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# Identification of the functional regions of the yeast activator ADR1

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# Identification of the functional regions of the yeast activator *ADR1*

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# IDENTIFICATION OF THE FUNCTIONAL REGIONS OF THE YEAST ACTIVATOR *ADR1*

BY

Lynne Taylor Bemis

Bachelor of Science, University of New Hampshire, 1979

DISSERTATION

Submitted to the University of New Hampshire

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in

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ABSTRACT

IDENTIFICATION OF THE FUNCTIONAL REGIONS OF THE YEAST
ACTIVATOR ADR1

by

Lynne T. Bemis
University of New Hampshire, May, 1989

ADR1, a transcriptional activator from the yeast *Saccharomyces cerevisiae*, is required for activation of the glucose-repressible alcohol dehydrogenase, ADH II (encoded by the *ADH2*gene). The *ADR1* gene encodes a protein which binds to an upstream activation sequence in the *ADH2* promoter. Several methods were used to locate functional regions of the ADR1 protein.

The *adr1-1* mutation was identified as a C to G transversion resulting in a nonsense codon at the eleventh codon of ADR1. tRNA-suppressors which substituted an amino acid at the eleventh codon of *adr1-1* resulted in a functional *adr1-1* protein, indicating that the translational start of *ADR1* occurs at the first AUG of the

*ADR1* transcript. The *adr1-1* 5.1 kb RNA was found to be two - to three - fold less abundant than the *ADR1* transcript. This observation is a further instance of a nonsense mutation early in a gene leading to decreased stability of the RNA and confirms that the 5.1 kb transcript is the *ADR1* mRNA.

A series of C-terminal truncations of the *ADR1* gene were constructed and used to investigate functional regions of *ADR1*. A C-terminal truncation leaving only 151 N-terminal amino acids (ADR1-151) and deleting part of a "Zn-binding" DNA-binding finger motif was not capable of transcriptional activation. In contrast, the ADR1-220 protein containing the complete "Zn-binding" DNA-binding finger motif was capable of transcriptional activation. However, none of the truncated ADR1 proteins were as transcriptionally active as the entire ADR1 protein.

An ADR1 protein containing at least 506 N-terminal amino acids was required for glycerol growth. Mutations in the *GLP1* gene were identified that allowed strong glycerol growth, while remaining dependent on the presence of a functional *ADR1* gene. This newly identified function of *ADR1* suggests that *ADR1* has a more extensive role in the regulation of nonfermentative growth in *S. cerevisiae* than previously expected.

To All My Family

especially

Mary and Ken Taylor

Kristen and Gordon Bemis

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Many people in the Biochemistry department have helped me achieve my goals. I would like to thank Clyde Denis for his efforts on my behalf and for his dedication to our research. I would also like to thank the technicians who have assisted me, Gail, Ann, and Steve; to the graduate students, Joel, Tom, Dan, Debbie, and Sue, keep having fun on all those long hours in the lab. And, special thanks to Bob Vallari for all the editing, proof reading and trips to Concord.

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# INTRODUCTION

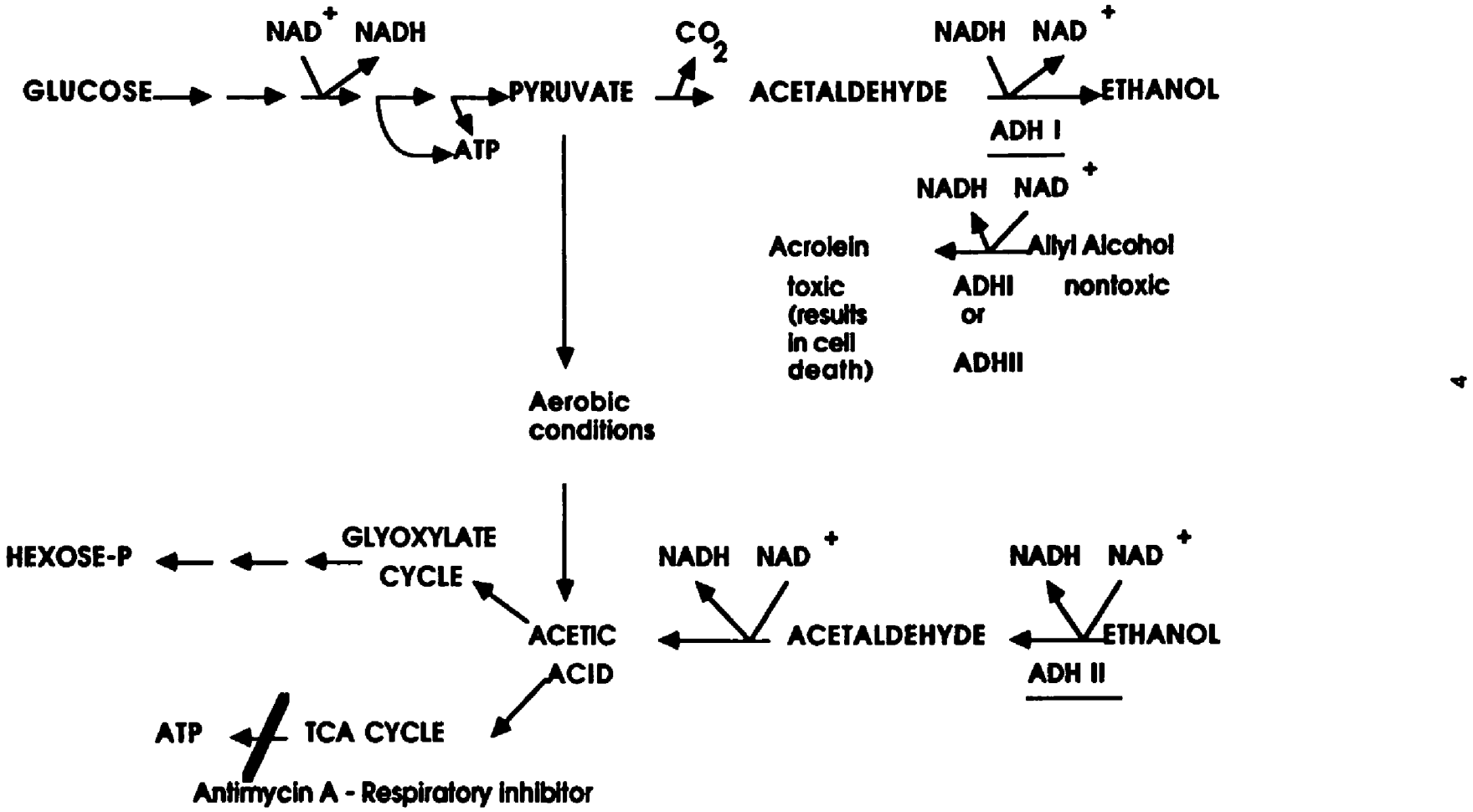
Regulatory mechanisms have been studied in many eukaryotic and prokaryotic organisms. *Saccharomyces cerevisiae* is an organism particularly amenable to the study of regulatory mechanisms because it has a short generation time and a well-characterized genome. One of the most well studied systems of cellular control is that of carbon catabolite repression and the regulatory mechanisms necessary for that control. Carbon catabolite repression, in both prokaryotes and eukaryotes, is a regulatory mechanism used to block the use of alternative carbon sources until the bulk of available glucose is utilized. Systems regulated by carbon catabolite repression in the yeast *Saccharomyces cerevisiae,* include the galactose utilization pathway (Matsumoto *et al.*, 1983; Oshima, 1982), the sucrose utilization pathway (Grossman and Zimmerman, 1979) cytochrome C (Zitomer et al., 1979) and the glucose-repressible alcohol dehydrogenase II (Ciriacy, 1975a).

There are four NAD-linked alcohol dehydrogenase isozymes ADH I, II, III (Lutstorf and Megnet, 1968) and ADH IV (Paquin and Williamson, 1986) in yeast. ADH I and ADH II are cytoplasmic enzymes while ADH III is found in the mitochondria ( Fowler *et al.*, 1971; Ciriacy, 1975a). The recently identified enzyme, ADH IV, is not well characterized at this time. The ADH I isozyme, encoded by the *ADH1* gene, converts acetaldehyde to ethanol under fermentative growth conditions (Figure 1). The glucose-repressible isozyme, ADH II, is encoded by the *ADH2* gene which is transcribed only in the absence of glucose (Denis *et al.*, 1981). *ADH2* is therefore a gene subject to carbon catabolite repression. In the absence of glucose and under aerobic conditions, ADH II converts the ethanol accumulated during fermentative growth to acetaldehyde (Wills, 1976). Although, ADH I and ADH II are 95% identical at the protein level (Wills and Jornvall, 1979) the glucose-repressible isozyme ADH II has a lower Km for both ethanol and $NAD^+$ than does ADH I (Wills, 1976). ADH III, the mitochondrial isozyme, is encoded in the nucleus and is targeted to the mitochondrial membrane by a leader sequence (Young and Pilgrim, 1987). The mitochondrial ADH is 85% homologous to ADH I and ADH II at the protein level.

The availability of yeast strains lacking one or more of the ADH isozymes has facilitated the characterization of individual ADH

isozymes and the factors which control their expression. Mutated ADH isozymes have been identified by growth on allyl alcohol, an unsaturated alcohol which is nontoxic to yeast. In the presence of ADH activity allyl alcohol is readily oxidized to acrolein, a toxic unsaturated aldehyde (Figure 1) (Lutstorf and Megnet, 1968; Ciriacy, 1975a ). Only cells possessing a reduced level of ADH activity can grow in the presence of allyl alcohol. ADH isozymes have been separated and identified by electrophoresis of crude extracts. Analysis of mutant strains have been used to identify three classes of mutations, *adh1*, *adh2*, *adh3* (originally called *adc1*, *adr2*, and *adm*, respectively) (Ciriacy, 1975a). A fourth mutation *adr1-1*, initially thought to be an *ADH2* mutation, was later identified to occur in the regulatory allele *ADR1*(Ciriacy, 1975b). All four genes *ADH1*, *ADH2*, *ADH3*, and *ADR1*, were found to be unlinked and observed to show Mendelian segregation. Yeast strains were constructed which contained mutations in both *adh1* and *adh3* but not in *ADH2.* The only ADH activity in these strains is derived from the glucose-repressible ADH. These strains were used to select for mutations at *ADH2* and mutations in regulatory genes controlling *ADH2* expression.

Figure 1 ALCOHOL DEHYDROGENASES IN METABOLIC PATHWAYS



The increase in ADH II activity under nonfermentative growth conditions was shown to result from an increase in *ADH2* mRNA levels (Denis *et al.*, 1981). In contrast, the *ADH1* mRNA was repressed about fivefold during growth in medium containing a nonfermentable carbon source (Denis *et al.*, 1983).

The *ADH1* and *ADH2* genes have been cloned (Williamson *et al.*, 1980; Williamson *et al.*, 1981) and sequenced (Bennetzen and Hall, 1982; Russell *et al.*, 1983a). Comparison of DNA sequence analysis shows 90% homology between *ADH1* and *ADH2* confirming earlier evidence that at the protein level ADH I and ADH II are 95% homologous . The structural genes and parts of the promoter regions of ADH I and ADH II are similar. The TATAA boxes of *ADH1* and *ADH2* are located 91 to 107 base pairs prior to the mRNA start sites and the sites of translational initiation are located 40 to 60 base pairs to the 3' side of either the *ADH1* or *ADH2* mRNA (Bennetzen and Hall, 1982; Russell, *et al.*, 1983a). The location of the TATAA box and the mRNA start site of *ADH1* and *ADH2* are similar to the location of these transcriptional elements in other known yeast genes (Sentenac and Hall, 1982; Russell *et al.*, 1983a).

The sequences 5' to the TATAA box of the *ADH2* gene, originally

designated *ADR3* (Ciriacy, 1976), are significantly different from those 5' to the *ADH1* gene (Russell *et al.*, 1983b). The $ADR3^{C}$ mutations located *cis* to *ADH2* block catabolite repression of *ADH2*. The $ADR3^{C}$ mutations were later sequenced and shown to result from Ty (yeast transposable element) insertions in the promoter region of *ADH2* (Williamson *et al.*, 1981; Wiliamson *et al.*, 1983) or from an increase in the number of A residues in a poly A tract upstream of the *ADH2* transcriptional start site (Russell *et al.*, 1983b) (Table 1). The Ty insertions alter the regulation of *ADH2* such that it is no longer under the control of glucose-repression. Ty insertions have been shown to disrupt the normal regulation of other structural genes, including Ty insertions that were localized upstream from the iso-2-cytochrome C gene (Errede *et al.*, 1980). The Ty insertions and the mutations at the polyA tract in the upstream region of *ADH2* identify a region required for normal regulation of *ADH2*.

The upstream region of *ADH2* required for the glucose-repression phenomenon was further characterized by placing the *ADR3* region (*ADH2* upstream) upstream from a reporter gene.

Table 1. ADH activities for strains containing mutations at one or more regulatory allele.

| Relevant genotype | ADH II activities (mU/mg)[a] glucose | ethanol |
|---|---|---|
| *ADR1* | 8 | 2400 |
| *adr1-1* | 3 | 15 |
| *ADR1-5c* | 280 | 6600 |
| *ADR1, ADR3c* (Ty)[b] | 783 | 1590 |
| *ADR1, ADR3c* (+poly dA)[b] | 463 | 4000 |
| *ADR1, ccr1* | 2 | 30 |
| *ADR1, cre1* | 175 | 2200 |
| *adr1-1, cre1* | 155 | 730 |
| *ADR1, cre2* | 77 | 2900 |
| *adr1-1, cre2* | 70 | 718 |
| *ADR1, ccr4* | 12 | 40 |
| *ADR1, ccr4, cre1* | 23 | 500 |
| *ADR1,ccr4, cre2* | 6 | 110 |

[a]One unit of ADH activity reduced 1 uM of NAD in one minute at 22°C (Denis *et al.*, 1981). All enzyme activities are taken from (Denis, 1984) except as noted.

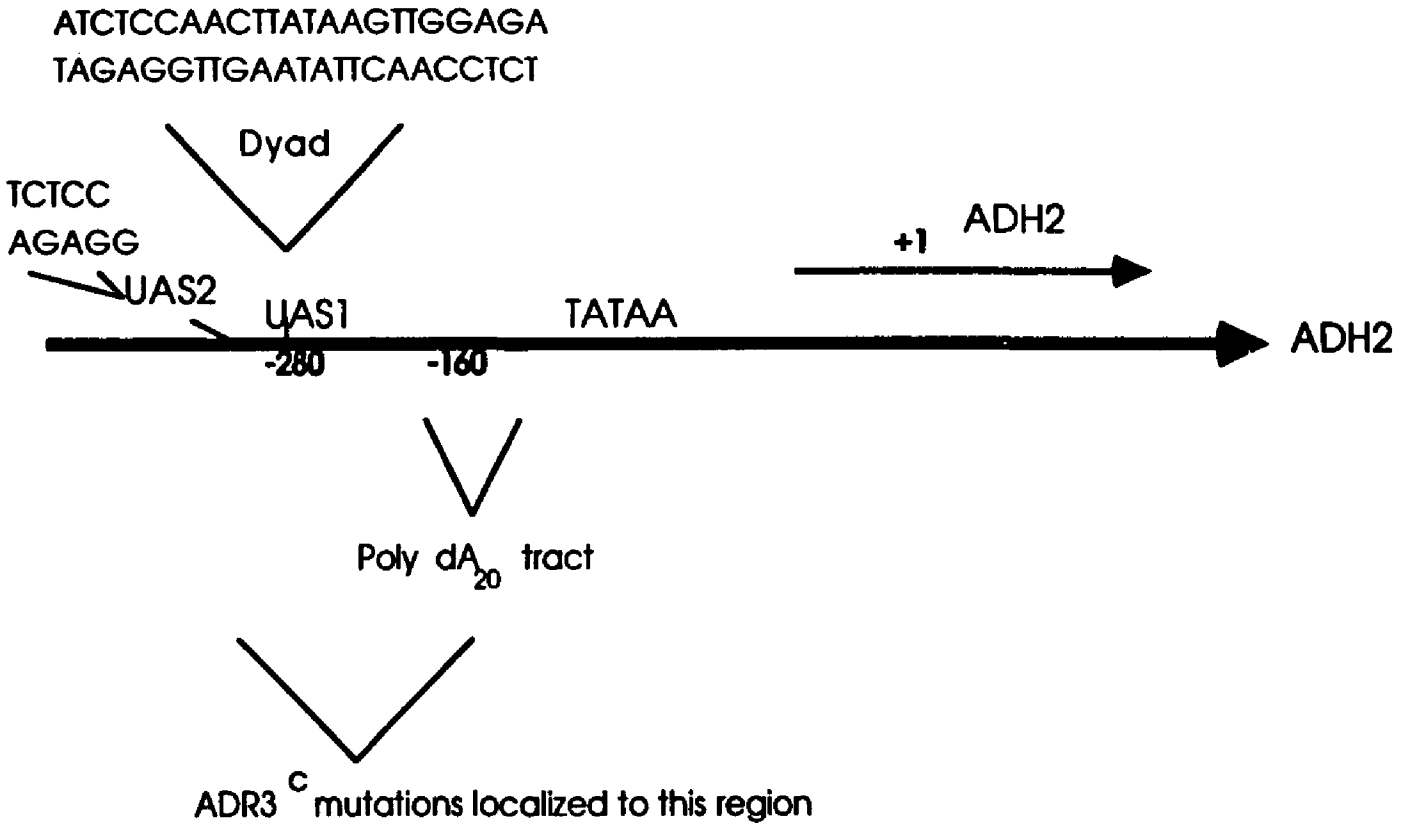
[b]Values taken from Ciriacy, 1979.

All activities are taken from strains which are *adh1*, *adh3*, and *ADH2*.

The reporter gene encoded the fermentative ADH , *ADH1* (Beier and Young, 1982). Synthesis of the reporter gene (*ADH1*) was shown to depend upon the presence or absence of glucose. These experiments confirmed that the upstream region of *ADH2* is required for the glucose repression signal. Deletion analysis of *ADR3* sequences upstream from the reporter gene identified a region necessary for activation of the reporter gene but did not detect a region that when deleted releases *ADH2* from glucose repression (Beier and Young, 1985). These analyses support the hypothesis that a trans-activating element acts through the *ADR3* (*ADH2* upstream) sequences as opposed to a trans-repressing element.

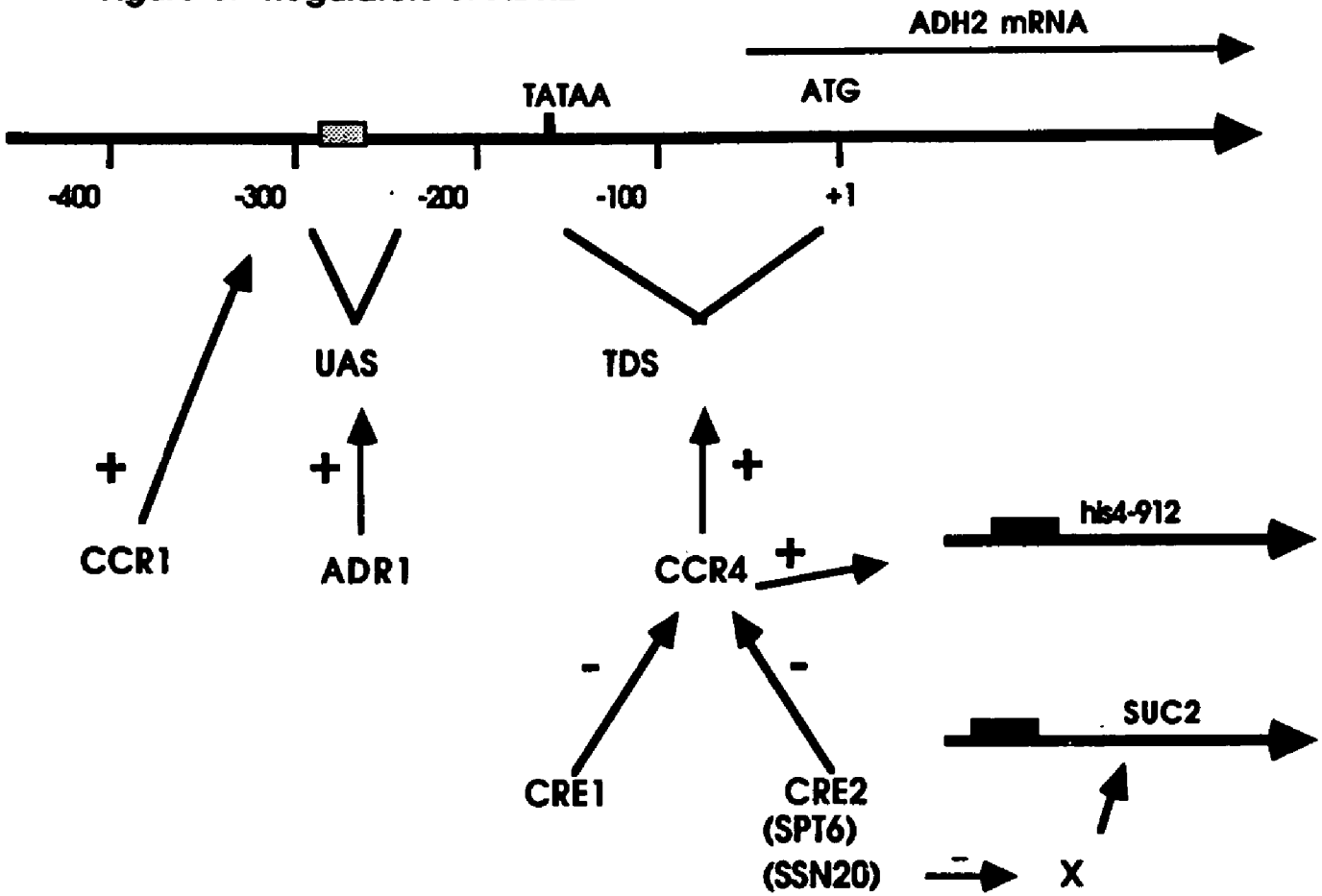
Upstream Activation Sites (UAS) have been characterized in many inducible systems in yeast (Guarente and Ptashne, 1981; Guarente, Yocum, and Gifford, 1982; Struhl, 1982). The region of *ADH2* ( -210 to -320 base pairs) containing the upstream activation sites of *ADH2* has seven copies of the sequence A/TGGAGA, a 22 base pair dyad, and a poly dA tract of 20 base pairs (Figure 2). The 22 bp dyad is required for *ADR1*-dependent activation of *ADH2* transcription (Shuster *et al.*, 1986; Eisen *et al.*, 1988). Deletions of the poly dA tract did not affect *ADH2* expression and thus may not be important to transcriptional regulation of *ADH2* (Shuster *et al.*, 1986).

**Figure 2. Regulatory regions of the ADH2 gene**



A variety of regulators that control *ADH2* expression have been identified by genetic means. *ADR1,* an activator of *ADH2* transcription, was identified by recessive *adr1* mutations that block *ADH2* expression (Ciriacy, 1975b) and by semi-constitutive $ADR1^{c}$ mutations that allow low levels of ADH II expression on glucose (Ciriacy, 1975a). The *ADR6* allele is an activator of *ADH2* expression and was identified by recessive *adr6* mutations that blocked Ty-induced *ADH2* expression. *ADR6* is thought to act downstream from the TATAA box of *ADH2* (Taguchi and Young, 1987) and appears to be a DNA binding protein (O'Hara *et al.*, 1988). *ADR6* is not specific to *ADH2* expression since it is required for sporulation (Taguchi and Young, 1987). The *CCR1* gene (Ciriacy, 1977), allelic to *SNF1*(Denis, 1984), encodes a protein kinase required for the expression of several glucose-repressible genes (Carlson and Celenza, 1986). It has been suggested that *CCR1* acts through or with *ADR1* to exert its regulatory effect on *ADH2* (Denis, 1984; Denis, 1987) (Figure 3). More recent evidence suggests that *CCR1* acts independently of *ADR1* (D. Audino, pers. comm.). Other regulators of *ADH2* expression include *CRE1*,*CRE2*, and *CCR4*. These genes are pleiotropic regulators which appear to act at the *ADH2* TATAA sequence or downstream from the TATAA sequence (Denis and Malvar, in prep.). *CCR4* is an

Figure 3. Regulators of ADH2



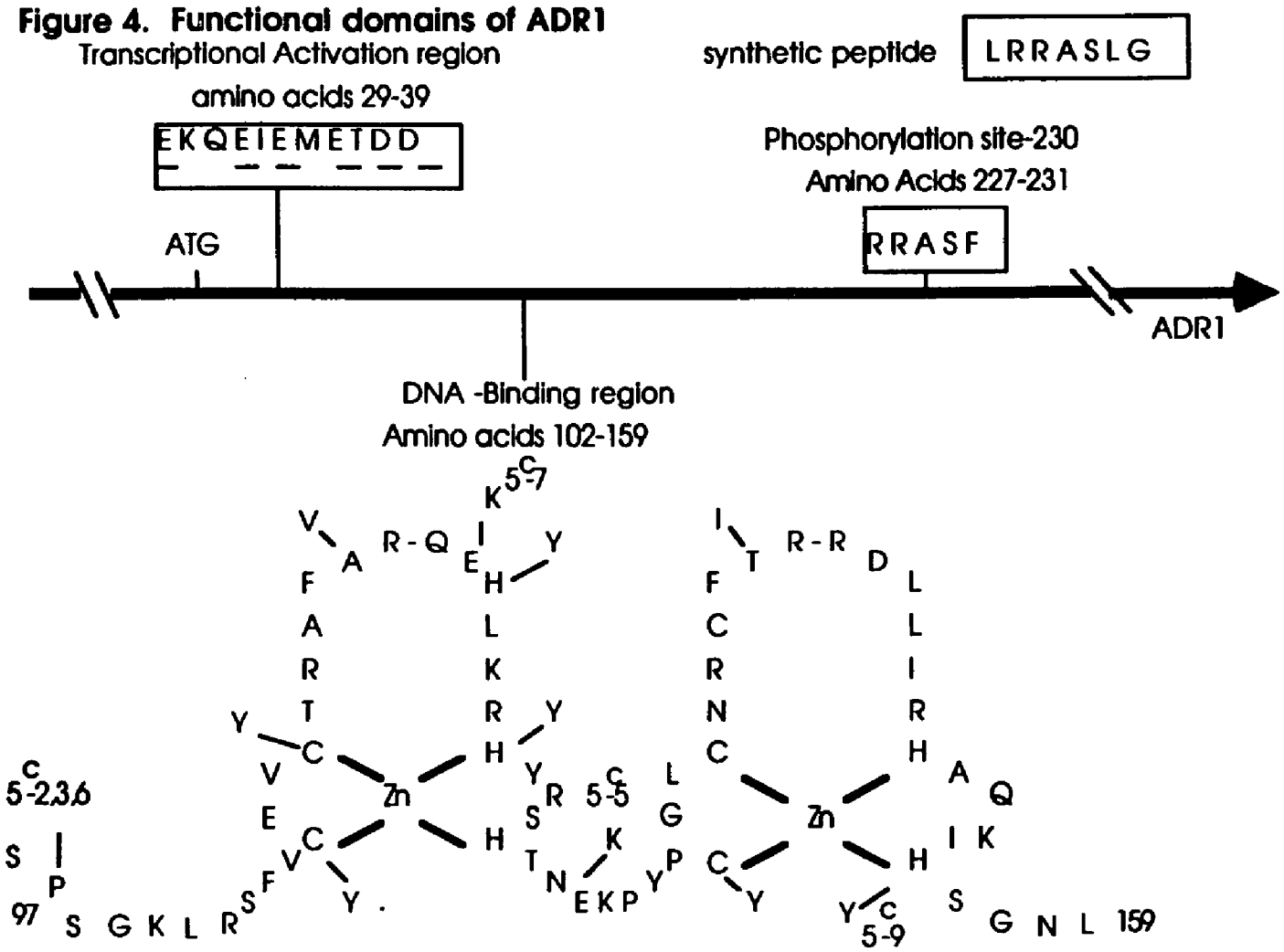
activator of *ADH2* and is controlled by the negative effectors *CRE1* and *CRE2* (Denis, 1984). *CRE2* is allelic to *SPT6*, which acts to regulate *his4-912 delta*,and to *SSN20*, a regulator of the *SUC2* gene. The effects of the *CRE* genes on *ADH2* expression are mediated through *CCR4* as are their effects on *his4-912* delta expression (Denis and Malvar, in preparation). *ADR1* and *CCR4* are independent activators of ADH2 expression and both are required for the full activation of *ADH2* (Table 1). *cre1* mutations result in altered cell morphology and the *ccr4* allele in combination with the *cre1* allele can restore normal cell morphology. Other than *CRE1* and *CRE2*, a repressor specific to *ADH2* has yet to be conclusively identified although the *ADR4* gene may act as a repressor. The phenotype of *ADR4* is not clearly understood because *adr4* relieves glucose repression only in the presence of the *ADR1-5$^{C}$* allele but not in the presence of the *ADR1* allele (Ciriacy, 1979).

The most well studied of these regulators is *ADR1*. The *ADR1* allele has been cloned (Denis and Young, 1983) and sequenced (Hartshorne *et al.*, 1986). The open reading frame encodes a protein of 153 Kilodaltons (1323 amino acids, 3969 base pairs). The *ADR1* protein has been identified by antibody precipitation and corresponds to the size predicted by sequence analysis of the open reading frame

(R. Vallari, pers. comm.). DNA sequence analysis and comparison with other proteins have identified several potentially important regions. These include, an acidic amino acid region which may be important to transcriptional activation, a DNA binding domain, and multiple potential phosphorylation sites (Hartshorne *et al.*, 1986). Mutations that fall within the DNA binding domain at amino acids 102-159 (Blumberg *et al.*, 1987, Denis in prep.) have been characterized (Figure 4) and reduce or block activation of *ADH2*.

The acidic amino acid region of *ADR1* has been identified by analogy to regions found in other yeast activators. This region of eleven amino acid residues (between residues 29 and 39, Figure 4) contains six acidic residues, 4 Glu and 2 Asp. Transcriptional activating segments separate from DNA binding regions have been characterized in the yeast transcriptional activators *GAL4* (Ma and Ptashne 1987a) and *GCN4* (Hope and Struhl 1986; Hope *et al.*, 1988), and these regions are noted for their abundance of acidic residues. In other experiments, random DNA fragments from *E. coli* were ligated in place of the yeast *GAL4* transcriptional activation region. Several of the fragments were able to allow *GAL4* to activate transcription (Ma and Ptashne, 1987a). These *E. coli* DNA fragments were found to encode peptide regions with a relatively large concentration of acidic amino

**Figure 4. Functional domains of ADR1**



acid residues. A deletion of one or more of the GCN4 acidic residues in its transcriptional activation region did not completely disrupt activation. However, a gradual loss of transcriptional activation was observed with the increasing deletion of this region of GCN4 (Hope and Struhl, 1986). Similar results were observed with mutations in the GAL4 acidic segment (Gill and Ptashne, 1987). The deletion experiments suggest that a decrease in negative charge results in a corresponding decrease in transcriptional activation and that overall acidity of the region is important, not the specific amino acid content. The absence of identified mutations in the acidic region of *ADR1* could result from the fact that single amino acid changes in this region would not result in a detectable phenotype due to the lack of a rigid sequence requirement for this region.

Several *adr1*-type mutations suppressing the *ADR1-5$^C$* mutation have been cloned and sequenced (Denis, in prep.). These mutations occur in a region of *ADR1* homologous to the DNA binding region of transcription factor IIIA (TFIIIA) of *Xenopus laevis* (Hartshorne *et al.*, 1986; Blumberg *et al.*, 1987; Denis, in prep.) (Figure 4). TFIIIA has nine repeat units of a finger motif which has been shown to bind DNA (Fairall, *et al.*, 1986). Each finger has two cysteines and two histidines which act as ligands to bind $Zn^{2+}$ (Diakun *et al.*, 1986). The twelve

amino acids between the cysteines and histidines are thought to loop out to form the DNA-binding finger (Miller *et al.*, 1985). *ADR1* contains two such DNA-binding finger motifs in tandem located between amino acid 102 and 159 (Figure 4). C-terminal truncations leaving only 151 amino acids and disrupting the second $Zn^{2+}$ coordination site block ADH II activity (Bemis and Denis, 1988). Mutations in the finger region of *ADR1* have been characterized (Blumberg *et al.* 1987; Denis, in prep.). The mutations were selected by their ability to suppress the *ADR1-5$^{C}$* mutation (Denis, in prep.) or by their ability to reduce ADH II activity in strains carrying an ADR1-ß-galactosidase fusion protein (Blumberg *et al.*, 1987). The amino acids which have been mutated are designated with an arrow and the amino acid replacement is noted in Figure 4. The mutations found in the conserved DNA-binding finger region blocked or reduced *ADR1* activation of *ADH2*. DNA binding studies using a synthesized peptide modeled on the finger region of *ADR1* show that a single finger motif is capable of binding DNA in a nonspecific manner and binding of DNA is enhanced by coordination with $Zn^{2+}$ (Parraga *et. al.*, 1988). Moreover, a single finger is not capable of sequence specific binding.

The *ADR1$^{C}$*- type mutations which result in increased ADH II expression under glucose-repressed conditions have also been

sequenced. Each point mutation occurs at a cAMP-dependent protein kinase (cAPK) consensus sequence in which serine-230 is the presumed phosphoacceptor (Denis and Gallo 1986; Cherry *et al.*, 1989). *ADR1*$^{c}$ mutations have been shown to result in decreased phosphorylation of residue 230 by cAPK (Cherry *et al.*, 1989), suggesting that *ADR1* is more active when it is less phosphorylated.

The focus of this thesis is the identification of the functional regions of *ADR1*. Functional regions are the areas of this large protein (153 Kd) responsible for a specific action or interaction of the protein. The following questions were addressed in this dissertation:

Does the *adr1-1* mutation inactivate a specific domain required for *ADR1* control over *ADH2* expression? This was the first question, broached at a time when it was still unclear whether *ADR1* specified a protein product. Cloning and sequencing of the *adr1-1* allele identified the *adr1-1* mutation as a nonsense mutation and confirmed that *ADR1* encoded a protein product. The *adr1-1* mutation was found to be a serine codon mutated to a stop codon at amino acid 11. The translational start of *ADR1* was inferred by the identification of the *adr1-1* mutation. It was concluded that the first possible AUG codon is the translational start site as predicted by the nucleic acid sequence (Hartshorne *et al.*, 1986). The serine residue at amino acid eleven was

found not to be important to ADR1 function since it could be replaced by several other amino acids without affecting ADR1 activity. The instability of the *adr1-1* mRNA as compared to the *ADR1* mRNA was also analyzed (Section I).

The second question addressed by this thesis concerned the separation of the functional domains of ADR1. In multifunctional proteins it is often possible to identify separate regions of the protein responsible for individual functions. This method was used with much success in regulatory proteins such as the glucocorticoid receptor (Danielsen *et al.*, 1986) and the yeast regulatory proteins GAL4 (Keegan et al., 1986) and GCN4 (Hope and Struhl, 1986). C-terminal truncations of ADR1 were used to identify functional regions. Using this technique the transcriptional activation region, a DNA-binding domain, a site required for activation on glucose, and a region required for growth on glycerol were all identified.

The last questions asked in this thesis involved further characterization of the sites involved with activation on glucose and the region required for glycerol growth. An internal deletion of the phosphorylation site at amino acid 230 was constructed to determine if the loss of this site affected glucose activation. The role of *ADR1* in glycerol growth was addressed by genetic methods to determine which

factor or factors were being affected by ADR1 during growth of yeast on glycerol.

SECTION 1

# CHARACTERIZATION OF THE *adr1-1* MUTATION

## Introduction

The *adr1-1* mutation was first identified by Ciriacy (1975b). The mutation was identified following growth of yeast on medium containing allyl alcohol. Allyl alcohol, which is nontoxic, is converted to the toxic aldehyde acrolein by ADH. Only strains that are deficient in ADH activity are able to survive in the presence of allyl alcohol. The *adr1-1* mutation has since been found to inhibit transcription of *ADH2* (Denis *et al.*, 1981). Yeast cells containing the *adr1-1* mutation are thus able to grow on allyl alcohol, providing that the ADH isozymes ADH I and ADH III are also absent (Table 1). Recessive mutations that suppress *ADR1-5$^{C}$* function and recessive mutations in *ADR1-lacZ* fusion genes have also been isolated (Blumberg *et al.*, 1987; Denis, in prep.). Characterization of different *ADR1* clones often made use of the *adr1-1* phenotype and thus it

was imperative that the mutation be analyzed. The first chapter in this thesis focuses on the characterization of the *adr1-1* allele and discusses the implications resulting from its identification.

Table 2. *Saccharomyces cerevisiae* strains used to characterize the *adr1-1* allele

| Strain | Genotype |
|---|---|
| 43-2B | *MATa adh1-11 adh3 ura1 his4* |
| 411-40 | *MATa adh1-11 adr1-1::YRp7-ADR1-411 adh3 ura1 his4* |
| 500-16 | *MATa adh1-11 adr1-1 adh3 ura1 trp1 his4* |
| 3C4 | same as 500-16 except *adr1-1::YRp7-adr1-1-23A* |
| 4B1 | same as 500-16 except *adr1-1::YRp7-adr1-1-4-23A* |
| 11-2 | same as 500-16 except *adr1-1::YRp7-5'adr1-1-3'ADR1* |
| C9 | same as 500-16 except *adr1-1::YRp7-5'ADR1-3'adr1-1* |
| 17-3c | *MATa leu2-u trp1 ade5,7-u adh1-11 adr1-1* |
| 19-6d | *MATa leu2-u trp1 ade5,7-u his5-u adh1-11 SUP88* |
| 21-5c | *MATa leu2-u trp1 ade5,7-u adh1-11 adr1-1 SUP79* |

## Materials and Methods

### Strains and conditions of growth

Yeast strains are described in Table 2. Yeast were grown overnight in YEP medium (2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 1% yeast extract, 20 mg each of adenine and uracil, per liter) supplemented with either 8% glucose or 3% ethanol. YD plates contained YEP medium, 2% glucose, and 2.5% agar. Minimal plates lacking tryptophan contained synthetic complete media, 2% glucose, 2.5% agar, and amino acids except tryptophan (Williamson *et al.*, 1981).

### Localization of the *adr1-1* mutation

The *adr1-1* mutation was mapped within the 5 to 6 kb of *ADR1* DNA using methods similar to those described by Denis and Gallo (1986). Two gapped plasmids were used (Plasmids A and B, Figure 5). The plasmid YRp7-ADR1-$5^{C}$-23A (Denis and Young, 1983), which contains the upstream region of *ADR1* and 2982 bp of the *ADR1* open reading frame, was deleted between base pairs +440 and +1075 upon cleavage with BclI (Plasmid A, Figure 5). Deletion of the 635 bp fragment removes the *ADR1-5*$^{C}$ point mutation at base pair 683 (Denis and Gallo, 1986). Plasmid YRp7-ADR1-411-BA, which contains a 4.8 kb *ADR1* insert including approximately 1600 bp of sequences upstream from *ADR1* to

+3200 bp of the *ADR1* structural gene, was cleaved with BclI which deleted a 2.2 kb region of *ADR1* extending from about -1200 bp to +1075 bp (Plasmid B, Figure 5).

The gapped plasmids were site-specifically targeted for integration (Orr-Weaver *et al.*, 1981) into the *adr1-1* allele in strain 500-16 (Denis and Gallo, 1986). Transformants carrying integrated copies of plasmids A and B were identified on minimal plates lacking tryptophan, followed by successive growth on non-selective YEP plates containing 2% glucose. Those colonies which did not lose the TRP+ phenotype conferred by the plasmid, and which presumably contained integrated plasmid, were analyzed further. Southern analysis (Southern, 1975) was used to determine the number of plasmid copies integrated into the genome while tetrad analysis (Mortimer and Hawthorne, 1969) verified integration at the *adr1-1* locus. The ADH II activities of all integrants were determined following growth on medium containing either glucose or ethanol and were compared to the activities of strains carrying the *ADR1* or *adr1-1* allele (strain 43-2B or 500-16, Table 3).

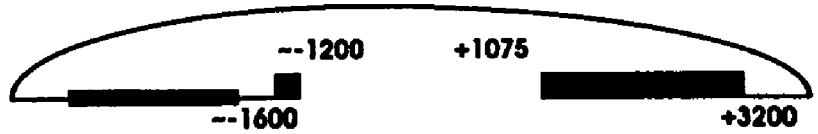
Figure 5. Plasmids Section 1.

*ADR1* sequences are represented by solid black boxes. Sequences from *adr1-1* plasmids are designated by grey boxes. Solid black lines designate the *TRP1* marker gene from *S. cerevisiae*. Thin black lines represent pBR322 sequences and are not drawn to scale. Numerical designations refer to *ADR1* sequences only.

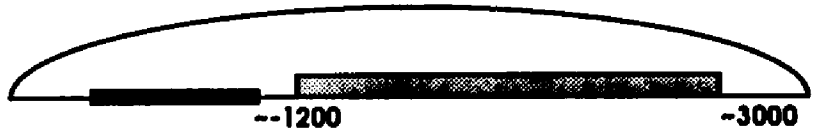
Figure 5. Plasmids (Section 1)

A YRp7-ADR1-5$^{c}$-23A-BclI △

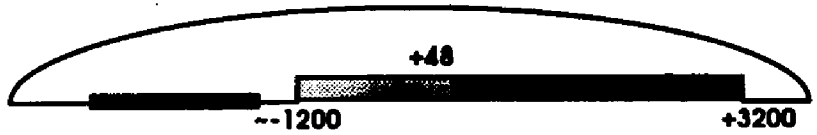

B YRp7-ADR1-411BA-BclI △



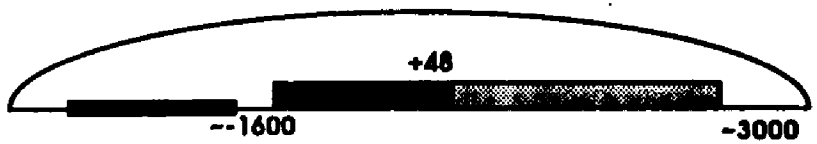

C YRp7-adr1-1-23A




D YRp7-5'adr1-1-3'ADR1




E YRp7-5'ADR1-3'adr1-1



The *adr1-1* allele was cloned by plasmid rescue (Orr-Weaver *et al.*, 1983), from the integrant 3C4 (Table 2). Subsequent transformation of the strain 500-16 (*adr1-1*) with the recovered plasmid demonstrated that the recovered plasmid (YRp7-adr1-1-23A, Plasmid C, Figure 5) was indeed carrying the *adr1-1* allele (strain 4B1, Table 3).

The YRp7-adr1-1-23A and the YRp7-ADR1-411-BA plasmids were used to construct two hybrid plasmids for further localization of the *adr1-1* mutation. Plasmid D (Figure 5) contained the 5' region of the *adr1-1* plasmid (YRp7-adr1-1-23A) to base pair +48 or *adr1-1* and the 3' region of the *ADR1* plasmid (YRp7-ADR1-411-BA). Plasmid E (Figure 5) contained the 5' region of the *ADR1* allele from plasmid YRp7-ADR1-411-BA to base pair +48 and the 3' region of the *adr1-1* allele from plasmid YRp7-adr1-1-23A.

The *adr1-1* mutation was identified by sequence analysis (Sanger *et al.*, 1977) following digestion of YRp7-adr1-1-23A plasmid DNA with PvuII and EcoRI and subcloning the fragments into M13 phage mp18 and mp19.

Table 3. ADH II enzyme activities (Section 1)

| Strain | *ADR1* genotype | Integrated *ADR1* plasmid | ADH II activity mU/mg[a] glucose | ethanol |
|---|---|---|---|---|
| 43-2B | *ADR1* | none | 5 | 2500 |
| 500-16 | *adr1-1* | none | 2 | 9 |
| 13 integrants[b] | *adr1-1* | Plasmid A 635 bp deletion | 5 | 2200 |
| 2 integrants[b] | *adr1-1* | Plasmid A 635 bp deletion | 3 | 20 |
| 11 integrants[c] | *adr1-1* | Plasmid B 2200 bp deletion | 2 | 10 |
| 4B1[d] | *adr1-1* | Plasmid C (*adr1-1*) | nd[e] | 4 |
| 11-2[f] | *adr1-1* | Plasmid D 5'*adr1-1*/3'*ADR1* | nd[e] | 4 |
| C9[g] | *adr1-1* | Plasmid E 5'*ADR1*/3'*adr1-1* | nd[e] | 2500 |

[a]Yeast that were inoculated into YEP medium containing 8% glucose had been pregrown on YEP plates supplemented with 8% glucose, while yeast inoculated into YEP medium containing 3% ethanol had been pregrown on YEP plates supplemented with 2% glucose.

[b]Integrants derived from transformation of strain 500-16 with Plasmid A.

[c]Integrants derived from transformation of strain 500-16 with Plasmid B.

[d]Strain 4B1 carrying rescued plasmid YRp7-adr1-1-23A (Plasmid C) integrated at adr1-1.

[e]nd - not determined

[f]Strain 11-2 carrying hybrid plasmid D, 5' region of adr1-1 to base pair +48 ligated to the 3' region of ADR1 at base pair +49.

[g]Strain C9 carrying hybrid plasmid E, 5' region of ADR1 to base pair +48 ligated to the 3" region of adr1-1 at base pair +49.

tRNA-Suppressors

Strains carrying the *adr1-1* allele and the UGA-tRNA-suppressors *SUP88* and *SUP79* (D. Hawthorne personal communication) were constructed using standard yeast genetic techniques (Table 4). The *SUP4* (E. Schmidt, pers. comm.) and *SUP3e* (Hottinger *et al.*, 1982) alleles, present on shuttle vectors containing the *S. cerevisiae TRP1* gene and pBR322 sequences, were used to transform strains carrying the *adr1-1* allele.

Table 4. ADH II activities in the presence and absence of UGA-tRNA suppressors

| Strain | UGA suppressor (amino acid insert) | ADH II activity (ethanol)[a] mU/mg |
|---|---|---|
| segregants (*adr1-1*)[b] | no SUP | 12 |
| segregants (*adr1-1*)[b] | *SUP88* leucine | 96 |
| 19-6D (*ADR1*) | *SUP88* leucine | 2500 |
| segregants (*adr1-1*)[c] | no SUP | 4 |
| segregants (*adr1-1*)[c] | *SUP79* arginine | 44 |
| 500-16 | no SUP | 10 |
| 500-16-1AS (*adr1-1*)[d] | *SUP3e* serine | 216 |
| 500-16-5A (*adr1-1*)[e] | *SUP4* tyrosine | 205 |

The ADH II activities were shown to be statistically significant at the 0.95 confidence level for the leucine inserting genomic tRNA-suppressors and at the 0.99 confidence level for the arginine inserting tRNA-suppressor using the t-test.

[a] Yeast were pregrown on YEP plates supplemented with 2% glucose before inoculation into YEP medium containing 3% ethanol.

[b]Average of ten segregants from the cross 19-6d with 17-3c.

[c]Average of eleven segregants from the cross of 21-5c with 17-3c.

[d]Strain 500-16 transformed with SUP3e-UGA inserting serine.

[e]Strain 500-16 transformed with SUP4-UGA inserting tyrosine.

Northern analysis

Northern analysis of total RNA was conducted as previously described (Denis and Gallo, 1986). Densitometric analysis of the *ADR1* mRNA, rRNA, and *URA1* mRNA was conducted using an EC-210 densitometer. The amount of rRNA and *URA1* mRNA were used as internal standards of the RNA present.

Fragments of DNA were isolated from agarose gels following restriction digestion and electrophoresis and used as hybridization probes. Probes were labeled with [∂ -$^{32}$P]dATP using a Random Primed DNA labeling kit from Boehringer Mannheim (Indianapolis, IN).

## Results

Localization and identification of the *adr1-1* mutation

The *adr1-1* mutation was localized by transformation of yeast harboring the *adr1-1* allele. Two plasmids with different size deletions of the *ADR1* DNA sequences (Materials and Methods) were targeted for site-specific integration. Upon integration of the plasmid into the *adr1-1* locus the plasmid deleted sequences would be filled in with information from the genomic *adr1-1* allele (Orr-Weaver *et al.*, 1981). Fifteen transformants

containing an integrated copy of plasmid A (Figure 5), the YRp7-ADR1-5$^{C}$-23A BclI deleted plasmid, were characterized. Thirteen displayed the wild-type *ADR1* phenotype (Table 3), while two failed to derepress ADH II, a characteristic of the *adr1-1* phenotype (Table 3). These results indicate that the *adr1-1* mutation is not located in the deleted region (+440 bp to +1075 bp), but is located either to the 5' or 3' side of the deletion. The two transformants displaying the *adr1-1* phenotype probably resulted from plasmid integrations in which the size of the original 635 bp gap had been increased (Clancy *et al.*, 1984).

The location of the *adr1-1* mutation was further investigated by transforming strain 500-16 with linearized plasmid B (Figure 5) lacking *ADR1* sequences from -1200 bp to +1075 bp. Eleven transformants carrying integrated copies of the plasmid were identified. All transformants were deficient in ADH II activity (Table 3), indicating that the *adr1-1* mutation was located within the -1200 bp and +1075 bp gap. The mutation causing the *adr1-1* phenotype was thus localized to the 5' side of the BclI site at base pair +440. Further localization was conducted using YRp7-adr1-1-23A (Plasmid C, Figure 5), a plasmid rescued from integrant 3C4 (Table 2). Hybrid plasmids were constructed using plasmid YRp7-adr1-1-23A and YRp7-ADR1-411-BA (Materials and Methods). Transformants carrying plasmid D integrated into the genome (Figure 5: *adr1-1*

sequences 5' to +48 bp) expressed low ADH II activities (strain 11-2, Table 3) while those carrying plasmid E (Figure 5: *ADR1* sequences 5' to +48 bp) expressed wild type ADH II activities (strain C9, Table 3). The hybrid plasmid experiments indicated that the *adr1-1* mutation was contained either within the 5' noncoding region or within the first 48 base pairs of the putative *adr1-1* open reading frame.

The nucleotide sequence of the *adr1-1* allele between base pairs -121 and +54 was determined to correspond to that previously observed for the *ADR1* gene (Hartshorne *et al.*, 1986) with one exception: a C to G transversion was observed at base pair +32 in *adr1-1*. Such an alteration would lead to the replacement of a UCA serine triplet with a UGA stop signal.

UGA-tRNA-suppressors are capable of suppressing the *adr1-1* mutation

UGA-tRNA-suppressor studies were undertaken to confirm that the C to G transversion and the resultant nonsense codon were the cause of the *adr1-1* phenotype. Two UGA-suppressors of genomic origin were combined with the *adr1-1* allele by making the appropriate crosses. *SUP88* would result in the insertion of leucine (Hawthorne, 1981) while *SUP79* is thought to insert arginine (D. Hawthorne, pers. comm.). In addition, plasmid borne tyrosine-inserting (E. Schmidt, pers. comm.) and

serine-inserting (Hottinger *et al.*, 1982) UGA-tRNA-suppressors were also combined with the *adr1-1* allele. The results of the *adr1-1* UGA-tRNA-suppressor studies are compiled in Table 4. In all cases the presence of a UGA-tRNA-suppressor caused an increase in ADH II activity, indicating that the *adr1-1* allele did in fact contain a UGA nonsense mutation. In contrast, no increase of *ADR1*-dependent ADH II activity was found in the presence of the UGA-tRNA-suppressors (Table 4, 19-6D). The arginine UGA-tRNA-suppressor appeared to restore the *ADR1* phenotype to the lowest extent, whereas the tryrosine and serine suppressors were the most effective in restoring *ADR*1 function (Table 4).

RNA analysis

Nonsense mutations which occur early in a translated sequence may result in less stable mRNAs (Chang and Kan, 1979; Losson and Lacroute, 1979; Kinniburgh *et al.*, 1982; Pelsy and Lacroute, 1984). In order to analyze the effect of *adr1-1* on the stability of the putative 5.1 kb *ADR1* mRNA, total yeast RNA from strains 500-16 (*adr1-1*) and 411-40 (*ADR1*) grown on glucose-containing medium was examined by Northern analysis. Figure 6 shows a typical Northern blot in which the levels of *adr1-1* and *ADR1* mRNA are compared. *ADR1* mRNA was found to be two-to three-fold more abundant than *adr1-1* mRNA (Figure 6). The decrease in the amount of *adr1-1* mRNA was not due to defective ADH II expression, for

yeast containing the *ccr1-1* allele, which also blocks ADH II synthesis, express normal levels of *ADR1* mRNA (Denis and Gallo, 1986). A similar decrease in *adr1-1* mRNA levels relative to *ADR1* mRNA levels was also observed when yeast strains were grown on ethanol containing medium (Figure 7).

Figure 6. Northern analysis of *adr1-1* mRNA levels.

Total cellular RNA was loaded in each lane and probed with an *ADR1* probe as described in Materials and Methods (Section 1): A-RNA from strain 500-16 (*adr1-1*) B-RNA from strain 411-40 (*ADR1*) C-duplicate of lane A at higher concentration D-duplicate of lane B at higher concentration. The inserts show the amount of rRNA or *URA1* RNA in each lane and these were used to standardize the levels of total *ADR1/adr1-1* RNA loaded.

Figure 6

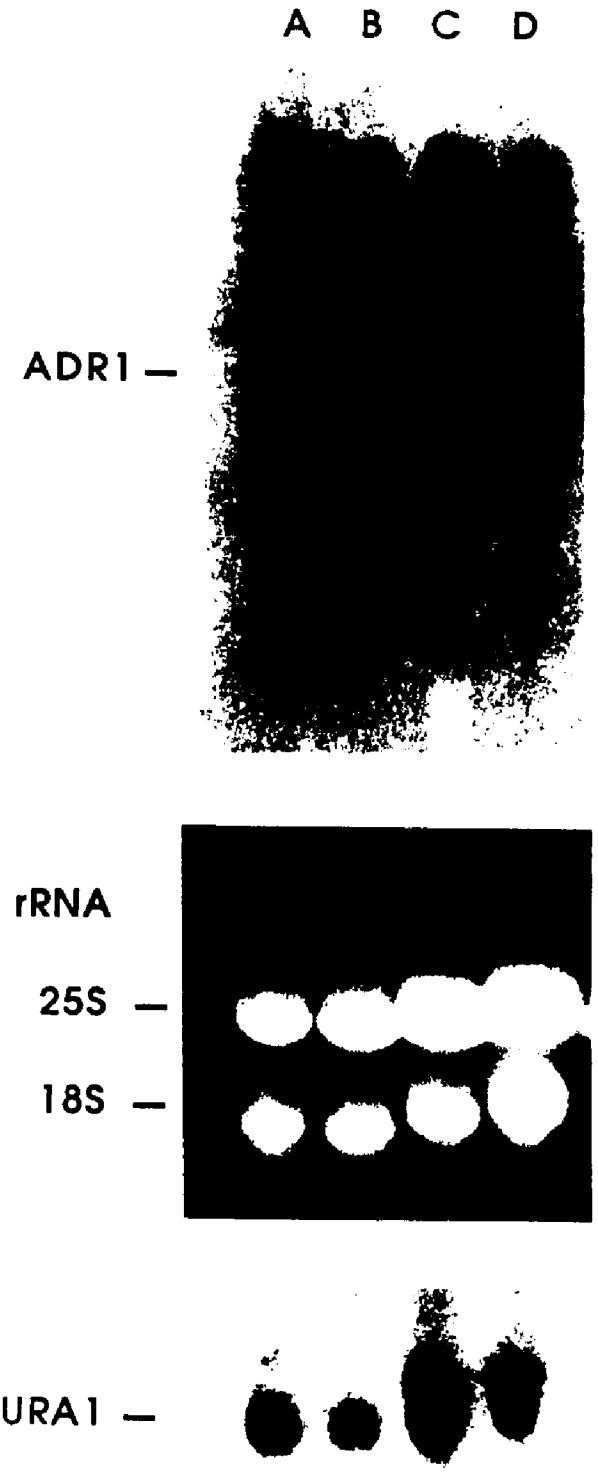
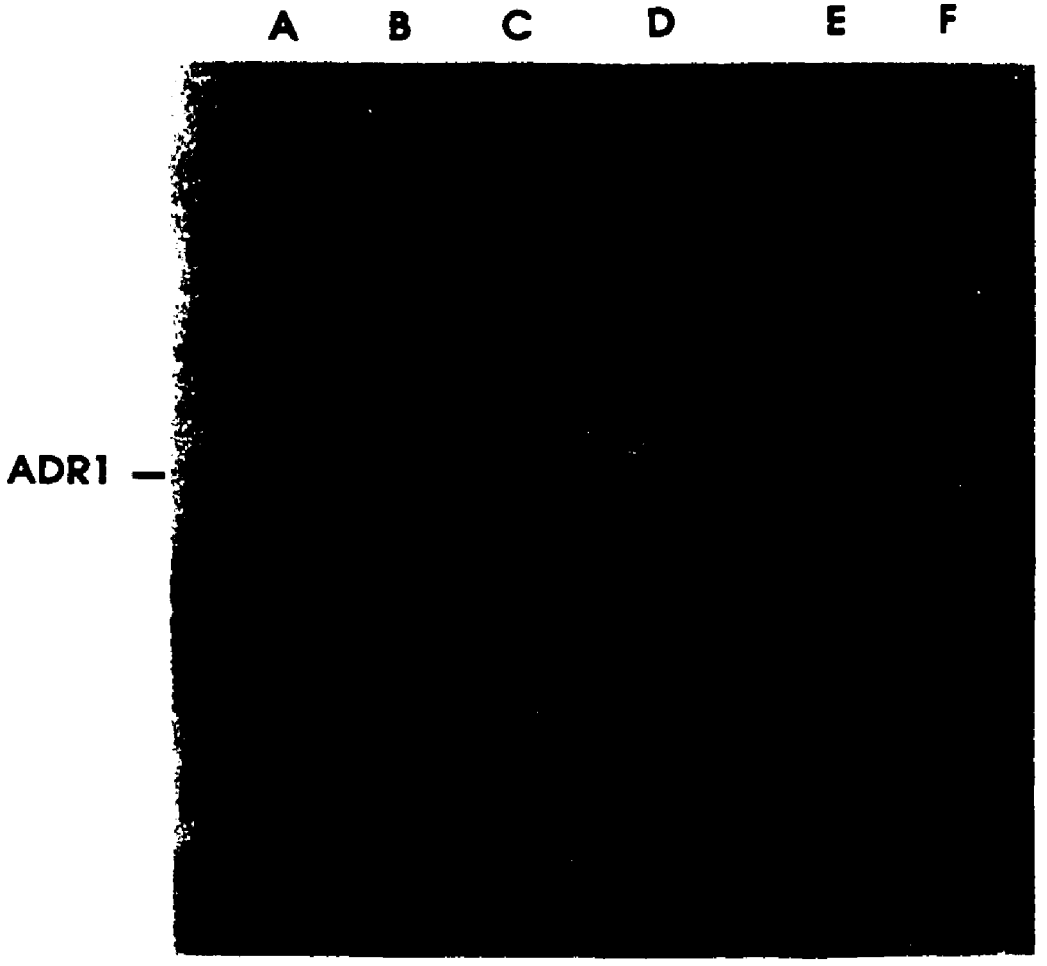
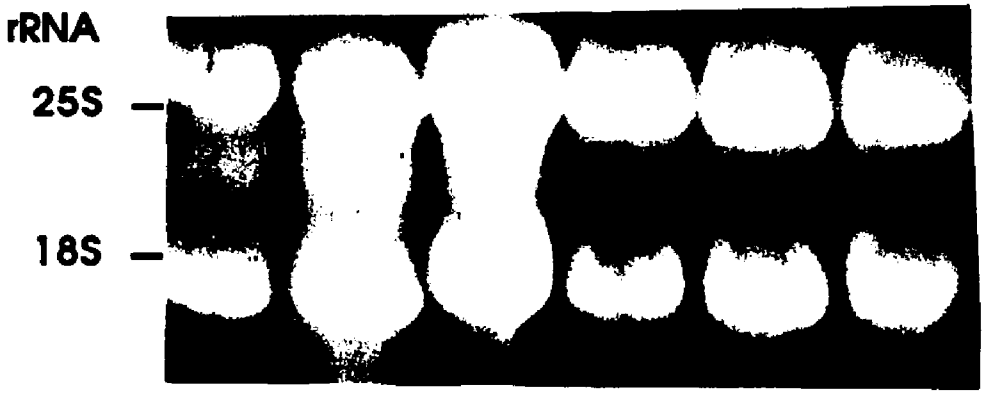


Figure 7. Northern analysis of *ADR1/adr1-1* mRNA levels from cells grown on glucose- or ethanol- containing medium.

Total cellular RNA was loaded in each lane and probed with an *ADR1* probe as described in Materials and Methods (Section 1): A and B-RNA from strain 500-16 (*adr1-1*) from cells grown overnight in 3% ethanol-containing medium C- RNA from strain 500-16 (*adr1-1*) from cells grown overnight in 8% glucose-containing medium D and E- RNA from strain 411-40 (*ADR1*) grown overnight in 3% ethanol-containing medium F- RNA from strain 411-40 (*ADR1*) grown overnight in 8% glucose-containing medium. The insert shows the amount of rRNA in each lane. The amount of rRNA was used to standardize the levels of total *ADR1/adr1-1* RNA loaded.

Figure 7



## Discussion

Cloning and sequencing of the *adr1-1* allele identified a UGA nonsense mutation at the 11th codon of ADR1. The identification of the *adr1-1* mutation was confirmed by the use of UGA-tRNA-suppressors to restore *ADR1* function. These results indicate that the *ADR1* gene does in fact encode a protein product and that the first AUG codon in the *ADR1* sequence is the site of translational initiation. The *adr1-1* mRNA is less stable than the *ADR1* mRNA, this observation allowed confirmation that the previously identified 5.1 kb transcript (Denis and Gallo, 1986) is the *ADR1* transcript.

There are two explanations which could account for the low level of ADH II activity in strains carrying both the *adr1-1* mutation and the UGA-tRNA-suppressors. One possibility is that read-through of the normal UGA stop codon at base pair +3970 (Hartshorne *et al.*, 1986) yields a larger than normal protein having diminished *ADR1* function. This seems unlikely since the UGA-tRNA-suppressors were observed to have no effect on wild-type *ADR1* activity. An alternative explanation is that suppressors have variable efficiencies for individual nonsense codons. The variability of suppression by tRNA-suppressors is influenced by the context surrounding the nonsense codon, the acceptability of the amino acid inserted at the mutation site, the position of the mutant codon within the

locus, and the level of expression of the suppressor (Sherman, 1982). A serine inserting UGA-tRNA-suppressor, which restores the original *ADR1* sequence, did suppress the mutation with the highest efficiency (10% of wild-type). The tyrosine inserting UGA-tRNA-suppressor suppressed the *adr1-1* mutation at levels roughly similar to the serine suppressor, while leucine and arginine suppressors resulted in somewhat lower ADH II activities. The inherent variability of tRNA-suppressors would indicate that all four amino acid substitutions resulted in functional ADR1 proteins. The placement of a serine residue at codon 11 in the ADR1 protein is thus believed nonessential for ADR1 function.

The first 5' AUG codon is normally the one used for translational initiation in eukaryotes (Kozak, 1983), although there are exceptions in which one or more small open reading frames are present prior to the site of translational initiation. The mechanism which allows reinitiation of translation at internal open reading frames is not clear at this time (Kozak, 1987). Translation could reinitiate at an AUG following the *adr1-1* mutation. The next closest AUG is out of frame at base pair +83 and 5 triplets away is a UAG stop codon. Following the second AUG is a third AUG that is in-frame with *ADR1*. If reinitiation occurred here, an *ADR1* protein with an N-terminal deletion of 34 amino acids would result. Because the first 34 amino acids contain part of an acidic region proposed

to be involved in transcriptional activation (Bemis and Denis, 1988; Figure 1) an *adr1-1* reinitiation product is likely to be nonfunctional.

The reduced levels of the 5.1 kb *adr1-1* transcript confirm that the previously identified 5.1 kb transcript (Denis and Gallo, 1986) is indeed the *ADR1* mRNA. The ability of nonsense mutations to decrease the level of mRNA appears to be a common phenomenon. Several alleles of the human $\beta^{0}$ globin gene have been identified which contain nonsense mutations within the first 180 bp of the structural gene (Chang and Kan, 1979; Orkin and Goff, 1981; Kinniburgh *et al.*, 1982). These mutations also show reduced levels of mRNA as compared to the normal allele. Nonsense mutations in N-terminal regions resulting in unstable mRNAs have also been identified in the *ura1* (Pelsy and Lacroute, 1984), *ura3* (Losson and Lacroute, 1979), and *cyc1* (Zitomer *et al.*, 1979) genes of *S. cerevisiae*. Nonsense mutations in the C-terminal region of *ura1* have no effect on its mRNA stability (Pelsy and Lacroute, 1984). A mechanism similar to transcriptional polarity in *E. coli* has been suggested to explain the translational protection evidenced by the comparison of mRNA stability in *ura1* alleles which contain polar nonsense codons (Pelsy and Lacroute, 1984). A single base pair change resulting in a nonsense codon would block further translation of the mRNA and further protection of the mRNA by ribosomes or by the newly formed protein product (Maquat and Daar,

1988). The *ADR1* mRNA is normally present at very low levels (less than 0.01% mRNA) in the cell and may represent a class of rapidly degraded transcripts (Santiago *et al.*, 1986).

# SECTION 2

# IDENTIFICATION OF FUNCTIONAL REGIONS OF ADR1

## Introduction

Transcriptional activators have several functions in common. They must be capable of interacting with DNA and they must also be able to interact with the transcriptional machinery. Transcriptional activators may also interact with other protein factors. GAL4, for example, binds the repressor GAL 80 which prevents GAL4 transcriptional activation of the galactose utilization genes (Ma and Ptashne, 1987b). The various functions required by a transcriptional activating protein are often carried out by distinct regions of the protein. Deletion analysis of transcriptional activators may be used to define the location of such domains.

Early investigations suggested that deletion of the C-terminus of ADR1 (amino acids 305 to 1323) results in a truncated version of the ADR1 protein which is still capable of activating *ADH2* transcription (Denis and

Young, 1983). Further deletions of ADR1 from the C-terminus were constructed to identify functional regions of ADR1 (these experiments are described in the Appendix). An internal deletion of ADR1 was also constructed that spanned the region of ADR1 suggested to be required for bypassing glucose control.

## Methods and Materials

### Internal deletion of amino acids 220 to 262

Plasmids were constructed by ligation of fragments from plasmid YRp7-ADR1-23A (Denis and Gallo, 1986), pUC18 (obtained from New England Biolabs, Beverly MA), and pTRP1 (obtained from M. Wigler, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY).

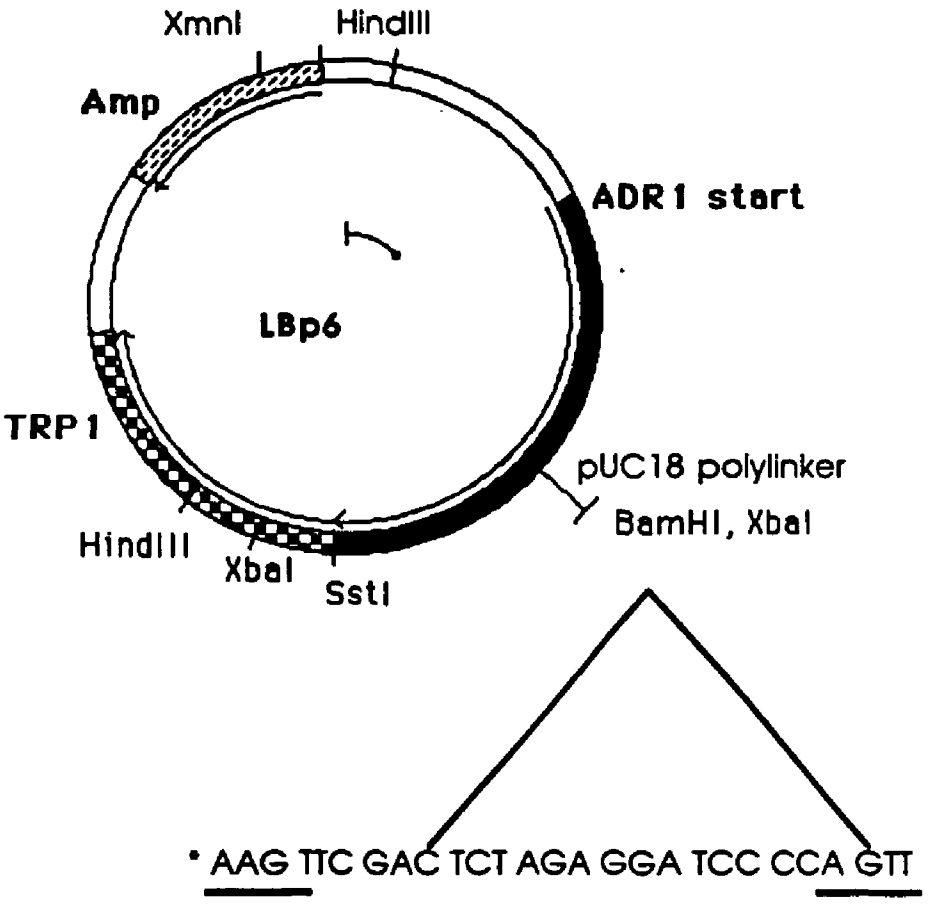
A fragment of *ADR1* extending from the XmnI site at base pair +786 to the SstI site at base pair +1713 was ligated into the pUC18 polylinker region at the SmaI and SstI sites to generate plasmid LBp45. Plasmid LBp45 was cleaved at the unique SalI site and the 5' overhang was filled in to form blunt ends. The resultant plasmid was then restricted with the enzyme HindIII. Restriction of the YRp7-ADR1-23A plasmid with HindIII and ScaI allowed the isolation of a fragment containing 346 bp of pBR322 and the entire 5' region of *ADR1* to base pair +661. The fragment containing the 5' region of *ADR1* was then ligated into the LBp45 plasmid which had

been filled in and restricted as described above. The resulting plasmid, LBp8, contains the 5' region of *ADR1* at the HindIII site of pUC18 to the ScaI site of *ADR1* at base pair 661, the polylinker region of pUC18 from the filled in SalI site, 19 base pairs of the pUC18 polylinker to the SmaI site, and base pairs 786 to 1713 of *ADR1* (Figure 8). The LBp8 plasmid contains a deletion that would result in an ADR1 protein lacking amino acid residues 220 to 262. Seven additional amino acids are added after amino acid 220 due to the polylinker of pUC18 (Figure 8).

Expression of plasmid LBp8 in yeast required the addition of the yeast autonomously replicating sequence (ARS) contained on the *TRP1* gene fragment (Tschumper and Carbon, 1980). Plasmid pTRP1 carries the *TRP1* gene inserted at the EcoRI site of pUC18. A fragment extending from the XmnI site to the SstI site containing the entire *ADR1* gene from plasmid LBp8 was ligated into the pTRP1 plasmid at the SstI and XmnI sites. The ligation of these two fragments repairs the ampicillin gene of the pUC18 vector and forms LBp6. Plasmid LBp6 contains the *TRP1* gene, all of the 5' *ADR1* sequences, and the *ADR1* open reading frame to base pair 1713 with a deletion between base pairs 661 and 786 (Figure 8).

Transformation of plasmid LBp6 into the yeast strain 500-16 was by the lithium acetate method (Ito *et al.*, 1983). Plasmid LBp6 was restricted with

Figure 8: Internal deletion of amino acids 220-253 of ADR1




*ADR1 sequences are underlined

**Plasmid LBp8 is the same as LBp6 except that it lacks the TRP1 fragment.

the enzyme NruI at base pair 1411 (in wild type *ADR1* the NruI site is at base pair 1517) to allow site directed integration into the *ADR1* gene (Orr-Weaver *et al.*, 1981; Denis and Gallo, 1986).

## Results

### Expression of the internally deleted plasmid LBp6 in yeast strain 500-16

Plasmid LBp6 was transformed into strain 500-16 and transformants carrying free plasmid or integrated plasmid were identified (Denis and Gallo, 1986). The presence of plasmid LBp6 in yeast strain 500-16 allowed growth on antimycin A. This result suggests that when present in high copy, plasmid LBp6 allows activation of *ADH2* expression under glucose growth conditions (Table 5). This observation is in contrast to our previous result which suggested that the phosphorylation region is required for *ADR1* to bypass glucose control.

A single integrant of plasmid LBp6 in strain 500-16 were also isolated. The ADH II activity of these strains have been determined under glucose growth conditions and is similar to that of a strain carrying an $ADR1^{C}$ allele (Table 5).

Table 5. ADH II enzyme activities (Section 2)

| | Plasmid | ADH II activity mU/mg glucose | ethanol |
|---|---|---|---|
| *ADR1-5*[c] | none | 280 | 6600 |
| plasmid LBp6 | LBp6 | 400 | nd[a] |
| integrated LBp6 | LBp6 | 100 | 5000 |

[a]nd-not determined

## Discussion

The goal of the internal deletion experiments described in this section was to obtain a better understanding of the role of the region defined by amino acids 220 to 253 of ADR1. The results presented in Appendix for ADR1-220 and ADR1-253 suggested that the region between residues 220 and 253 was necessary for enhancing ADR1 activity but was not required for ADR1 activity. Mutations in the region between amino acids 227 and 231 (a phosphorylation site) (Cherry *et al.*, 1989) allow ADH II to bypass glucose repression. Two models have been proposed to explain the function of this region: this region may be required to enhance ADR1 activity or these mutations prevent a repressor from binding to this region. Other models are also possible as described below. The deletion leaving

just the N-terminal 220 amino acids of ADR1 was subject to glucose repression but was also capable of transcriptional activation (Bemis and Denis, 1988; Appendix). These results suggest that the absence of the phosphorylation region does not allow escape from a negative effector.

The internal deletion removing just the region between amino acids 220 and 262 leaves the C-terminus of ADR1 intact. Therefore the region of *ADR1* to which constitutive mutations have been localized is deleted. ADH II activity in strains carrying the internally deleted *ADR1* was no longer subject to glucose repression which was similar to what was observed in strains carrying an $ADR1^{C}$ mutation.

The above results, suggest that the phosphorylation region may in fact interact with a negative effector and when this region is absent the negative effector can not act through this portion of ADR1.

Another plausible role for this region is that it could be required for dimerization of ADR1. Foot print analysis of ADR1 bound to the promoter region of *ADH2* is consistent with the supposition that ADR1 binds as a dimer to this region (Eisen *et al.*, 1988). ADR1 monomers may bind separately to UAS1 but once bound may interact, possibly through the phosphorylation region. This hypothesis is supported by the fact that ADR1 can bind to one half of the inverted repeat sequence but only half of UAS1 is inactive as a UAS (Yu *et al.*, 1989).

The phosphorylation region could be required for proper folding of the ADR1 protein. The complete deletion of this region results in a less active protein than does a point mutation in this region. A point mutation might be expected to have a different effect upon the folding of the protein than the deletion of a large region. This model suggests that the phosphorylation region is required for interactions within the ADR1 protein rather than interaction with a repressor or another monomer of ADR1.

The contrasting evidence between the ADR1 protein containing only 220 amino acids and the internal deletion of the region between amino acids 220 and 262 could be due to the low level of *ADH2* activation detected in strains carrying ADR1-220. The ADH II activity in these strains is very low under derepressed conditions and any bypassing of glucose repression (typically at a level 5-10% of derepressed activity) may not be detectable under glucose growth conditions. The addition of the C-terminus may assist the N-terminal 220 amino acids to a higher level of *ADH2* activation. In agreement with this, we previously determined that no C-terminal truncation of ADR1 is as active as the entire ADR1 protein (Appendix).

Further experiments involving this region are planned and include the creation of internal deletions throughout this region. The LBp6 plasmid has seven amino acids present due to the pUC18 polylinker region which

would not be present in ADR1. The seven amino acids include an Asp which would provide a negative charge and might be expected to reduce the activity of ADR1 in a manner similar to that of a phosphorylated serine. This, however, could not account for the observed increase in activity. Another consideration when planning future experiments is the recent identification of several other *ADR1$^{C}$* mutations in this region. The recently identified mutations were not localized to the phosphorylation site between 227 and 231. These mutations include a serine to an arginine change at amino acid 232 and a tyrosine to an asparagine or a cysteine change at amino acid 239 (pers. comm. Dan Chase and Susan Fontaine). The sites of these newly characterized mutations have also been deleted in the LBp6 plasmid. Internal deletions and studies of the actual ADR1 protein will help to clarify the remaining questions concerning the regulation of ADR1 through the region C-terminal to amino acid 220.

## SECTION 3

# THE EFFECT OF *ADR1* ON GLYCEROL GROWTH

## Introduction

The transcriptional activator *ADR1* has been shown to be required for the use of glycerol as a carbon source (Bemis and Denis, 1988; Appendix). The previously described C-terminal truncation analysis of ADR1 indicated that ADR1-truncated proteins capable of transcriptional activation could grow on ethanol-containing medium (Table 2, Appendix). In contrast, only ADR1-truncated proteins which contained at least 506 N-terminal amino acids could utilize glycerol-containing medium (Table 2, Appendix). A yeast strain with constitutive ADH II activity and an *adr1-1* allele (Figure 3, Appendix) could not grow on glycerol-containing medium although it could grow on ethanol-containing medium. These results suggest that *ADR1* is

required for growth on glycerol-containing medium regardless of ADH II activity. The requirement of *ADR1* for glycerol growth is the first indication that *ADR1* acts in a metabolic pathway other than that directly regulating *ADH2*.

Catabolism of glycerol is poorly understood in *S. cerevisiae* although two glycerol catabolizing enzymes, glycerol kinase and glycerol 3-phosphate dehydrogenase, have been identified (Sprague and Cronan, 1977). The glucose repressible enzymes glycerol kinase and glycerol 3-phosphate dehydrogenase are not regulated by *ADR1* (Bemis and Denis, 1988; Appendix).

Evidence presented here suggests that *ADR1* is required for glycerol growth and that a recessive mutation at the *GLP1* locus must be present for strong glycerol growth.

## Materials and Methods

### Yeast Strains

Glycerol growth characteristics were studied using strains isogenic to 43-2B and 411-40. The genotypes of these strains are given in Table 6.

Growth Conditions

Strains were tested for growth on glycerol by inoculation into YEP media (1% yeast extract, 2% Bacto-Peptone, 20 mg/l adenine, and 20 mg/l uracil) supplemented with 3% glycerol. Strains that were able to grow in YEP 3% glycerol after two days were considered "strong glycerol growers". Strains able to grow in YEP 3% glycerol after 4-7 days of incubation were classified as "glycerol growers". Strains unable to grow on glycerol after 10 days of incubation in YEP 3% glycerol were termed "glycerol-non-growers". Strains were initially tested on YEP 3% glycerol plates ( YEP 3% glycerol media and 2% bacto agar) in which the same glycerol growth characteristics were detected. Plate analysis was not definitive and needed to be confirmed by growth in liquid glycerol-containing medium. All growth conditions were conducted at 30$^{o}$C.

Medium containing alternative carbon sources was prepared as described above by substituting either 2% lactate, 2% pyruvate, or 3% acetate for the 3% glycerol.

Table 6. Isogenic strains used for characterization of glycerol growth.

| Strain | relevant genotype |
|---|---|
| 43-2B* | *MATə adh1-1 adh3 ura1 his4* |
| 43-2B-R* | *MATə adh1-11 adh3 ura1 his4 glp1* |
| R234* | *MATə adh1-11 adh3 ura1 his4 ADR1-5$^{C}$* |
| R234-R* | *MATə adh1-11 adh3 ura1 his4 ADR1-5$^{C}$ glp1* |
| R234-7* | *MATə adh1-11 adh3 ura1 his4 adr1-5$^{C}$* |
| 411-40** | *MATa adh1-11 adh3 adr1-1:YRp7-ADR1-411 ura1 his4* |
| 660** | *MATa adh1-11 adh3 adr1-1:YRp7-ADR1-5$^{C}$-23A ura1 his4* |
| 500-16** | *MATa adh1-11 adh3 adr1-1 trp1 ura1 his4* |
| 68-3D | *MATa adh1-11 adh3 trp1 ura1 his4* |
| 68-3D-R | *MATa adh1-11 adh3 trp1 ura1 his4 glp1* |

*Strains isogenic to 43-2B

**Strains isogenic to 411-40

# Results

## Glycerol Growth of Isogenic Strains in Liquid Culture

Figure 3 (Appendix) shows the pattern of glycerol growth for 411-40 (*ADR1*, *ADH2*) and the isogenic strain ΔSPH6 (*adr1-1*,. *ADH2-S6*). Strains having a functional *ADR1* (411-40) were found to be capable of slowly utilizing glycerol. In contrast strains lacking a functional *ADR1* (ΔSPH6) were observed to be unable to utilize glycerol as a carbon source (Figure 3, Appendix).

The isogenic strains 43-2B and R234 (Table 5) could not grow on glycerol at first but could adapt to growth on glycerol after 5-7 days (Figure 9). Thus, adaptation to glycerol growth by 43-2B and R234 appears to be a different phenotype than that observed in the 411-40 isogenic series which grows slowly on glycerol (Figure 3, Appendix; Figure 10). Single colonies from adapted cultures of 43-2B and R234 were isolated to determine if the ability to grow on glycerol is reversible. The putative glycerol-adapted strains isolated in this manner were then grown on nonselective media (glucose containing) for several generations. The glycerol-adapted phenotype did not revert back to being poor glycerol growers after several generations of nonselective growth. This result

**Figure 9**

**Growth of strains isogenic to 43-2B on 3% glycerol**

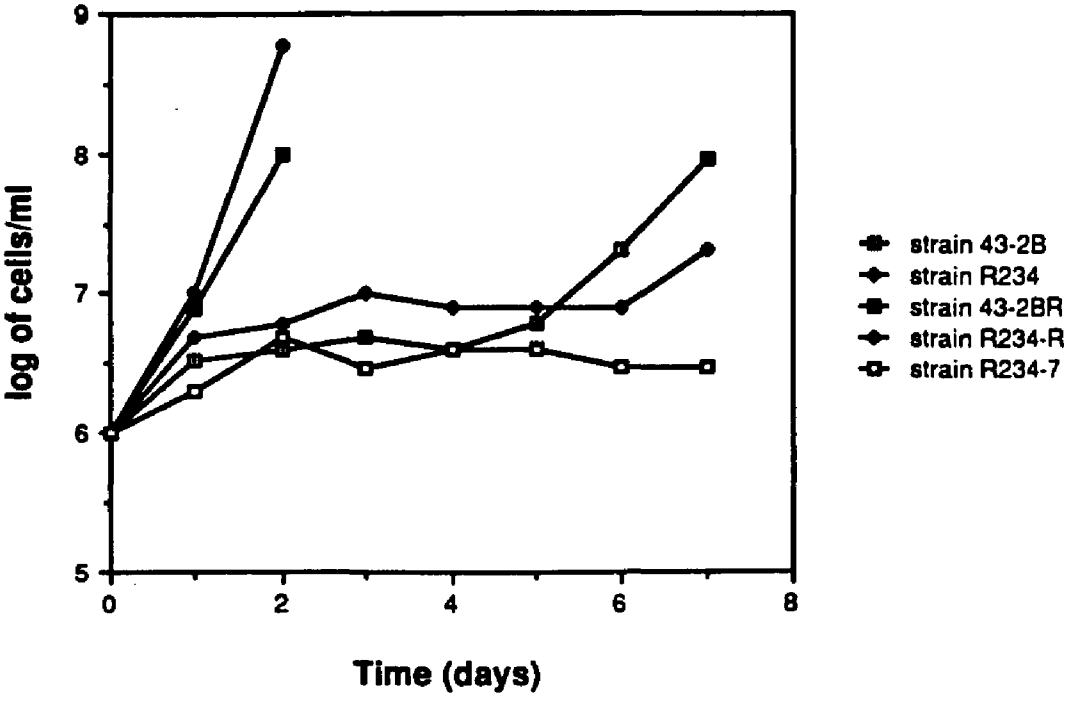
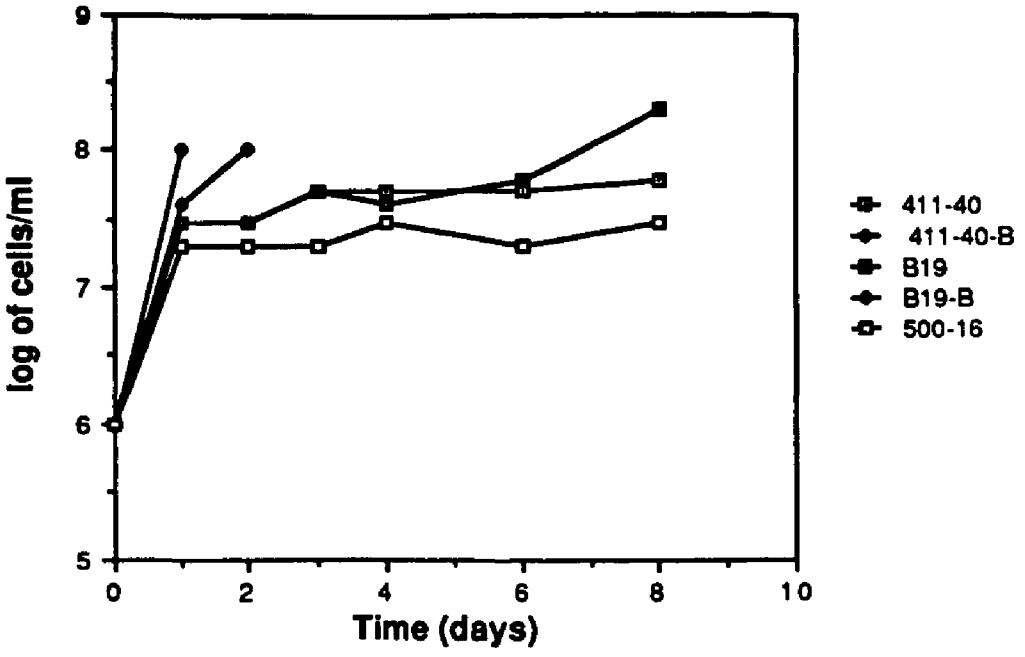


**Figure 10**

**Growth of strains isogenic to 411-40-B on 3% glycerol**



suggests a genetic alteration had occurred in those strains (43-2B-R and R234-R). The strains that were "strong glycerol growers" were called 43-2B-R and R234-R (R indicates revertant on glycerol). These strains grew strongly on glycerol after two days (Figure 9). The isogenic strain R234-7 (*ADR1-5$^{C}$-7*) lacking a functional *ADR1* could not adapt to glycerol growth (Figure 9), suggesting that ADR1 is required for reversion to the strong glycerol growth phenotype.

Strong glycerol growth is due to genetic alteration

To confirm that strong glycerol growth was due to a nuclear genetic alteration and not a cytoplasmically inherited alteration genetic studies were undertaken. The strains 43-2B-R and R234-R were crossed to 567-5d, a "strong glycerol grower". Glycerol growth segregated 4:0 in tetrads from these crosses (Diploids 111 and 112, Table 7) suggesting that the strong glycerol growth observed in 567-5d and in 43-2B-R and R234-R is of a similar nature. In another cross the strong glycerol growth seen in 567-5d segregated 2:2 against the "glycerol grower" phenotype of 43-2B-m1 (Table 8).

In the cross 411-40 to 43-2B-R (Diploid 68, Table 7) "strong glycerol growers" segregated 2:2 further indicating that the mutation which allows glycerol growth is a nuclear mutation rather than a cytoplasmically inherited trait.

The segregant 68-3d was used in further studies because it is adaptable to glycerol growth on plates and in liquid culture. The strain 68-3d-R was isolated following growth of 68-3d in liquid glycerol containing media. The culture of 68-3d was monitored daily by cell counts. When cells were counted after 6 days of growth in glycerol media, two different cell types were detectable, one round and the other elongate. The two cell types were separated by micromanipulation and allowed to grow on YEP 2% glucose. The isolates were screened for ability to grow on glycerol. The cells that were elongate were found to be "strong glycerol growers". The round cells were determined to be "glycerol-non-growers". The "strong glycerol grower" isolate was designated 68-3d-R and subjected to further analysis.

Table 7. Segregation of glycerol growth

| Cross | Parental strains | genotype | Glycerol nongrowers/Glycerol growers complete tetrads 4:0 | 3:1 | 2:2 | 1:3 | 0:4 |
|---|---|---|---|---|---|---|---|
| 65 | 411-40 / 43-2B | *GLP1/GLP1* • | 8 | | | | |
| 68 | 411-40 / 43-2B-R | *GLP1/glp1* | | 1 | 7 | | |
| 69 | 411-40 / R234-R | *GLP1/glp1* | | | 7 | | |
| 77 | 612-4A / 68-3D | *GLP1/GLP1* | 7 | | | | |
| 78 | 612-4A / 68-3D-R | *GLP1/glp1* | | | 15 | | |
| 63 | 43-2B / 567-5D | *GLP1/glp1* | | 2 | 4 | | |
| 111 | 43-2B-R / 567-5D | *glp1/glp1* | (6 tetrads separated only 2 complete) | | | | 2 |
| 112 | R234-R / 567-5D | *glp1/glp1* | (15 tetrads separated only 4 complete) | | | | 4 |

• glp1 is the designation of the mutation allowing strong growth on glycerol.

Table 8. Glycerol growth is independent of *ADH2* but not *ADR1*.

| Strain | relevant genotype | Growth on ethanol | Growth on glycerol |
|---|---|---|---|
| 411-40 | *ADR1/ADH2* | + | +/- |
| 500-16 | *adr1-1/ADH2* | - | - |
| ΔSPH6 | *adr1-1/ADH2-S6* | + | - |
| 216-2a | *ADR1/ADH2* | + | + |
| 216-2b | *ADR1/ADH2* | + | - |
| 216-2c | *ADR1/adh2-m1* | - | + |
| 216-2d | *ADR1/adh2-m1* | - | - |
| 216-7a | *ADR1/adh2-m1* | - | + |
| 216-7b | *ADR1/adh2-m1* | - | + |
| 216-7c | *ADR1/ADH2* | + | - |
| 216-7d | *ADR1/ADH2* | + | - |

Strong glycerol growth is the result of a recessive mutation.

Segregation of the mutation resulting in strong glycerol growth indicated that the mutation was in a nuclear gene but it was unclear if the mutation was recessive or dominant. In order to answer this question the following crosses were conducted. Strain 68-3d-R and strain 612-4a (glycerol grower) were crossed to form diploid 78. Diploid 78 did not grow strongly on glycerol (Table 9). In addition, tetrad analysis of diploid 78 yielded a strong glycerol growth segregation pattern of 2:2 (Table 7). When strain 612-4a was crossed to 68-3d to form diploid 77, the diploid grew slowly on medium-containing glycerol (Table 9). Upon tetrad analysis of diploid 77 no segregants were "strong glycerol growers" (Table 7). These results confirm that a recessive nuclear mutation results in ability to grow well on glycerol-containing medium. Segregants from two tetrads of diploid 78 were back crossed to 68-3d-R or 43-2B-R in order to show that strong glycerol growth was recessive (diploids of tetrads 78-11 and 78-16, Table 9). The nuclear mutation resulting in strong glycerol growth was designated *glp1* because it allows strong growth on glycerol, lactate, and pyruvate. The wild type allele is designated *GLP1*. Diploids listed in table 9 are "strong glycerol growers" if both parents carry the *glp1* allele.

Table 9. Diploid Strains used to characterize recessivity of GP1.

| Diploid | Parent strains | Genotype | Growth on Glycerol |
|---|---|---|---|
| 77 | 68-3d/612-4a | *GLP1/GLP1* | - |
| 78 | 68-3d-R/612-4a | *glp1/GLP1* | - |
| | 68-3d-R/78-11a | *glp1/glp1* | + |
| | 43-2B-R/78-11b | *glp1/GLP1* | - |
| | 68-3d-R/78-11c | *glp1/glp1* | + |
| | 43-2B-R/78-11d | *glp1/GLP1* | - |
| | 43-2B-R/78-16a | *glp1/glp1* | + |
| | 68-3d-R/78-16b | *glp1/glp1* | + |
| | 68-3d-R/78-16c | *glp1/GLP1* | - |
| | 43-2B-R/78-16d | *glp1/GLP1* | - |
| 101 | 43-2B-R/612-1d-2 | *glp1/GLP1, ccr4* | - |
| 102 | 43-2B-R/612-1d | *glp1/GLP1, CCR4* | - |
| 103 | 78-16b/553-4b | *glp1/GLP1, CCR4* | - |
| 104 | 78-16b/553-4b-10 | *glp1/GLP1, ccr4* | - |
| 107 | 78-16b/B19-B* | *glp1/?* | + |
| 108 | 78-16b/411-40-B | *glp1/glp1* | + |
| 113 | 78-17a/411-40 | *GLP1/GLP1* | - |
| 114 | 78-17a/411-40-B | *GLP1/glp1* | - |
| 115 | 78-17a/B19* | *GLP1/?* | + |
| 116 | 78-17a/B19-B* | *GLP1/?* | + |

*Strain B19 carries 21 copies of *ADR1*-642 integrated into the genome.

Mutation to strong glycerol growth on YEP 3% glycerol plates

Strains isogenic to 411-40 and to 43-2B were plated to single colonies on YEP 3% glycerol plates. Two colony types were observed in those strains containing a completely functional *ADR1* or *ADR1-5$^{C}$* allele. Strains lacking *ADR1* could not mutate to strong glycerol growth and displayed only small colonies. Strains carrying the *glp1* allele *ie.* 43-2B-R, contained only large colonies. Strains that could revert to strong glycerol growth formed two colony types: those that were larger like those that carried the *glp1* allele and smaller colonies as found in strains carrying the *GLP1* allele.

Large colonies from strains 411-40 and 500-16:B19 (B19) were isolated and found to result in strong glycerol growth. When strains isolated as big colonies, designated (B for big or larger), were crossed to the strain 78-16b (*glp1*) the diploids were "strong glycerol growers" indicating that they failed to complement the *glp1* allele. Diploids formed by crossing 78-17a (*GLP1*) with 411-40-B or 411-40 were poor growers on glycerol (Table 9). The diploids formed when 78-17a (*GLP1*) was crossed to 500-16:B19 (B19) and B19-B, however, were strong glycerol growers. This latter result is puzzling since strain B19 by itself is not a strong glycerol grower. The lack of complementation between 411-40-B and 78-16b indicates that the mutation in 411-40-B allowing strong glycerol growth is at the *GLP1* locus.

Glycerol growth is independent of *ADH2* but requires *ADR1*

Previous studies indicate that strain 411-40 (*ADR1, ADH2*) grows weakly on glycerol containing medium (Appendix). Strain ΔSPH6 (*adr1-1, ADH2-S6*) which has constitutive ADH II activity due to deletion of the *ADH2* promoter does not utilize glycerol as a carbon source (Appendix). Further indication that ADH II enzyme activity is not linked to glycerol growth was derived from strains lacking ADH II activity (containing the *adh2-m1* allele). Two tetrads from diploid 216 formed between strains 43-2B-m1 (adh2-m1, GLP1) and 567-5d (ADH2, glp1) were analyzed. Strong glycerol growth segregated 2:2 and did so independently of the *adh2-m1* allele (Table 8). The suggestion that *ADR1*, and not *ADH2*, is required for glycerol utilization was therefore confirmed by the independent segregation of *adh2-m1* and strong glycerol growth.

Effect of the *glp1* mutation on Lactate, Pyruvate, and Acetate growth

The effect of the *glp1* mutation on growth on other nonfermentative carbon sources was also tested. Strains isogenic to 411-40 which carried a functional or non-functional *ADR1* gene grew equivalently on medium containing acetate (Figure 11 and 12). Although strain 411-40-B appears by cell counts to grow almost as well on acetate-containing medium as its isogenic counterparts the cells appeared small and irregularly shaped

**Figure 11**

**Growth of strains isogenic to 411-40 on 3% acetate**

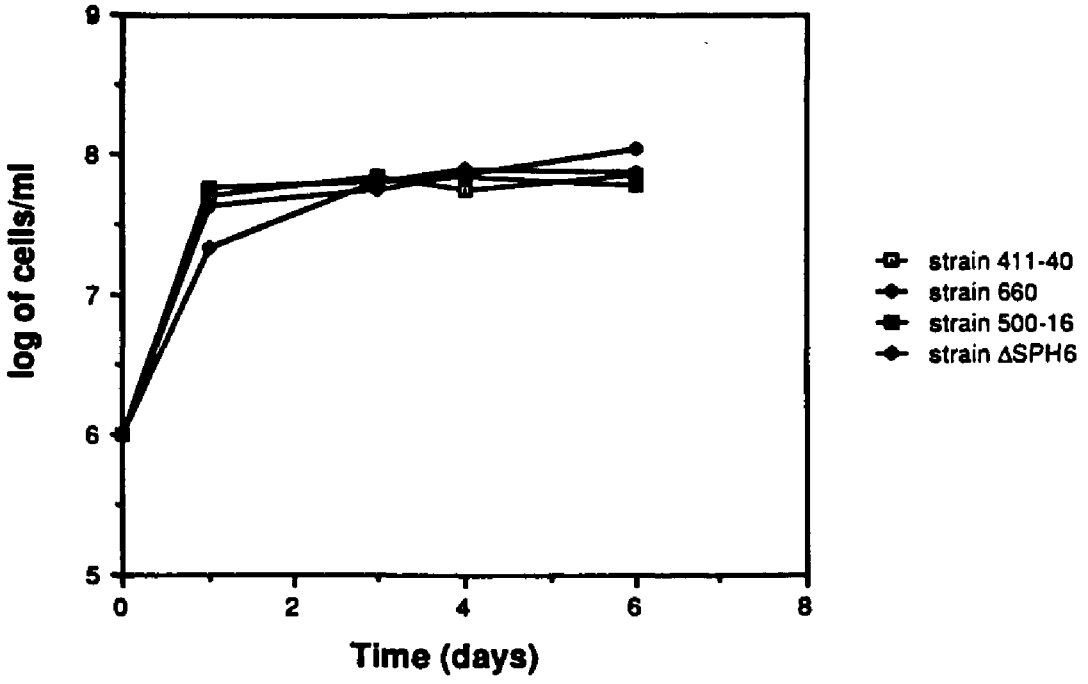
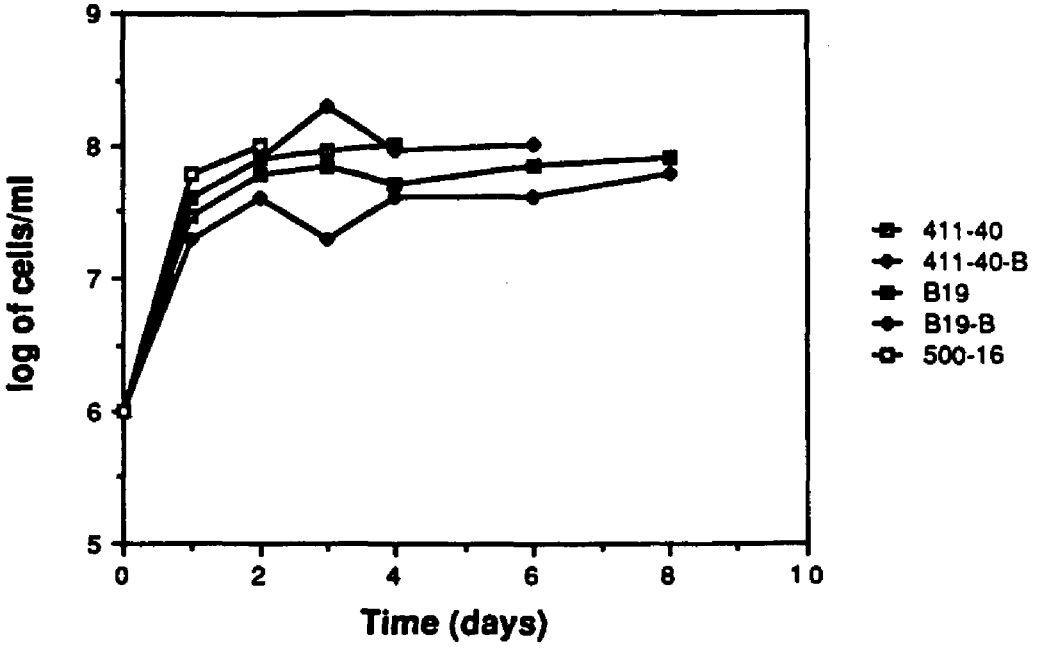


**Figure 12**

**Growth of strains isogenic to 411-40B on 3% acetate**



suggesting that these growth conditions were stressful. Strain B19-B grew slightly better than its parental strain on acetate-containing medium (Figure 12).

Strains 43-2B-R and R234-R which carry the *glp1* alleles grew slightly better than their respective parental strains when grown on acetate-containing medium (Figure 13). The *glp1* mutation in these strains may augment growth on acetate containing medium.

Strains isogenic to 43-2B grown on lactate-containing medium show growth similar to that observed when these strains were grown on glycerol-containing medium (Figure 14 as compared to Figure 9). Strains 43-2B and R234 which carry the *GLP1* allele did not grow as well on medium containing lactate as did strains 43-2B-R and R234-R which carry the *glp1* allele. After 5-7 days of growth in lactate-containing medium strains carrying the *GLP1* allele adapt in a manner similar to that observed for the same strains in glycerol-containing medium (Figure 14 as compared to Figure 9).

Growth in pyruvate-containing medium of strains isogenic to 43-2B carrying either the *GLP1* allele or the *glp1* allele was similar to growth in glycerol- or lactate-containing medium. However, adaptation of strains carrying the *GLP1* allele was observed after 7 days of growth in pyruvate-

**Figure 13**

**Growth of strains isogenic to 43-2B on 3% acetate**

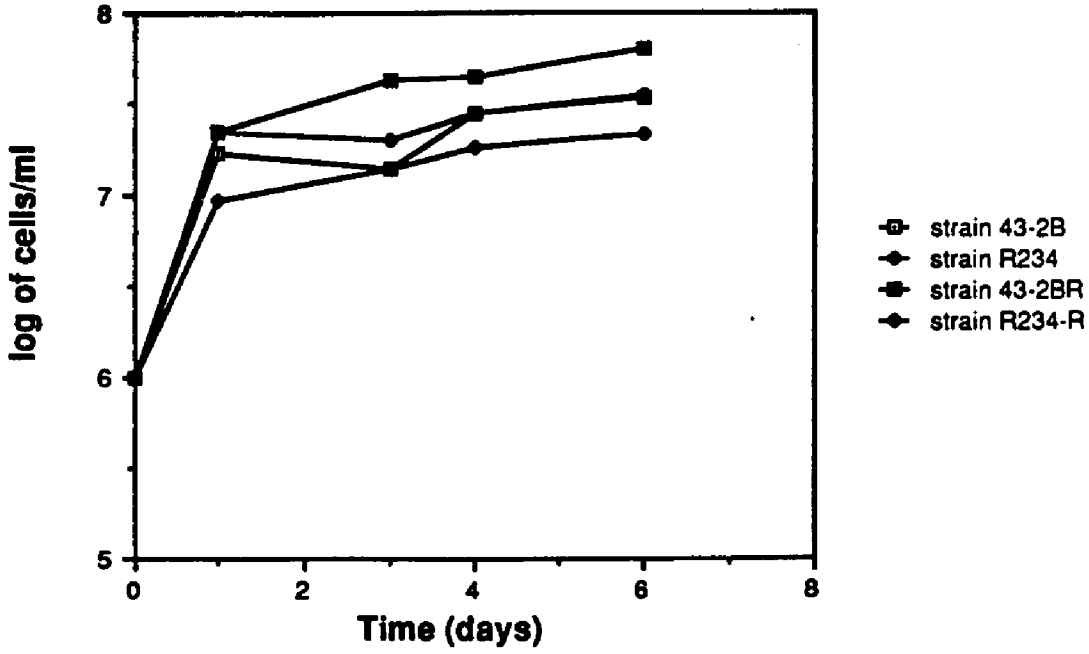


**Figure 14**

**Growth of strains isogenic to 43-2B on 2% lactate**

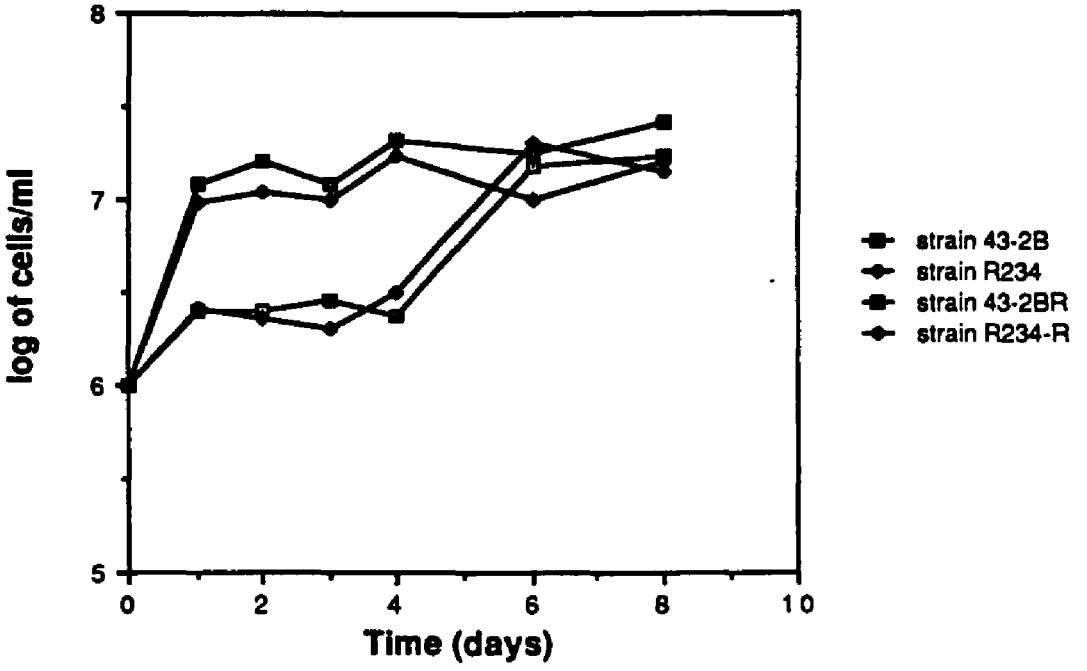
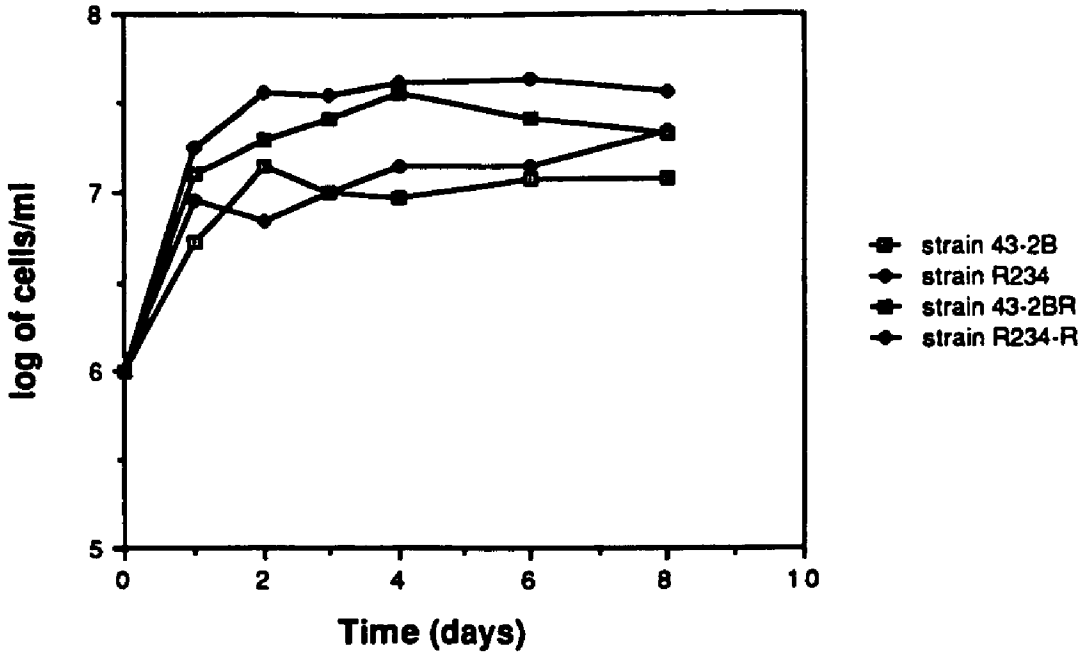


**Figure 15**

**Growth of strains isogenic to 43-2B on 2% pyruvate**



containing medium rather than 5-7 days observed for the same strains in glycerol- or lactate-containing medium (Figure 15).

Strains isogenic to 411-40 carrying a *GLP1* allele grown on lactate did not adapt to growth but grew slowly similar to glycerol growth in the same strains (Figures 16, 17, and 18 as compared to Figure 10). Strain B19-B (*glp1*) grew strongly on lactate-containing medium as it did on glycerol-containing medium. In contrast, strain 411-40-B (*glp1*) which grew strongly on glycerol-containing medium could not grow on 2% lactate and grew weakly on 1% lactate (Figures 17 and 18). Independent selection of the mutant *glp1* alleles may explain the discrepancy between lactate growth and glycerol growth of strain 411-40-B as compared to strain 43-2B-R.

Growth of strains isogenic to 411-40 on pyruvate-containing medium suggested that a functional *ADR1* gene was needed for growth, a result similar to that found for these strains in glycerol and lactate-containing medium (Figure 19 as compared to Figure 16). B19-B (*glp1*) grew slightly better than its respective parental strains (*GLP1*) in pyruvate-containing medium (Figure 20).

**Figure 16**

**Growth of strains isogenic to 411-40 on 2% lactate**

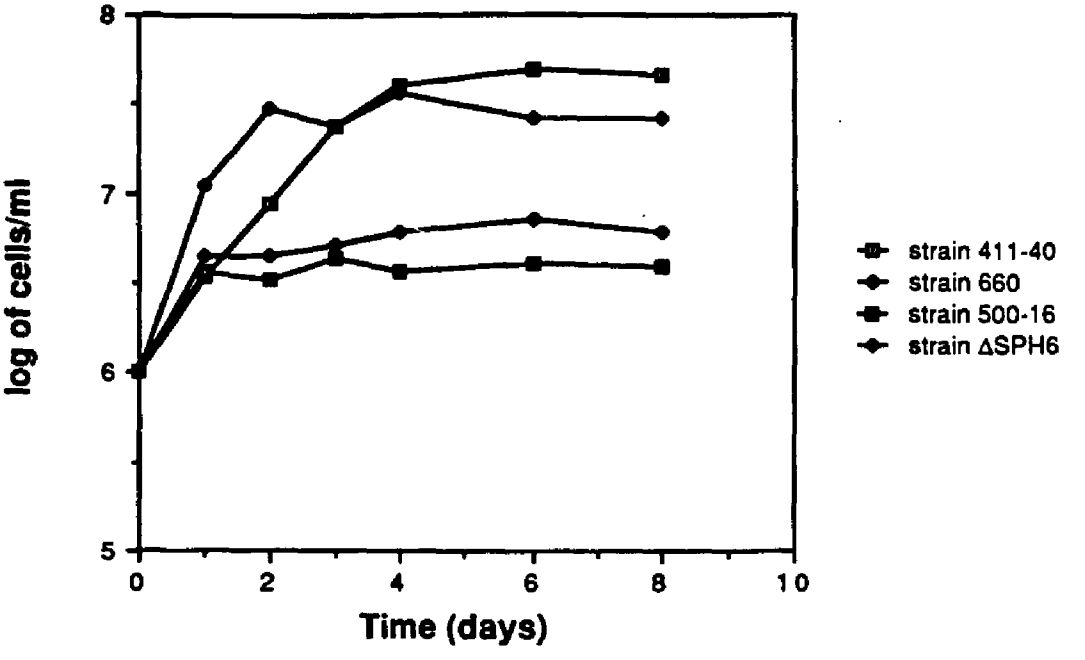


**Figure 17**

**Growth of strains isogenic to 411-40-B on 2% lactate**

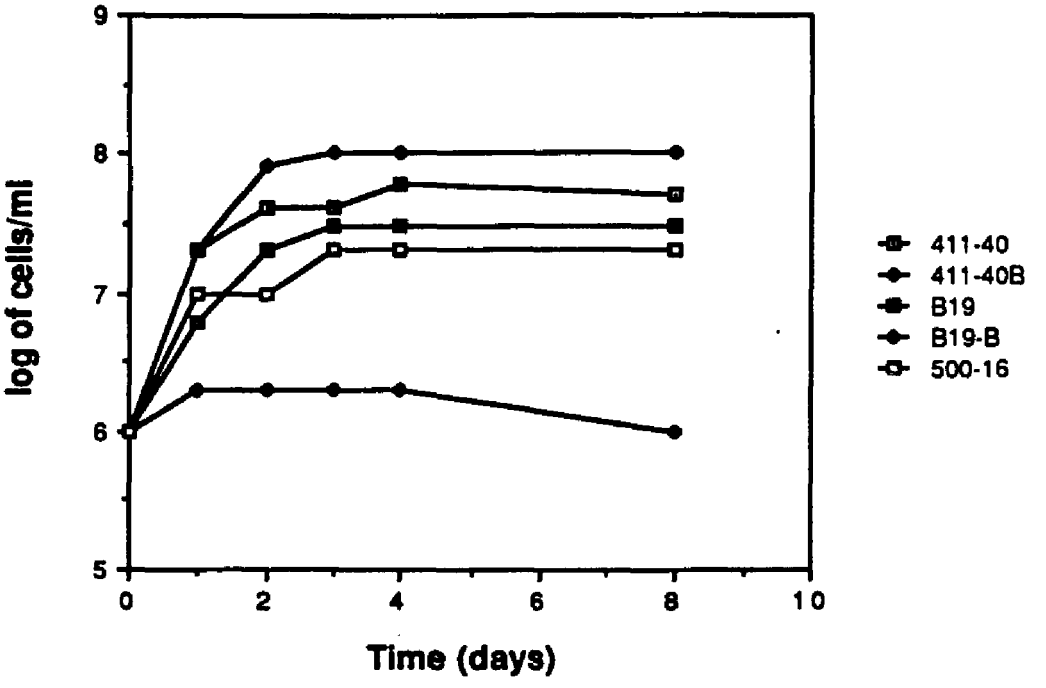


**Figure 18**

**Growth of strains isogenic to 411-40-B on 1% lactate**

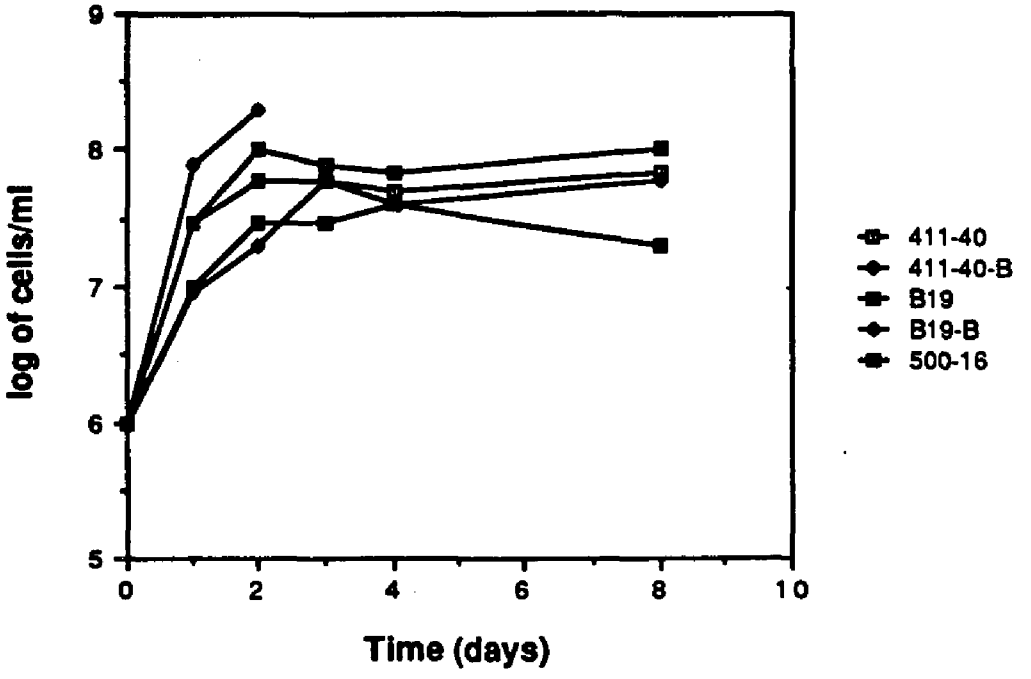


**Figure 19**

**Growth of strains isogenic to 411-40 on 2% pyruvate**

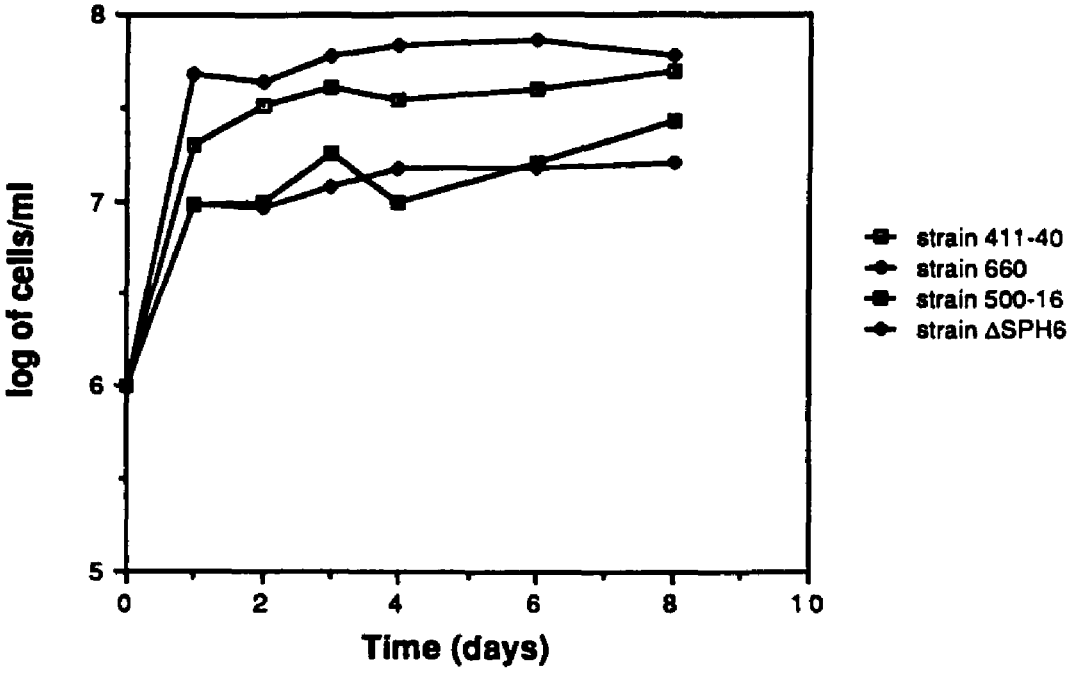
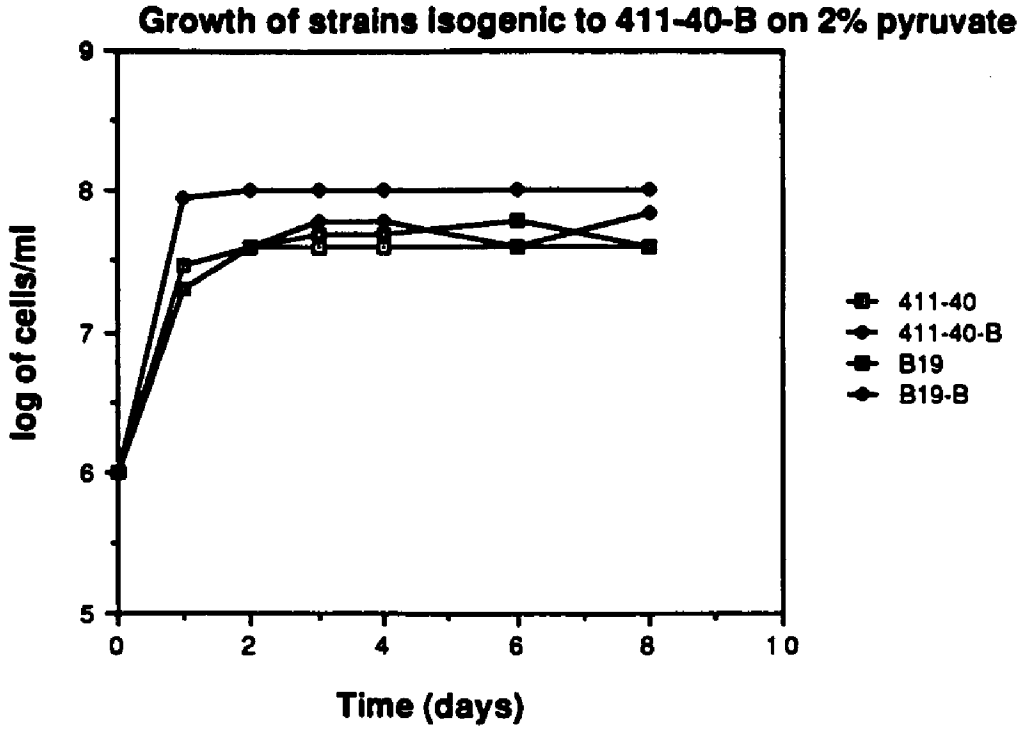


**Figure 20**


Growth of strains isogenic to 411-40-B on 2% pyruvate
log of cells/ml
9
8
7
6
5
0
2
4
6
8
10
Time (days)
411-40
411-40-B
B19
B19-B

## Discussion

A mutation allowing *S. cerevisiae* to grow well when the sole carbon source is either glycerol, lactate, or pyruvate has been identified. The mutation, designated "*glp1*" is recessive and allows glycerol growth only in the presence of a functional *ADR1* gene encoding at least 506 N-terminal amino acids (Bemis and Denis, 1988; Appendix). Furthermore, a functional *ADR1* gene was required for growth in either lactate- or pyruvate-containing medium.

Previous studies of glycerol growth in wild type *S. cerevisiae* present conflicting evidence as to whether wild type yeast can grow on glycerol. Sprague and Cronan (1977) state that wild type yeast can utilize glycerol as a sole carbon source while Wills *et al.*, (1984) find that wild type yeast can not grow on glycerol as a sole carbon source. Earlier confusion may stem from variations in growth conditions and different genetic backgrounds among strains. In our hands, strain 567-5d grew very well on glycerol whereas most of our other strains did not grow well. Strain 567-5d was found to already carry a *glp1* mutation (Table 7). All results presented in this study were conducted with isogenic strains under growth conditions of YEP media plus 3% glycerol at 30$^{o}$C. The results included here suggest that *S. cerevisiae* is able to rapidly metabolize glycerol as a carbon source when the strain carries at least 506 amino acids of ADR1 and the recessive

mutation *glp1*. Wild type strains utilize glycerol at a very slow rate unless a mutation occurs in the *GLP1* gene.

*ADR1* has not been implicated in the regulation of glycerol kinase and glycerol 3-phosphate dehydrogenase because the enzyme activities of these two proteins were not altered in a strain carrying the *adr1-1* allele (Bemis and Denis, 1988; Appendix). The *GLP1* allele is thought to act at a later stage of glycerol metabolism because metabolism of lactate and pyruvate can be affected by the *glp1* mutation.

In contrast to the requirement of a functional *ADR1* gene for growth on glycerol-, lactate-, and pyruvate-containing medium, a functional *ADR1* gene is not required for growth on ethanol-containing medium as long as there is constitutive ADH activity (Bemis and Denis, 1988). Similarly, a functional *ADR1* gene is not required for growth on acetate containing-medium. The presence of the *GLP1* allele or a mutated *glp1* allele does not affect growth on ethanol-containing medium. However, the presence of the *glp1* mutation may enhance growth on acetate-containing medium.

Ethanol is metabolized to acetaldehyde by ADH, the acetaldehyde is then metabolized to acetate by acetaldehyde dehydrogenase. Acetate is metabolized directly into acetyl CoA and used as an energy and carbon source in the TCA cycle (tricarboxylic acid). The fact that yeast readily metabolize ethanol and acetate regardless of the presence of *ADR1* or

*GLP1* suggests that the newly identified function of *ADR1* is probably not involved with the regulation of enzymes that are required to metabolize ethanol and acetate. The increase in growth on acetate in strains containing the *glp1* mutation may be due to increased transport across the mitochondrial membrane but does not require *ADR1*.

The *GLG* mutations previously characterized by Wills *et al.*(1984) may be allelic to *GLP1* but it is unclear if any of the *GLG* mutations require *ADR1* for activity or if they are recessive. The *GLG* mutations do allow growth on glycerol, lactate, and pyruvate (C. Wills, per. comm.).

The *GLG* mutations may be involved in a mitochondrial shuttle mechanism necessary for transport of reducing equivalents across the mitochondrial membrane. If the *glp1* mutation increases transport of metabolites into the mitochondria, growth on glycerol, lactate, pyruvate, and acetate might be enhanced.

It has previously been observed that when the mitochondrial porin gene is disrupted yeast slowly adapt to glycerol growth and an 86 kd protein accumulates (Dihanich *et al.*, 1987). The adaptation to glycerol growth by yeast with a disrupted porin gene is not permanent. Porin minus yeast grown on nonselective media must readapt to glycerol growth in the absence of other carbon sources. Alleles required to regulate porin have not been identified although they are postulated to exist. The porin gene

encodes the only known mitochondrial pore for entry of metabolites, of molecular weight 6000 or less. The 86-kd protein that accumulates is not porin nor is it the 90-kd heat shock protein of yeast (Dihanich *et al.*, 1987). ADR1 is required for yeast to adapt to glycerol growth in strains isogenic to 411-40 and 43-2B. ADR1 could be one of the as yet unidentified regulators of genes involved with mitochondrial transport.

Heat shock proteins and glucose-repressible proteins have been implicated in translocation of proteins across membranes when cells are stressed (Deshaies *et al.*, 1988). Growth on glycerol is a metabolic stress to yeast and thus might be expected to invoke one of the known stress responses.

One model for the function of *ADR1* in the utilization of glycerol is as a transcriptional activator of a gene (Y) necessary for interaction with *GLP1*. *GLP1* would be the repressor of gene (Y) which ADR1 activates or it could be a repressor of a gene (X) needed to interact with gene (Y). *ADR1* would not directly interact with *GLP1* but would regulate some factor (Y) that was in turn needed for growth on glycerol.

Future experiments are required to determine how *ADR1* influences *glp1* and what role *GLP1* normally plays in the metabolism of *S. cerevisiae*. The disruption of *ADR1* in a *glp1* strain would confirm that *ADR1* is required for glycerol growth. The disruption of *ADR1* in a *glp1*

strain would provide a strain necessary for further experiments. This *adr1* strain with the *glp1* mutation could be mutagenized and screened for "strong glycerol growers". These mutations would be in either the gene (Y) or gene (X) of the above model. Isolation of the *glp1* allele would be very difficult because of the recessive nature of the mutation and the absence of a good method for identifying differences between strong and slow glycerol growers. The gene (Y) of the model could also be cloned by overexpression using an appropriate plasmid library. It would be expected that overproduction of Y would bypass the need for *ADR1*. Another experimental approach would be to search a yeast gene library with the *ADH2* dyad sequence which is required for ADR1 binding. This would be an interesting approach because ADR1 may activate other genes that have yet to be identified.

# SECTION 4

## GENERAL DISCUSSION

The goal of this research was to identify functional regions of the transcriptional activator ADR1. This was accomplished by analyzing several facets of ADR1 function.

The *adr1-1* mutation, which blocks *ADR1* activation of *ADH2*,was identified as a nonsense codon at amino acid 11. This result confirms that the *ADR1* open reading frame encodes a protein product and that translation of the ADR1 protein begins at the first AUG in the *ADR1* transcript. The *adr1-1* mutation was found to be suppressible by tRNA-suppressors carrying serine, tyrosine, leucine, or arginine aminoacyl residues, indicating that serine-11 is not essential to ADR1 function.

The second section of this thesis used C-terminal deletions of the *ADR1* gene to detect functional regions of the ADR1 protein. Such analyses have been conducted for other transcriptional activators including

the E1A protein (Krippl *et al.*, 1985), *GAL4* (Johnston *et al.* 1986), and *GCN4* (Hope and Struhl. 1986). The C-terminal deletion analyses led to the identification of a transcriptional activation domain and identification of a region of *ADR1* required for glycerol growth.

The third section of this thesis concerned the requirement of *ADR1* for growth on glycerol. Previous observations suggested that the sole function of ADR1 was the transcriptional activation of *ADH2*. The requirement of *ADR1* for growth on glycerol suggests that *ADR1* may also be involved in some function other than *ADH2* regulation.

The upstream region of *ADR1* had been sequenced to base pair -610 by Hartshorne *et al.*, in 1986. The 5' end of the *ADR1* mRNA had been mapped to base pair -509 (Blumberg *et al.*, 1988) and found to contain an unusually long untranslated leader sequence relative to the translational start site identified in this thesis. Other long 5' untranslated leader sequences have been identified on yeast genes. The *GCN4* mRNA contains small open reading frames in its 5' untranslated sequence which have been implicated in translational control of *GCN4* (Mueller and Hinnebusch, 1986). Translational regulation of *ADR1* in a manner similar to that found for *GCN4* has been ruled out by the lack of small open reading frames in the *ADR1* untranslated region. The *ADR1* mRNA could be regulated by another translational regulatory mechanism or through the

stability of its mRNA.

The possibility that *ADR1* is translationally regulated or is controlled by differences in RNA stability has not been extensively analyzed. Little variation in total *ADR1* RNA levels under glucose or ethanol growth conditions suggests that differences in mRNA stability are not controlling ADR1 function. It has been shown using an ADR1-ß-galactosidase hybrid protein that ß-galactosidase protein levels do not vary between glucose and ethanol growth conditions (Blumberg *et al.*, 1988). More recent data suggest that actual ADR1 protein levels may vary by five- to -ten-fold under the two growth conditions (R. Vallari, pers. comm.). It is also possible that the 5' untranslated region plays no regulatory role but may act in slowing general translation of the *ADR1* mRNA under all growth conditions.

Santiago *et al.*, (1986) have classified mRNA of *S. cerevisiae* into two groups on the basis of mRNA stability. The first group, very stable transcripts, include transcripts having a half life greater than ca. 20 minutes. The class of less stable mRNAs have stabilities less than 20 minutes. While function does not appear to be related to stability, stability was inversely proportional to transcript length. For example, the actin mRNA has a half life of 77 minutes and is 1.3 kb in length. The *PPR1* mRNA has a half life of 2 minutes and is 2.9 kb in length, 1 kb smaller than *ADR1*. If *ADR1* belongs to this less stable class of mRNA it is likely that

the *ADR1* half life would be less than 2 minutes. Overall stability of the transcript may be a mechanism by which mRNA species are maintained at desired levels in the yeast cell. Too high of an ADR1 concentration is known to cause mitochondrial DNA loss and petite formation (Cherry and Denis, 1989).

The presence of the nonsense mutation at codon 1,1 in the *adr1-1* mRNA results in decreased stability of the *ADR1* mRNA. Decreased stability of mRNA by nonsense mutations has been characterized for several other alleles containing nonsense mutations in the 5' region of the structural gene. It is unclear at this time if the instability results from a translational block due to the ribosomes being stalled or if it is due to increased nuclease action on the mRNA. The most likely explanation is a combination of ribosomal stalling and nuclease degradation (Daar and Maquat, 1988).

Transcriptional activating proteins require a region of acidic nature that may be important for interaction with the transcriptional machinery (Ptashne, 1986; Struhl, 1987). Amino acid sequence homology comparison of ADR1 to other transcriptional activators suggests an acidic transcriptional activation region at amino acids 29 through 39 (Figure 3). Initial studies suggested that the commonality between the transcriptional activation regions in GCN4 and GAL4 is the overall acidic nature of the

regions. Recent evidence suggests that not only must the amino acids be of an acidic nature, but the acidic region must form an amphipathic alpha helix (Giniger and Ptashne, 1987). The acidic region of ADR1 has not been characterized by deletion analysis or by mutational analysis. It is possible that single mutations occurring at the acidic region in *ADR1* would not destroy the phenotype if the overall shape or charge of the region is important.

Regulation of transcription by proteins which bind specifically to DNA is a common regulatory mechanism well studied in both eukaryotes and prokaryotes alike. The ADR1 "Zn-binding" DNA-binding finger motif has been characterized by sequence analysis (Hartshorne *et al.*, 1986), mutational analysis (Blumberg *et al.*, 1987; Denis *et al.*, in prep.), deletion analysis (Bemis and Denis, 1988), and DNA binding gel retardation studies (Eisen *et al.*, 1988). The "Zn-binding" DNA-binding finger domain was originally identified in transcriptional factor TFIIIA from *Xenopus* oocytes (Miller *et al.*, 1985). The binding fingers of TFIIIA have nine repeats of a 30 amino acid finger that are coordinately stabilized by cysteine and histidine residues through $Zn^{2+}$ linkages (Diakun *et al.*, 1986; Frankel *et al.*, 1987). A "Zn-binding" DNA-binding finger of ADR1 (amino acids 130-159) was synthesized and studies confirm that metal binding occurs and drives the folding of the peptide (Parraga *et al.*, 1988). These results agree with

similar experiments on synthesized peptides of TFIIIA finger regions (Frankel et al., 1987) and with studies that suggest $Zn^{2+}$ is tetrahedrally coordinated between histidine and cysteine residues in peptides of this nature (Diakun *et al.*, 1986). ADR1 has two "Zn-binding finger" regions. The presence of both of these regions are required for ADR1 binding to the *ADH2* gene(Bemis and Denis, 1988; Appendix ; Parraga *et al.*, 1988).

Mutations localized to the DNA-binding domain of *ADR1* have been characterized. Mutations of the amino acids proposed to be directly involved in "Zn-binding" block ADR1 activity while mutations in other residues of the finger reduce activity to varying degrees (Blumberg *et al.*, 1987; Denis *et al.*, in prep.).

The *adr1-5$^{C}$-x* mutations (Figure 3) which were isolated as second site mutations of the *ADR1-5$^{C}$* allele have been identified. The *adr1-5$^{C}$-7* mutation was identified at amino acid 117 in the finger tip of the first DNA-binding finger and blocks activity of *ADR1*. The *adr1-5$^{C}$-5* mutation was localized to amino acid 129 which is in the linker region between the two fingers. This change causes a less severe effect on *ADR1* function with respect to its ability to activate *ADH2*. *The adr1-5$^{C}$-2,-3*, and *-6* mutations were all localized to amino acid 97 causing a proline to a serine change in all three alleles (D. Mullaney, 1988). A proline residue is commonly found 8 amino acids from the first finger in "Zn-binding" DNA-

Binding proteins. The proline at amino acid 97 is nine residues away from the first finger region in ADR1, but other than the possibility of creating a bend in the polypeptide chain, little is known about the importance of this residue.

Several cAMP-dependent protein kinase phosphorylation consensus sequences have been identified in ADR1 by sequence analysis (Hartshorne *et al.*, 1986). The *ADR1-2$^{C}$*, *5$^{C}$*, and *7$^{C}$* mutations which allow *ADH2* expression during glucose growth were localized to the phosphorylation sequence near amino acid 230 (Figure 3) (Denis and Gallo, 1986; Cherry *et al.*, 1989). *ADR1* mutations localized to the phosphorylation sequence allow partial *ADH2* activation during glucose repression (Strains R234, Table 1). *In vitro* phosphorylation studies of ADR1, ADR1-2$^{C}$, 5$^{C}$, and 7$^{C}$ fusion proteins confirm that the mutations near the phosphorylation site at amino acid 230 reduce the phosphorylation of ADR1 (Cherry *et al.*, 1989). The *ADR1-7$^{C}$* mutation completely blocks phosphorylation at this site because the phosphoacceptor serine-230 is mutated to a leucine (Figure 3). The *ADR1-2$^{C}$* and *5$^{C}$* mutations cause a phenylalanine to serine conversion at amino acid 231 and an arginine to lysine substitution at amino acid 228, respectively. Both changes appear to increase the Km of phosphorylation but do not completely block possible phosphorylation at serine-230 (Cherry *et al.*, 1989). The *in vitro*

phosphorylation studies of the ADR1 and ADR1$^{C}$ fusion proteins indicate that ADR1 is also phosphorylated by cAMP-dependent protein kinase at a site other than the site at amino acid 230 because even the ADR1-7$^{C}$ protein is a phosphoprotein (Cherry *et al.*, 1989).

The C-terminal truncated ADR1 protein (220 amino acids, Appendix) lacking the phosphorylation site at amino acid 230 was found to be capable of transcriptional activation of *ADH2* but not able to allow escape of *ADH2* from glucose repression. Alternative explanations for this result may be that the deletion of such a large region (1103 amino acids) of ADR1 may alter interactions necessary for the escape from glucose repression or that the increase in ADH II activity during glucose growth conditions may be undetectable due to the reduced function of the ADR1-220 protein. In contrast to the C-terminal truncation leaving only 220 amino acids, a small internal deletion removing the amino acids between 220 and 262 of ADR1 results in an ADR1 protein able to escape glucose repression. The deletion of this region may remove a site required for interaction with a repressor or this region could have another function. Other functions proposed for this region are that it may be required to block dimerization of ADR1 monomers or that it may block the proper folding of the ADR1 protein until dephosphorylation occurs. Further experiments are planned to study the existence of a repressor. One experiment is to over express the

proposed repressor site on ADR1 in the expectation of titrating out the repressor.

CCR1 is not a cAMP-dependent protein kinase and is proposed to be an activator of *ADH2*. CCR1 would therefore not be the kinase directly phosphorylating *ADR1*, but might be required for the regulation of a phosphatase that controls ADR1. Alternatively CCR1 may phosphorylate a protein that ADR1 acts with.

The cAMP-dependent protein kinase of yeast is composed of regulatory subunits encoded by *BCY1* (Toda *et al.*, 1985) and catalytic subunits encoded by the *TPK* alleles, *TPK1*, *TPK2*, and *TPK3* (Toda *et al.*, 1987). A mutation at *BCY1* (*bcy1*) blocks regulation of the catalytic subunits allowing unregulated phosphorylation at cAMP-dependent phosphorylation sites. In strains carrying a *bcy1*allele, one functional *TPK*gene, and the *ADR1* gene ADH II activity was reduced under nonfermentative growth as compared to wild type ADH II activity. In strains of similar genotype except that an $ADR1^{C}$ allele was present the effect of unregulated cAMP dependent protein kinase activity was diminished. In strains in which all three cAMP dependent protein kinases are mutated or disrupted (*bcy1*, *tpk1*, *tpk2*, *tpk3w*) there was no effect on *ADR1* regulated *ADH2* activity. This result supports the hypothesis that *ADH2* may be regulated by complex mechanisms including both cAMP dependent and independent protein kinases. *ADR1* is suggested to be a

phosphoprotein (Cherry *et al.*, 1989; R. Vallari and D. Audino, pers. comm.) and may be phosphorylated by a complex and interactive method between several protein kinases under various conditions.

Phosphorylation may act as a signal for degradation of *ADR1* as it does for fructose-1,6-bisphosphatase (Lamponi *et al.*, 1987). Phosphorylation as a degradation signal is a model supported by the fact that under glucose growth conditions ADR1 is found to be present at very low levels as compared with ethanol growth conditions (R. Vallari, pers. comm.). This hypothesis is also consistent with the observation that $ADR1^{C}$ type mutations augment the activity of ADR1 under glucose repression but never to fully derepressed levels. It is possible that $ADR1^{C}$-type mutations may contain phosphorylation sites other than serine-230 which might allow altered protein degradation under glucose growth conditions.

Phosphorylation is the signal for many regulatory mechanisms. When the 60 kD protein pp60 from *Xenopus laevis* oocytes is phosphorylated it binds mRNA and blocks translation (Kick *et al.*, 1987). Upon dephosphorylation binding is destabilized and translation occurs. A mechanism of translational regulation for *ADR1* has purportedly been excluded because ADR1-ß-galactosidase fusion proteins appear to be equally abundant under glucose and ethanol growth conditions (Eisen *et al.*, 1988). However, the fusion proteins are capable of some escape from

glucose repression so the possibility of autoregulation of translation by *ADR1* is not completely ruled out.

Phosphorylation of the CREB protein, a protein isolated from rat brain and required to bind cAMP response elements, controls its dimerization and transcriptional activation (Yamamoto *et al.*, 1988). Phosphorylation of CREB enhances dimerization and transcriptional activation while phosphorylation of ADR1 is thought to inhibit activation of *ADH2*. The effect on dimerization of ADR1 by phosphorylation is unknown. The general trend for DNA-binding phosphoproteins is the more phosphorylated the protein the less able to bind nucleic acids (Radtke and Unteregger, 1988). ADR1 appears to follow this trend based on genetic evidence. However, it has not been determined if phosphorylation affects ADR1 binding to *ADH2* DNA.

A previously unidentified function of *ADR1*, involvement in utilization of glycerol, has been characterized. The natural environment of *S. cerevisiae*, grapes, would provide sugars as the major carbon source available for yeast metabolism. In the absence of glucose *S. cerevisiae* can use ethanol which has been excreted following the fermentation of glucose. Metabolism of glucose and ethanol are thus highly regulated processes in yeast. Growth on nonfermentative carbon sources uncommon outside of the laboratory probably were not important enough for yeast to

develop separate highly controlled pathways for utilization. Growth of wild-type yeast on glycerol as the sole carbon source is a very stressful condition for yeast and the cells grow slowly (Wills *et al.* 1984; Bemis and Denis, 1988). Strains capable of rapid growth on glycerol were identified and isolated (approximately 1 in $1x10^6$ cells). These strains were shown to carry the *glp1* mutation, a mutation which allows growth on glycerol, lactate, pyruvate, and acetate. *glp1* is a nuclear gene requiring *ADR1* for activity. The *GLP1* gene may be allelic to one of the *glg* mutations previously characterized by Wills *et al.*, (1984). The *glg* mutations are putative enhancers of transport across the mitochondrial membrane (Wills *et al.* 1984). Transport is thought to be important in the adaptation to glycerol growth in the porin minus yeast described by Dihanich *et al.*, (1987).

Isolation of the *GLP1* gene may be difficult because the mutation is recessive. Genetic studies are in progress and cloning strategies to isolate the gene or genes regulated by *ADR1* or *GLP1* are being contemplated. The isolation of the *GLP1* gene itself would be complicated because the only detectable phenotype would be loss of glycerol growth. Another important experiment would be to disrupt the *ADR1* gene in a *glp1* strain to confirm the requirement of *ADR1* to glycerol growth.

*GLP1* has been proposed to be important in transport across the

mitochondrial membrane. The porin gene and the 86 Kd protein gene (Dihanich *et al.*, 1987) have been cloned. Increased information about the mitochondrial membrane proteins may provide insight into the role of *GLP1*.

The results of this thesis extend our understanding of the various functions of *ADR1* and suggest further avenues of exploration. Investigations of *ADR1* and its interactions with other alleles will be of great interest to the subject of transcriptional activators and their universal role in gene regulation.

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# APPENDIX

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# Identification of Functional Regions in the Yeast Transcriptional Activator ADR1†

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**The transcriptional activator ADR1 from *Saccharomyces cerevisiae* is a postulated DNA-binding protein that controls the expression of the glucose-repressible alcohol dehydrogenase (*ADH2*). Carboxy-terminal deletions of the ADR1 protein (1,323 amino acids in length) were used to localize its functional regions. The transcriptional activation region was localized to the N-terminal 220 amino acids of ADR1 containing two DNA-binding zinc finger motifs. In addition to the N terminus, a large part of the ADR1 sequence was shown to be essential for complete activation of *ADH2*. Deletion of the putative phosphorylation region, defined by *ADR1*$^{c}$ mutations that overcome glucose repression, did not render *ADH2* expression insensitive to glucose repression. Instead, this region (amino acids 220 through 253) was found to be required by ADR1 to bypass glucose repression. These results suggest that *ADR1*$^{c}$ mutations enhance *ADR1* function, rather than block an interaction of the putative phosphorylation region with a repressor molecule. Furthermore, the protein kinase CCR1 was shown to affect *ADH2* expression when the putative phosphorylation region was removed, indicating that CCR1 does not act solely through this region. A functional *ADR1* gene was also found to be necessary for growth on glycerol-containing medium. The N-terminal 506 amino acids of ADR1 were required for this newly identified function, indicating that *ADH2* activation and glycerol growth are controlled by separate regions of ADR1.**

Eucaryotic transcriptional regulators tend to be large proteins having molecular masses greater than 70,000 daltons (20, 24, 27, 35). Their large size suggests the presence of multiple domains, each being wholly or partly responsible for imparting one or more biological activities to the protein. Deletion and mutation analyses, together with sequence homology comparisons, have proven useful in identifying and localizing functional regions. These approaches have been notably successful in studies of the simian virus 40 T antigen (19, 32), the glucocorticoid receptor (6, 23, 28), GAL4 (17, 21, 26), and GCN4 (14).

The transcriptional activator ADR1, a protein of 151,000 daltons (12), functions in controlling the expression of the glucose-repressible alcohol dehydrogenase (ADH II; encoded by the *ADH2* gene) from *Saccharomyces cerevisiae* (4). Two functional regions of ADR1 have been identified by sequence comparisons and mutation analysis. One of these regions is highly homologous to the zinc-containing DNA-binding fingers previously identified in transcription factor IIIA of *Xenopus laevis* (29). This region lies between amino acids 99 and 155 of ADR1 (12) and contains two such zinc fingers. Although not yet demonstrated to be a DNA-binding protein, ADR1 is located in the nucleus (1) and has been shown to control *ADH2* expression through a 22-base-pair (bp) segment of dyad symmetry upstream of the *ADH2* gene (31). Deletion analysis indicates that the first 304 N-terminal amino acids of ADR1 are sufficient to cause partial derepression of *ADH2* (10), whereas the first 151 amino acids are not sufficient (12). Furthermore, most mutations that inactivate *ADR1* occur in the zinc finger region (1; C. L. Denis et al., manuscript in preparation). These results confirm the importance of the finger region to ADR1 function.

The second functional region in ADR1 consists of a cAMP-dependent protein kinase phosphorylation recognition site, located between amino acids 227 and 231 (22). Mutations which allow ADR1 to bypass glucose control and activate *ADH2* transcription have been localized to this region (9; J. Cherry et al., manuscript in preparation). These *ADR1*$^{c}$ mutations (constitutive for *ADH2* expression) have been postulated to increase ADR1 function under repressed conditions by decreasing ADR1 phosphorylation (9). This could occur by blocking interaction of ADR1 with a negative effector or by enhancing the ability of ADR1 to activate transcription. It has also been suggested that activation of *ADH2* upon derepression is regulated by a glucose-dependent dephosphorylation of ADR1 (9). The *CCR1* gene, encoding a protein kinase (2, 7) required for *ADH2* derepression (5), may play a role in controlling ADR1 phosphorylation state (8, 9).

We have undertaken a series of progressive carboxy-terminal deletions of ADR1 to obtain a clearer understanding of its structure and function. The questions which we address include the following. (i) What part of ADR1 is required for *ADH2* activation? (ii) Is the putative phosphorylation region the site through which the glucose signal is mediated? (iii) Does CCR1 act through the phosphorylation region of ADR1? We report here that ADR1 contains several functional regions and identify a region specifically required for growth on glycerol. We also present data showing that the putative phosphorylation region, while not being absolutely required for ADR1 activation of *ADH2*, does play a positive role in ADR1 function.

## MATERIALS AND METHODS

**Yeast strains.** Strain 500-16 (*MATa adh1-11 adh3 adr1-1 ura1 trp1 his4*) was used for yeast transformations. Other strains used in this study are: 521-10 (*MATa adh1-11 adh3 adr1-1 trp1::YRp7-ADR1-311 ura1 his4*), 205-5d (*MATα adh1-11 adh3 ccr1-1 trp1 ura1*), and 500-16-C3 (same as

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500-16, except that 15 copies of *ADR1-220* are integrated at the *trp1* locus).

**ADR1 plasmids.** All *ADR1* genes were derived from plasmids YRp7-ADR1-411, YRp7-ADR1-23A, and YRp7-ADR1-311 (9, 10) and were present on YRp7 vectors. Each contains at least 1.2 kilobases of *ADR1* upstream DNA, which is sufficient for normal *ADR1* expression (8). Truncated *ADR1* gene sequences end at the restriction sites and base pair numbers shown in Fig. 1. Each gene is designated by the number of amino acids of ADR1 protein sequence it encodes (e.g., *ADR1-282* encodes the first 282 N-terminal amino acids of *ADR1*). In most cases, non-ADR1 amino acids are present on the carboxy termini of the truncated ADR1 polypeptides: *ADR1-1323* encodes the wild-type coding sequence. *ADR1-1068* encodes an extra 81 amino acids derived from pBR322 sequences from bp 385 to 623. *ADR1-642* encodes an extra 16 amino acids derived from the complementary strand of pBR322 from bp 376 to 326. *ADR1-506* encodes an extra 32 amino acids derived from pBR322 sequences from bp 975 to 1071. *ADR1-304* encodes an extra 7 amino acids from the complementary strand of pBR322 that runs from bp 374 to 353. *ADR1-282* encodes an extra 22 amino acids derived from bp 185 to 251 of the *TRP1* gene. *ADR1-272* encodes 23 extra amino acids that run from bp 2036 to 2105 of pBR322. *ADR1-253* encodes 10 extra amino acids from bp 974 to 1004 of pBR322. *ADR1-220* encodes no extra amino acids, and *ADR1-151* encodes an extra 235 amino acids derived from pBR322 sequences extending from bp 568 to 1273.

**Growth conditions, assays, and transformations.** For ADH assays, yeast cells were grown to mid-logarithmic phase at 30°C in YEP medium (2% Bacto-Peptone [Difco Laboratories], 1% yeast extract, 20 mg each of adenine and uracil per liter), supplemented either with 8% glucose or 3% ethanol. ADH activity assays were performed as previously described (10), and all values represent the average of at least three separate determinations. Yeast transformations were conducted by either the protoplast (10) or lithium acetate method (16). The methods used for identifying integration of the *ADR1* genes and for determining the number of copies integrated into the genome have been described previously (8). Integration of the YRp7 plasmids carrying the *ADR1* genes was site directed to the *trp1* locus by cutting the plasmids in the interior of the *TRP1* gene with *Mst*II or *Bgl*II.

For transformations conducted with plasmids containing the *ADR1-648* and *ADR1-282* genes, only a single integrant was obtained in each case, carrying 21 and 29 tandem copies, respectively. Isolation of fewer numbers of iterated *ADR1-648* and *ADR1-282* genes per cell was conducted by selecting for deintegration events, as previously described (8). No difference in *ADH2* expression has been observed between strains carrying the same number of *ADR1* genes, whether derived from deintegration or by a primary transformation event (8).

## RESULTS

Carboxy-terminal deletions of the *ADR1* gene were used to localize functional regions of the ADR1 protein (Fig. 1). Truncated *ADR1* genes, present either on plasmid vectors or as stable integrants at the *trp1* locus (8), were analyzed. Genes were stably integrated into the genome so that the gene dosage per cell could be accurately determined. This approach allows the *ADR1* dosage dependence of *ADH2* expression to be studied (8). Five criteria were used to compare the functionality of the truncated *ADR1* genes: (i) ability to allow derepression of *ADH2*, (ii) maximal level of *ADH2* expression, (iii) ability to overcome glucose repression, (iv) ability to sustain growth on a nonfermentative carbon source, and (v) insensitivity to a defect in the *CCR1* gene.

**ADR1 activating region.** Wild-type and deleted *ADR1* genes were stably integrated at the *trp1* locus and assayed for their ability to activate *ADH2* expression (Table 1). *ADR1* genes coding for at least the N-terminal 220 amino acids of ADR1 were capable of allowing *ADH2* derepression. ADR1-220 lacks the putative phosphorylation site implicated in glucose control of ADR1. All functional *ADR1* genes at a single or low dosage conferred an ADH2 phenotype that remained glucose repressed (Table 1). Conversely, no region of ADR1 was identified which, when removed, rendered *ADH2* resistant to glucose repression. ADR1-151, containing only one complete DNA-binding zinc finger, did not allow *ADH2* expression.

Increases in *ADR1* gene size did not show a proportional increase in the ability to activate *ADH2*. This feature is observed most clearly when single dosages of the different genes are compared (Table 1). These variations could be the result of differences in protein or mRNA stability between the various *ADR1* constructs.

To better estimate the relative function of the various-sized *ADR1* genes in a way that would eliminate possible differences in their protein and mRNA stability, the maximal ability of the *ADR1* genes to activate *ADH2* was measured. The maximal activation ability of ADR1 is based on previous observations that high *ADR1* dosages saturate *ADH2* expression during nonfermentative growth in which *ADH2* copy number is the limiting factor (8). By using the value for maximal activation of *ADH2*, a comparison could be made between the functions of different-sized ADR1 proteins that is independent of their relative protein stability. A range of dosages for the indicated *ADR1* genes integrated at the *trp1* locus were obtained, and the resultant ADH II activities were determined. Figure 2A illustrates the effect of varying *ADR1* dosage on *ADH2* derepression for three *ADR1* alleles. Values for maximal ADH II activity were determined by using an Eadie-Hofstee plot and are given in Fig. 2B as a function of ADR1 polypeptide length. For *ADR1-253*, *-304*, *-506*, and *-1068*, the ranges of dosage obtained were not sufficiently broad to allow calculation of maximal ADH II activities. It was observed that by progressively removing parts of the C terminus of ADR1, the ability of ADR1 to maximally activate *ADH2* expression also progressively

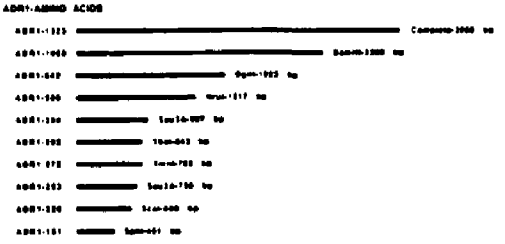


FIG. 1. Comparison of deleted *ADR1* genes. Each *ADR1* construct is designated on the left by the number of amino acids present in its coding sequences. To the right are given the restriction sites and base pair numbers at which *ADR1* DNA sequences end. *ADR1-1323* represents the complete *ADR1* gene as present on YRp7-ADR1-411 (10).

decreased. A major decrease in activity was observed when ADR1 was shortened from 282 to 272 amino acids. These results indicate that a large part, if not all, of the ADR1 protein is required to modulate ADR1 activation of *ADH2*. It should be noted that the $K_{1/2}$, defined as the number of *ADR1* genes required to give half the maximal ADH II activity, was similar for ADR1-1323, -642, -272, and -220 ($K_{1/2}$ = 2 to 3 genes), whereas the $K_{1/2}$ for ADR1-282 was much greater ($K_{1/2}$ = 14 genes). The similarity in $K_{1/2}$ for the four polypeptides suggests that their protein stabilities were not significantly different.

**Region of ADR1 required to bypass glucose repression.** Glucose repression of *ADH2* can be overcome by increased dosage of the *ADR1* gene (8, 15; Table 1). To define the region of ADR1 required to bypass glucose repression, plasmids bearing the *ADR1* genes were transformed into *S. cerevisiae*, and ADH II enzyme activities were determined after growth on medium containing both glucose and the respiratory inhibitor antimycin A. Antimycin A restricts growth to those cells capable of fermentation and, in this case, the cells which are expressing the *ADH2* gene. Because *ADR1* concentration is limiting for *ADH2* expression when cells are grown on glucose (8), the presence of the inhibitor selects for cells carrying copy numbers of *ADR1* plasmid sufficient to bypass glucose repression. *ADR1-151*, which was incapable of activating *ADH2* under derepressed conditions, did not allow growth on medium containing antimycin A (Table 2). *ADR1-220* resulted in poor growth in the presence of antimycin A (data not shown) because of the low levels of ADH II enzyme activity expressed (8 mU/mg; Table 2). *ADR1-220* was, however, capable of activating *ADH2* during derepression (Table 1). Increased expression of all ADR1 polypeptides longer than ADR1-220 bypassed glucose repression and allowed for increased ADH II levels (greater than 80 mU/mg; Table 2). The 2- to 10-fold difference in ADH II activity under glucose-repressed conditions between the *ADR1-1323* and the *ADR1-304*, *-282*, *-272*, and *-253* alleles is most likely a result of a comparable 5- to 8-fold decrease in transcriptional activation ability, as seen under derepressed conditions (Table 1). ADR1-220 appears, therefore, to lack part of the region required to bypass glucose repression.

The above results obtained using plasmid-bearing *ADR1*

TABLE 1. Effect of truncated *ADR1* genes on *ADH2* expression

| Gene[a] | Copies | ADH II activity (mU/mg) on | |
|---|---|---|---|
| | | Glucose | Ethanol |
| *ADR1-1323*[b] | 1 | 5 | 2,400 |
| | 96 | 680 | 7,400 |
| *ADR1-1068* | 1 | 2 | 8 |
| | 3[c] | 2 | 530 |
| *ADR1-642* | 1 | 8 | 1,400 |
| | 21 | 160 | 4,400 |
| *ADR1-506* | 1 | 5 | 120 |
| | 12 | 22 | 1,000 |
| *ADR1-304* | 1 | 5 | 800 |
| *ADR1-282* | 5 | 5 | 680 |
| | 29 | 28 | 1,800 |
| *ADR1-272* | 1 | 2 | 370 |
| | 20 | 56 | 1,000 |
| *ADR1-253* | 1 | 2 | 220 |
| | 5 | 7 | 760 |
| *ADR1-220* | 1 | 1 | 120 |
| | 18 | 5 | 360 |
| *ADR1-151* | 6 | 2 | 10 |
| | Plasmid[d] | ND[e] | 20 |
| *adr1-1* | 1 | 2 | 10 |

[a] The number after the *ADR1* designates the number of *ADR1*-encoded amino acids present. All strains are isogenic to S00-16, except for *ADR1-304*, as determined by Denis and Young (10).

[b] Yeasts were pregrown on YEP plates supplemented with 3% glucose before inoculation into YEP medium containing 4% glucose.

[c] Yeasts were pregrown on YEP plates supplemented with 2% glucose before inoculation into YEP medium containing 3% ethanol.

[d] As determined by Denis (8).

[e] The *ADR1-151* gene was retained on the YRp7 plasmid.

[f] ND, Not done.

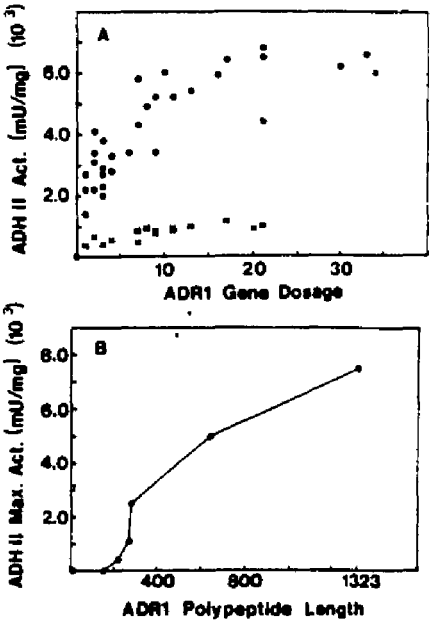


FIG. 2. Effect of *ADR1* dosage on *ADH2* expression and maximal ADH II activity as a function of ADR1 polypeptide length. (A) ADH II enzyme activity (Act.) is given as a function of *ADR1* dosage. ADH II activity was determined after growth on ethanol-containing medium as described in footnote c of Table 1. Values represent the average of at least three determinations. *ADR1* dosages were determined as described in Materials and Methods. The values for *ADR1-253* are as determined by Denis (8). Symbols: ●, *ADR1-1323*; ○, *ADR1-642*; ▲, *ADR1-272*. (B) Maximal ADH II activity (Max. Act.) is given as a function of ADR1 polypeptide length. Maximal ADH II activity was obtained from an Eadie-Hofstee plot of ADH II enzyme activity under derepressed conditions as a function of *ADR1* dosage. The polypeptide lengths (in amino acids) that are plotted, from left to right, are as follows: 0, 151, 220, 272, 282, 642, and 1,323.

TABLE 2. ADR1 regions involved in bypassing glucose control and in growth on nonfermentative carbon sources

| Gene | ADH II activity (mU/mg)[a] on glucose and antimycin A | Relative growth[b] on: | | ADH II activity (mU/mg) on ethanol |
|---|---|---|---|---|
| | | Glycerol | Ethanol | |
| *ADR1-1323* | 760 | + | + | 2,400 |
| *ADR1-1068* | 300 | + | + | 830 |
| *ADR1-642* | 530 | + | + | 1,400 |
| *ADR1-506* | 210 | + | + | 1,000 |
| *ADR1-304* | 100 | + | + | 800 |
| *ADR1-282* | 78 | + | + | 1,800 |
| *ADR1-272* | 81 | + | + | 1,000 |
| *ADR1-253* | 96 | + | + | 760 |
| *ADR1-220* | 8 | + | + | 360 |
| *ADR1-151* | NG | + | + | 10 |
| *adr1-1* | NG | + | + | 10 |
| *adr1-1 ADH2-56* | 200 | + | + | 2,200 |

[a] Growth was in YEP medium supplemented with 4% glucose and 0.1 mg of antimycin A per ml. All *ADR1* genes were carried on YRp7 plasmids in strain 500-16 (*adr1-1*) with the following exceptions: *adr1-1* was strain 500-16 without a plasmid, and *adr1-1 ADH2-56* is strain SPH6, generously provided by J. Cherry. It was strain 500-16 carrying an integration at the *trp1* locus of the *ADH2* gene derived from plasmid YRp7-ADH2-Bsh (37), whose control sequences upstream of −176 bp have been deleted. After growth on medium containing antimycin A, strains were checked to ensure that antimycin A growth was dependent on plasmid sequences and not due to mutations that had arisen in genomic sequences. NG, No growth.

[b] Growth was in YEP medium supplemented with either 3% glycerol or ethanol. All strains contained integrated *ADR1* genes. For each *ADR1* allele, the dosage tested was that which resulted in an ADH II activity during growth on ethanol as near as possible to 1,500 mU/mg, as indicated in the last column, to minimize differences in *ADH2* expression. Growth to densities of $2 \times 10^8$ to $5 \times 10^8$ cells per ml (+) and growth to $1 \times 10^7$ to $3 \times 10^7$ cells per ml (+) are indicated.

genes to bypass glucose repression are confirmed when a comparison is made of the relative abilities of known dosages of integrated *ADR1* genes to bypass glucose repression (Table 1). Fifteen copies of *ADR1-220* allowed 1.10 the level of ADH II activity under conditions of growth on glucose as a comparable dosage of ADR1-272, whereas under derepressed conditions, *ADR1-220* allowed about 1.2 to 1.3 the maximal *ADH2* expression as allowed by *ADR1-272* (Table 1; Fig. 2). In contrast, although 21 copies of *ADR1-642* under repressed conditions allowed three times as much ADH II activity as did 20 copies of *ADR1-272*, this is probably due to decreased transcriptional activation by *ADR1-272* relative to *ADR1-642*. *ADR1-642* allowed four times as much maximal ADH II activity under derepressed conditions as did *ADR1-272* (Table 1; Fig. 2). These results, therefore, are consistent with the interpretation that the ADR1-220 polypeptide, relative to larger ADR1 polypeptides, lacks sequences which are required to bypass glucose repression.

**Site of CCR1 interaction with ADR1.** The ADR1-220 protein, which was capable of allowing *ADH2* gene expression, lacks a putative phosphorylation site believed to be the site through which the protein kinase CCR1 acts, albeit indirectly (8, 9). By the proposed model, *ADH2* expression elicited by ADR1-220 would be expected to be unresponsive to defects in the *CCR1* gene if indeed *CCR1* acted through this site. To test this hypothesis, strains containing *ADR1-220*, *ADR1-304*, and *ADR1-1323* genes were constructed which carried the *ccr1-1* allele (5). *ADH2* derepression was blocked in each case by the *ccr1-1* allele (Table 3). These results indicate that CCR1 does not transmit its signals solely through amino acids 220 through 1323 and suggest that the putative phosphorylation site at amino acid 230 is not the only requirement for CCR1 control.

TABLE 3. Effect of *ccr1* mutation in conjunction with *ADR1* genes

| Relevant genotype | ADH II activity (mU/mg) on ethanol |
|---|---|
| *ADR1*[a] | 2,500 |
| *ADR1 ccr1*[a] | 50 |
| *ADR1-304*[b] | 800 |
| *ADR1-304 ccr1*[b] | 6 |
| *ADR1-220*[c] | 360 |
| *ADR1-220 ccr1*[c] | 5 |

[a] As determined by Denis (8).
[b] See Table 1.
[c] Average of four segregants from diploid 521-10 × 205-5d.
[d] Average of three segregants with 15 copies of *ADR1-220* from diploid 500-16-C3 × 205-5d.

**ADR1 domain required for growth on glycerol as a carbon source.** Each of the *ADR1* genes capable of activating *ADH2* allows cells to grow on ethanol-containing medium (Table 2). However, this was not found to be the case for growth on glycerol-containing medium. Yeast cells containing a complete *ADR1* gene grew rapidly to an initial cell density of $1 \times 10^7$ to $2 \times 10^7$ cells per ml on glycerol-containing medium (Fig. 3). This initial growth was due to the use of carbon sources other than glycerol in the YEP medium, since similar initial growth rates were observed in YEP medium lacking glycerol (Fig. 3). Further growth, to densities $3 \times 10^8$ to $5 \times 10^8$ cells per ml, required the presence of glycerol and an *ADR1* gene having at least 506 N-terminal amino acids (Fig. 3; Table 2). Strains carrying an *adr1-1* allele but also having ADH II enzyme activity due to the *ADH2-56* allele were unable to grow on glycerol-containing medium (Table 2), indicating that growth on glycerol was independent of ADH II activity levels. Reinoculation of cells grown to $3 \times 10^8$ to $5 \times 10^8$ cells per ml on glycerol-containing medium into fresh glycerol-containing medium resulted in growth patterns identical to those described above, indicating that glycerol growth is not due to mutation or adaptation. Glycerol kinase and glycerophosphate dehydrogenase, two enzymes responsible for glycerol metabolism (33), were assayed in cells containing the *ADR1* or the *adr1-1* allele, but no differences were observed (data not shown).

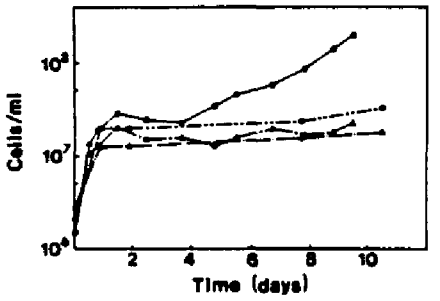


FIG. 3. Growth curves for cells grown on glycerol-containing medium. Symbols: ●, strain 411-40 (one copy of *ADR1-1323* as described in the legend to Fig. 1, also Table 1) grown on YEP medium supplemented with 3% glycerol; ▲, strain SPH6 (*adr1-1 ADH2-56*) grown on YEP medium supplemented with 3% glycerol; ○, strain 411-40 grown on YEP medium; △, strain SPH6 grown on YEP medium. Strain SPH6 is described in footnote *a* of Table 2.

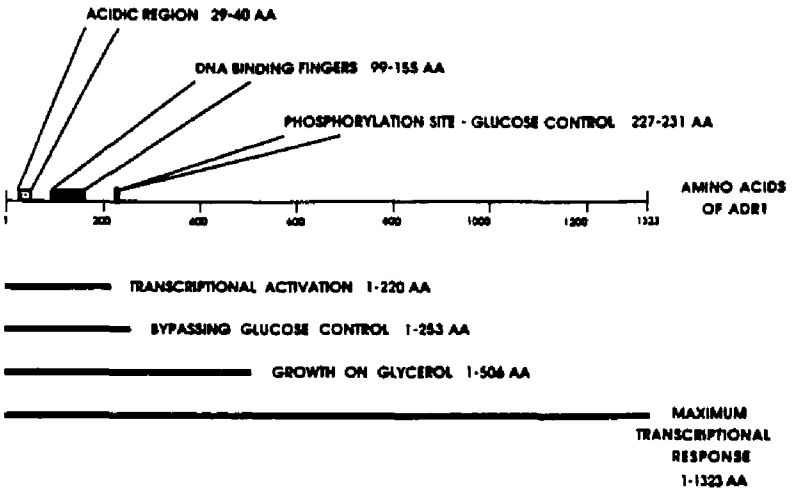


FIG. 4. Functional regions of the ADR1 protein. Solid lines beneath the ADR1 polypeptide indicate regions which have been identified as important in ADR1 function. Regions identified by sequence homology comparisons and mutation analysis are indicated above the line representing the ADR1 polypeptide. The numbers after the lines refer to the amino acid regions indicated.

## DISCUSSION

The large size of the transcriptional activator ADR1 suggests that it contains multiple domains, each potentially having one or more functions. Several of the regions which have been identified are depicted in Fig. 4. The deletion analysis experiments described in this paper confirm the importance of the putative DNA-binding region of ADR1 (amino acids 99 through 155) to ADR1 function. Deletion into this region resulted in an ADR1 polypeptide unable to allow *ADH2* derepression. While this result is consistent with a previous deletion analysis of *ADR1* (12), the possibility remains that such an observation could be the result of decreased levels of ADR1-151 protein in the cell, rather than intrinsic inactivity of ADR1-151. However, this is probably not the case. When an ADR1-151–β-galactosidase fusion protein was expressed to the same levels as longer ADR1-β-galactosidase fusions, as measured by β-galactosidase activity, the ADR1-151–β-galactosidase protein was unable to activate *ADH2*, whereas the longer fusion polypeptides were able to (R. Vallari and J. Cherry, personal communication). Our results thus support the hypothesis that ADR1-151 is inactive as a result of its lack of two complete zinc finger regions. In this regard, it is interesting to note that all proteins which share homology to the DNA-binding fingers of transcription factor IIIA contain at least two such fingers (3, 29, 30, 36), implying that multiples of such regions are required for function.

Although we have not been able to show directly that ADR1 binds to *ADH2* DNA, it is probable that it does bind DNA on the basis of its homology to transcription factor IIIA. If this were the case, the first 220 amino acids of ADR1 must contain at least the recognition sites for binding and the ability to activate transcription. Transcriptional activation regions have been mapped relative to the DNA-binding regions for several other eucaryotic regulators. For the yeast GCN4 (14) and GAL4 (25) activators, the two regions are physically distinct. For the glucocorticoid receptor, however, it appears that both regions may occur in the same or overlapping segments of the protein (13, 28). The GCN4 and GAL4 activation regions coincide with an acidic part of the polypeptide that has been postulated to be a contact point with RNA polymerase II or another transcriptional component (34). ADR1 also contains a single acidic region, amino acids 29 through 40 (50% acidic), within the first 220 amino acids (Fig. 4).

Whereas the first 220 amino acids of ADR1 were sufficient to cause at least some derepression of *ADH2*, no shortened ADR1 protein was as functional as ADR1-1323 (Fig. 2B). Progressive carboxy-terminal deletions caused progressive decreases in the ability of ADR1 to maximally activate *ADH2*. These differences in maximal activation of *ADH2* are not due to differences in protein stability, indicating that a large part, if not all, of ADR1 is required for normal modulation of its transcriptional function. These findings are in contrast to the results of a previous study using plasmid *ADR1* gene constructs, which suggested that an ADR1-506 polypeptide retained wild-type function (12). The interpretation of the earlier results was complicated, however, by the fact that not all cells contained plasmids and that *ADR1* dosage per cell was not known. Although we are unable to define the function of amino acids 220 through 1323 of ADR1 as they pertain to *ADH2* control, it is possible that this region provides contact points to itself or other regulatory proteins that improve the efficiency of ADR1 activation but are not required for transcriptional activation. It is also possible that parts of the C terminus are required to ensure proper folding of the N terminus. Without the whole original C terminus, the N-terminal domain, although partially active, would form a functionally impaired structure.

A second region of importance to ADR1 function has been defined by characterization of *ADR1*$^c$ mutations that allow *ADR1* to bypass glucose control. These mutations occur within the cAMP-dependent protein kinase phosphorylation recognition sequence (22) between amino acids 227 and 231

(9; Cherry et al., in preparation). *ADR1*$^c$ mutations could bypass glucose repression, either by enhancing ADR1 transcriptional activity or by blocking ADR1 interaction with a negative effector that acts at the putative phosphorylation region. The ADR1-220 protein lacking the site of the *ADR1*$^c$ mutations was shown to be capable of activating *ADH2* under derepressed conditions, indicating that the putative phosphorylation site is not absolutely required for ADR1 function. However, ADR1-220 still conferred a glucose-repressed ADH2 phenotype. Therefore, our studies do not support the hypothesis that ADR1 is inactivated under repressed conditions by the binding of a repressor at the putative phosphorylation region.

Instead, the effect of increased *ADR1* dosage on glucose-repressed ADH II activity supports the model that the putative phosphorylation region plays a positive role in ADR1 function. Increased levels of ADR1-220 as compared with ADR1-253 and larger ADR1 polypeptides displayed diminished ability (at least 10-fold) to bypass glucose control. In contrast, under derepressed conditions, ADR1-220 was only two- to threefold less functional than ADR1-253 and ADR1-272 (Table 1; Fig. 2B). Also, the $K_d$ values for ADR1-220 and ADR1-272 were similar, suggesting that the difference in function under repressed conditions was not a result of a difference in protein stability. On the basis of these results, the region between amino acids 220 and 253 appears to be required for increased dosages of *ADR1* to activate *ADH2* under glucose conditions. The fact that ADR1-220 is partly active under derepressed conditions may indicate that the transcriptional state of *ADH2* during derepression is sufficiently enhanced by other induced transcriptional factors to allow a shortened ADR1 to be sufficient for *ADH2* expression. Under repressed conditions, however, these other factors would be expected to be less active, and a larger ADR1 polypeptide would be required to bypass glucose repression.

We believe, therefore, that the *ADR1*$^c$ mutations bypass glucose control by enhancing ADR1 activity. Such a mechanism could be envisioned to occur by increased DNA binding, by augmented contacts with other regulatory proteins or transcriptional components, or by counteracting a negative domain in the N-terminal 220 amino acids. The control of ADR1 should be compared with that proposed for the control of yeast transcriptional activator GAL4, in which GAL4 is inactivated by an interaction with the negative effector GAL80 (18, 26). The region of GAL4 to which GAL80 binds, however, is also required by GAL4 for transcriptional activation. Similarly, for ADR1 we propose that phosphorylation would inactivate ADR1 by interfering with a required function of the phosphorylated region.

The protein kinase CCR1 has been suggested to act through the putative phosphorylation region of ADR1 in its positive control of *ADH2* (9). However, deletion of the ADR1 phosphorylation region did not render *ADH2* derepression independent of the *CCR1* mutation (Table 3). This result implies that CCR1 may control ADR1 through the first 220 amino acids at other potential phosphorylation sites or that CCR1 does not act through ADR1 at all.

The requirement of ADR1 for growth on glycerol implicates ADR1 as a more global regulator of nonfermentative growth than had previously been thought. However, the identity of the factor through which ADR1 controls growth on glycerol is unknown. A factor important to growth on glycerol, other than the enzymatic activities of glycerol kinase and glycerophosphate dehydrogenase, could be the process of transporting reducing equivalents or other molecules across the mitochondrial membrane (5, 11). Amino acids 304 through 506 of ADR1 are required for growth on glycerol, though they are not essential for *ADH2* activation. These results suggest that ADR1 control of growth on glycerol is modulated by as yet undefined regulators which require this region of the ADR1 protein.

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