 and UAS2 were separated from each other than when they were juxtaposed (Table 10). This suggests that separation of the UAS1 and UAS2 elements at least partially relieves the UAS2-dependent repression seen in the earlier study. Unfortunately, we were unable to clone the UAS1 element alone into this promoter. Therefore, we do not know to what extent the LexA-ADR1 fusions were repressed at the LR2-1 promoter. Juxtaposition of the two elements was capable of repressing the activity of the LexA-ADR1 fusions which contained all three activation domains nearly two-fold below the activity observed when the elements were separated (Table 10). This supports the notion that the inability of the three domains together to be repressed in the LR2L reporter was a consequence of abnormal exposure of TADI. The high activities of our LexA-ADR1 fusions at this promoter (LR2-1) when the fusion is bound through UAS1, supports the idea that these same fusions were repressed at both pADCY4 and ADCY5. The three-fold repression seen earlier for TADI and TADII when expressed in the same fusion, was not seen in this assay.

Previous studies of the functional regions of ADR1 identified two domains located between amino acids 227-239 and
Table 10 UAS2 repression is dependent upon distance from UAS1.

<table>
<thead>
<tr>
<th>LexA-ADR1 Fusion</th>
<th>LR2-1</th>
<th>LR21</th>
<th>Fold Repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR1 1-1323</td>
<td>1100</td>
<td>630</td>
<td>1.7</td>
</tr>
<tr>
<td>ADR1 1-642</td>
<td>1580</td>
<td>780</td>
<td>2.0</td>
</tr>
<tr>
<td>ADR1-5° 1-642</td>
<td>1900</td>
<td>920</td>
<td>2.1</td>
</tr>
<tr>
<td>ADR1 1-397</td>
<td>300</td>
<td>330</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Yeast strains were grown in the presence of 8% glucose as the sole carbon source. Fold repressions were calculated as the ratio of the activity from the LR2-1 promoter divided by the activity from the LR21 promoter. Standard errors of the means were less than 15%.
which inhibited ADR1 activation potential (Cook et al 1994). Several lines of evidence suggest that the UAS2-dependent repression we observe does not work through either of these inhibitory domains. First, the ADR1−5Δ mutation when present in the LexA-ADR1-147-359 construct does not relieve UAS2-dependent repression (Table 11). Secondly, TADIII, which does not contain either of these inhibition domains is still repressed by UAS2. Further, it appears that this repression is non-specific as many other activation domains, when tethered adjacent to the UAS2 element, were repressed by UAS2. The diminished ability of UAS2 to repress the activity of ADR1 when physically distanced from the UAS1 element also indicates that the repression is non-specific and may involve a simple occlusion mechanism.
Table 11 ADR1<sup>c</sup> mutation does not relieve UAS2-dependent repression.

<table>
<thead>
<tr>
<th>LexA-ADR1 Fusion</th>
<th>β-Galactosidase Activity</th>
<th>LR2L</th>
<th>Fold Repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR1 147-359</td>
<td>20</td>
<td>7.8</td>
<td>2.6</td>
</tr>
<tr>
<td>ADR1-5&lt;sup&gt;c&lt;/sup&gt; 147-359</td>
<td>110</td>
<td>26</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Yeast strains were grown in the presence of 8% glucose as the sole carbon source. Fold repressions were determined as described in Table 8. Standard errors of the means were less than 12%.
Discussion

The results presented here demonstrate two activities both of which function through the UAS2 element. Under conditions of glucose repression, the UAS2 element is capable of repressing the transcriptional activity of ADR1. It had been shown earlier that expression of LexA-ADR1 hybrid molecules containing activation domains from ADR1 were capable of activating transcription of a heterologous promoter which contained the LexA operator as the sole upstream element (Cook et al. 1994). The UAS2 element from ADH2, when cloned into this promoter in a position adjacent to the binding site for the fusions is capable of repressing this activity. This repression was independent of the particular activation domain present in the fusion. Additionally, the UAS2 element was capable of repressing the transcriptional activity of several other activators. The degree of repression varied depending on which activation domain of ADR1 was present. The strongest repression was seen for TADII and when TADII and TADIII were present in the same fusion. LexA-ADR1 fusions which contained all three ADR1 activation domains were not repressed by UAS2 when the fusion was bound to the LexA operator. This would suggest that at the LexA operator TADI was interfering with UAS2 repression. In support of this, a fusion which contained all three activation domains as well as a point mutation in TADI was repressed by UAS2 when the fusion bound the LexA operator. This mutation (E117A) occurs at the tip of the first
zinc finger in the DNA-binding domain. As yet it has been impossible to separate the DNA-binding function of TADI from the activation function. It is possible that the DNA-binding function of this region influences the ability of the activation domain to induce transcription.

Several lines of evidence suggest that UAS2 repression is non-specific. First, UAS2 was capable of repressing activation domains other than those found in ADR1. This repression did not operate through either of the two inhibitory domains identified in ADR1. Second, UAS2 was capable of repressing the activity of other transcription factors including other yeast factors as well as one factor of bacterial origin. Third, physical separation of the UAS2 element from UAS1 relieved repression. It is possible that the factor which binds UAS2 is a general repressor which shows a preference for acidic activators as all the activators we tested had at least one activation domain which was acidic.

It is interesting to note that there is another element which shares a great deal of homology with UAS2 located in the promoter of ADH2 between the UAS1 element and the TATA box. This element, when deleted from the chromosomal locus allowed ADH2 to bypass glucose repression (Schuster et al.). Another deletion encompassing the same number of residues just upstream of this site did not relieve repression, suggesting that this activity is not a result of moving the UAS elements closer to the transcription start site. Deletion of the UAS2-
like element allows \textit{ADH2} to bypass glucose repression suggesting that ADR1 is active under both repressed and derepressed conditions and that these elements function to repress the activity of ADR1. The activity of LexA-ADR1 fusions co-expressed with hybrid genes under the control of \textit{ADH2} promoter sequences which included this site as well as UAS1 and UAS2 were compared with activities from the same promoter which did not contain UAS2 (Figure 3). In this case when we compared the activities of the LexA-ADR1 fusions from the two promoters we saw no UAS2-dependent repression (Table 7). These promoters did show, however, a UAS2-dependent increase in transcriptional activity under derepressed conditions. This evidence, although indirect, suggests that these elements may work together to repress transcription at \textit{ADH2} when cells are grown in the presence of glucose. This repression might entail a looping mechanism similar to that seen in vivo between two spatially separated gal operators in the repression of the gal operon of \textit{E. coli} (Choy and Adhya 1992).

We also found that the UAS2 element was capable of dramatically increasing the activity of a LexA operator-bound ADR1 fusion under derepressed growth conditions. This increase was independent of the particular ADR1 activation domain and was seen irrespective of the number of activation domains present. The increase in activation in every case was synergistic as we saw little activation function by the UAS2
element alone. In addition to ADR1 activation domains, UAS2 also derepressed the activity of the activation domains of the yeast activator CCR4 and the bacterial activator B42. This element was not, however, capable of affecting the activity of the GAL4 activator when it was fused to LexA. In these experiments the LexA-GAL4 fusion is over-expressed. Therefore, the inability of UAS2 to increase the transcriptional activity of GAL4 activation domains is not due to GAL80 repressor binding.

Separating the UAS1 and UAS2 elements caused at best a two-fold decrease in activation function of the LexA-ADR1 hybrids when the strains were grown under derepressed conditions (Table 10). This effect requires further study but suggests that the mechanism of synergistic activation between UAS1 and UAS2 is complex and may be comprised of a combination of the models discussed earlier.

Future Prospectives:

We have shown that the UAS2 element is able to increase the rate of transcription when placed near the binding site for ADR1 in a heterologous promoter. This element likely functions by contacting some factor involved in the formation of the transcription complex rather than influencing the ability of ADR1 to bind template as the UAS2 element can augment transcriptional activity irrespective of the particular activation domain of ADR1 expressed as well as
increasing the activity of other activators. Furthermore, the UAS2 element can increase transcription slightly under derepressed conditions when present as the sole upstream element. In addition, the spacing between the UAS elements has a minimal effect on the ability of UAS2 to increase the activity of certain LexA-ADR1 fusions. Spacing between the UAS1 and UAS2 elements had the greatest effect on the activity of the smaller LexA-ADR1-1-397 fusion, and therefore, it is possible that the protein which binds UAS2 may form a ternary complex with ADR1 and a third factor to effect an increase in transcription. Bringing the UAS2 element closer to the binding site for the LexA-ADR1-1-397 fusion may allow the three proteins to form this complex structure. If this is the case, there should be a distance of separation between the two elements beyond which the UAS2 element will no longer affect the activity of full-length ADR1. Because we do not have a promoter which contains only the UAS1 element upstream of a GAL1-lacZ hybrid gene, we first need to make this construction. This will permit us to determine the activity of the LexA-ADR1 fusions when bound to UAS1 in the absence of other promoter elements and will allow us to better analyze the derepressive effects of UAS2 seen with plasmids LR21 and LR2-1. Alternatively, we can use the p1840 reporter as our estimate of LexA-ADR1 activity and insert the UAS2 element into this promoter at various distances from the LexA operator. This would eliminate the need to express the DNA-
binding portion of ADR1 and will allow us to determine the
effect of spacing upon the individual activation domains of
ADR1 as well as those of other activators when fused to LexA.

We have shown that the UAS2 element does not increase the
transcriptional activity of all activators as the activity of
a LexA-GAL4 fusion was unaffected by the presence of UAS2. The
GAL genes are regulated by the activity of GAL4 and are
normally repressed by the presence of glucose but require
galactose to become induced. In our studies GAL4 activity is
repressed two-fold by UAS2 but is not significantly affected
by the presence of the UAS2 element in the absence of glucose.
This would suggest that GAL4 and ADR1 induce transcription by
different mechanisms. It would be interesting to further
investigate the specificity of UAS2. UAS2 is able to increase
the activity of another acidic activator (LexA-B42), which
suggests a specific interaction between UAS2 and a particular
class of activator. Insertion of the UAS2 element into the
control region of other genes which are activated by the
absence of glucose may therefore influence the transcription
rate of these genes.

The mechanism of repression by UAS2 appears to be more
general. UAS2 was able to repress the activity of each
activator tested although to varying extents. This repression
was relieved when the UAS2 element was physically separated
from the activator-binding element. This suggests a simple
model where the UAS2-binding protein represses the activity of
an adjacent site by an occlusion mechanism. The avidity of repression is then dependent of the ability of the repressor to compete with the activator for binding sites which overlap on the template. This model can be further investigated by constructing a promoter which contains the LexA operator separated from the UAS2 element as discussed earlier. In this way we can investigate the effect of the physical separation of UAS2 from other activators when fused to LexA. Alternatively, we can insert the UAS2 element into the control regions of other genes in a position adjacent to known UAS elements for that particular promoter. If repression is caused by occlusion of the UAS element, UAS2 should be able to repress the activity of these promoters in a glucose-dependent fashion.

Cloning of the UAS2-binding protein has obvious benefits, and is currently being attempted in our laboratory. Perhaps most importantly, this will allow us to determine if the same protein binds UAS2 under both repressed and derepressed conditions through disruption of the wild-type allele.
REFERENCES


ADRR mutations enhance the ability of ADRR to activate transcription by a mechanism that is independent of effects on cyclic AMP-dependent protein kinase phosphorylation of Ser-250

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Four ADRR mutations that occur close to Ser-250 of the xanthine oxidoreductase transcriptional activator ADRR and which greatly enhance the ability of ADRR to activate ADRR expression under glucose-repressed conditions have been shown to reduce or eliminate cyclic AMP-dependent protein kinase (cAPK) phosphorylation of Ser-250 in vitro. In addition, unregulated cAPK expression in two blocks ADRR dephosphorylation in an ADRR-dependent fashion in which ADRR mutations display decreased sensitivity to unregulated cAPK activity. Taken together, these data have suggested that ADRR mutations enhance ADRR activity by blocking cAPK phosphorylation and inactivation of Ser-250 mutations, defining 10 different amino acid changes, that were located in the region defined by residues 222 through 250 of ADRR. Three observations, however, indicate that the ADRR phenotype is not simply equivalent to a lack of cAPK phosphorylation. First, some of these newly isolated ADRR mutations affected the ability of yeast cAPK to phosphorylate corresponding synthetic peptides modeled on the 222 to 250 region of ADRR in vitro. Second, we observed that strains lacking cAPK activity did not display enhanced ADRR expression under glucose-repressed conditions. Third, when Ser-250 was mutated to a nonphosphorylatable residue, lack of cAPK activity led to a substantial increase in ADRR expression under glucose-repressed conditions. Thus, while cAPK controls ADRR expression and ADRR is required for this control, cAPK acts by a mechanism that is independent of effects on ADRR Ser-250. It was also observed that deletion of the ADRR region resulted in an ADRR phenotype. The ADRR region, therefore, involved in maintaining ADRR in an inactive form. ADRR mutations may block the binding of a repressor to ADRR or alter the structure of ADRR in such that transcriptional activation regions become unmasked.

The regulation of the glucose-repressible alcohol dehydrogenase (ADH2) gene, encoded by the ADHR gene from Saccharomyces cerevisiae, is mediated by several pathways. The SRE1 protein kinase and the RIG1 gene constitute one pathway (10, 33), but their site of action at ADHR has not been identified. The transcriptional activator ADHR acts independently of SRE1 (34) and binds to the upstream activation sequence, UAS1, located between bp -221 and -31 of the ADHR promoter (59). Part of the glucose regulation of ADHR is mediated by control of ADHR translation (57a). A third regulatory network whose factors have not been identified may act through UAS2, which is just upstream of UAS1 (59).

The importance of ADHR in the glucose control of ADHR is evidenced by a class of ADHR mutations (ADHR) causing enhanced ADHR transcription under repressed conditions (8, 11). These mutations do not affect ADHR RNA (12) or protein levels (37a) and must activate ADHR by a posttranscriptional mechanism. Four ADHR mutations have been identified and found to occur between amino acids 221 and 231 of ADHR in a conserved cyclic AMP-dependent protein kinase (cAPK) phosphorylation consensus sequence (RRASF, where Ser-300 is the phosphorylated) (6, 12). ADHR has been found to be a substrate for cAPK in vitro; cAPK phosphorylates both Ser-250 and some other site on the N-terminal side of Ser-250 (19). The ADHR mutations were shown to decrease or eliminate cAPK phosphorylation of ADHR at Ser-250 in vitro. These data suggest that in S. cerevisiae the ADHR alterations enhance ADHR activity by interfering with the cAPK phosphorylation of Ser-250.

Subsequent genetic analysis indicated that unregulated cAPK activity, the result of disrupting the B 511 cAPK regulatory gene, reduced ADHR expression (9). The effect of the b 511 disruption was relieved, albeit incompletely, by ADHR mutations (6). These results provided support for the model that cAPK inactivated ADHR function by phosphorylating Ser-250, although Ser-250 appeared not to be the only site of cAPK inactivation. More recently it has been demonstrated that all of the effects of cAPK on ADHR expression are mediated by ADHR (14). Two other protein kinases, SRE1 and SIR1, were observed to affect ADHR expression independently of both ADHR and cAPK (10).

In this study, we have characterized 17 additional ADHR alleles with the expectation of furthering our understanding of how these mutations activate ADHR. Using synthetic peptides, we observed that not all ADHR mutations affected the cAPK phosphorylation of Ser-250 in vitro. More importantly, lack of cAPK activity did not affect an ADHR phenotype in vivo. These data argue for a model in which ADHR mutations alter the structure and function of ADHR independent of cAPK phosphorylation of Ser-250. In addition, when Ser-250 could not be phosphorylated, a lack of cAPK was observed to result in enhanced ADHR function. Accordingly, cAPK may act through another protein that mediates ADHR activity through another protein that mediates ADHR activity.
MATERIALS AND METHODS

Yeast strains. Yeast strains are listed in Table 1. Strains containing the ADRI alleles 9 through 20 were isogenic to 315-1D (32).

Identifying ADRI mutations. Strains containing dominant mutations that allowed ADH2 expression under glucose growth conditions were obtained by V. Williamson in the course of a previous study (32). Each mutant was an independent spontaneous isolate and was shown not to result from Ty insertions at either the ADH2 or ADRI locus (32). Tetrad analysis was used to determine whether the dominant mutations mapped to the ADRI locus. ADRI alleles were rescued from S. cerevisiae and localized to the region between bp -440 and +1076 as described previously (6, 12). For each different ADRI allele, the region between bp +440 and +1076 was sequenced to identify the ADRI lesion.

cAPK phosphorylation of synthetic ADRI peptides. Peptides were synthesized and isolated as described previously (13). The yeast TPK1 (57) enzyme was provided by M. Zoller, and the porcine heart cAPK was provided by S. Taylor. Conditions for determining the K_m of phosphorylation of each peptide were as described previously (15). The unique size of phosphorylation for each peptide, except ADRI-222-234 (F2315), was determined to be Ser-250 by chymotrypsin analysis as described previously (13). Peptide ADRI-222-234 (F2315) was found to be stoichiometrically phosphorylated on serine, but the precise size of phosphorylation was not determined.

Growth conditions and ADHII assays. Conditions for growth of cultures on YEP medium (2% Bacto Peptone, 1% yeast extract, 20 mg of adenine and uracil per liter) have been described previously (12). ADHII enzyme activities were assayed as previously described (10, 15). Yeast transformations were conducted by the Li acetate procedure (22).

Gene disruptions and constructions. The BY4741 gene disrupted with the URA3 gene was used as previously described (36). The trp1, ura3, leu2, and the trg1, leu2 through trp1 alleles were specifically selected as suppressors of the hyperinduced phenotypes (inability to grow at 30°C after a shift to 35°C for 1 h, dark colony color, and reduced growth on noninducing carbon sources) as previously described (5, 31). Genetic analysis was conducted as previously described (5) to verify that a trg1 allele was responsible for suppressing the unregulated cAPK activity caused by the trp1 allele.

cAPK enzyme activity was determined as described previously (56).

ADRI 222-232 was constructed as follows. The XnoII-SacI fragment of ADRI (bp 782 to 1715) was ligated into pUC18 at the XhoI (blunt ended) and SacI sites to generate plasmid Lb45. The HindIII-SacI fragment of YEp7-ADRI (22A (12), extending from 1.5 kb upstream of ADRI to bp +661, was subsequently ligated into Lb45 restricted with HindIII and SalI (filled in with a Klavey fragment). The resultant plasmid, Lb58, contained the 5' region of ADRI and the region encoding ADRI amino acids 1 to 220 placed in frame to residues 262 to 571. Between residues 250 and 262 were seven additional amino acid residues derived from the pUC18 polylinker (FDSRGSP). Lb58, after addition of the TRP1 gene, was targeted for integration at the adri-1 locus in strain 500-16 following cleavage with NruI at bp 1517. Identification of integrants by genetic and Southern analysis has been described previously (21). Integration of the plasmid Lb58 (TRP1) at the adri-1 locus generates a complete ADRI 222-232 allele as well as a truncated adri-1 allele. adri-1 does not express a functional ADRI protein, since it contains a nonsense mutation in its 11th codon (5).
RESULTS

Identification of 17 new ADR1 mutations. Twenty independently isolated strains containing dominant mutants which allowed glucose-insensitive ADH2 expression and which were not the result of Ty transformation at either the ADH1 or ADH2 locus (27) were analyzed. Ten amino acid changes that affected the function of the ADR1 locus were identified, and 2 had been previously reported (data not shown). Of these, 17, 16 were rescued and sequenced. All 17 mutations were from single base pair changes (Table 3). Ten different amino acid changes that affected the function of the ADR1 locus were identified, and 8 new mutations were obtained previously (6, 12) that substitute amino acids in the region of residues 25 to 39 of ADR1 (Fig. 1). These new mutations occurred in the same region of ADR1 as did the previous four ADR1 mutations that were contained in the Ser-259 (6, 12). The occurrence of all 21 known ADR1 mutations in this region containing Ser-259 (6, 12) suggests that this is the only domain of ADR1 which can be mutated to allow ADH1 to bypass the effects of glucose repression.

The effect on ADH2 expression of each of the different ADR1 mutations is given in Table 3. The mutations resulted in ADH1 II enzyme activity levels ranging from 70 to 250 mU/mg under glucose-growth conditions. Under ethanol-growth conditions, each of the mutations resulted in similar ADH1 II enzyme activity levels (about 2,500 mU/mg). The observation that under glucose-growth conditions, the ADR1-25 (A25P) (i.e., Ser-25P) displayed enhanced ADH2 expression relative to ADR1-18 (S250L) suggests that structural alterations in the ADR1 region more important to enhancing ADH2 expression were not a significant factor. These results also suggest that the potential for CAPK phosphorylation of Ser-250 (as in ADR1-18).

Only some ADR1 mutations affect CAPK phosphorylation of synthetic ADR1 peptides. Our previous analysis indicated that ADR1-18 (S250L) and ADR1-23 (S251H) reduced the phosphorylation by CAPK of ADR1 Ser-250 in vivo (6). This was expected on the basis of other studies that defined the substrate recognition determinants of CAPK (4, 24). Several of the newly identified ADR1 mutations appeared to work. However, to similarly affect CAPK phosphorylation, it is known that some mammalian CAPK substrates, e.g., the CK1B protein (19) and protein-1,6-hexosamines (17), are phosphorylated effectively at the sequence PKP, a sequence identical to that found in ADR1-23 and ADR1-18. Also, it has been shown that a protein with this 7 position (relative to the phosphorylation site) is essential for or an effect on phosphorylation of synthetic ADR1 peptides (16, 17, 20). In addition, the yeast CAPK regulatory subunit HCY1, a known in vivo substrate for yeast CAPK, contains a phospho-acceptor site in its - 13 position, resulting in a sequence RRASVSG (19) which is very similar to that of ADR1-23 (RRSS122) (Table 4). We therefore examined whether the ADR1 mutations were likely to affect CAPK phosphorylation by using synthetic peptide models on the sequence containing Ser-250 of ADR1.

Synthetic peptides have generally been found to be excellent substrates for mammalian CAPK and to be phosphorylated with kinetics similar to those of the natural protein substrates (25). Moreover, synthetic peptide models of the ADR1 sequence at Ser-250 have previously been shown to be excellent substrates for yeast CAPK (TPK1) enzyme (13). ADR1-222-234, which is phosphorylated with a K, of 6.8 mM by the yeast TPK1 enzyme (Table 4), was chosen as our parent substrate (13). Eight different ADR1 peptide analogs were synthesized, each containing an amino acid change corresponding to an ADR1 mutation, and the ability of each peptide to be phosphorylated by yeast CAPK was analyzed. Some ADR1 mutations causing alterations in previously known important recognition determinants of CAPK (e.g., R227L, S250K, S235L, and S2351L) had very dramatic effects on CAPK phosphorylation of Ser-250. These alterations increased the K, for phosphorylation by 20- to 400-fold (Table 4). These results are in agreement with our previous data demonstrating that R227L and S235L reduce the ability of CAPK to phosphorylate Ser-250 in vivo (6). In contrast, other ADR1 mutations had very little or no effect on CAPK phosphorylation (e.g., A232P, S221L, A232L, and A231S) (Table 4). In fact, we observed no correlation between CAPK recognition of the ADR1 peptide analogs and the ability of the ADR1 or ADR1 peptide to activate ADH2 under glucose-growth conditions (Table 3). These results do not appear to be due to some recognition feature of the yeast TPK1 enzyme, since similar effects on Ser-250 phosphorylation were also obtained with the distantly related porcine CAPK (Table 4). In addition, the recently determined crystal structure of mouse CAPK clearly indicates that the A232P change would not be expected to affect CAPK phosphorylation (26). These data do not support the model that the only effect of the ADR1 mutations on ADR1 function is to alter CAPK phosphorylation of Ser-250.
For the remaining three ADR\(^II\) mutations, S390I, Y229C, and Y230F, cAPK did not phosphorylate the peptide 227-234 containing the S390I change (data not shown). We did not analyze peptides corresponding to the two alterations at Tyr 229, but it has been observed that yeast cAPK phosphorylates ADR\(^I\) peptide 225-234 with a K\(_d\) of 15 \(\mu M\), a value that is only twice that found for ADR\(^I\) peptide 225-241 (7.5 \(\mu M\)). Sequences from 225 to 241 appear, therefore, to have only a small effect on cAPK phosphorylation of Ser 225.

Lack of cAPK activity does not confer an ADR\(^II\) phenotype. Because the results described above were conducted in vitro, we acknowledge the possibility that the ADR\(^II\) alterations could result in gross changes in the structure of the full-length ADR\(^I\) protein that would affect cAPK phosphorylation of Ser 225 in vivo. To address this question, we analyzed in vivo the effects of a lack of cAPK activity on ADR\(^I\) expression. If the ADR\(^II\) phenotype were due solely to reducing cAPK phosphorylation of Ser 225, then strains containing no measurable cAPK activity would be expected to exhibit an ADR\(^II\) phenotype. We investigated this possibility by measuring the level of ADR1 expression in strains containing deletions in two of the three TPK genes and a mutation in the third TPK gene (tpk2 allele). Such strains lack measurable cAPK activity (reference 5 and Materials and Methods above) and are known to be refractory to the effects of disrupting the BCR1 gene, a disruption that would otherwise cause underphosphorylation of ADR1 (5). Our analysis of strains lacking cAPK activity indicated that the levels of ADR1 expression under glucose growth conditions were unaffected from those found in wild-type strains (Table 5). These results were observed regardless of the combination of deleted and mutated TPK alleles (Table 5 and data not shown). Under ethanol growth conditions, however, lack of cAPK activity relieved the reduction in ADR1 expression caused by unregulated cAPK activity (e.g., compare strains isogenic to 410-1e [Table 5]), indicating that strains containing disruptions in two TPK genes and a mutation in the third behaved as though they lacked cAPK activity.

This is consistent with the idea that under glucose growth conditions, trace levels of cAPK activity that fall below our detection capabilities may persist and be sufficient to maintain glucose repression of ADR1. Several observations suggest that this is not the case. First, similar strains lacking detectable cAPK activity in an ADR1\(^\beta\) background containing a nonphosphorylatable residue at Ser 125 resulted in a 4- to 15-fold enhancement of ADR1 expression under glucose growth conditions (see below) (Table 6). Second, strains which contain the tpk2\(_{-}\) allele and the disrupted tpk3\(_{2}\) and tpk1\(_{2}\) alleles have been found to be defective in glucose repression of mitochondrial functions (30). Moreover, the CTTI gene (encoding catalase T) displays elevated levels of expression under glucose-growth conditions in a tpk2\(_{2}\) tpk1\(_{2}\) background (2). These three observations suggest that there is insufficient cAPK activity in strains carrying a tpk2\(_{-}\) allele to maintain completely the effects of glucose repression. Finally, we have observed that strains containing disruptions in all three TPK alleles (presumably viable because of the presence of another mutation, such as sak1\(_{16}\) [16]) were fully glucose repressed for ADR1 expression (Table 5). Our results indicate that lack of cAPK activity does not confer an ADR1\(^II\) phenotype and that the ADR1 mutations do not enhance ADR1 function by interfering with cAPK phosphorylation of Ser 225. It remains possible that Ser 225 is phosphorylated by a protein kinase other than cAPK and that the ADR1 mutations interfere with the role of this enzyme.

cAPK inactivates ADR1 through a site other than Ser 225. Because cAPK phosphorylation of Ser 230 cannot be solely responsible for maintaining ADR1 in an inactive state, we surmised that the effects of cAPK may be observed only when ADR1 is in an activated form such as that which occurs under ethanol growth conditions. Factors other than decreased cAPK phosphorylation would contribute to the activation of ADR1 under derepressed conditions. To test this idea, we repeated the experiment described above with...
strains carrying the ADR1-7 allele. We presumed that the ADR1-7 (S220L) mutation causes a conformational change in ADR1 that may mimic ADR1 structure, either partially or completely, under ethanold growth conditions. In such a case, the effect of a lack of cAPK activity on ADR1 activity and ADH2 expression might become discernible. Strain 409-TC carrying the ADR1-7 allele and a single functional TPK gene (TPK2) was analyzed (Table 6). ADH2 expression decreased about twofold under glucose growth conditions when the BCI gene was disrupted (6) (Table 6). When the remaining TPK gene was mutated (allele spk3d11) to generate a yeast strain containing a measurable cAPK activity (data not shown), ADH2 expression increased to a level that was 15-fold higher than that found in the parental strain containing a functional TPK3 allele. ADH2 expression also increased under ethanol growth conditions (Table 6). A similar increase (eightfold) in ADH2 expression under glucose growth conditions was obtained with another strain carrying the ADR1-7 allele and lacking cAPK activity as demonstrated with a second set of ethanold strains (Table 6), confirming that these results are not strain dependent. These results are in splendid agreement with the view that the ADH1 gene is regulated by the cAPK activity, which is activated in a similar manner. A strain lacking the cAPK activity in an ADR1-7 background expressed the ADR1 gene, the ADH2 activity under glucose growth conditions, and ethanol growth conditions to the same extent, strain containing the ADR1-7 allele (Table 6). These observations are not limited to ADR1 alleles, since similar results were obtained in an ADR1-7 containing strain that carried only spk1 or spk3 gene disruption, and a spk1 allele (Table 6). In addition, we tested whether the BCI disruption results in high activities under glucose growth conditions by forming strain 407-TC, which lack cAPK activity, with a plasmid containing the BCI gene (6). Elevated levels of ADH2 expression were still observed in a strain with the ADR1-7 allele that lacked cAPK activity but contained a functional BCI gene (Table 6).

In order to examine whether the effects described above were the result of the ADR1 alleles or were caused by a general increase in ADH2 transcription, the effects of a lack of cAPK activity on a strain carrying the cpl1 allele was also analyzed. cpl1 mutations under glucose growth conditions enhance ADH2 transcription in an ADR1-independent manner to a level similar to that observed with an ADR1-7 allele (14). Deletion of the BCI1 gene in cpl1 background resulted in a 25% reduction in ADH2 expression in glucose-grown conditions, as compared with that of the parental strain (Table 6). When the TPK3 allele was also mutated, a return to a level of ADH2 expression comparable with that observed in the strain containing cAPK activity was obtained (Table 6). These results are essentially the same as those observed with a strain carrying the wild-type BCI1 and ADR1 genes (Table 5). The ADH1 gene expression that is observed with lack of cAPK in an ADR1-7 background is, therefore, not the result of a general elevated level of ADH2 transcription. Instead, it is dependent on the ADR1-7 allele. These results suggest that cAPK inactivates ADH2 transcription through a site separate from Ser-220 of ADR1 and that the structure of the region around Ser-220 (as in the ADR1-7 allele) determines this effect of cAPK on ADH2 expression.

Deletion of the ADR1 region confers an ADR1 phenotype. The results described above suggest a lack of correlation between ADR1 mutations and cAPK phosphorylation of Ser-220 but do reveal the importance of the sequence of the ADR1 region in ADH1 function. An internal in-frame deletion between amino acids 220 and 262 of ADR1, the entire ADR1 region, was constructed and integrated as a single copy into the genome to address this question. The resulting ADR1-2036 allele was found to result in an ADH1 phenotype that was very similar to that obtained with the ADR1 mutations (Table 3). Under glucose growth conditions, ADH1 expression in the strain carrying the deletion was observed to be 20-fold higher than that found in a wild-type strain. We also observed that this effect of ADR1-2036 on ADH1 expression was not a result of a corresponding increase in the level of ADH1 protein as quantitated by immunoprecipitation of ADR1-2036 (data not shown) (6). The observation that the deletion of the residues between 220 to 262 resulted in elevated ADH1 expression suggests, therefore, that the ADR1 region plays a regulatory role in ADH1 function. These results suggest the model that
the ADRR mutations enhance ADR1 activity by altering the structure of the region and thereby its contact with another protein or another region of ADR1.

**DISCUSSION**

Our previous studies indicated that ADRR mutations reduce or eliminate cAPK phosphorylation of Ser-230 in vitro. Unregulated cAPK was subsequently shown to inhibit ADR1 expression in an ADR1-dependent manner. ADRR mutations displayed reduced sensitivity to unregulated cAPK activity. These results suggested that cAPK inactivates ADR1 by inhibiting ADR1 function through phosphorylation of Ser-230.

The data we present here, however, do not support the model that the ADRR mutations inactivate ADR1 function by solely affecting cAPK phosphorylation. We observed that several newly characterized ADRR mutations did not affect the ability of cAPK to phosphorylate Ser-230 on synthetic peptides modeled on the ADR1 region. Most importantly, we found that strains lacking cAPK activity did not overexpress ADR1, suggesting that the lack of cAPK caused an increase in ADR1 activity. This result supports the observation that the cAPK regulation of ADR1 does not occur strictly through phosphorylation of Ser-230. Thus, the previous model suggesting that Ser-230 of ADR1 was the site of this mechanism is probably incorrect.

Our previous papers (6, 16, and the present data [Tables 3 and 4]) clearly indicate that cAPK inhibits ADR1 expression. This inhibition is physiologically relevant and is not an artifact of unregulated cAPK activity. This inhibition is observed by the observation that lack of ADR1 activity augmented ADR1 expression in an ADR1 background. We now have also established that ADR1 function is required for inactivation of unregulated cAPK activity on the ADR1 promoter. These results imply that the ADR1 promoter phosphorylation of either ADR1 directly or in an ADR1 region required for ADR1 activity. The role of the other ADR1 promoter protein, however, remains unknown. The two other protein kinases (SNF1 and SIC1) which control ADR1 expression are not involved in the inactivation of cAPK activity. Both proteins have been shown to act independent of cAPK and ADR1 in controlling ADR1 expression. This previous observation that ADR1 expression was low was due to unregulated cAPK activity, not ADR1 activity. It has been postulated that Ser-230 was the site of cAPK regulation in vivo. This clearly cannot be the case as described above. ADR1 protein production instead is due to bypassing the effects of unregulated cAPK on ADR1 expression by altering ADR1 structure and hence responsiveness at other sites. Alternatively, the mutated proteins may reduce cAPK effects by affecting the functions of proteins required for ADR1 action. In either case, the mechanism by which ADRR mutations activate ADR1 is distinct from that exercised by cAPK in controlling ADR1 expression.

In addition, because a lack of cAPK activity did not release ADR1 expression from glucose-repression in an ADR1 background, cAPK does not appear to mediate the glucose-to-ethanol transition in controlling ADR1 expression. Other factors must first turn on these effects. This suggestion is consistent with the observation that ADR1 must be in an altered structural state for cAPK to affect ADR1 expression. cAPK appears, therefore, to control the level of ADR1 activity but not necessarily the carbon source regulation of ADR1.

How do ADRR mutations enhance ADR1 function? They do not affect cAPK phosphorylation of Ser-230. ADRR alterations could be affecting the activity of a protein kinase other than cAPK to phosphorylate Ser-230. However, the phosphorylation state of Ser-230 may be only one factor that contributes to the integrity and function of the ADR1 region, since we observed that several ADRR mutations resulted in higher ADR1 expression than ADR1, which cannot be phosphorylated at Ser-230 (Table 3). Because we demonstrated that deletion of the ADR1 region (removal of amino acids 270 to 262) conferred an ADR1 phenotype, the region where these mutations are located represents an inhibitory domain of ADR1. This domain may bind a repressor (see Fig. 2B), which maintains ADR1 in an inactive state through inhibition of its ability to activate transcription. ADR1 alterations would prevent the repressor from binding to ADR1. Alternatively, the ADR1 domain may block the ability of another region of ADR1 to activate transcription through an intragenic interaction (Fig. 2A). We favor the latter model for two reasons. There is no genetic evidence indicating the presence of a repressor that binds ADR1. Also, overexpression in S. cerevisiae of the protein encompassing the ADR1 region was not observed to affect ADR1 expression, suggesting that there is no titratable repressor that binds to ADR1 (unpublished observations). In either case, the fact that all ADRR mutations were observed to occur in a 13-amino-acid stretch between residues 239 and 263 indicates that this region is structurally important in maintaining ADR1 in an inactive state.

Many other enzymatic transcriptional activators contain regions which serve to inhibit their ability to carry out transcription. These include the yeast activators GAL4 (25, 27).
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APPENDIX B
Dissection of the ADR1 Protein Reveals Multiple, Functionally Redundant Activation Domains Interspersed with Inhibitory Regions: Evidence for a Repressor Binding to the ADR1′ Region†

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The yeast transcriptional activator ADR1 is required for expression of the glucose-repressible alcohol dehydrogenase gene (ADH2), as well as genes involved in glycerol metabolism. The N-terminal half of the ADR1 protein was shown to contain three separate transactivation domains, including one (TADII) that encompasses the zinc finger DNA-binding domain. While TADII and TADIII were shown to be functionally redundant in activating ADH2 expression, deletion of only TADII impaired ADR1 control of glycerol metabolism genes. None of these activation domains appeared to be carbon source regulated when removed, increased ADR1 activity; one was localized to the site of ADR1′ mutations (residues 227 to 239) that allow glucose-insensitive ADH2 expression. The 227-to-239 region blocked ADR1 activity independently of the TAD present on ADR1, ADR1 DNA binding, and specific ADH2 promoter sequences. In addition, this region inhibited the function of a heterologous transcriptional activator. These results are consistent with the existence of an extragenic factor that binds the ADR1′ region and represses ADR1 activity and suggest that other factors are responsible for aiding ADR1 in the carbon source regulation of ADH2.

In Saccharomyces cerevisiae, the transcriptional activator ADR1 is required for transcription of ADH2 (13), which encodes glucose-repressible alcohol dehydrogenase (ADH2), and genes required in glycerol metabolism (2, 31) and for peroxisome function and biogenesis (56). ADR1 binds to a palindromic sequence (designated UAS1) located between bp −271 and −294 upstream of the ADH2 translational start site (15) and does so as two monomers (35). In addition to ADR1 binding to UAS1, full activation appears to require binding of an unidentified factor to UAS2, a sequence just upstream of UAS1 (43). ADR1, a protein of 1232 amino acids, contains several functional regions. These include a nuclear localization sequence which encompasses amino acids 1 to 16 (39), a zinc finger DNA-binding domain encompassing amino acids 99 to 155 (39), and at least two regions involved in transcriptional activation, amino acids 76 to 172 and 262 to 642 (2, 39).

In addition to these regions required for ADR1 function, amino acids 227 to 239 of ADR1 play a role in inhibiting ADR1′ activity (17). This regulatory region was identified following the isolation of dominant ADR1′ mutations within residues 227 to 239 which allowed glucose-insensitive ADH2 expression (5, 16, 17). Deletion of the 220-to-262 region also caused an ADR1′ phenotype (17), indicating that this region functions to inhibit ADR1′ activity. ADR1′ mutations do not affect ADR1 RNA (5, 16) and protein abundance (57, 47) or ADR1 ability to bind DNA (5, 37). Two mechanisms have been proposed to explain how the 227-to-239 region inhibits ADR1′ function (17). The 227-to-239 region may intramurally bind another region of ADR1 and inhibit or facilitate an ADR1′ activation domain. Alternatively, a repressor may bind the 227-to-239 region and inhibit ADR1′ function.

The role of ADR1′ in the carbon source regulation of ADH2 has also been studied. ADR1′ mRNA levels are repressed twofold by glucose (16, 40), presumably as a result of increased mRNA degradation (7), and glucose represses ADR1 translation as an additional five to eightfold (49). CAM-dependent protein kinase also inhibits ADR1′ function (5, 12), although whether it phosphorylates ADR1′ directly or another factor remains unclear (17).

In the present study, we investigated the functional and regulatory regions in ADR1. Deletion analysis, in conjunction with in vitro transcription assays, was used to identify functional regions. By coupling these two techniques with analysis of both the ADR1 and ADR1′ proteins, we were able to identify three transcriptional activation regions in ADR1, none of which were carbon source regulated, and demonstrate that the ADR1′ mutations enhance ADR1′ activity independently of the transcriptional activation domain present, ADR1 binding to DNA, or the ADH2 promoter context. Moreover, the 227-to-239 region of ADR1′ was found to inhibit a heterologous transcriptional activator. These data support a model in which a repressor binds the 227-to-239 region of ADR1 and inhibits ADR1′ function.

MATERIALS AND METHODS

Yeast strains. Strain 300-16, MATa ura1 his4 trpl ade2-1 ade1-11 ade1-1 ade2, was used for the integration of deleted and truncated alleles of ADR1. Strain 237-1b-19, MATa ura1 his4 trpl kan1 ade2-1 ade1-11 ade1-1 ade2 trpl1, was used for transformation.

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with plasmids expressing Lex::ADRI fusion proteins. Strain 237-b is the same as 237-b-10, except that it carries \( A D R I \). Strain EGY18, \( N a t A u r a l a u r a l t r p 1 e x a 1 L e u 2 \), was also used for transformation with plasmids expressing Lex::ADRI fusion proteins. Fusion proteins expressed in 237-b-10 gave results similar to those obtained with strain EGY18.

Plasmid constructions. (i) \( A D R I \) deletions. All \( A D R I \) deletion alleles were made with \( A D R I \) sequences derived from plasmid CD10 (formerly designated YP7-ADR1-25A (\( A D R I I \), CD10-5') (\( A D R I I \), CD10-5') or CD10-5' (\( A D R I I \), CD10-5') (15) inserted into the pUC18 or pUC19 vector. All contained at least 1.2 kb of \( A D R I \) upstream DNA, which is sufficient for normal \( A D R I \) expression (11). Each \( A D R I \) gene is designated by the number of \( A D R I \) amino acids it encodes (e.g., \( A D R I - 5 7 1 \) encodes the N-terminal 571 amino acids of \( A D R I \)), and deletions are given in terms of \( A D R I \) amino acids flanking deleted residues (e.g., \( A D R I - 2 6 9 5 8 \) encodes the complete \( A D R I \) protein containing an in-frame deletion of amino acids 263 to 287). Sequencing of selected junctions was done as previously described (35) when proteins were not visualized by Western (immunoblot) analysis or were of an unpredicted size. Visualization of \( A D R I \) proteins was done by Western analysis as previously described (40).

(ii) C-terminal truncations. The construction of plasmids containing the \( A D R I - 6 2 2 \), \( A D R I - 2 9 2 \), \( A D R I - 2 6 2 \), and \( A D R I - 2 2 0 \) alleles has been described previously (2). The \( A D R I - 7 9 7 \) versions of these deletion alleles were constructed exactly as described previously for the wild-type alleles, except that \( A D R I - 7 9 7 \) sequences were derived from plasmid CD10-5'. The \( A D R I - 1 0 5 8 \) allele was reconstructed in the present study by digesting CD10 (which contains the entire \( A D R I \) gene plus other yeast-derived sequences 3' to \( A D R I \)) with BanHI (cuts at \( A D R I \) bp +3200), filling in the ends with the large subunit of DNA polymerase (Klenow), and religating. This deletion allele encodes one extra non-\( A D R I \) amino acid (a leucine). \( A D R I - 7 0 9 \) was constructed by digesting CD10 with BanHI (cuts at \( A D R I \) bp +3219) and religating; four non-\( A D R I \) amino acids, SKO, are encoded at the C terminus. \( A D R I - 5 7 1 \) was constructed by ligating a 4.8-kb \( A u r I - S r l 1 \) fragment isolated from CD10 which contains the TRP1 gene and the N-terminal 571 codons of \( A D R I \) into pUC19 digested with \( A u r I \) and \( S r l 1 \); the extra amino acids, KFYGIL, derived from the pUC19 polynucleotide are encoded at the C terminus. \( A D R I - 3 2 9 \) was constructed by digesting plasmid \( A D R I - 5 7 1 \) with NdeI (at \( A D R I \) bp +987) and PmaI (at \( A D R I \) bp +1193), filling in the recessed ends with Klenow, and religating. This introduced a stop codon at \( A D R I \) codon 330. The \( A D R I - 5 7 1 \) version of \( A D R I - 1 0 5 8 \) and the \( A D R I - 7 9 7 \) versions of \( A D R I - 7 0 9 \), \( A D R I - 5 7 1 \), and \( A D R I - 3 2 9 \) were constructed exactly as described above, except that \( A D R I - 7 9 7 \) sequences were derived from CD10-5' or CD10-5'.

(iii) Internal deletions. The construction of \( A D R I - 2 2 0 / 2 6 3 \) has already been described (17). \( A D R I - 2 6 2 / 2 8 8 \) was constructed by first ligating a 2.3-kb XbaI (cuts at \( A D R I \) bp +661)-BanHI (cuts in 3' vector sequences) fragment derived from CD10 into pUC18, which was digested with XbaI and BamHI, to produce WC28. A 3.9-kb \( A u r I \) (cuts in pBR322 vector sequences)-XmaI (cuts at \( A D R I \) bp +786) fragment derived from CD10 was then ligated into WC28, which was digested with \( A u r I \) and HindIII (in the pUC18 polynucleotide) to produce ADRI-262/288. The protein encoded by this plasmid contained one non-\( A D R I \) amino acid (a threonine) derived from the pUC18 polynucleotide between \( A D R I \) residues 262 and 288. ADRI-1:262/288 was constructed by replacing the 3.9-kb XmaI (cuts in the TRP1 gene)-SacI (cuts at \( A D R I \) bp +1713) fragment in plasmid ADRI-571 with the analogous XmaI-SacI fragment isolated from ADRI-262/288. ADRI-262/288 was constructed by first transferring the 4.6-kb BamHI fragment from CD10 (formerly called YPH-ADR1-571 (11), which contains \( A D R I \) bp +157) or SacI-BamHI (cuts at \( A D R I \) bp +765) fragment derived from CD10 into plasmid ADRI-571, which was digested with SstI and Smal (cuts in the pUC19 polynucleotide) to produce plasmid WC13. A 0.7-kb fragment was subsequently isolated from ADRI-571 following digestion with NdeI (cuts at \( A D R I \) bp +587), which recessed ends were filled in with Klenow, and religating with Sall (cuts in the pUC19 polynucleotide). This 0.7-kb fragment was ligated into plasmid WC13, which was first digested with BanHI (cuts in the pUC19 polynucleotide) and whose ends were made flush with Klenow, and then Sall (also cuts in the pUC19 polynucleotide) to produce ADRI-1:262/288. ADRI-1:262/288 was constructed by first ligating a 3.5-kb XmaI-XmaI (cuts at \( A D R I \) bp +765) fragment derived from CD10 into plasmid ADRI-571, which was digested with SstI and Smal (cuts in the pUC19 polynucleotide) to produce plasmid WC13. A 0.7-kb fragment was subsequently isolated from ADRI-571 following digestion with NdeI (cuts at \( A D R I \) bp +587), whose recessed ends were filled in with Klenow, and digestion with Sall (cuts in the pUC19 polynucleotide). This 0.7-kb fragment was ligated into plasmid WC13, which was first digested with BanHI (cuts in the pUC19 polynucleotide) and whose ends were made flush with Klenow, and then Sall (also cuts in the pUC19 polynucleotide) to produce ADRI-1:262/288. 

(iv) Lex::ADRI fusions. Lex::ADRI fusion proteins were constructed by inserting ADRI fragments into polynucleotide sites in vector LexA(1-382)+PL or, in some cases, LexA(1-382) under the control of the ADRI promoter (4, 33). We introduced slight alterations in the polynucleotide sites in plasmid LexA(1-382)+PL (pLCA021-1) to facilitate in-frame fusions of LexA and ADRI sequences (Fig. 1A). Plasmid LexA(1-382) was constructed by replacing the EcoRI-BamHI fragment derived from pGEM(32F)+(+) (Promega, Madison, Wis.)

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FIG. 1. Plasmids used in the study of transcriptional activation by LexA-ADRI fusion proteins. (A) LexA operator (op)-controlled reporter plasmids. Plasmid 1840 was used to measure transcriptional activation by LexA-ADRI fusion proteins, the second and third plasmids were used to assay the activity of LexA-ADRI fusions to bind DNA, and the fourth plasmid was used as a control.

pLexA202-3 was constructed by filling in the EcoRI site in pLexA202-2 with Klenow. pLexA202-4 was constructed by replacing the EcoRI-SalI fragment of pLexA202-1 with an EcoRI-SalI fragment derived from pGEM-3Zf(+) (+). pLexA202-5 was constructed by filling in the EcoRI site in plasmid pEC202 (a kind gift of Roger Brent). For LexA-ADRI fusion proteins that contain ADRI amino acid 1, ADRI sequences were derived from plasmid JS119, which contains an NcoI site introduced by site-directed mutagenesis at the start of translation (5). Plasmid LexA-ADRI-1-1223 was constructed by isolating a 4.4-kb NcoI-BstEII fragment (ADRI bp +1 to +4370) from plasmid AK52 which contains the synthetic NcoI site and the entire ADRI gene. Following Klenow treatment, the 4.4-kb fragment was ligated into pLexA202-1, which was digested with EcoRI and blunt ended with Klenow. The LexA-ADRI-1-1223 protein contains the four intervening residues, RPEF, derived from polynucleotide sequences between LexA and ADRI residues. LexA-ADRI-1-642 was constructed by digesting plasmid JS119 with EcoAI (cuts at ADRI bp +1193), blunt ending with Klenow, and digesting with NcoI (cuts at ADRI bp +1). This 1.2-kb fragment was inserted into pLexA202-2, which was digested with XhoI, blunt ended with Klenow, and then digested with NcoI. The LexA-ADRI-1-359 protein contains the nine intervening residues ELIFGDFEST and the seven non-ADRI amino acids VDVQLSP at the C terminus. LexA-ADRI-1-230 was constructed by inserting a 668-bp BglII-SalI fragment (ADRI bp +18 to +660) into pLexA202-7 (identical to pLexA202-2, except that it contains only the first 87 amino acids of LexA), which was first digested with SalI, blunt ended with Klenow, and then cut with BamHI. The LexA-ADRI-1-220 protein contains the 13 intervening residues RPEFELGTGNSIT and the 6 non-ADRI amino acids FDLOPS derived from ADHI terminator sequences at the C terminus. LexA-ADRI-1-485-359 was constructed by inserting a 630-bp BglII-BamHI fragment (ADRI bp +440 to +1074) isolated from CD10 into pLexA202-1, which was digested with BamHI. The LexA-ADRI-1-485-359 protein contains the 6 intervening residues RPEFEPFG and the 16 extra C-terminal residues PSTCRQANSGRISYDL. LexA-ADRI-1-359-740 was constructed by first ligating a 2.1-kb BglII-BamHI fragment (ADRI bp +440 to +720) isolated from CD20 into pLexA202-3, which was digested with BamHI to produce plasmid DC11. DC11 was then digested with EcoRI, which cuts at ADRI bp +2119 and +3185, treated with Klenow, and religated to produce LexA-ADRI-1-359-740. The LexA-ADRI-1-359-740 protein contains the 7 intervening residues RPELIPFG and the 16 extra C-terminal residues ILOSSGSGDLQPS. LexA-ADRI-1-359-396-597-740 was constructed exactly as described for LexA-ADRI-1-359-740, except that ADRI sequences were derived from plasmid ADR-1-359-507+71B. LexA-ADRI-1-482-1323 was constructed by digesting CD30 with BstEII (ADRI bp +1570), blunt ended with Klenow, and then recutting with BglII (cut at ADRI bp +1923); this 2.4-kb fragment was then ligated into pLexA202-3, which was cut with SalI, blunt ended with Klenow, and then cut with BamHI. The LexA-ADRI-1-1323 protein contains the seven intervening residues RPELIPFG and the wild-type stop codon. LexA-ADRI-1-220-1323 was constructed by inserting a 3.7-kb SalI-BstEII fragment (ADRI bp +660 to +4570) fragment isolated from CD20 that had been blunt ended with Klenow into pLexA202-3, which was digested with BamHI and treated with Klenow. The LexA-ADRI-1-220-1323 protein contains the nine intervening residues RPELIPFGDH and the wild-type stop. LexA-ADRI-1-220-262-359 was constructed in the same manner as LexA-ADRI-1-484-148-359, except that ADRI sequences were derived from plasmid ADR-1-220-262 (17). LexA-ADRI-1-148-262-285-359 was similarly constructed, except that ADRI sequences were derived from plasmid ADR-1-262-285. LexA-ADRI-1-148-262 was constructed by ligating a 342-bp BglII (ADRI bp +440)-XmnI (ADRI bp +785) fragment into pLexA202-1, which was first digested with SalI, blunt ended with Klenow, and then digested with BglII. The LexA-ADRI-1-148-262 protein contains the 6 intervening residues RPEFPG and the 20 extra C-terminal residues FRPAKLIPGEFLMY OFY. LexA-ADRI-1-304 was constructed by ligating a 458-bp BglII (ADRI bp +440)-BamHI (cuts at ADRI bp +912) fragment isolated from YRP7-ADRI-311 (11) into pLexA202-1, which was digested with BamHI. The LexA-ADRI-1-304 protein contains the six intervening residues...
PPEPFG and the six extra C-terminal residues VH S G P S. 

LexA-ADR1-418-337 was constructed by removing the LexA fragment from pLexA-ADR1-418-359 that contains codons 145 to 337 and ligating this into LexA-202-c cut with EcoRII. The LexA-ADR1-418-337 protein contains the six intervening residues PPEPFG and the nine extra C-terminal residues GSSGSDPS. LexA-ADR1-263-359 was constructed by taking a 785-bp fragment from LexA-ADR1-265-359 cut with BamHI (cuts at the junction of codons 220 and 265) and SalI (cuts 3' to ADR1 sequences) and ligating it into pLexA-202-c cut with BamHI and SalI. The LexA-ADR1-263-335 protein contains the 12 intervening residues PPEEFECGRGS and the 16 extra C-terminal residues PSTCRCQNSGRISYDL. Plasmids LexA-ADR1-418-337, LexA-ADR1-418-335, LexA-ADR1-5' 1-322, and LexA-ADR1-5' 1-337, and LexA-ADR1-415-202-268-359 were constructed exactly as described for their wild-type counterparts, except that ADR1-5' or ADR1-7 sequences were derived from C107, C107, or JC105 (8).

LexA-ADR1-B42 was constructed by inserting an Smal-HindIII fragment (codons 148 to 262) from LexA-ADR1-418-260-289 and LexA-202-289 at the EcoRI site located between LexA and B42 sequences in plasmid pMA57, which contains only the LexA binding domain of LexA (residues 1 to 87) (27, 33).

LexA-ADR1-418-260-289-339 was cut with EcoRI and the 418-260-289-339 plasmid was constructed in the same manner, except that sequences were derived from LexA-ADR1-418-262-257-359. The LexA-ADR1-B42 protein contains the extra eight amino acids RPELGFIRH between LexA and ADR1 residues and the six extra amino acids TLEPFG between ADR1 and B42. LexA-ADR1-418-262 and its ADR1-7 counterpart were derived from the corresponding LexA-ADR1-418-242 constructs by cutting with EcoRIII and NotI, filling in with Klenow, and religating. The LexA-ADR1-418-262-339 protein contains the eight intervening residues RPELGFIRH and the nine extra C-terminal residues TLEPFGDD.

The transcriptional activity of LexA-ADR1 fusion proteins was measured by assaying β-galactosidase activity as an indicator of LexA expression from the 1840 reporter plasmid, which contains only the LexA operator upstream of a UAS-less GAL1 promoter controlling the lexA gene (16). Other variants of the 1840 reporter plasmid that were used (also diagrammed in Fig. 1B) included JK101, which contains two LexA operator sites placed between the GAL4 UAS and the TATA sequence of the GAL4 promoter (25), Δ20B, which is the same as JK101 except it lacks the LexA operator (42), and LR141, which is the same as 1840 except that it contains no LexA operator sites (42). The abundance of LexA-ADR1 fusion proteins was determined by Western analysis with a LexA antibody as previously described (40).

Transformations. All yeast transformations were conducted by using the lithium acetate method as previously described (23). Linearized ADR1 and ADR1' deletion plasmids were site specifically targeted for integration at either the ADR1 or the ADR1' locus. The methods used to identify integration of the plasmids and determine the number of ADR1 or ADR1' genes integrated have been previously described (11).

RESULTS

Carboxy-terminal deletions of ADR1 and ADR1' proteins. We previously used C-terminal deletions of the ADR1 protein to identify regions of the protein which affected the ability of ADR1 to activate ADR2 transcription when removed (2). We extended these studies by constructing the following four additional C-terminal deletion alleles to better define the importance of residues between residues 263 and 320 of ADR1: ADR1-1086, ADR1-1069, ADR1-571, and ADR1-329. These truncated ADR1 genes were targeted for integration at the m2 locus, and a range of integrated dosages were obtained and analyzed as described previously (2). The activity of each of the C-terminal truncations at a single dose and the dosage required to reach half-maximal ADH3 activity (defined as K50) are given in Table 1. Except for ADR1-571, ADR1-329, and ADR1-282, the K50 values were quite similar, suggesting that the proteins' stability and ability to bind DNA were unaffected by the C-terminal truncations. The higher K50 values for ADR1-571, ADR1-329, and ADR1-282 are presumably due to protein stability, as evidenced by their lower concentrations as determined by

| Table 1: ADH3 enzyme activity in strains containing single gene copies of ADR1 or ADR1' truncated alleles |
|---------------------------------|-----------------|
| **Defect** | **Δ[ADH3]** |
| ADR1-1132 | 2.49 | 3.3 |
| ADR1-1069 | 2.49 | 3.3 |
| ADR1-1086 | 1.75 | 1.4 |
| ADR1-142 | 1.26 | 1.7 |
| ADR1-329 | 1.26 | 1.7 |
| ADR1-282 | 1.26 | 1.7 |
| ADR1-571 | 1.26 | 1.7 |
| ADR1-329 | 1.26 | 1.7 |

* The strain number denotes the number of ADR1 or ADR1' gene inserted into the genome. All strains are isogenic to S1565.

* ADH3 activity values were measured following growth of yeast strains in YEP medium containing 5% ethanol. Yeast cells were grown to mid-log phase and then treated with ADH3 activity as a function of ADR1 or ADR1' gene dosage by using the Enzolite computer program.

* K50 values are from reference 2.

* ND, not done. A single copy of the ADR1-329 gene integrated into the genome was not obtained.

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FIG. 2. Effects of ADR1-709 and ADR1-7-709 gene dosage on ADHIII activity. (a) ADHIII activity is given as a function of ADR1-709 (C) and ADR1-7-709 (D) gene dosage in strains isogenic to 500-16 and containing multiple copies of these two alleles. Growth was in YEP medium supplemented with 2% ethanol. Determination of ADHIII activity and ADR1 dosage is described in Materials and Methods. ADHIII activity values represent averages of at least three separate determinations, and standard errors of the means were less than 5%. All strains were isogenic to 500-16. The fact that ADR1-7-709 had a higher K<sub>t</sub> than ADR1-709 was distinctive for this construction. All of the other ADR<sup>+</sup> C-terminal truncations displayed lower K<sub>t</sub> values than did their ADR1 counterparts. (b) ADHIII activity is given as a function of ADR1-709 (C) or ADR1-7-709 (D) gene dosage following growth in YEP medium supplemented with 8% glucose. ADHIII activities and ADR1 dosage were determined as described for panel a. Standard errors of the means were ±2%, except when activity values were <10 mU/mg. Strains containing 33 and 41 copies of ADR1-7-709 alleles were not used in this analysis because ADHIII activity did not increase linearly at these high gene dosages for this allele. This finding was also obtained with other highly active ADR<sup>+</sup> deletion alleles.

Western analysis and/or immunoprecipitation (data not shown). Because of the apparent differences in protein stability between different ADR1 proteins, we used the maximal activation ability of the ADR1 proteins to obtain a more meaningful comparison of their intrinsic activities.

The maximal ability of each ADR1 allele to activate ADH2 under ethanol growth conditions was determined by plotting ADH1 gene dosage against ADHIII activity. A graphical representation of a typical analysis is depicted in Fig. 2a. Under nonfermentative growth conditions, when high ADR1 protein concentrations saturate the ADH2 promoter, maximal ADHIII activity becomes the direct measure of the activating potential of the ADR1 protein independent of its protein concentration, stability, or DNA-binding capability (2, 11). In Fig. 3, maximal ADHIII activity was plotted as a function of ADR1 polypeptide length. In agreement with previous results (2, 39), no single region of ADR1 was solely responsible for ADR1 activation ability. Instead, sequential removal of the following five regions of ADR1 significantly reduced the ability of ADR1 to activate: residues 709 to 1068, 329 to 751, 262 to 282, 220 to 262, and 151 to 220 (required for DNA binding) (39). Removal of residues 1068 to 1323 increased the ADR1 activation potential, suggesting that the region between 151 and 220 inhibits the function of ADR1.

A similar set of deletions using the ADR<sup>+</sup> allele was constructed to identify sequences important to the ability of the ADR<sup>+</sup> mutation to enhance the function of ADR1. The maximal ADHIII activities under ethanol growth conditions, as determined for each truncated ADR<sup>+</sup> allele, were plotted as a function of ADR<sup>+</sup> length in Fig. 4. In each case, the ADR<sup>+</sup> allele allowed a higher maximal level of ADH2 expression than did the corresponding ADR1 allele (Fig. 4).

We extended this comparison of ADR1 and ADR<sup>+</sup> proteins under glucose growth conditions. The relative abilities of ADR1 and ADR<sup>+</sup> to activate ADH2 were contrasted by plotting ADHIII activity as a function of ADR1 or ADR<sup>+</sup> gene dosage for strains containing multiply integrated copies of carboxy terminally deleted ADR1 and ADR<sup>+</sup> alleles. A sample of this analysis is shown in Fig. 2b, in which the ADR<sup>+</sup>-709 allele at each gene dosage allowed greater ADHIII activity than did the comparable ADR1-709 allele. A similar analysis was conducted with other ADR1 and ADR<sup>+</sup> alleles. To compare the activation abilities of the ADR1<sup>+</sup> proteins with those of their ADR1 counterparts, we obtained in each case a value for the increase in ADHIII activity as a
FIG. 4. Maximal ADHII activity as a function of ADRI and ADRII polyepitide lengths. Maximal ADHII activities were calculated for ADRII carboxyl-terminal deletion proteins (dashed line) as described in the legend to Fig. 2 and are compared to values for ADRI deletion proteins (solid line) taken from Fig. 3. The polypeptide lengths plotted for ADRII proteins are as follows (left to right): 262, 698, 751, 709, 1,568, and 1,233 amino acids.

function of gene dosage. This value would, therefore, indicate how well the ADRI or ADRII protein could activate under glucose-repressed conditions over a range of concentrations and minimize reliance on the data obtained at any particular dosage. For each pair of ADRII and ADRII alleles, the ratio of the increase in ADHII activity as a function of gene dosage is compared in Table 2. In each case, the ADRII protein derivative allowed greater ADHII activity over a range of gene dosage than did the corresponding ADRII form. On the basis of the results presented in Fig. 4 and Table 2, ADRII-262, therefore, contains sequences sufficient to make ADRII more functional than its ADRI counterpart, and residues 262 to 1533 appear not to be absolutely required for the ADRII mutation to overcome the effects of the 277-2059 inhibitory region. Residues 1068 to 1323 appear to play a role in aiding the function of the ADRII protein, since the ratio of ADRII to ADRI activity under glucose growth conditions was greatest for the full-length protein. Alternatively, the 227-2059 inhibitory region may not function as well with each activation region.

**TABLE 2.** Relationship of ADRII to ADRII activity under glucose growth conditions for C-terminal truncations of the ADRI and ADRII proteins.

<table>
<thead>
<tr>
<th>C-terminal truncation</th>
<th>Ratio of ADRII to ADRII activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRII-320-953</td>
<td>4.2</td>
</tr>
<tr>
<td>ADRII-953-320</td>
<td>0.4</td>
</tr>
<tr>
<td>ADRII-1068-320</td>
<td>3.3</td>
</tr>
<tr>
<td>ADRII-751-320</td>
<td>9.3</td>
</tr>
<tr>
<td>ADRII-751-320</td>
<td>6.3</td>
</tr>
<tr>
<td>ADRII-751-320</td>
<td>2.0</td>
</tr>
<tr>
<td>ADRII-751-320</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Yeast strains that were isogenic to 500-16 and contained multiple copies of ADRI and ADRII deletion alleles were grown in YEP medium supplemented with 10% glucose. ADRII or ADRII activity was determined by calculating ADRIII enzyme activity as a function of ADRI or ADRII gene dosage under glucose-grown conditions. A sample graphical representation of this comparison is given in Fig. 2b.

Internal deletions of ADRII amino acids 220 to 574. The results shown in Fig. 3 indicate that ADRII amino acids 220 to 574 are of primary importance for activation of ADHII2 transcription, since removal of residues 220 to 574 reduced ADRII activation ability 10-fold, compared with a 1.5-fold reduction following removal of residues 574 to 1223. Internal deletions spanning residues 220 to 574 were constructed to identify functional regions in this area of the protein. ADRII genes with in-frame internal deletions were then directed to the adri-1 locus to create single copies of full-length ADRII alleles containing the internal deletions. The activation ability of each of these deletions, when present as a single copy in the genome, was assayed following growth in either glucose- or ethanol-containing medium. Table 3 shows that single small deletions across the 220-574 region in the full-length ADRII protein did not severely impair ADRII activation ability. Deletion of residues 262 to 288, encompassing a putative activation domain identified by C-terminal deletion analysis, had no effect on ADRII function. Deletion of residues between 330 and 507 or 507 and 574, encompassing a second putative activation region, reduced ADHIII levels about 1.7-fold. Deletion of both the 262-288 and 330-507 regions resulted in a more severe effect, in which the ADRII protein was only one-third as functional as the full-length protein. These results suggest that the ADRII protein contains multiple, functionally redundant activation regions, deletion of any one of which has little effect on ADRII function. Western analysis indicated that the ADRII protein concentration was not reduced for those deletions which displayed reduced ADRII activity (data not shown), suggesting that the reductions in activity were due to losses of activation ability rather than effects on protein stability.

In contrast, deletion of two separate regions was found to enhance ADRII activation ability, particularly under glucose growth conditions: residues 220 to 263 (17), the site of the

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**ABR1** mutations, and residues 262 to 330 (ala 262 to 330). ADR1 protein abundance under glucose growth conditions, as determined by Western analysis or immunoprecipitation, was not affected by any of the later three deletions (15, data not shown). Since deletion of residues 262 to 330 did not cause a similar elevation of **ADH2** expression (Table 3), residues 258 to 301 appear to be critical within the 262 to 350 region for inhibition of ADR1 function. Because deletions with two different C-terminal endpoints (262 to 350 and 262 to 350) resulted in similar phenotypes, it is also unlikely that the elevation of ADR1 activity is the result of an artificial construction of a new protein domain. Deletion of residues 262 to 350 in combination with an ADR1-7 mutation, however, resulted in only an additive increase in function over that observed with just the ADR1-7 allele (Table 3). This result suggests that the ADR1-7 mutation and the deletion of residues 258 to 350 may allow enhanced ADR1 function by a common mechanism. 

We were unable, however, to reproducibly obtain multiple integrated copies of full-length ADR1 alleles containing the internal deletions. Because the ADR1-571 proteins retained most of the activation ability of the full-length ADR1 protein, we determined the effect of the small internal deletions across the 262 to 350 region within the ADR1-571 protein. This analysis would also allow assessment of the effect of removal of residues in this region without the presence of the C-terminal half of the protein. Deletion of residues 330 to 397 in the ADR1-571 protein resulted in significantly reduced activation when either a single gene copy or maximal ADH31 activity was compared (Table 3). Deletion of residues 359 to 397 also reduced ADR1-571 function but to a lesser extent. In addition, deletion of the 262 to 258 region had a small effect on ADR1-571 activity but not nearly to the extent observed for the 262 to 350 deletion. These results indicate that the 262 to 263 and 220 to 258 regions of ADR1 are important to its function, especially in the absence of the latter half of the ADR1 protein. We also found that under glucose growth conditions, the ADR1-1-1-262-258-571 and ADR1-1-262-397-571 proteins were nine- and fourfold more active, respectively, than ADR1-7 when ADH2 activity as a function of gene dosage was compared in strains containing a single copy of each (data not shown). Since deletion of residues 262 to 288 did not similarly increase ADR1 activation ability under glucose growth conditions (data not shown), these results support the identifications of residues 262 to 300 as encompassing a region that inhibits the ability of ADR1 to activate.

To summarize the results obtained by deletion analysis of the ADR1 and ADR1-7 proteins and to combine these results with previous findings, several regions important to ADR1 function were identified. A transcriptional activation domain must be present in the N-terminal 220 amino acids (2), and it most likely centers around residues 76 to 172 (39) since these regions by themselves can activate **ADH2** expression. Residues 262 to 288, 330 to 397, and 709 to 1006 were also found to be important for activation of transcription. However, deletion of any one of the latter three regions in the full-length protein had, at most, a twofold effect on ADR1 activity, indicating that these sequences are functionally redundant. The C-terminal region from residues 571 to 1353 also contributed to the ADH2 promoter activity of the ADR1-7 protein. Two regions which increased ADR1 activity when removed were localized to residues 227 to 299 (17) and 258 to 330. Moreover, the ADR1-7 mutation enhanced ADR1 activity even when the C-terminal 262 to 1353 region of ADR1 was removed.

Only ADR1 amino acids 399 to 574 are required for growth on glycerol as a carbon source. In an earlier study, it was found that a functional ADR1 gene is required for S. cerevisiae to grow in medium containing glycerol as the sole carbon source irrespective of **ADH2** expression. The minimum ADR1 protein that allowed growth under glycerol growth conditions contained the N-terminal 156 amino acids (2). To further identify the ADR1 region involved in controlling glycerol metabolism, we tested the effects of deleting ADR1 amino acids between positions 220 and 574 on this function. Strains expressing full-length ADR1 proteins that contained deletions extending from positions 220 to 574 were grown in YEP medium supplemented with 3% glycerol, and their ability to grow to densities between 5 x 10^6 and 5 x 10^7 cells per ml were determined (2). Deletion of residues between positions 399 and 574 and 570 to 574 eliminated the ability of ADR1 to allow S. cerevisiae to grow on glycerol as a carbon source (Table 3). Sequences in the 399 to 574 region appear to be essential for ADR1 activation of genes involved in growth on glycerol and also appear to be the sequence most important for **ADH2** expression (Tables 3 and 4). These results are in agreement with the finding that ADR1-220 and ADR1-262 showed reduced expression of glycerol kinase (involved in glycerol catabolism) compared with that obtained with ADR1-1-1-262-571.

Use of LexA-ADR1 fusion proteins to define transcriptional activation domains of ADR1. The above described deletion analysis analyzed principally the loss of ADR1 function when a region was deleted. Because of the possibility of artifactual results derived from deletions of proteins, we used a second method to identify ADR1 activation regions. Regions of ADR1 involved in activation of transcription were defined by determining whether different regions of ADR1, when fused to the full-length LexA protein from *Escherichia coli*, were capable of converting LexA into a yeast transcriptional activator (Fig. 5). These studies would allow ADR1 activation ability to be assayed separately from the context of the **ADH2** promoter and independently of ADR1 binding to DNA. Fusion proteins under control of the
FIG. 5. Transcriptional activation by LexA-ADRI fusion proteins. (A) Solid bars represent ADRI protein residues fused to the complete LexA protein, except for LexA-1-220, in which LexA amino acids 1 to 87 were fused to the ADRI portion, and the numbers above the bars refer to ADRI amino acids. Strain 237-1b-10 was grown in minimal medium lacking uracil and histidine and supplemented with 0.5% glucose (Gluc) or with 2% ethanol and 2% glycerol (Ethanol). Each strain carried the 1890 reporter plasmid, which contains one LexA-binding site (Fig. 1B) (5). $beta$-Galactosidase activities (in units per milligram) represent averages of at least three separate determinations, and the standard errors of the means were <.20%, except when activity values were less than 20 Umg, in which case they were <30%. The LexA-ADRI fusion proteins were about 50-fold more abundant than single-copy ADRI as judged by Western analysis. (B) The transactivation ability of ADRI amino acids 143 to 359 was analyzed by use of LexA-ADRI fusion proteins as described for panel A.

ADRI promoter were expressed from 2µm plasmids in strains containing a reporter plasmid which contained one LexA operator placed upstream of a UAS-less GAL1-locZ reporter gene (plasmid 1840, 4). Each of the LexA-ADRI fusion proteins, when expressed in S. cerevisiae, had the expected protein size and relative equivalent protein abundance, asverified by Western analysis with an antibody directed against LexA (data not shown). Because the fusion of different segments of proteins to LexA may, in some cases, affect the ability of LexA to bind DNA (20), the interpretation of our results was done in a semiquantitative manner.

A LexA-ADRI fusion protein containing the complete ADRI protein (amino acids 1 to 122) was able to activate transcription of the locZ reporter gene (Fig. 5A). We further localized the activating regions in ADRI by analyzing LexA-ADRI fusion proteins containing smaller ADRI fragments. C-terminal truncations of ADRI were found to reduce LexA-ADRI activity in much the same manner as they affected the ability of ADRI to activate $ADH1$ (compare Fig. 5 and Table 1 with Fig. 5A).

The results of these analyses indicated that 30 levels of transcription were allowed by amino acids 1 to 22, and 11 to 359, while an apparently stronger activating region in ADRI was detected in amino acids 359 to 720. Sequences between 54 to 507 were required for the 556-to-715 region to activate transcription. Residues C-terminal to ADRI amino acid 427, while found to be important in the C-terminal deletion analysis, appeared to be unable to activate transcription in the LexA assay (Fig. 5A). These results verify that multiple activation regions function in ADRI. The same results were obtained when only the LexA DNA binding domain (residues 1 to 87) was fused to the ADRI segments (data not shown). The ability of LexA-ADRI fusion proteins to activate transcription was also unaffected by the presence of the full-length, wild-type ADRI protein (data not shown). LexA-ADRI fusions were also incapable of activating the LR181 locZ reporter gene (Fig. 1B) that lacked LexA operators (data not shown).

Results of the deletion analysis shown in Fig. 3 and Tables 3 and 4 suggested that within the 148-to-359 fragment of ADRI there exist regions that both inhibit and activate ADRI function. We analyzed more closely the 148-to-359 region with the use of LexA-ADRI fusions (Fig. 5H). The activating residues in this fragment were found to reside within amino acids 263 to 359, and sequences between positions 262 and 288 and 304 and 359 were most critical to this activation region. Removal of the inhibitory region encompassing residues 227 to 239 allowed increased activation (compare LexA-ADRI 148-359 with LexA-ADRI 148-200263-359), supporting the identification of the 220-to-263 region as an inhibitory domain (17) (see Table 6).

The following three nonoverlapping activation domains were therefore identified by using this in vivo transcription assay: residues 1 to 220, 263 to 359, and 297 to 720 (residues 399 to 507 were the most important to the latter region). These three regions correspond to the following three of the four regions important to ADRI function that were identified as described above by use of C-terminal and internal deletions of ADRI: residues 76 to 176 (39), 262 to 288, and 304 to 507.

To determine whether any of the ADRI activation domains are carbon source regulated, the activation ability of each of the LexA-ADRI fusions was assayed under nonfermentative growth conditions (Fig. 5). None of the regions of ADRI appeared to be carbon source regulated in that no substantial decrease in activation ability was observed under nonfermentative growth conditions. LexA-ADRI protein concentrations under nonfermentative growth conditions were generally two- to fourfold less than under glucose growth conditions, a result of the use of the glucose-induced ADRI promoter (14), which appeared to be the cause of the lower values observed under nonfermentative growth conditions than under glucose growth conditions. It should be noted that for LexA-1-220, there was no difference in protein concentration between the two growth conditions (data not shown).

Because the inability of some of our LexA-ADRI fusion proteins to activate transcription may have been due to changes in LexA protein conformations that affected their binding to DNA, we tested the abilities of the inactive LexA-ADRI fusion proteins to bind the LexA operator by use of a transcriptional repression or interference assay (20). This assay incorporates reporter plasmid JK101, which

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contains two LexA operators placed between an upstream UASg activator element and a downstream lacZ reporter gene (Fig. 1B). The binding of inactive LexA-ADRI fusion proteins to the LexA operator site interfered with the ability of endogenous GAL4 protein to activate transcription from UASg, albeit at a lower level (Table 5). Fusion proteins LexA-ADRI-145-262, LexA-ADRI-145-359, and LexA-ADRI-145-359/262-740 reduced the ability of GAL4 to activate the LexA gene at least threefold, indicating that LexA-ADRI activity in each of these proteins bound well to the LexA operator. The C-terminal residues of ADRI between positions 42 and 1222, when fused to LexA, interfered with GAL4 activation to a lesser extent. The LexA-ADRI-642-1222 fusion may bind poorly at the LexA operator. Alternatively, it may contain a weak activation domain that can act synergistically with the GAL4 activator, resulting in intermediate enzyme levels. We have observed the latter behavior with other activator sequences fused to LexA (Table 5, LexA-ADRI-145-337, unpublished data).

The ADRI mutation can enhance ADRI activity when ADRI is fused to LexA. The results of the deletion analysis identified the N-terminal 62 amino acids of ADRI as being minimally required for enhanced activation of the ADRI protein. To better define how the ADRI protein enhances activation and to determine whether ADRI promotes other than the ADRI-binding site are required for the ADRI effect, LexA-ADRI-15 (or LexA-ADRI-7) fusion proteins were expressed and assayed for the ability to activate transcription of a lacZ reporter gene (Table 6). The LexA-ADRI-1-5-142, LexA-ADRI-5-145-359, LexA-ADRI-5-145-359, and LexA-ADRI-7-145-262/255-359 fusion proteins were found to be better activators than the same fusion proteins containing wild-type ADRI residues (Table 6). The observed smaller difference between LexA-ADRI-1-5-142 and its ADRI counterpart than for the other constructs may have occurred because these two constructs resulted in nearly maximal levels of lacZ expression. Similar results were obtained when LexA(AS7) ADRI fusions were compared to their ADRI counterparts (data not shown). The only activation domain present in LexA-ADRI-15-145-359 appears to be in the 255 to 359 region (Fig. 5), the ADRI mutation does not require the 60 to 172 activation region to enhance transcription. The ADRI mutation does not also require other elements of the ADRI fusions to overcome the 237 to 259 inhibitory region and convert ADRI into a more active protein. These results and those described above indicate, therefore, that the ADRI mutation can enhance the activity of ADRI independently of the activation domain which is present in the ADRI protein. This conclusion is consistent with the model in which a repressor binds 227 to 239 and inhibits ADRI.

We tested this model further by determining whether the ADRI-145-262 polypeptide segment could replace the activity of a heterologous transcriptional activation domain. LexA-ADRI-145-262 and its ADRI-17 counterpart were fused in frame with B42, an E. coli polyepitide that can activate transcription in S. cerevisiae (27, 28) (Fig. 6). LexA-ADRI-7-145-262-B42 was found to be eightfold more active in allowing lacZ gene expression than was LexA-ADRI-145-262-B42. The LexA-ADRI-B42 protein concentration, as assayed by Western analysis, was about 15-fold greater than that of LexA-ADRI-17-145-262. In contrast, LexA-ADRI-17-145-262 was inactive as LexA-ADRI-145-262-B42 (Fig. 6). Both LexA-ADRI-17-145-262 and its ADRI counterpart were also completely inactive (activities were less than 1%) with a reporter plasmid containing eight copies of the Lex operator, a reporter which augments other LexA-ADRI proteins' activation potential by about 10-fold (data not shown). These results indicate that ADRI-17-145-262 does not contain its own activation region. LexA-B42 was also more active than LexA-ADRI-B42, but it is difficult to compare the activities of these polypeptides because of differences in the distance of B42 from the LexA-binding domain. These experiments confirm that the 143 to 252 region containing the ADRI region can act to repress transcription independently of the activator to which it is attached.

**DISCUSSION**

ADRI contains at least three activation domains. The results presented here identify three regions of ADRI that are important to its transcriptional activation (Fig. 7). Previous analyses indicated that amino acids 16 to 172 were sufficient to allow 100% of the activation ability of ADRI (59). We confirm that residues 1 to 220 are capable of converting...
LEXA into a yeast transcriptional activator, albeit one with weak activation ability. Sequences from positions 138 to 262 were incapable, by themselves, of activating transcription, implying that the zinc finger DNA-binding region of ADR1, residues 76 to 172, contains the activation function in position 1 to 270. The 76 to 172 region, designated TAD1, contains no sequence similarities to other known activation regions, such as an enrichment of acidic residues (5). It has been suggested that residues 76 to 52 could form an amphiphatic α-helix (39), a structure that has been proposed for yeast activation sequences (21, 22).

A second transactivation domain was localized to residues 263 to 359 (TADII). This region alone could convert LEXA into a strong transactivator (Fig. 5B). Residues 263 to 359 were critical for the function of this region when analyzed for both ADH2 transcription by using a number of C-terminal and internal deletions and for LEXA-ADR1 mediated transcription. The 263 to 341 region is rich in acidic amino acids (29G) and is similar to the type of transactivation domains previously identified as potentially forming an amphiphatic α-helix (GAL4 [27] and VP16 [9]). More recent evidence, however, indicates that the GAL4 activation domain may form a β-sheet structure and that its excess acidity may not be important to its function (26, 41). Removal of residues 304 to 359 and, to a lesser extent, 337 to 359 also impaired the function of this activation domain when it was fused to LEXA (Fig. 5B). Moreover, LEXA-ADR1-271-312-359-378 was active (Table 6), confirming the presence of an activation domain in residues 283 to 359. The 263 to 359 region may contain two separate activation domains. It is also possible that deletion of the 262 to 288 region reduced the activity of LEXA-148-359 by bringing the 227 to 239 inhibitory domain closer to an activation region located in the 258 to 359 region.

A third region of importance to ADR1 activation was located between sequences 330 and 571, and its deletion reduced ADR1 activation of ADH2 (Tables 3 and 4). We tentatively assigned residues 339 to 571 to TADIII, since LEXA-ADR1-339-571 was a potent transactivator and removal of residues 399 to 507 eliminated the activity of this fusion. Deletions within TADIII also made ADR1 incapable of allowing growth on glycerol as a carbon source, whereas deletions within TADII did not have a similar effect. Because of this difference and the finding that deletions in the TADIII region had more severe effects on ADR1 activation of ADH2 than did deletions in TADII (Tables 3 and 4), it appears that TADIII is more important to ADR1 activation than is TADII. It should be noted, however, that ADR1-1330-507-571 may have less activity than ADR1-1397-507-571 because of removal of residues 330 to 359 (important to TADII) and 399 to 507 (important to TADIII).

A fourth putative activation domain may lie within residues 709 to 1068, since deletion of this region reduced ADR1 activation of ADH2 by about 45% (Fig. 3). Because this region by itself could not convert LEXA into a transactivator, it is possible that the C terminus of ADR1 is required for the structural integrity of the N-terminal segment of the protein. The C terminus may, for instance, bind other factors that stabilize contacts that the transactivation domains make.

This is consistent with the finding that the 330 to 507 deletion had a 10-fold effect on the ability of a single copy of ADR1-571 to activate ADH2 but had only a 1.5-fold effect on ADR1-1323. It is also possible that our LEXA-542-1323 fusion contains latent activation ability that is either masked by inhibitory domains or not functional in the in vivo transcription assay system that we used.

Other transcriptional activators have been shown to contain multiple activation domains (29) that coordinate to allow synergistic activation of transcription (30). The ADR1 activation domains, however, appear more functionally redundant than synergistic. Deletion within TADIII alone or
TADII alone reduced ADHII activity by at most, 25% (Table 3). While we could not ascertain the effect of deleting TADII on ADHII expression, LexA-ADR1-220-1323 was capable of activating transcription nearly as well as LexA-ADR1-1323 (Fig. 5A). Only when two or more of the activation regions were deleted did a more substantial reduction in ADHII expression occur (Fig. 3 and Tables 3 and 4).

It is unclear, however, whether each of these activation domains functions through the same mechanism.

The ADRII inhibitory region. Three regions, when deleted, resulted in increased ADR1 activity under nonfermentative conditions (1988 to 1323, 288 to 330, and 220 to 263, Fig. 3 and Table 3), while removal of residues 220 to 263 and 288 to 330 also allowed increased ADHII expression under glucose growth conditions. An analysis of 21 independent ADRII mutations which could allow glucose-insensitive ADHII expression identified the 227-239 region as the sole site for such mutations (17). Because no mutations were obtained in the 288-330 region, single-point mutations probably cannot sufficiently alter the structure of the 288-290 region to affect the activity of ADRII. When the 263-330 deletion was combined with an ADRII mutation, ADHII expression was not much greater under glucose growth conditions than that observed with the ADRII mutation alone (Table 3). These two regions may, therefore, act through the same mechanism to inhibit ADRII activity. One possible model would be that the 288-330 region provides a structural scaffold for the adjacent 227-239 sequence. Only a gross disturbance in 258 to 330 would alter the 227-239 region.

The 227-239 region clearly inhibited ADRII activity irrespectively of the activation domain present on ADR1. For instance, ADR1-262 was more active than ADR1-262, in which only TADII (residues 76 to 172) appears to function, and LexA-ADR1-262-1323-259 was more active than LexA-ADR1-145-259, in which only TADII (residues 262 to 359) is present. Most importantly, we showed that the 147-262 region could inhibit the activity of a heterologous transcriptional activator (Fig. 6). The finding that LexA-ADR1 was more active than LexA-ADR1 with both LexA-1(203), which contains a dimerization domain, and LexA-1(87), which does not, suggests that enhanced dimerization is not the reason why the ADRII protein is more active than ADRII. One previous model describing how the 227-239 region inhibits the function of ADRII was that this region makes an intrasteric contact with another domain of ADR1 and blocks the activation function (17). This seems unlikely in light of the facts that all regions of ADRII, except residues 148 to 262, can be deleted without eliminating the ability of the ADRII protein to be more active than its ADR1 counterpart and that the 147-262 region can inhibit an activator other than ADR1. Since the 148-262 region is itself contains no activation function, we favor a second model (17) in which an extragenic repressor protein binds the 227-239 region and it is the repressor which reduces the function of the ADR1 protein. Such a mechanism would occur independently of other ADRIII motifs and the proteins which bind to them; this is consistent with the finding that the ADRIII region could enhance the activity of a heterologous DNA-binding protein (Table 6). Genetic analyses initiated to identify such a repressor have not, however, been successful (6,7,10).

A possible explanation for how the ADRII protein functions is that the difference observed between the ADR1 and ADRII proteins under glucose growth conditions mimics, in part, the glucose-to-ethanol transition for the activation of ADR1. The fact that LexA-ADR1 is not carbon source responsive in promoting Lex operator-controlled gene expression suggests that LexA-ADR1 requires other factors to promote its full activation upon a switch to a nonfermentable carbon source. That is, removal of the putative repressor from ADR1 probably requires other proteins. These factors may be brought to ADR1 via their binding to other elements of the ADRII promoter which are not present in the simplified Lex operator-controlled plasmids.

It is clear, therefore, that ADR1 is a highly complex protein consisting of multiple activating and inhibiting regions and the potential to interact with a number of proteins. Such a structure has been observed for c-Jun (1) and GAI (4,24). Within the 220-359 region alone, the juxtaposition of activation and inhibitory domains suggests that only a detailed structural analysis of ADR1 will elucidate how these regions interact to modulate ADR1 activity and contacts.

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