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Regulation of the yeast transcriptional activator ADR1

W James Cook
University of New Hampshire, Durham

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Cook, W. James, Ph.D.

University of New Hampshire, 1993

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

REGULATION OF THE YEAST TRANSCRIPTIONAL ACTIVATOR ADR1

by

W. James Cook
B.S., University of Utah, 1988

DISSERTATION

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This dissertation has been examined and approved.

Dissertation Director, Clyde Denis
Associate Professor of Biochemistry
and Molecular Biology

John J. Collins
Assistant Professor of Biochemistry
and Molecular Biology

Anita S. Klein
Associate Professor of Biochemistry
and Molecular Biology

Andrew P. Laudano
Associate Professor of Biochemistry
and Molecular Biology

Subhash C. Minocha
Professor of Plant Biology

11-13-92
Date

Dedicated to

my Grandfather Henry Cook

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ABSTRACT

REGULATION OF THE YEAST TRANSCRIPTIONAL ACTIVATOR ADR1

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W. James Cook
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Glucose repression of the ADH2 gene from Saccharomyces cerevisiae is mediated by the abundance and activity of the transcriptional activator ADR1. The focus of this dissertation is to characterize the mechanisms by which glucose controls the ability of ADR1 to activate ADH2 transcription.

Glucose results in a two-fold decrease in the steady state levels of ADR1 mRNA. This glucose-dependent reduction in steady state ADR1 mRNA was shown to be due to an increased rate of ADR1 mRNA degradation. The unusually long, 510 nucleotide 5′ untranslated region of the ADR1 mRNA appeared to mediate this glucose-dependent ADR1 mRNA decay.

To better understand the posttranslational control of ADR1 activity by glucose, the ADR1^c mutations which allow glucose-insensitive ADH2 expression were analyzed. The mechanism by which three mutated genes (saf1, saf2, and saf3) suppressed the ADR1^c phenotype was investigated. Each of the mutated saf genes was found to suppress ADR1^c

activity by reducing ADR1^c transcription 5- to 8-fold under glucose growth conditions. The SAF genes were also required for ADR1 transcription under glucose conditions, indicating that these genes are not specifically involved in ADR1^c function. A deletion analysis conducted on an ADR1^c protein indicated that no ADR1 residues outside the site of the ADR1^c mutations were specifically required for ADR1^c function. ADR1^c mutations enhanced the activity of ADR1 proteins that contained either of two separate activation domains.

Deletion analysis also allowed for the improved mapping of the functional domains in ADR1. ADR1 was found to contain multiple domains required for transcriptional activation, and these activation domains were found to be separated by residues that inhibit activation. One inhibitory region encompasses the site of the ADR1^c mutations. This array of alternating activating and inhibitory regions in ADR1 suggests that ADR1 activity is modulated by interactions between different positive and negative domains in ADR1.

GENERAL INTRODUCTION

In the yeast Saccharomyces cerevisiae, glucose is the carbon source of choice. Given a mixture of glucose and either disaccharides or nonfermentative carbon sources (e.g. ethanol, lactate or glycerol), the glucose will be metabolized first (Suomalainen and Oura 1971). This preference is mediated by the repression of genes not directly involved in glucose metabolism, a phenomenon termed glucose repression or catabolite repression (Magasasik 1961). Genes repressed by glucose include those required for growth on disaccharides, nonfermentative carbon sources, the glycolytic shunt, the tricarboxylic acid cycle, respiration, gluconeogenesis, mitochondrial function, and peroxisomal function (for reviews see Gancedo and Gancedo 1986; Wills 1990; and Gancedo 1992). Glucose repression allows yeast to cope effectively with changes in the carbon source present in their environment.

While many of the mechanisms of catabolite repression have been characterized in Escherichia coli (for review see Botsford 1981), the underlying mechanisms of glucose repression are poorly understood in yeast. However, it is known that this repression in yeast works through different mechanisms than in bacteria. The degree of glucose repression in yeast depends on the enzymes affected and the

strain used. Enzyme activities measured in glucose-repressed or derepressed (absence of glucose) growth conditions show differences ranging from 800-fold for invertase to less than 10-fold for aconitase, cytochrome c oxidase or isocitrate dehydrogenase (Gancedo 1992). While the mechanisms of glucose repression of metabolic enzymes in yeast appear to act primarily at the level of transcription (Gancedo 1992), glucose also appears to exert effects on a limited number of enzymes at the level of mRNA decay (Zitomer and Nichols 1979; Lombardo et al. 1992) and at the level of protein degradation (Gancedo and Serrano 1989; Chiang and Schekman 1991). It should be noted, however, that the effects of glucose on posttranscriptional processes may be additional effects and, therefore, glucose repression is generally considered to control metabolic enzymes at the level of transcription.

The study of glucose repression in yeast is facilitated by the ability to isolate mutations by genetic means that specifically affect this regulatory process. The vast array of mutants that are involved in glucose repression are summarized in recent reviews (Gancedo and Gancedo 1989; Wills 1990; Gancedo 1992). These mutations fall into two categories: those that cause constitutive expression of glucose-repressible enzymes (glucose repression mutants), and those that cause an inability to derepress glucose-repressible enzymes (glucose derepression mutants). The

isolation of mutants deficient in either glucose repression or derepression has provided the means to address the mechanisms of glucose control of metabolic genes. From a multitude of studies, the following general features have been deduced:

(1) Glucose affects transcription of metabolic genes by modifying the activity of the trans-acting elements that control metabolic gene expression. The trans-acting elements that have a positive role in gene expression are termed transcriptional activators as these factors function to activate transcription of target genes.

(2) Glucose acts in most cases by modifying the activity of transcriptional activators rather than by affecting the transcription of these activators. This glucose effect is predicted to typically occur by a posttranslational modification of an activator or some factor involved in transcriptional activator function. In some cases, however, the protein expression of transcriptional activators is affected (Thireos et al. 1984; Lamphier and Ptashne 1992).

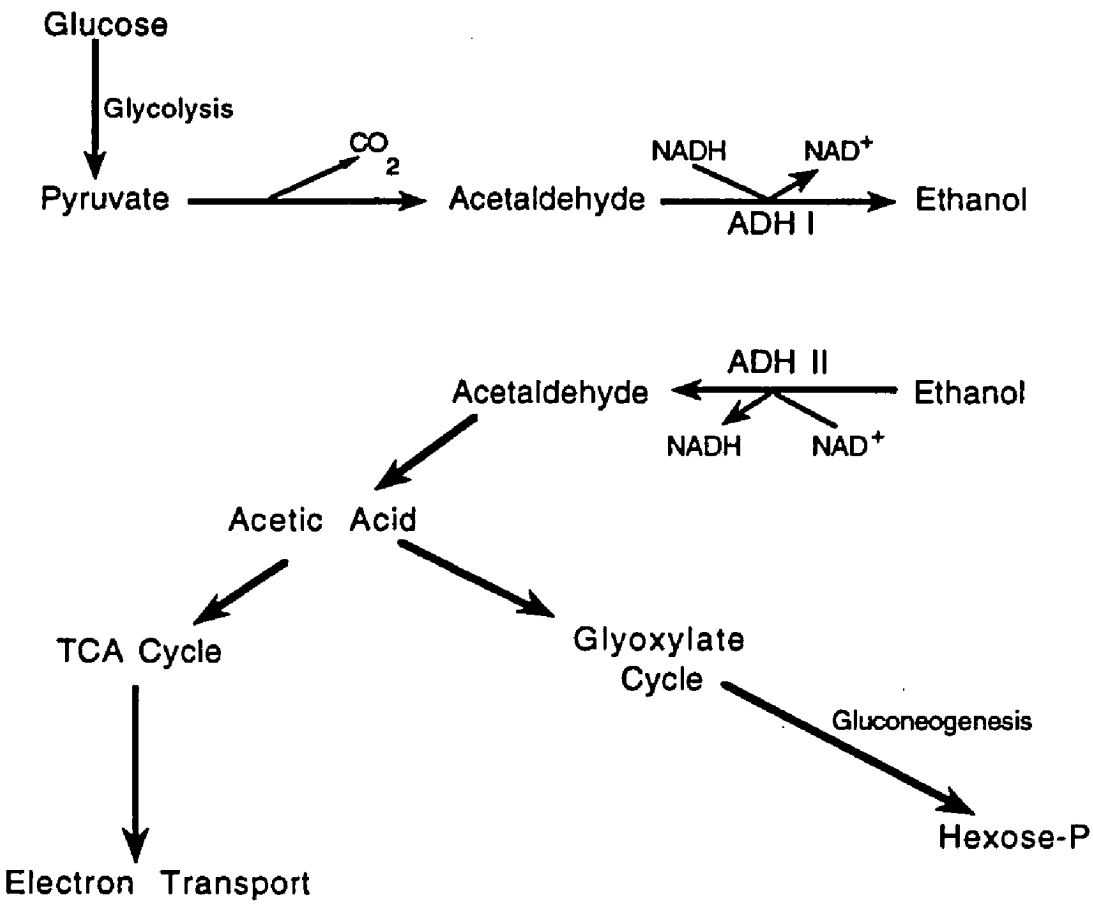
(3) The glucose repression of many genes involves multiple regulatory trans-acting genes, and these trans-acting factors are shared by a variety of metabolic gene expression systems. Some elements have negative effects on some systems and positive effects on others (Silve et al. 1992).

The purpose of this dissertation research is to further the understanding of gene regulatory mechanisms in

eukaryotes. The system of study is the regulation of the glucose-repressible alcohol dehydrogenase (ADH II encoded by the ADH2 gene) in S. cerevisiae. The ADH II enzyme functions in the utilization of ethanol as a carbon and energy source (Figure 1). When glucose is removed from the growth medium and yeast are forced to grow on a nonfermentable carbon source such as ethanol, ADH2 transcription is derepressed and ADH II activity increases 500-fold (Ciriacy 1975). DNA sequences located -176 to -453 bp upstream of the ADH2 translation start site were shown to be required for regulated ADH2 transcription (Beier and Young 1982; Figure 2). Within this region are two upstream activating sequences, UAS1 and UAS2, that were shown to mediate the regulation of ADH2 by glucose. In addition, elements near the ADH2 TATA box appear to mediate regulation independently of the UAS elements (Denis and Malvar 1990).

A number of trans-acting factors have been identified that act to control ADH2 transcription including ADR1 (Ciriacy 1975), CCR4 (Denis 1984), REG1 (C. L. Denis unpublished observations), SNF1 (Ciriacy 1977), SPT10 and SPT6 (Denis 1984), SW11-3 (Peterson and Herskowitz 1992), and TYE1-5 (Ciriacy et al. 1991). The transcriptional activator ADR1 plays a pivotal role in ADH2 expression in that its function is controlled by glucose (Ciriacy 1975). ADR1 controls other glucose-repressed genes besides ADH2, including genes involved in glycerol metabolism (Bemis and

Figure 1. Alcohol dehydrogenases in yeast metabolic pathways.



Denis 1988), mitochondrial function (Cherry and Denis 1989), and peroxisomal function (Simon et al. 1991).

ADR1 has been cloned (Denis and Young 1983) and sequenced (Hartshorne et al. 1986). The ADR1 protein contains 1323 amino acids as deduced from the DNA sequence (Hartshorne et al. 1986) and has been identified in immunoprecipitated yeast extracts as having a mass of 150 kD, which agrees with sequence data (Vallari et al. 1992). The ADR1 protein contains four functional motifs that are common to transcriptional activators (for review see Guarente 1987): a nuclear localization sequence, a DNA binding region, transcriptional activation regions, and a region involved in regulation (Figure 3).

Nuclear localization of ADR1 is dependent on amino acids 1-16 (Thukral et al. 1989). ADR1 binds to UAS1 through a zinc-finger DNA-binding motif located between amino acids 102 and 159 (Yu et al. 1989, Taylor and Young 1990). These two zinc-fingers in ADR1 are homologous to the Cys_2His_2 family of zinc-fingers first identified in TFIIIA (Miller et al. 1985). Other members of the Cys_2His_2 family include Sp1 (Kadonaga et al. 1987), Zif268 (Pavletich and Pabo 1991), and Krox-20 (Nardelli et al. 1991). Mutations in the ADR1 zinc-fingers led to an adr1 phenotype indicating that this region is essential for ADR1 function (Blumberg et al. 1987, Cook et al. 1993). ADR1 binds through the zinc fingers to UAS1 which is a 22 bp inverted repeat (Yu et al.

Figure 2. Regulatory regions in the ADH2 gene.

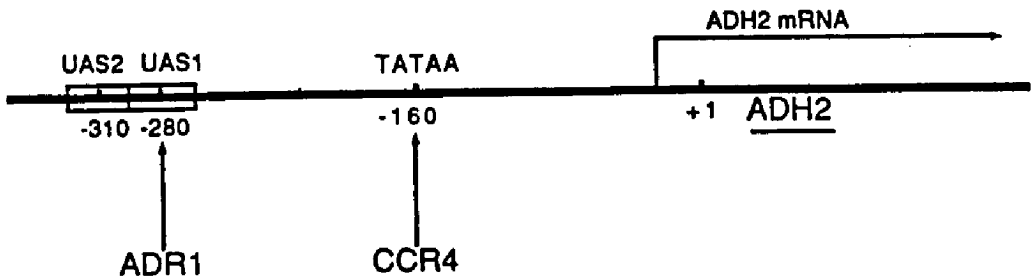
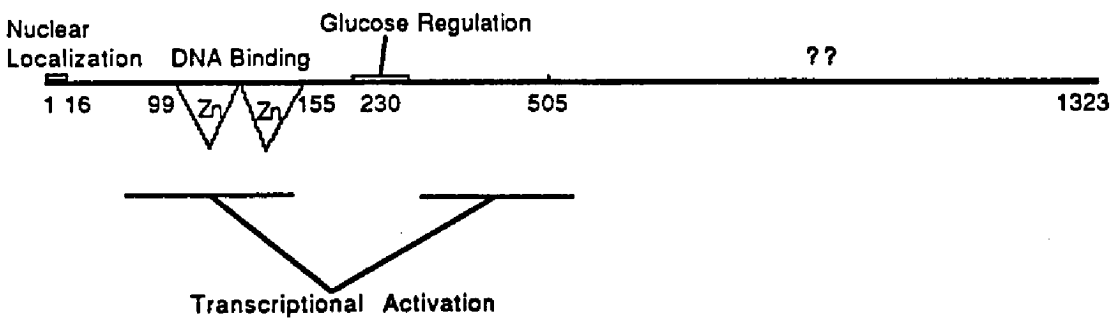


Figure 3. Functional regions in the ADR1 protein.



1989). The binding of ADR1 to this region of dyad symmetry implicates an oligomeric state of DNA-bound ADR1. In a previous study, however, ADR1 appeared to bind as two independent monomers to each half UAS1 site (Thukral et al. 1991).

ADR1 contains two broad regions that are involved in transcriptional activation (Figure 3). Transcriptional activation domains are regions of transcriptional activator proteins that interact with other protein components of the transcriptional machinery to activate transcription. One of the activation domains in ADR1 overlaps the zinc-finger DNA-binding region. The identification of this activation domain is based on results which showed that ADR1 deletion proteins that contained amino acids 76-172 (Thukral et al. 1989) or 1-220 (Bemis and Denis 1988) were able to activate ADH2 transcription. Single amino acid changes within residues 77-155 induced by ethyl-methyl-sulfonate (EMS) mutagenesis caused decreases in activation independently of DNA binding (Cook et al. 1993). Deletion analysis also indicated that the region between amino acids 262 and 642 contained activating residues (Thukral et al. 1989; Bemis and Denis 1988). Activation domains in other yeast transcriptional activators including GAL4 (Ma and Ptashne 1987), GCN4 (Hope and Struhl 1986), HAP4 (Forsburg and Guarente 1989) and MCM1 (Bruhn et al. 1992) contain an abundance of acidic amino acid residues. Acidic activation

domains are predicted to adopt an amphipathic alpha-helical structure (Giniger and Ptashne 1987). However, other yeast activation domains appear to not conform to these characteristics (Bruhn et al. 1992). Like ADR1, many other transcriptional activators contain multiple activation domains (Mitchell and Tjian 1989).

The region around ADR1 amino acid 230 appears to mediate the glucose regulation of ADR1 activity. The involvement of this region in glucose regulation was first postulated upon the identification of dominant mutations, designated ADR1^c for constitutively activating ADR1, which caused ADH II activity to be 50- to 70-fold higher under glucose growth conditions (Denis and Gallo 1986; Cherry et al. 1989; Denis et al. 1992; Table 1). The ADR1^c mutations encode ADR1^c proteins which contain single amino acid changes within residues 227-239. Within this region is the sequence Arg-Arg-Ala-Ser-Phe, which conforms to a cAMP-dependent protein kinase (cAPK) phosphorylation consensus sequence with the serine at 230 representing the putative phosphoacceptor. The ADR1^c alleles in 21 spontaneous mutant strains have been sequenced and all contain point mutations that change amino acids within the Ser-230 region. Deletion of the Ser-230 region also caused an ADR1^c phenotype, indicating that this region acts to inhibit activity (Denis et al. 1992). *In vitro* studies showed that cAPKphosphorylates Ser-230 and that some ADR1^c proteins have

Table 1. ADH II activity in selected ADR1 strains.

a)

ADR1 allele	ADH II Activity (mU/mg)[a] Glucose[b]	Ethanol[c]
[d]ADR1	5	2500
[e]adr1-1	2	10
[f]16X ADR1	60	5100
[f]65X ADR1	360	8300
[f]96X ADR1	680	8000
[d]ADR1-5[c]	370	3400
[d]ADR1-7[c]	250	2800

All strains are isogenic to 500-16 (Denis 1987).

[a] One unit of ADH II activity reduced 1 μM of NAD in one minute at 22°C (Denis et al. 1981).

[b] Yeast were grown in YEP medium supplemented with 8% glucose.

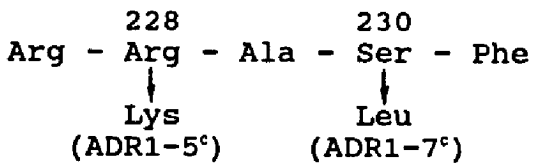
[c] Yeast were grown in YEP medium supplemented with 3% ethanol.

[d] Values taken from Cherry et al. (1989).

[e] Values taken from Bemis and Denis (1988).

[f] Values taken from Denis (1987).

b) Identity of the ADR1-5[c] and ADR1-7[c] mutations.



reduced or undetectable levels of Ser-230 phosphorylation. In a genetic study, deletion of the gene encoding the regulatory subunit of cAPK, BCY1, resulted in hyperactive cAPK activity that caused decreased ADR1 activation of ADH2. The same bcy1 deletion had less effect in a strain carrying the ADR1-7^c allele (Ser-230 changed to Leu, Cherry et al. 1989). Since glucose caused a transient rise in cAMP levels which resulted in increased cAPK activity, phosphorylation of protein substrates by cAPK appears to represent at least one mechanism of glucose repression (Tortora et al. 1984). Taken together, these results prompted Cherry et al. (1989) to propose a simple model in which glucose induces the cAPK phosphorylation of ADR1 Ser-230, an event that inactivates ADR1. The ADR1^c proteins were predicted to escape glucose inactivation by disrupting cAPK phosphorylation of Ser-230.

Other data suggest that the simple model of cAPK inactivating ADR1 by phosphorylation may not be correct. Lack of cAPK activity under glucose conditions did not result in increased ADH2 expression (Denis and Audino 1991) which would be expected if ADR1^c mutations only perturbed cAPK phosphorylation of Ser-230. Moreover, in vitro data incorporating the use of synthetic peptides modeled on the putative ADR1 phosphorylation region indicated that not all ADR1^c mutations affect Ser-230 phosphorylation (Denis et al. 1992). Therefore, while cAPK inactivation of ADR1 remains an attractive hypothesis, the ADR1^c mutations may cause

effects that are independent of Ser-230 phosphorylation state.

The *ADR1*^{c} mutations do not affect *ADR1* mRNA levels (Denis and Gallo 1986) or ADR1 protein levels (Taylor and Young 1990; Vallari et al 1992). *In vitro* gel retardation studies showed that ADR1 in yeast extracts from both glucose and ethanol grown cells bound UAS1 equally. ADR1^c bound UAS1 to the same extent as ADR1 regardless of carbon source (Taylor and Young 1990). This indicates that if glucose does induce the phosphorylation of ADR1, phosphorylation does not affect DNA binding. ADR1 probably binds UAS1 constitutively while the *ADR1*^{c} mutations exert their influence on the ability of ADR1 to activate transcription.

Northern analysis of *ADR1* mRNA in various strains suggested that *ADR1* mRNA expression was not affected by carbon source (Denis and Gallo 1986). However, ADR1 protein abundance appears to play a role in *ADH2* activation since overexpression of *ADR1* either on plasmid vectors or as gene copies multiply integrated into the yeast genome allowed *ADH2* expression to escape glucose repression. Under glucose growth conditions, a linear increase in *ADR1* gene dosage resulted in a corresponding linear increase in ADH II activity (Denis 1987). An initial analysis of carbon source control of ADR1 protein levels using immunoprecipitation techniques suggested that glucose causes a decrease in ADR1 protein expression (Vallari et al. 1992). The *ADR1*^{c}

mutations did not affect ADR1 protein levels (Taylor and Young 1990; Vallari et al. 1992), suggesting that the putative glucose suppression of ADR1 activity through effects at the Ser-230 region must represent a regulatory mechanism separate from control of ADR1 protein abundance. Taken together, these results indicate that glucose repression of ADH2 mediated by ADR1 probably occurs through a combination of unrelated mechanisms. Modification of ADR1 at the Ser-230 region or control of ADR1 protein abundance by themselves cannot account for the full effects of glucose repression upon this system.

Studies conducted in other labs on the glucose regulation of ADR1 indicated that the fore-mentioned conclusions may not generally be true of all yeast strains. In some yeast strains that are related to those in our lab, ADR1 mRNA levels were shown to be equal or slightly higher under ethanol growth conditions than glucose growth conditions in agreement with our lab's results. However, other unrelated strains expressed 10- to 20-fold greater ADR1 mRNA levels in derepressed as compared to repressed conditions (Blumberg et al. 1988). In another study, ADR1 protein levels in a strain unrelated to strains in our studies were shown to not change according to carbon source (Taylor and Young 1990). Despite the contradictions in ADR1 control shown in these other studies, ADH2 was regulated by carbon source in a manner similar to our studies. Such

variation in published reports disclose the need for further characterization of the mechanisms of glucose control of ADR1 in one series of well defined strains.

The focus of this dissertation is to decipher the mechanisms by which glucose controls ADR1 activation of ADH2 transcription. The approaches taken to address this question were four-fold:

(1) Characterize the effect of glucose on ADR1 protein abundance. This project was conducted in conjunction with Dr. Robert Vallari. While Dr. Vallari identified the glucose control of ADR1 protein synthesis in a defined yeast strain, I analyzed the effect of carbon source on ADR1 mRNA expression in this strain. Results from these experiments indicated that glucose caused a two-fold decrease in ADR1 mRNA levels. In an effort to identify the ADR1 mRNA sequences that mediate the glucose control of ADR1 translation, the unusually long 5′ untranslated region (UTR) of the ADR1 mRNA was replaced. The 5′ UTR of the ADR1 mRNA was not involved in glucose control of ADR1 translation, but rather these mRNA sequences mediated the glucose control of ADR1 mRNA stability. This study is described in Chapter 1 and in a published manuscript by Vallari et al. which can be found in the Appendix.

(2) Characterize the glucose-induced posttranslational control of ADR1 activity by identifying other genes involved in this process. A genetic screen had previously identified

three mutations, designated saf1, -2, and -3, which suppressed the ability of the ADR1-5^c allele (encodes an Arg-to-Lys change at amino acid 229; Table 1) to allow glucose-insensitive ADH2 expression. Characterization of these three mutations indicated that the SAF genes are involved in the glucose-dependent transcription of ADR1. This study is described in Chapter 2.

(3) Identify the regions in the ADR1^c proteins that are required for the enhanced activation of ADH2. Findings from a deletion analysis together with an in vivo transcription assay indicated that no specific region of ADR1 was required for the ADR1^c effect suggesting that the ADR1^c mutations may act through a mechanism that is independent of residues other than the 227-239 inhibitory region. This study is presented in Chapter 3.

(4) More precisely map the functional domains in ADR1 using both deletion analysis and an in vivo assay system. Results from this analysis indicated that the ADR1 protein contains multiple activation domains, and these activation domains are separated by regions that inhibit activation. The implications of this array of alternating activating and inhibitory regions upon ADR1 activity and the possible role of glucose in modulating interactions between different domains in ADR1 are addressed in Chapter 3.

CHAPTER 1

EFFECTS OF GLUCOSE ON ADR1 PROTEIN ABUNDANCE

Introduction

Preliminary investigations into the effects of carbon source on ADR1 protein expression indicated the ADR1 protein levels are decreased 10- to 16-fold under glucose growth conditions compared to ethanol growth conditions (R. Vallari pers. comm.). Glucose could exert control on ADR1 protein expression at four levels: mRNA synthesis, mRNA degradation, protein synthesis, and protein degradation. Early reports showed that ADR1 is not controlled at the level of mRNA expression (Denis and Gallo 1986, Denis 1987). However, these conclusions were contradicted by other studies that showed that in some strains, ADR1 mRNA levels were 10- to 20-fold lower under glucose compared to ethanol conditions (Blumberg et al. 1988). Therefore, an investigation of the carbon source regulation of ADR1 mRNA expression in an established yeast strain was conducted.

Results presented in the accompanying manuscript by Vallari et al. (Appendix) indicated that glucose reduces the steady state levels of ADR1 mRNA two-fold. In contrast to this slight effect, glucose reduces the rate of ADR1 protein synthesis 5- to 8-fold. In this chapter, the following

questions are addressed: (i) Is the two-fold decrease of ADR1 mRNA under glucose conditions due to a repression of ADR1 transcription or to an increase in ADR1 mRNA degradation? (ii) What are the ADR1 mRNA sequences that mediate the glucose control of ADR1 translation rate?

One of the best characterized examples of translational control in eukaryotes occurs in the regulation of the yeast transcriptional activator GCN4. GCN4 is a positive effector of amino acid biosynthesis genes in S. cerevisiae that is regulated in response to amino acid starvation (Thireos et al. 1984). Since only approximately 6% of the sequenced eukaryotic genes have 5′ UTRs longer than 150 nt (Kozak 1984), the most striking feature of the GCN4 mRNA is its 577 nt long 5′ UTR (Thireos et al. 1984). The 5′ UTR of the GCN4 mRNA contains four AUG codons which control translational initiation (Thireos et al. 1984, Miller and Hinnebusch 1989). Deletion of the GCN4 5′ UTR or replacement of this region with a GAL1 promoter (Hinnebusch 1985) caused GCN4 protein expression to be constitutive.

Like GCN4, ADR1 function is regulated by its protein abundance. The ADR1 mRNA also contains an unusually long (510 nt) 5′ UTR (Hartshorne et al. 1986). However, the ADR1 5′ UTR lacks the multiple AUGs that are found in the GCN4 5′ UTR. I sought to examine if the ADR1 5′ UTR was involved in translational control by replacing this region in the ADR1 mRNA with mRNA sequences derived from another yeast 5′ UTR.

Results presented here show that the control of ADR1 protein synthesis does not involve the ADR1 5′ UTR. Instead, data is presented that suggests a role for this UTR is in promoting glucose-dependent ADR1 mRNA decay.

Materials and Methods

Yeast Strains and tetrad analysis. Strains used in this study were isogenic to 500-16 (MAT**a**, adh1-11 adh3 adr1-1 ura1 trp1 his4) except RY260 (MAT**a** ura3-52 rpb1-1), JC44-5a (MATα, adh1-11 his4 rpb1-1), JC88-1b (MAT**a** adh1-11 ADR1-5c ura3 his3 his4 rpb1-1 saf1), and JC105-6c (MATα adh1-11 adr1-1::GADR1 his4 rpb1-1). Tetrads were dissected as described (Mortimer and Hawthorne 1969).

Growth Conditions and Assays are described in Vallari et al. (1992). ADH II activity values represent the average of three separate determinations.

Plasmid constructions and Protein Analysis

Vallari et al. (1992) describes the construction and transformation of the G-ADR1 plasmid, and measurements of ADR1 protein levels by immunoprecipitation and Western analysis.

Northern Analysis and mRNA Degradation:

Total RNA was extracted by lysing cells with glass beads as described in Vallari et al., or by the hot phenol/SDS method (Schmitt et al. 1990). Northern analysis is described in Vallari et al. For U1 snRNA Northern analysis, a U1 snRNA oligonucleotide (a kind gift of J. Warner, Albert Einstein College of Medicine, Bronx, NY) was used as a probe. The U1 snRNA oligonucleotide was labeled as described (Sambrook et al. 1989), and hybridizations with the U1 oligonucleotide probe were conducted according to the NEN (New England Nuclear, Boston, MA) manufacturer's specifications with the appropriate temperature modifications (Sambrook et al. 1989). Densitometric analysis was conducted using an E-C 610 (E-C Apparatus, St. Petersburg, FL) or a GS300 (Hoefer Scientific, San Francisco, CA) densitometer. RNA concentrations were quantitated by spectrophotometric measurements of absorbance at 260 nm. ADH2 and ADR1 mRNA levels were normalized to the amount of rRNA present as described previously (Denis and Young 1983).

RNA degradation studies were conducted using the temperature-sensitive RNA polymerase II allele rpb1-1 present in strain Y260 (a gift of the A. Jacobson lab, Univ. Massachusetts Medical School, Worcester, MA) as described (Parker et al. 1991). Total RNA was prepared using the hot phenol/SDS method. Graphical quantitation of mRNA half-lives was conducted as described (Parker et al. 1991).

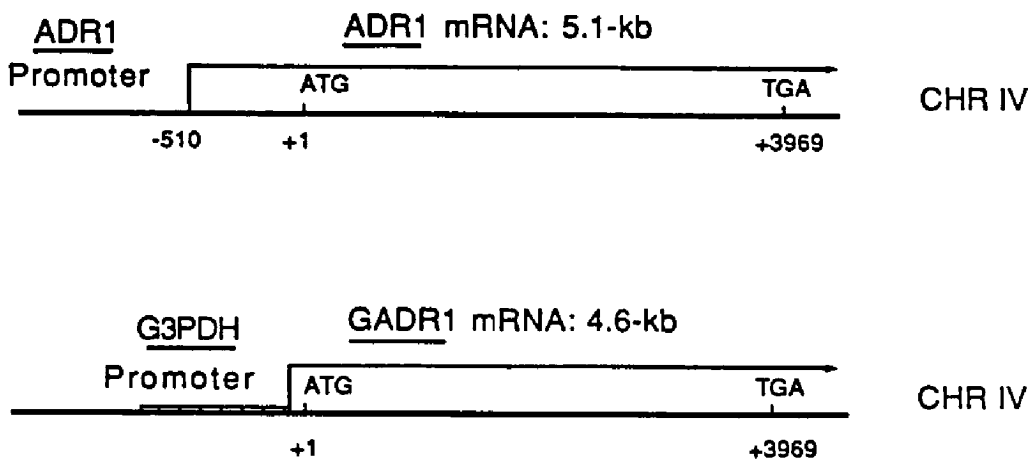
Results

To understand the mechanism by which glucose controls ADR1 protein synthesis, the regulatory role of the unusually long 5′ UTR of the ADR1 mRNA was analyzed. The ADR1 5′ UTR was removed by replacing the 5′ promoter region and upstream sequences of ADR1 with a truncated promoter derived from the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene. The G-ADR1 mRNA expressed from the chimeric G-ADR1 gene contained a 5′ UTR of approximatly 30 nt (Figure 4). The construction of the yeast strain containing the G-ADR1 allele and subsequent effects on ADH II activity, ADR1 mRNA levels, and ADR1 protein levels are described in the paper by Vallari et. (Appendix).

To summarize the findings reported in Vallari et al., a yeast strain containing the G-ADR1 allele expressed ADH II enzyme activities which were 10-fold higher under glucose conditions and 2-fold higher under ethanol conditions as compared to the wild-type (see also Figure 4). This increased ADH II activity was caused by a 15-fold increase in G-ADR1 mRNA expression; the increased mRNA levels were commensurate with an approximate 15-fold increase in ADR1 protein levels. ADH II activities and ADR1 protein levels in the G-ADR1 strain were similar to that measured in a strain that contained 16 integrated copies of ADR1. These results indicated that while G-ADR1 caused increased ADR1 protein levels, glucose control of ADR1 translation was not

Figure 4. Replacement of *ADR1* 5' Noncoding Sequences

a)



b)

	ADH II Activity (mU/mg)	
	Glucose	Ethanol
Wild-type *ADR1*	5	2500
GADR1	50	4900
16X *ADR1*	60	5100

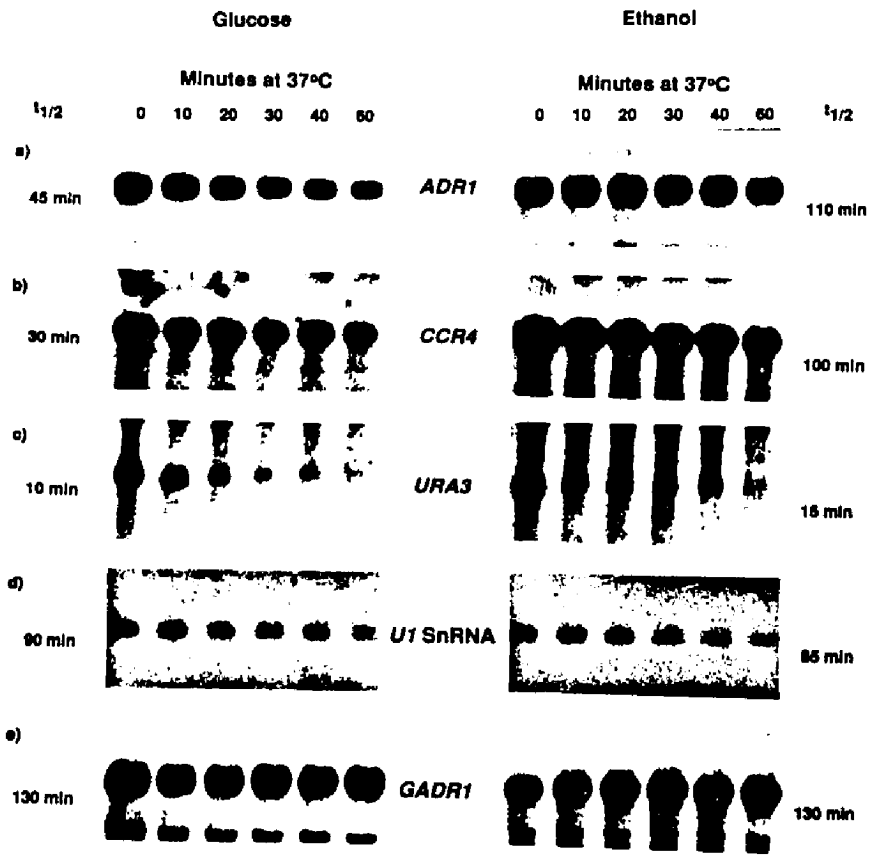
a. Strain GADR1 contains an altered ADR1 allele in which ADR1 promoter and 5' untranslated sequences were replaced with G3PDH promoter sequences.
b. Growth conditions were identical to those described in Table 1. Values represent the average of at least three separate determinations, and SEMs were less than 20% except for the wild-type ADR1 value under glucose conditions. All strains are isogenic to 500-16.

different from that found in other strains which overexpressed ADR1. Therefore, it was concluded that the glucose control of ADR1 translation is not mediated by the ADR1 5′ UTR.

An alternative role for the 5′ UTR might be in the control of ADR1 mRNA stability. While no example exists of a yeast 5′ UTR being involved in the control of mRNA stability, 5′ sequences have been implicated in promoting decay in prokaryotic mRNAs (Portier et al. 1987), in the human histone H3 mRNA (Morris et al. 1986), and in c-myc mRNA (Rabbitts et al. 1985). ADR1 mRNA stability was analyzed in strains containing a temperature-sensitive mutation in RPB1, the gene encoding the largest subunit of RNA polymerase II. The rpb1-1 allele allows normal mRNA synthesis at 26° but loses polymerase function at 36° (Nonet et. al 1987). At the permissive temperature of 26°, strains containing the rpb1-1 allele expressed ADR1 mRNA at levels equal to strains containing the wild-type RPB1 gene at 30° (data not shown).

Northern analysis was conducted on ADR1 mRNA levels in glucose and ethanol grown cells extracted at indicated time intervals after shifting cells to 37° (Figure 5). ADR1 mRNA degradation was influenced by carbon source as calculations of ADR1 mRNA decay rates indicated a half-life of 45 min under glucose conditions as compared to 110 min under ethanol growth conditions (Figure 5a). In contrast, the

Figure 5. Carbon source control of ADR1 mRNA decay rate. Levels of selected RNAs were measured in strains containing a temperature-sensitive mutation in the RNA polymerase II gene (allele rpb1-1). Following growth to mid-log phase in medium containing either 8% glucose or 3% ethanol at the permissive temperature of 26°C, cultures were abruptly shifted to 37°C. Samples were subjected to Northern analysis using the indicated probes. Equivalent amounts of total RNA were loaded in each lane as verified by inspection of rRNA bands in a duplicate gel stained with ethidium bromide. Half-lives of selected mRNAs were quantitated by plotting the $\log_{10}$ percentage of mRNA remaining vs. time at 37°C as described (Parker and Jacobson 1990). Panel a) Decay rates of ADR1 mRNA were compared in strain JC44-5c between cells grown in glucose- or ethanol-containing medium. Fifteen μg of total RNA were loaded in each lane. The ADR1 probe used is described in Figure 6. Panel b) Strain JC88-1b was used to compare CCR4 mRNA decay rates between glucose and ethanol conditions. For the glucose grown cells, 30 μg of total RNA were loaded in each lane while 15 μg were loaded in each lane for the ethanol grown samples. The CCR4 probe used is described in Figure 7. Panel c) Strain JC88-1b was used to analyze URA3 mRNA decay. Thirty μg of total RNA were loaded in each glucose grown lane while 15 μg were loaded from ethanol grown cells. The URA3 probe used is described in Figure 6. Panel d) Strain JC44-5c was used to analyze decay rates of U1 snRNA between glucose and ethanol growth conditions to verify that our method of normalizing RNA loadings based on rRNA bands was valid. A synthetic oligonucleotide complementary to U1 sequences was used as the probe. Fifteen μg of total RNA were loaded in each lane. Panel e) Strain JC105-6c, containing the GADR1 allele, was grown on glucose- or ethanol-containing medium to analyze the effect of replacing the 5′ untranslated leader sequence of ADR1 mRNA with G3PDH sequences. Fifteen μg of total RNA were loaded in each lane and probed with the ADR1 probe. The half-life values of 130 minutes were calculated by extrapolating the linear decay line beyond measured time points and, therefore, should be viewed as an approximate value. The reason for the reduced GADR1 signal at early time points in the ethanol grown samples is unexplained (see text). mRNA half-life values represent the average of three separate determinations which varied by approximately ± 25%.



decay rate of G-ADR1 mRNA was significantly slower than ADR1 mRNA as G-ADR1 mRNA decayed with a half-life of about 130 min. (Figure 5d). G-ADR1 mRNA was also completely stable under ethanol growth conditions as the ethanol half-life was also near 130 min. These results indicate that replacement of the 5′ UTR of the ADR1 mRNA with the 30 nt G3PDH leader sequence produces a stable transcript, and suggests that the ADR1 5′ UTR plays a role in glucose-dependent ADR1 mRNA decay.

To verify by the use of controls that our observation of carbon source influence on ADR1 mRNA decay was correct, and to determine if carbon source affected other mRNAs in a similar manner, degradation rates of other mRNAs were analyzed. The half-life of CCR4 mRNA was observed to be 30 min under glucose conditions and 100 min under ethanol conditions (Figure 5b). Decay rates of URA3 mRNA were also found to be under carbon source control as I measured a half-life of 10 min under glucose conditions and 15 min under ethanol growth conditions (Figure 5c). The half-life measurement of 10 min under glucose conditions is in agreement with previous reports (Bach et al. 1979; Kim and Warner 1983). The carbon source control of URA3 mRNA decay indicates that this observation is not unique to transcription factors.

As the normalization of mRNA loadings for the studies of mRNA decay were based on an rRNA standard, comparisons of

decay rates are dependent on a consistent rate of rRNA decay between both carbon sources. A previous study showed that while some rates of synthesis of rRNA were different, rRNA decay rates did not differ according to carbon source (Kief and Warner 1981). This is important in light of a study which showed that in strains containing the rpb1-1 allele, the rate of rRNA synthesis decreased rapidly after 30 min following a shift to the nonpermissive temperature in glucose grown cells (Nonet et al. 1987). To ensure that the carbon source-dependent differences in mRNA decay rates were not due to differing rates of rRNA decay, selected blots were reprobed with a synthetic oligonucleotide complementary to U1 snRNA. Expression of U1 snRNA was found to not be influenced by carbon source (J. Warner personal communication). Since it is a nuclear RNA and, therefore, not subject to mechanisms of cytosolic degradation, I predicted that U1 snRNA would be inherently stable. Results shown in Figure 5d indicate that, indeed, U1 snRNA was nearly equally stable under both glucose and ethanol growth conditions, indicating that measurements of different mRNA decay rates due to carbon source were not due to differences in rRNA decay. In addition, total RNA turnover rates are likely to be dependent on cell doubling time. The doubling time of cells is much faster when grown in glucose compared to ethanol conditions in strains containing a wild-type ADH1 gene. As strains used were lacking a functional ADH1 gene,

little difference in growth rates between glucose and ethanol inheres to these strains and, therefore, I was able to neglect effects of growth rate differences on our mRNA decay analysis.

Discussion

ADR1 control of ADH2 transcription is regulated by glucose through multiple mechanisms. These regulatory mechanisms include the control of ADR1 protein synthesis, and a minor control of ADR1 mRNA levels. The unusually long ADR1 5′ UTR was shown in this report to not be involved in this translational control. Subsequent experiments directed at measuring the degradation rate of ADR1 and G-ADR1 mRNA indicated that (i) ADR1 mRNA degradation is controlled by carbon source, and (ii) the glucose control of ADR1 mRNA decay is mediated through the ADR1 5′ UTR.

The small reduction in steady state ADR1 mRNA levels by glucose reported in Vallari et al. (Appendix) and in earlier studies (Blumberg et al. 1988, Denis 1987, Denis and Gallo 1986) is probably a result of this increased rate of decay under glucose growth conditions. A study of carbon source control of CCR4 and URA3 mRNA decay also showed that glucose increases the rate of decay of these mRNAs. Because steady state levels of CCR4 mRNA were found to be equivalent on both carbon sources (Malvar et al. 1992) and URA3 mRNA levels were elevated two-fold under glucose as compared to

ethanol growth conditions (unpublished observations), glucose must cause increased rates of transcription for these genes. For URA3, this is not an unexpected result since the synthesis of genes involved in pyrimidine biosynthesis would be expected to decrease on a poor carbon source such as ethanol.

Glucose may well increase the turnover rate of many mRNA species. Other reports have identified the relationship between translation rates and mRNA decay for numerous genes (Herrick et al. 1990, Santiago et al. 1986). Following this model, the increase in mRNA turnover in glucose grown cells as compared to ethanol grown cells may be related to increased translation rates under glucose conditions. However, the rate of ADR1 translation is decreased by glucose (Vallari et al. Appendix) and CCR4 protein synthesis is unaffected by carbon source (T. Malvar and C. L. Denis unpublished results), suggesting that this model does not apply to ADR1 and CCR4 mRNA decay. How carbon source control of mRNA decay influences the regulation of gene expression remains to be characterized.

The carbon source control of mRNA decay rates does not appear to be completely general. While SDH Id mRNA (Lombardo et al. 1992), the CYC1 mRNA (Zitomer and Nichols 1979), and some glycolytic mRNAs (Moore et al. 1991) were more stable in nonfermentative compared to glucose conditions, the decay rates of other glycolytic mRNAs were unaffected by carbon

source (Moore et al 1991). Findings reported here confirm that the glucose-dependent reduction in mRNA stability is not a general effect since the rate of G-ADR1 mRNA decay was unaffected by carbon source. Replacement of the long 5′ UTR of ADR1 mRNA with G3PDH sequences resulted in an extremely stable G-ADR1 mRNA under both glucose and ethanol conditions. While it was not determined whether the increased stability of G-ADR1 mRNA is caused by the loss of ADR1 mRNA sequences or by the addition of stabilizing G3PDH sequences, this result suggests that the 5′ UTR of the ADR1 mRNA plays a role in mRNA stability. Other studies investigating yeast mRNA sequences involved in controlling stability identified regions within the 3′ UTR as being responsible for decay (Lombardo et al. 1992, Parker and Jacobson 1990). For this reason, the observation that the 5′ UTR of the yeast ADR1 mRNA appears to be involved in decay represents a novel finding.

CHAPTER 2

IDENTIFICATION OF THREE GENES INVOLVED IN CONTROLLING THE GLUCOSE-DEPENDENT TRANSCRIPTION OF ADR1

Introduction

While results presented in the previous chapter indicate that glucose regulates ADR1 function by controlling ADR1 mRNA turnover and ADR1 translation, glucose also controls ADR1 by posttranslational effects. The posttranslational control of ADR1 activity was first postulated upon the identification of the $ADR1^{c}$ mutations which cause single amino acid changes in a cAMP-dependent protein kinase phosphorylation consensus sequence (Denis and Gallo 1986, Denis et al. 1992). The evidence in favor and against the role of Ser-230 phosphorylation in the control of ADR1 activity is presented in the Introduction. Even if phosphorylation of Ser-230 is not an important regulatory event, the identification of 21 constitutively activating mutations at or near Ser-230 clearly indicate a regulatory involvement for this region.

To characterize mechanisms by which the Ser-230 region is involved in ADR1 regulation, mutations that suppressed the ability of $ADR1\text{-}5^{c}$ to enhance ADH2 transcription were sought. Two general classes of mutations were predicted

that could suppress ADR1-5^c: mutations in other genes that are required for enhanced activity of ADR1-5^c, and second site mutations in the ADR1-5^c protein that counteract the effects of a mutation in Ser-230 region. Two methods were used to identify suppressor mutations. First, an ethyl-methyl-sulfonate (EMS) mutagenesis was conducted on a strain containing ADR1-5^c, and strains that exhibited decreased ADH II activity on glucose-containing medium were selected (D. L. Mullaney thesis; C. L. Denis unpublished results). Seven mutations were isolated that answered this selection. These seven mutations caused five different amino acid changes, all of which were mapped to the zinc-finger region of ADR1. However, these zinc-finger mutations had a similar suppressive effect on the ADR1 protein, indicating that the zinc-finger mutations were not specific to ADR1-5^c (Cook et al. 1993).

The second approach to isolating suppressor mutations in ADR1-5^c was to conduct a genetic screen that incorporated the petite forming phenotype of ADR1-5^c. Overexpression of ADR1 or the hyperactivity of an ADR1^c allele cause increased petite formation due to disruptions in the mitochondrial genome (Cherry and Denis 1989). While the genes responsible for this latter effect have not been identified, the ADR1-promoted induction of respiratory deficient daughter cells is analogous to the petite-forming pattern caused by elevated temperature and acriflavin. The petite-induction

phenotype of the ADR1-5^c allele at 37° was utilized to screen for mutations that suppressed the enhanced transcriptional activity caused by the ADR1-5^c allele (C. L. Denis unpublished results). In the present study, three new genes were identified using this screen that were found to be required for the glucose-dependent transcription of ADR1.

Materials and Methods

Yeast Strains. Yeast strains are listed in Table 2.

Growth conditions and assays. Conditions for growth of cultures on YEP medium (2% Bactopeptone, 1% yeast extract, 20 mg/liter adenine and uracil) have been described (Denis and Young 83). Yeast were grown on solid medium consisting of YEP medium plus 2% agar and were supplemented with 8% glucose (YD8 plates), 2% glucose (YD plates) or 3% ethanol (YETOH plates). YD plates supplemented with antimycin A were prepared as previously described (Denis and Young 1983). ADH II activity assays were conducted as described (Denis and Young 1983), and values represent the average of three separate determinations.

Tetrad Analysis. Dissection of tetrads was done as described (Mortimer and Hawthorne 1969).

Plasmid constructions. The construction and integration into the genome of plasmids ADR1-220/262 (Denis et. al 1992)

TABLE 2 Yeast Strains

Strain	Genotype
43-2B	*MATα adh1-11 adh3 ura1 his4*
R234	isogenic to 43-2B except *ADR1-5^c*
R234-14	isogenic to 43-2B except *ADR1-5 ^{c} saf1*
R234-15	isogenic to 43-2B except *ADR1-5^c saf 2*
R234-19	isogenic to 43-2B except *ADR1-5^c saf 3*
237-1b	*MATa adh1-11 ura3 his3 trp1 leu2*
JC1-2a	*MATa adh1-11 ADR1-5^c ura3 his4 trp1 saf2*
JC3-2a	*MATα adh1-11 ADR1-5^c ura3 his4 saf1*
JC7-2c	*MATa adh1-11 ADR1-5^c ura3 his3 saf3*
JC2-6b	*MATα adh1-11 ADR1-5^c ura3 his4 saf2*
JC32-4a	*MATα adh1-11 ADR1-5^c saf2 saf3*
JC3-6c	*MATa adh1-11 ADR1-5^c ura3 his4 saf1*
R308	isogenic to 43-2B except *ADR1-7^c*
628-3c	*MATa adh1-11 his4 saf1*
637-7b	*MATa adh1-11 ura3 his3 trp1 saf2*
642-4a	*MATa adh1-11 ura3 his4 trp1 saf3*
500-16-15	*MATa adh1-11 adh3 adr1-1::ADR1-220/262 ura1 his4 trp1*
628-1-6b	*MATα adh1-11 ura3 his4 trp1 saf1*
637-6a	*MATα adh1-11 ADR1-5^c ura1 his3 trp1 saf2*
644-9b	*MATα adh1-11 ADR1-5^c ura1 trp1 saf3*
R184	isogenic to 43-2B except *ADH2-2^c*
JC5-5c	*MATa adh1-Δ1::URA3 ADR1-5^c his4 leu2*
JC6-6b	*MATα adh1-Δ1::URA3 ADR1-5^c his4 saf1*
JC8-5c	*MATα adh1-Δ1::URA3 ADR1-5^c his4 saf3*
JC9-2b	*MATα adh1-Δ1::URA3 ADR1-5^c his4 saf2*
GADR1	isogenic to 500-16-15 except *adr1-1:: GADR1*
628-1-3b	*MATα adh1-11 ADR1-5^c ura3 his4 trp1 saf1*
RY260	*MATa ura3-52 rpb1-1*
JC44-5a	*MATα adh1-11 his4 rpb1-1*
JC88-1b	*MATa adh1-11 ADR1-5^c ura3 his3 his4 rpb1-1 saf1*
JC89-7c	*MATa adh1-11 ADR1-5^c ura3 his4 leu2 rpb1-1 saf2*
JC90-13d	*MATa adh1-11 ADR1-5^c ura3 his3 his4 leu2 trp1 rpb1-1 saf3*
HB23-3	*MATa adh1 adh3 ura3 his7 leu2 trp1 can1 sap3 ade3*
JC105-6c	*MATα adh1-11 adr1-1::GADR1 his4 rpb1-1*

and GADR1 (Chapter 1, Vallari et al. 1992) are described elsewhere.

Northern analysis and RNA degradation studies. These are described in Chapter 1.

Results

Isolation of suppressors of ADR1^c function

When strain R234 (ADR1-5^c) is placed at 37°, the frequency at which daughter cells become petite reaches 100%. Strain R234 therefore fails to grow on ethanol-containing medium at 37°. Using this phenotype, mutations in R234 that allowed for growth under these conditions were isolated (C. L. Denis unpublished results). Such mutants could arise from reduced ADR1-5^c activity which would result in decreased petite formation (Cherry and Denis 1989) or from defects in the genes through which ADR1-5^c and high temperature induce petite formation. The selection procedure also ensured that putative mutations in the ADR1-5^c allele would retain some activity in order to allow ADH2 expression, and hence growth of yeast under nonfermentative growth conditions. Of approximately 4 x 10^8 cells plated, eight spontaneous mutants were isolated that grew, albeit slowly, at 37° on medium containing ethanol.

Each of the eight mutant strains displayed decreased levels of ADH2 expression under glucose growth conditions as evidenced by the failure of these mutant strains to grow in

the presence of the respiratory inhibitor antimycin A. The presence of antimycin A forces yeast to ferment glucose which requires alcohol dehydrogenase activity (Ciriacy 1979). The antimycin A sensitivity of these mutations in an ADR1-5^c background proved to be a convenient marker for following the mutated alleles. Subsequent genetic analysis placed these eight recessive mutations into three complementation groups which were designated saf1, saf2 and saf3 for suppressor of ADR1 function. Following complementation and tetrad analysis, each SAF gene was found to not be allelic to ADR1, ADH2, SNF1 (CCR1), CCR4, or to each other (C. L. Denis unpublished results).

The saf mutations suppress ADR1-5^c-induced ADH II activity in repressed conditions but not in derepressed conditions. The effects of the saf1, -2 and -3 mutations on ADR1-5^c-dependent ADH2 expression were analyzed by measuring ADH II enzyme levels in each of the mutant strains (Table 3) Each of the saf1, -2 and -3 mutations lowered ADR1-5^c-dependent ADH II activity under glucose growth conditions approximately 80-fold, yet had no significant effect on ADH II activity under ethanol growth conditions (Table 3). Northern analysis of ADH2 mRNA levels in strains containing the ADR1-5^c allele and either the wild-type SAF gene or the saf1, -2, or 3 mutations showed that the lowered ADH II

TABLE 3

ADH II activity of strains containing the *saf* mutations

Relevant Genotype	Background Strain	ADH II activity (mU/mg) Glucose[a]	Ethanol[b]
ADR1-5^C SAF	R234	400 ±49	4800 ±800
ADR1-5^C saf 1	R234-14	4.3 ±2.0	4300 ±440
ADR1-5^C saf 2	R234-15	3.0 ±1.0	4800 ±600
ADR1-5^C saf 3	R234-19	6.6 ±1.7	4400 ±480
[c]*ADR1 SAF*	43-2B	5	2500
[d]*ADR1 saf 1*	segregants	5 (0-10)	3600 (2600-4000)
[e]*ADR1 saf 2*	segregants	4.5 (0-9)	2300 (2200-2400)
[f]*ADR1 saf 3*	segregants	2 (0-6)	3700 (2900-4800)
[g]*ADR1-5^C saf1 saf 2*	segs.	5 (0-10)	3750 (2590-4910)
[h]*ADR1-5^C saf1 saf3*	segs.	6	3900
[i]*ADR1-5^C saf2 saf3*	segs.	5.5 (0-11)	4260 (4070-4460)
[j]*ADR1-5^C saf1 saf2 saf3*	segs.	11 (6-18)	3200 (2400-4200)

ADH II enzyme activities represent the average of at least three separate determinations, and are given with ± S.E.M. when isogenic strains are compared and with ranges (in parentheses) when assay values of segregants are compared.

[a] Yeast were grown on YEP medium supplemented with 8% glucose.
[b] Yeast were grown on YEP medium supplemented with 3% ethanol.
[c] As reported in Cherry et al. 1989.
[d] Average of 5 segregants from cross: 237-1b x R234-14.
[e] Average of 2 segregants from cross: 237-1b x R234-15.
[f] Average of 4 segregants from cross: 237-1b x R234-19.
[g] Average of 2 segregants from cross: R234-14 x JC1-2a.
[h] One segregant from cross: JC3-2a x JC7-2c.
[i] Average of 2 segregants from cross: JC2-6b x JC7-2c.
[j] Average of 3 segregants from cross: JC32-4a x JC3-6c.

activity in the saf strains was caused by a lowering of ADH2 mRNA levels (data not shown).

It was also observed that in an ADR1-5^c genetic background, strains containing any of the three saf alleles grew better in ethanol-containing medium than strains containing wild-type alleles for all three SAF genes. This increased growth rate was attributed to a reduced rate of petite formation in strains containing SAF alleles (Cherry and Denis 1989; Denis 1987). The effects of the saf alleles on other phenotypes including mating response, sporulation, growth at elevated temperature on glucose-containing medium, and growth on medium containing glycerol as a carbon source were examined. No phenotypic differences were observed between isogeneic strains carrying the ADR1-5^c SAF genes and those containing the ADR1-5^c saf alleles in these tests except that strains carrying the saf alleles grew better on glycerol-containing medium than the parental strain, probably due to reduced petite formation as described above.

Following suitable crosses, saf mutations were isolated in a wild-type ADR1 background and their effects on ADH II enzyme levels were determined. No significant differences in ADH II activity were observed when comparing strains with the genotype ADR1 SAF to that of ADR1 saf under either glucose or ethanol growth conditions (Table 3). A value near 5 mU/mg is a basal level and, therefore, I would not be able to detect effects of the saf mutations on ADR1-

dependent ADH II activity under glucose conditions.

Further crosses were made to isolate strains containing multiple saf mutations in an ADR1-5^c background. Strains containing any two or all three saf alleles exhibited ADH II enzyme levels which were comparable to related strain containing a single saf mutation (Table 3). These results suggest that the SAF genes encode proteins that act in a common pathway or are components of a single protein complex. I detected no other phenotypic differences between strains harboring multiple saf mutations and strains harboring single saf mutations.

The above results suggested that the SAF gene products might be general glucose-dependent transcription factors with functions similar to such factors as GCR1 (Santangelo and Tornow 1990). In order to examine the effects of the saf mutations on the expression of a glycolytic gene, the levels of ADH1 mRNA were assessed in saf-containing strains. The saf mutations had no effect on the expression of ADH1. Also the saf mutations had no effect on the expression of URA3 or CCR4 which are two other genes expressed under glucose growth conditions (data not shown).

The ability of the saf mutations to suppress the increased ADH II activity under glucose-repressed growth conditions caused by other types of mutations in ADR1 or ADH2 was examined. All three saf mutations suppressed ADR1-7^c- and ADR1 220/262-dependent antimycin A resistance and

suppressed increased ADH II enzyme activity under glucose conditions (data not shown) indicating that these mutations have the same effect on these constitutively activating alleles as on ADR1-5^c. The effect of the saf alleles on increased ADH2 expression that is due to factors other than ADR1^c alleles was also examined. The saf mutations had no effect on the ADH2-2^c allele, which contains a Ty transposible element inserted between the ADH2 TATA box and the UAS elements (Williamson 1983), as also shown by antimycin A resistance and ADH II enzyme assays. These data strongly suggest that the SAF genes are specifically required for ADR1-dependent activation of ADH2 transcription. These genetic experiments were conducted in a collaborative effort by Dr. Denis and myself.

ADR1 mRNA levels are reduced in strains containing the saf alleles

The effects of the saf mutations on ADR1-5^c-dependent ADH2 expression under glucose-repressed conditions could be due to a reduction in levels of ADR1-5^c expression or loss of ADR1-5^c protein activity. To determine if the saf mutations affected ADR1-5^c expression, I analyzed ADR1-5^c mRNA levels using Northern analysis. A comparison of ADR1-5^c mRNA levels in isogeneic strains showed that the saf mutations caused a 5- to 8-fold drop in ADR1-5^c RNA levels when cells

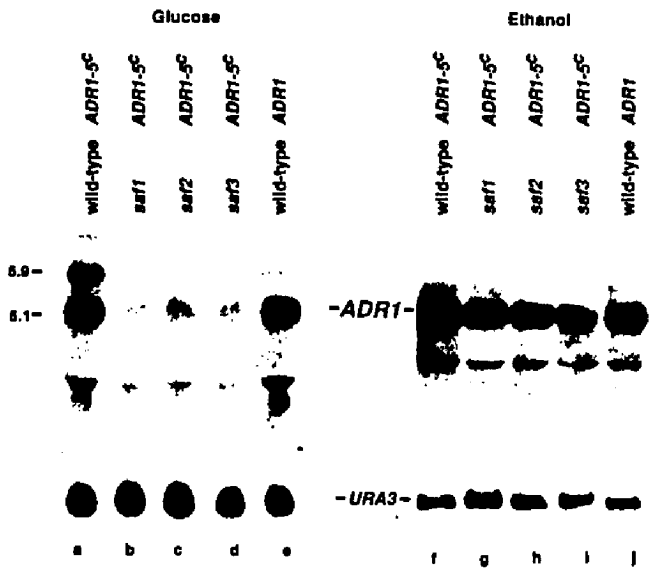


Figure 6. ADR1-5^c mRNA levels in strains carrying the saf mutations. Total RNA extracted from isogenic strains R234 (lanes a and f), R234-14 (lanes b and g), R234-15 (lanes c and h), R234-19 (lanes d and i), and 43-2B (lanes e and j) grown in YEP medium containing either 8% glucose or 3% ethanol was subjected to Northern analysis as described. Thirty μg of total RNA from each glucose grown sample were loaded in lanes a-e, and 15 μg of total RNA from each ethanol grown sample were loaded in lanes f-j. A 0.9 kb Eco R1 fragment containing ADR1 coding sequences from plasmid YRp7-ADR1-5^c-23A (Denis and Young 1983) was used as the radioactive probe. The glucose grown samples in lanes a-e were run on a separate gel from that for the ethanol grown samples in lanes f-j and, therefore, no comparisons may be made between autoradiograms from the separate carbon sources. Equal loading of total RNA was verified by using a duplicate gel stained for rRNA as described (Vallari et al. 1992), and by reprobing blots with a URA3 probe (a radiolabeled 1.1 kb Hind III fragment from plasmid YEp24 which contained the entire URA3 gene) as displayed in the lower panels. The ADR1 probe hybridizes non-specifically to 25S rRNA which is responsible for the band that migrates below the ADR1 band. The 5.9 kb ADR1 species most visible in lane a was identified as being caused by alternate termination of the ADR1 transcript as described in the text.

were grown under glucose conditions (Figure 6, lanes b, saf1, c, saf2, and d, saf3 compared to lane a, wild-type SAF). These data indicate that ADR1-5^c-dependent ADH2 expression is repressed under glucose growth conditions in strains containing the saf mutations due to this reduction in ADR1-5^c mRNA levels. The mechanism by which a 5- to 8-fold reduction in ADR1-5^c RNA levels results in an 80-fold reduction in ADH II activity is discussed below.

ADR1-5^c mRNA levels in this isogeneic series were also analyzed after growth in ethanol-containing medium. In contrast to what was observed under glucose growth conditions, the saf mutations caused only a 10-15% drop in ADR1-5^c mRNA in ethanol growth conditions (Figure 6 lanes g, saf1, h, saf2, and i, saf3 compared to lane f, wild-type SAF). This lowered expression of ADR1-5^c mRNA due to the saf mutations would be relatively insignificant with respect to ADR1-5^c activation of ADH2 in derepressed conditions since ADR1-5^c is not limiting under such growth conditions (Denis 1987). This data agrees with the observation that the saf mutations have little or no effect on activation of ADH2 expression under ethanol growth conditions (Table 3). The 10-15% drop in ADR1-5^c mRNA levels under nonfermentative growth conditions may be sufficient, however, to reduce the high level of petite formation observed in strain R234 and thus allow for the initial isolation of the saf mutations in Dr. Denis' mutant screen.

To determine if the saf alleles affected both ADR1 and ADR1-5^c mRNA levels, ADR1 mRNA levels in nonisogenic strains carrying saf alleles was analyzed by Northern analysis. Representatives of segregants containing a wild-type ADR1 allele and saf1 (Figure 7, lane a) saf2 (lane b) and saf3 (lane c) all exhibited a 4- to 10- fold drop in ADR1 mRNA levels compared to a related strain with the genotype ADR1 wild-type SAF (lane d) under glucose growth conditions. The saf mutations had a negligible effect on ADR1 mRNA levels under ethanol growth conditions (Figure 7, lanes e-h). These results indicate that the repressive effects of the saf mutations are not specific to the ADR1-5^c allele. The analysis of ADR1 steady state mRNA levels in these experiments indicated, therefore, that the SAF gene products are specifically involved in maintaining ADR1 mRNA levels under glucose growth conditions.

Removal of sequences 5′ to the ADR1 translation start site abolishes the saf1-induced reduction in ADR1 RNA levels

Reduced abundance of ADR1 and ADR1-5^c mRNA in mutants carrying the saf alleles grown under glucose growth conditions could occur by either a reduction in the rate of transcription of ADR1 mRNA or by an increased rate of ADR1 and ADR1-5^c mRNA decay. To distinguish between these two possibilities, I used an ADR1 chimeric gene in which all ADR1 5′ noncoding sequences, including the 510-nt

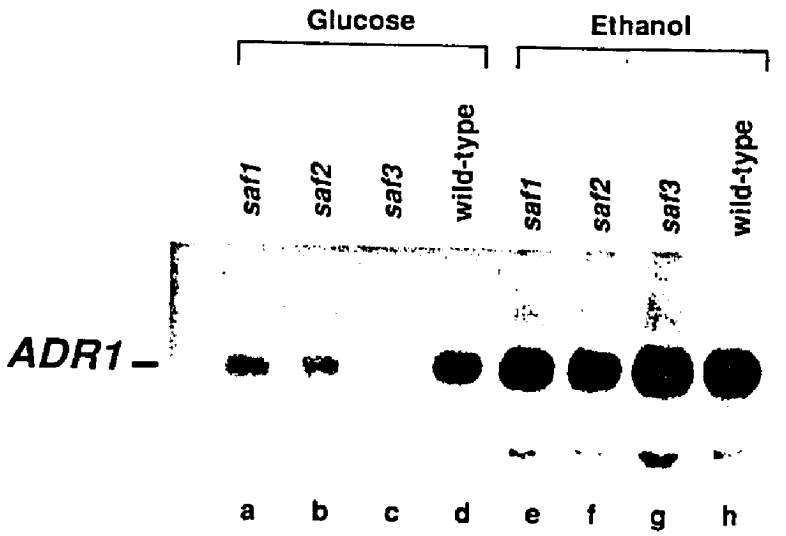


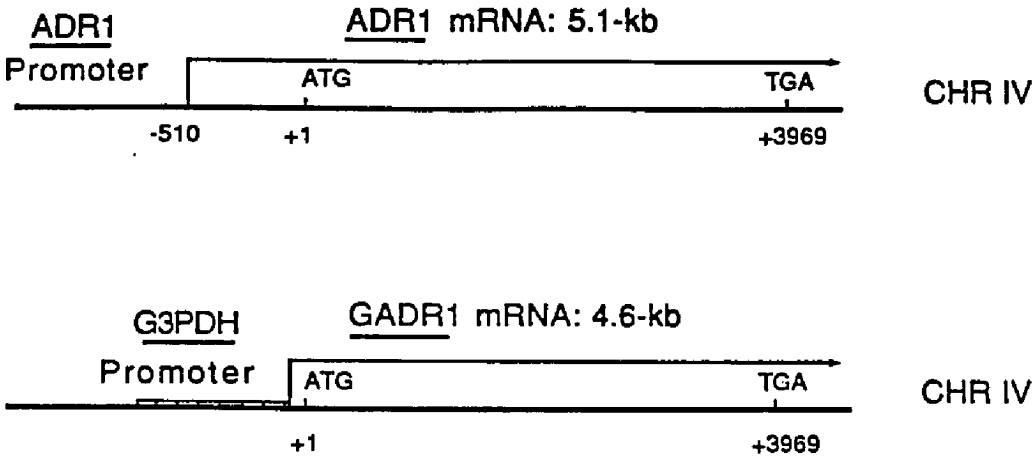
Figure 7. ADR1 mRNA levels in strains carrying the saf mutations. Twenty μg of total RNA extracted from either glucose or ethanol grown cells from strains 628-3c (lanes a and e), 637-7b (lanes b and f), 642-4a (lanes c and g), and 43-2B (lanes d and h) were subjected to Northern analysis using the ADR1 probe as described in Figure 6. Reprobing with a CCR4 probe and staining of a duplicate gel for rRNA bands showed that all samples were equally loaded except for lane g which was overloaded by 30% with respect to the other lanes. The CCR4 probe was a radiolabeled 1.5 kb Hind III-BamHI fragment from plasmid YRp7.3.5 (Denis and Malvar 1990) which contains CCR4 coding sequences.

untranslated leader sequence and the ADR1 promoter, were replaced with glyceraldehyde-3-phosphate dehydrogenase promoter sequences which lack UAS elements (allele GADR1; Rosenberg et al. 1990; see also Figure 8). The resultant mRNA expressed in strains containing GADR1 is approximately 500-nucleotides shorter than that synthesized in strains carrying the complete ADR1 gene (Figure 8; Vallari et al. Appendix).

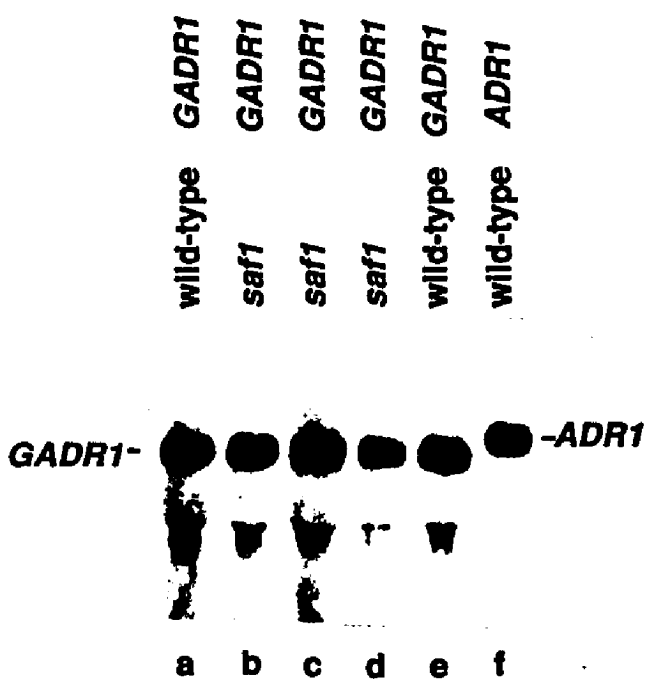
Using Northern analysis, I compared levels of GADR1 mRNA between wild-type SAF and saf1-containing strains grown under glucose conditions. In Figure 8 I show that the saf1 mutation had no effect on GADR1 expression (Figure 8b, lanes b, c and d, GADR1 saf1, compared to lands a and e, GADR1 SAF1). ADH II activity assays conducted on segregants from this cross confirmed that the saf1 mutation had no effect on GADR1-dependent ADH2 expression as both GADR1 SAF and GADR1 saf1 strains produced ADH II activity values in the 20-30 mU/mg range when grown in glucose-containing medium. These results indicate that the saf1 mutation represses ADR1 expression through the ADR1 5′ noncoding sequences. As data presented in Table 3 suggested that three SAF gene products act through a common pathway, the saf2 and saf3 mutations were predicted to also act through ADR1 5′ noncoding sequences.

Figure 8. Effects of the saf1 mutation on GADR1 expression. a) All ADR1 5′ sequences were replaced with G3PDH promoter sequences in strain GADR1 as described in Figure 4. b) Following growth in medium containing 8% glucose, 15 μg of total RNA isolated from segregants of the cross GADR1 x R234-14 which are either wild-type for the SAF1 allele (lanes a and e) or saf1 (lanes b,c,and d) and from strain 43-2B (lane f) were subjected to Northern analysis using the ADR1 probe as described. The two weakly hybridizing bands migrating below the GADR1 mRNA are, in decreasing size, due to non-specific hybridization to the 25S rRNA (migrating at 3.4 kb) and a truncated mRNA expressed from the adr1-1 allele (migrating at 3.0 kb). Integration of plasmid GADR1 was site-directed to the adr1-1 locus, an event which created both a GADR1 allele with the full complement of ADR1 coding sequences and an adr1-1 allele containing the wild-type ADR1 promoter and approximately 2.4 kb of adr1-1 coding sequences. Analysis of rRNA in a duplicate gel and reprobing of Northern blots with a URA3 probe indicated that total RNA loadings were nearly equivalent except for lane c which was overloaded by 25% and lane d which was overloaded by 20% with respect to the other lanes.

a)



b)



The saf mutations do no affect ADR1 mRNA stability

Data presented in Chapter 1 suggested that the 5′ untranslated region of the ADR1 mRNA mediates the control of ADR1 mRNA stability. Because the saf1 allele exerts its effects on ADR1 mRNA levels through these 5′ noncoding sequences, I determined whether ADR1 mRNA stability was being affected by the saf mutations. ADR1 mRNA stability was analyzed in strains containing the rpb1-1 allele as described in Chapter 1.

Northern analysis was conducted on ADR1 mRNA levels in glucose grown cells taken at indicated time intervals after shifting cells to 37°. In a wild-type SAF background, ADR1 mRNA (Figure 9a) and ADR1-5^c mRNA (data not shown) had half-lives of 45 min. The half-life of ADR1-5^c mRNA in strains containing saf1 was 65 min (Figure 9b), saf2 was 65 min (Figure 9c) and saf3 was 75 min (Figure 9d).

While these results indicated that the saf mutations did not increase the decay rate of ADR1-5^c mRNA under glucose growth condition, I was unable to explain some irregularities in the mRNA degradation profiles shown in Figure 9. I measured slightly higher ADR1-5^c mRNA half-lives in saf-containing strains than in wild-type SAF-containing strains. These half-life measurements may have been affected by the relatively lower ADR1-5^c mRNA levels at the early (0-20 min) time points. In another study using rpb1-1-containing strains, it was similarly observed that

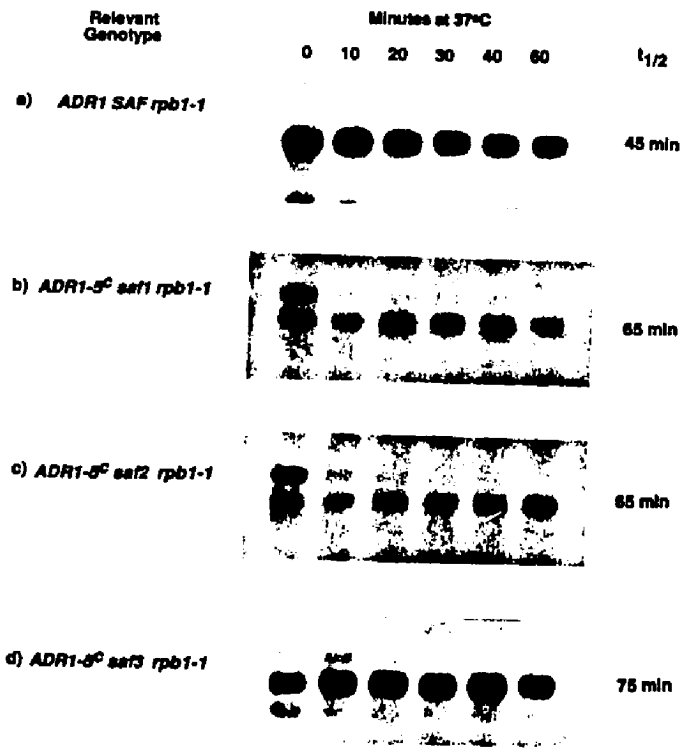


Figure 9. Effects of the saf mutations on ADR1 mRNA stability. Strains containing the temperature-sensitive RNA polymerase II mutation (rpb1-1) were grown on medium containing 8% glucose and subjected to mRNA decay analysis as described in Figure 5. Samples containing 15 μg of total RNA from strain JC44-5a (panel a), 30 μg from strain JC88-1b (panel b), 30 μg from strain JC89-7c (panel c), and 30 μg from strain JC90-13d (panel d) were extracted at the times indicated following shift to 37°C. For the strains containing the saf mutations (panels 5b,5c,and 5d), ADR1 mRNA half-lives were calculated by linear regression using the 10- or 20- to-60 minute time points. We observed that in strains containing the saf alleles, ADR1 mRNA levels were reduced at the early time points, an observation also noted for other mRNAs in another study (Moore et al. 1991) as described in the text. ADR1 mRNA half-live values represent the average of three separate determinations which varied by approximately $\pm$ 25%. Equivalent amounts of total RNA were loaded in each lane as verified by inspection of rRNA bands in a duplicate gel stained with ethidium bromide.

signal intensities at the earliest time points were also lower than expected in some experiments (Moore et al. 1991). To ensure that these irregularities were not due to inadequate thermal inactivation of the temperature-sensitive RNA polymerase II or to a general effect of the saf mutation on this inactivation, all blots were stripped and reprobed with CCR4 and URA3 probes. CCR4 and URA3 mRNAs, in contrast to ADR1-5^c mRNA, did not display the lower levels of RNA at the initial time points in strains containing the saf mutations (data not shown). I also calculated the half-life of URA3 mRNA in these strains and experimental conditions to be 10 min which is in agreement with previous measurements of the URA3 mRNA decay rate (Bach et al. 1979; Kim and Warner 1981). I compensated for the irregularities in mRNA signal reduction by using either the 10- or 20- to-60 min time points in half-life determinations. These results therefore indicate that ADR1-5^c mRNA stability is not dramatically decreased by the saf mutations.

These results show that under glucose growth condition, the 5- to 8-fold drop in ADR1 and ADR1-5^c mRNA levels caused by the saf mutations is not a consequence of increased mRNA degradation. Therefore, it can be concluded that the SAF gene products are required for ADR1 transcription.

Another lab strain in which glucose reduces ADR1 mRNA levels does not contain a saf mutation

The carbon source dependent differences in ADR1 mRNA levels in strains containing the saf alleles resemble the ADR1 mRNA expression differences seen in strain HB23-3 in which ADR1 mRNA abundance is controlled by glucose (Blumberg et al. 1988). Strain HB23-3 was examined for the presence of a saf mutation by complementation analysis. Crosses were made between the isogenic series of strains containing the saf alleles and strain HB23-3, and Northern analysis of ADR1 mRNA levels was conducted on the resulting diploids. Results shown in Figure 10 indicated that HB23-3 x ADR1-5^c saf1 saf2 or saf3 diploids all displayed high levels of ADR1 mRNA under glucose conditions indicating that HB23-3 does not contain a saf mutation. These results demonstrate that strain HB23-3 lacks a factor or factors responsible for ADR1 mRNA accumulation under glucose growth conditions since the reduced mRNA levels behaved as a recessive trait.

To determine if this 10- to 20-fold lowering of ADR1 mRNA by glucose in HB23-3 was due to a glucose-induced increase in the rate of ADR1 mRNA degradation, the appropriate crosses were made in an attempt to isolate a strain with the HB23-3 ADR1 mRNA phenotype and an rpb1-1 allele. I screened temperature-sensitive segregants (indicative of the rpb1-1 allele) from this cross by conducting Northern analysis of ADR1 mRNA levels between

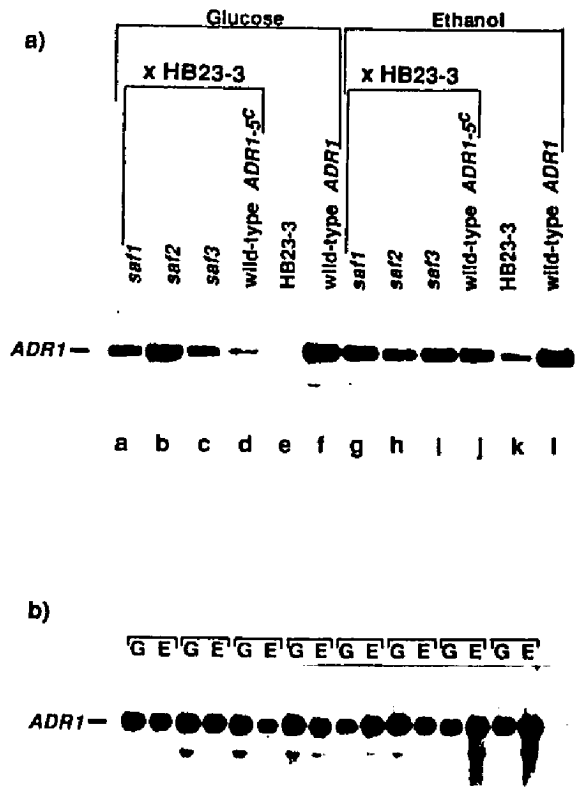


Figure 10. Analysis of a strain in which ADR1 mRNA is controlled by glucose. a) RNA was extracted from diploid strains created by crosses between HB23-3 and R234-14 (lanes a and g), HB23-3 and R234-15 (lanes b and h), HB23-3 and R234-19 (lanes c and i), and HB23-3 and R234 (lanes d and j), and also from haploid parental strains HB23-3 (lanes e and k) and 43-2B (lanes f and l). Total RNA was subjected to Northern analysis using the ADR1 probe described in Figure 6. Fifteen μg of total RNA were loaded in each lane. Reprobing the Northern blot with URA3 and inspection of rRNA bands in a duplicate gel indicated that lane b was overloaded 50%, lane e 75%, and lane f 50% with respect to the other samples. After normalizing ADR1 mRNA to these standards, the difference in ADR1 mRNA levels between glucose and ethanol grown cells for the diploids (lanes a,b,c,d vs. g,h,i,j) and Strain 43-2B (lanes f vs. l) ranged between 1.0- to 2.5-fold more ADR1 mRNA in ethanol grown cells. I was unable to detect ADR1 mRNA in strain HB23-3 grown under glucose conditions (lane e). b) Strain HB23-3 was crossed to JC44-5a in an effort to isolate strains that contained the rpb1-1 allele and that displayed the glucose reduction of ADR1 mRNA levels characteristic of strain HB23-3. Twenty μg of total RNA extracted from either glucose (G) of ethanol (E) grown cells from segregants created by this cross were subjected to Northern analysis using the ADR1 probe. When ADR1 mRNA levels were normalized to CCR4 mRNA and to rRNA bands in a duplicate gel, no differences in ADR1 mRNA levels between glucose or ethanol grown cells exceeded 2.5-fold.

glucose and ethanol grown cells. In the analysis of eight temperature-sensitive segregants, all failed to show any large (i.e. 10-fold) difference in ADR1 expression on the different carbon sources (Figure 10b). To investigate the possibility that the gene in HB23-3 responsible for the glucose-dependent 10- to 20-fold reduction in ADR1 mRNA was linked to RPB1 and, therefore, could not be recombined with the rpb1-1 allele, ADR1 mRNA was analyzed in two complete sets of tetrads. In all eight of these segregants analyzed, the observed differences in ADR1 mRNA between glucose and ethanol grown cells were similar to that presented in Figure 10b: no segregant displayed more than two-fold less ADR1 mRNA when grown under glucose as compared to ethanol conditions (data not shown). These results suggest that carbon source control of ADR1 mRNA levels in strain HB23-3 is due to multiple factors some of which are recessive to factors typically found in laboratory yeast strains.

Discussion

A novel genetic screen was utilized to identify mutations suppressing ADR1-5^c-induced petite formation at 37° (C. Denis unpublished results). Using this screen, strains that displayed diminished ADR1-5^c activity yet still expressed sufficient ADH II to allow growth on ethanol-containing medium were selected. The resulting mutants were

grouped into three complementation groups (saf1, saf2 and saf3). In the present study, the SAF gene products were found to be required for the transcription of ADR1 under glucose growth conditions. The role of the SAF genes in controlling ADR1 transcription is based on the following observations: (i) all three SAF genes were shown to be required for maintaining high ADR1 RNA levels under glucose growth conditions, (ii) the effect of the saf1 mutation on ADR1 steady state mRNA levels was relieved when ADR1 was expressed from another promoter, and (iii) the saf mutations had no effect on ADR1 mRNA stability.

In this study, I attributed a 5- to 8-fold decrease in ADR1-5^c expression due to the saf mutations as being responsible for an 80-fold decrease in ADH2 expression under glucose growth conditions. This conclusion was based on the observation that when the concentration of ADR1^c protein falls below that found in a strain carrying a single copy of the ADR1^c gene, ADR1^c-induced ADH2 expression becomes exponentially reduced (unpublished observations). Therefore, a small reduction in ADR1^c (or ADR1) RNA levels below that found in a wild-type strain has much larger effects on ADH II activity. Several mechanisms suggest themselves as to why this may occur including differential translation of ADR1 mRNA dependent on its abundance, cooperative ADR1 protein-protein interaction at the ADH2 promoter, cooperative binding of ADR1 to a required

transcription factor (Thukral et al. 1991), and preferential binding of ADR1 at sites other than the ADH2 promoter. Studies investigating the cooperative nature of GAL4 activation have identified cooperative DNA binding (Giniger and Ptashne 1988) and competition between different binding sites for limiting amounts of activator (Baker et al. 1984) as being responsible for similar discrepancies.

ADR1 mRNA levels appear to be similar under both glucose-repressed and derepressed growth conditions for most wild-type strains tested indicating that glucose does not exert a large measure of control over ADR1 mRNA expression (Blumberg et al. 1988, Denis 1987, Denis and Gallo 1986). Yet the saf mutations affected ADR1 transcription under glucose growth conditions but had little effect under ethanol conditions. While the physiological role of the SAF genes is unclear, two models suggest themselves. The SAF genes may be required for basal transcription of ADR1 under glucose growth conditions. This model is consistent with an ADR1 promoter that lacks upstream activation sequences (UAS) or with SAF products acting as in a UAS-independent fashion. Alternatively, the SAF gene products may act through UAS elements to elevate ADR1 mRNA above a minimal glucose-repressed level. An analysis of the structural features of the ADR1 promoter region would be needed to decipher which of these models is correct.

Since the saf mutations affected ADR1 transcription

under glucose growth conditions and affected petite formation under ethanol conditions, the SAF gene products appear to be expressed under both carbon sources. Various mechanisms might explain how the saf mutations have vastly different effects on ADR1 transcription depending on carbon source, including the possibility that the function of the SAF gene products may be redundant in derepressed conditions. Another possibility is that the SAF products are similarly required in either carbon source yet glucose has pronounced effects on the activity of the mutated saf products. Glucose might affect the stability or alter the conformation of the saf products, or the contacts made between the saf products and other components of the transcriptional machinery may have more stringent requirements for structural integrity in repressed conditions.

Under ethanol growth conditions, it was observed that a small 10-15% reduction in ADR1-5^c mRNA in the saf-containing strains had negligible effects on ADH2 expression. This reduction in ADR1-5^c mRNA may be sufficient, however, to suppress ADR1-5^c-induced petite formation. It should be noted that the saf-containing strains grew very slowly on ethanol-containing medium at 37°, suggesting that the suppression of petite induction by the saf mutations was slight. Other mechanisms may also exist that are responsible for petite suppression including the possibility

that the saf mutations affect other genes involved in petite induction. Throughout the analysis of the saf mutations, no direct evidence was encountered to suggest that these mutations influence genes other than ADR1 and ADH2. For example, they did not affect URA3, CCR4, or ADH1 mRNA levels or Ty-mediated gene expression. Indirect evidence, however, suggests that the saf mutations have pleiotropic effects. I have shown that removal of ADR1 5′ noncoding sequences (GADR1 allele) resulted in the removal of the saf1 effect on ADR1 expression (Figure 8). It was also observed that strains containing the SAF GADR1 alleles grew faster than strains containing the saf1 GADR1 alleles on medium containing glucose (unpublished observations). This observation suggests that the saf1 mutation has a deleterious effect on cell growth and, therefore, controls genes other than ADR1. Cloning the SAF genes will allow further characterization of the roles and functions of these factors.

CHAPTER 3

IDENTIFICATION OF FUNCTIONAL AND REGULATORY REGIONS IN THE ADR1 AND ADR1^c PROTEINS

Introduction

In the previous chapter, a genetic screen was used with the intent of isolating mutations that suppressed the enhanced activity of the ADR1-5^c gene. The only suppressor mutations isolated in this study were found to cause a reduction in both ADR1-5^c and ADR1 transcription. In another mutagenic study that also had the goal of isolating suppressor mutations of ADR1-5^c, mutations in the zinc-finger region were isolated (D. L. Mullaney thesis; Cook et al. 1993). These mutations principally affected DNA binding and were not specifically detrimental to ADR1-5^c function. Therefore, a different approach was taken to identify the regions in the ADR1-5^c protein that are required for enhanced activity. A deletion analysis, coupled with an *in vivo* transcription assay, was used to identify these required regions.

The regions in the ADR1-5^c protein required for enhanced activity of this mutated protein were predicted to fall into two groups. First, an activation domain in ADR1 might be specifically required for the ADR1-5^c effect. If

this proved to be correct, it would indicate that the inhibitory function of the 227-239 region involved the suppression of a specific activation domain. Alternatively, a non-activating region of ADR1 might be required for the ADR1-5^c effect. Such an interacting region might be analogous to the DSSLD amino acid sequence in the CREB protein that was suggested to synergize with a cAPK phosphorylation site at Ser-133 to enhance CREB activity (Gonzalez et al. 1991).

To compare the effects of deletions on the ability of the ADR1^c proteins to enhance activation, deletions were constructed in both ADR1^c and ADR1 proteins. Following measurement of the activation ability of the deleted ADR1^c and ADR1 proteins, it was possible to ascertain if the deleted ADR1^c protein was still more active than the deleted ADR1 counterpart. If the deleted ADR1^c and ADR1 proteins were equal in their activation ability, it would be predicted that a region in the ADR1^c protein was removed which was required for the ADR1^c effect.

While a major objective of this deletion analysis was to identify regions required for ADR1^c function, a second objective was to better characterize the functional regions in the wild-type ADR1 protein. The activating regions in ADR1 have not been thoroughly characterized. While two broad regions that appear to have activating residues have been identified (Figure 3), the residues that are directly

required for activation have not been localized. In addition, while the 227-239 region has been identified as playing an inhibitory role, other regions in ADR1 may also function to inhibit activity. Therefore, objectives of the deletion analysis in this chapter were the following: (i) localize the activating and inhibitory regions in ADR1, and (ii) identify regions in the ADR1^c proteins that are required for enhanced activation.

Materials and Methods

Plasmid Constructions:

ADR1 deletions: All ADR1 deletion alleles were constructed with ADR1 sequences derived from plasmids 23A-65 (ADR1), 23A (ADR1-5^c) or 3II3-7^c (ADR1-7^c) (Denis and Young 1983, Cherry et al. 1989) inserted into pUC18 or pUC19 vectors. All contain at least 1.2 kb of ADR1 upstream DNA, which is sufficient for normal ADR1 expression (Denis 1987). Restriction sites used to isolate specific ADR1 deletion fragments are diagramed in Figure 11. Each ADR1 gene is designated by the number of ADR1 amino acids it encodes (e.g. ADR1-571 encodes the N-terminal 571 amino acids of ADR1), and internal deletions are given in terms of ADR1 amino acids flanking deleted residues (e.g. ADR1-262/288 encodes the complete ADR1 protein containing an in-frame deletion of amino acids 263-287). The following non-ADR1 amino acids were present on the C-terminal of the truncated

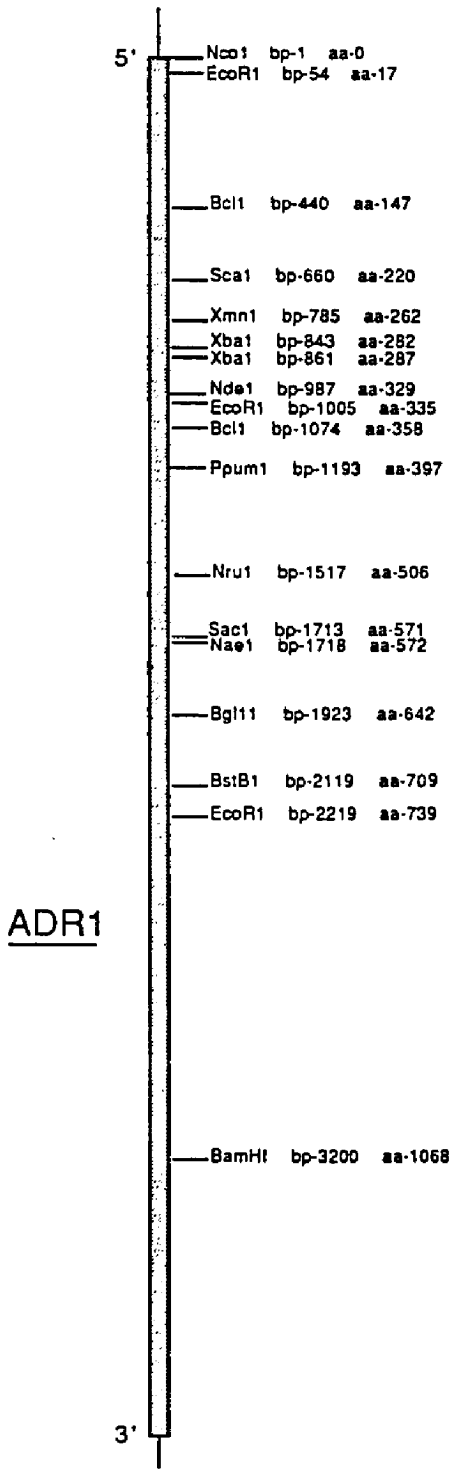


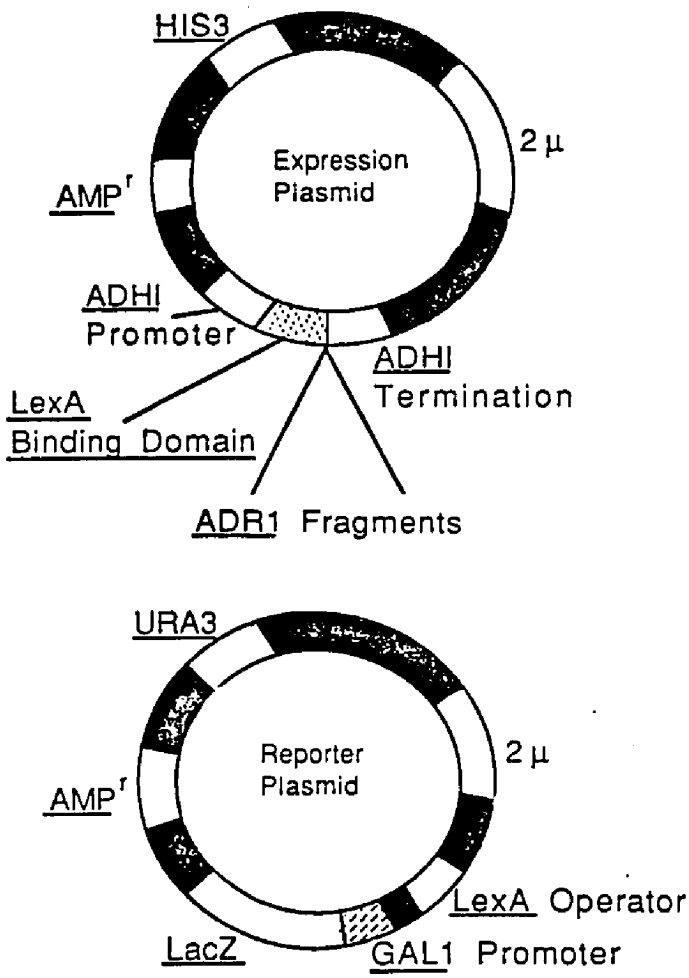
Figure 11. Restriction enzyme cut sites in the ADR1 gene.

Sites shown were used to make ADR1 deletion derivatives analyzed in this study. Sites are described by restriction endonuclease used, by base pair number (bp) denoting the 3' end of ADR1 DNA sequences cut, and by the amino acid number (aa) that corresponds to the cut site.

ADR1 proteins: ADR1-1068 contains an extra L, ADR1-709 contains SKQF, ADR1-571 contains RYPGIL, and ADR1-329 contains SGDRPLGLLL. In some cases non-ADR1 amino acids were present between deletions: ADR1-262/288 contains a T between amino acids 262 and 288, and ADR1-262/330 contains a GI between 262 and 330. Ligations involving blunt-ended fragments were done using the large subunit of E. coli polymerase (Klenow fragment) as described (Sambrook et al. 1989). In some cases, calf-intestinal phosphatase was used as described by the manufacturer (New England Biolabs, Beverly MA) to dephosphorylate compatible ends in the vectors. Sequencing of selected junctions was done as described (Sanger et al. 1977) when proteins were not visualized or were of an unpredicted size. Visualization of proteins was conducted by Western analysis as described (Vallari et al. 1992).

LexA-ADR1 fusions: To construct LexA-ADR1 fusion proteins, ADR1 fragments were inserted into polylinker sites in vector LexA(1-202)+PL (Ruden et al. 1991, see Figure 12). Restriction sites in ADR1 that were used are shown in Figure 11. For LexA-ADR1 fusion proteins that contain ADR1 amino acid 1, ADR1 sequences were derived from plasmid JS119 which contains a Nco1 site introduced at the start of translation (Cherry et al. 1989). All plasmids encoding fusions of ADR1-1-642 are derived from plasmid W153, which has been described (Cook et al. 1993). Other LexA-ADR1 fusion

Figure 12. Expression plasmid LexA(1-202)+PL and reporter plasmid 1840 used in the LexA transcription assay system.



The LexA(1-202)+PL (Ruden et al. 1991) expression vector contains an ampicillin drug resistance gene (AMP^r), a yeast HIS3 marker, a 2μ replication origin, and ADH1 promoter and termination sequences that flank the complete LexA coding sequences. A polylinker site is located between the 3' end of the LexA sequences and the ADH1 terminator. The 1840 reporter plasmid (Brent and Ptashne 1985) contains the yeast URA3 marker, and a LexA operator binding site located 5' to a GAL1 promoter-driven LacZ reporter gene. Binding of LexA-ADR1 fusion proteins to the LexA operator brings ADR1 activating residues into position to activate the LacZ reporter gene.

proteins contain intervening extra amino acids derived from LexA(1-202)+PL polylinker sequences between LexA and ADR1 residues, and extra amino acids derived from the ADH1 terminator attached to the ADR1 C-terminus: LexA-ADR1-1-220 contains the 13 intervening residues RPEFELGTRGSIT and the 6 extra C-terminal residues FDLQPS; LexA-ADR1-148-359 contains the 6 intervening residues RPEFPG and 16 C-terminal residues PSTCRQANSGRISYDL; LexA-ADR1-148-262 contains the 6 intervening residues RPEFPG and the C-terminal residues RGSVDLQPS; LexA-ADR1-263-359 contains the 12 intervening residues RPEFECGTRGSP and the 16 C-terminal residues PSTCRQANSGRISYDL; and LexA-ADR1-1-18,337-642 contains the 12 intervening residues RPELIPGDRSIT and the C-terminal residues PSTCSQANSGRISYDL. The activation ability of selected LexA-ADR1 fusion proteins was measured by assaying β-galactosidase activity as an indicator of LacZ expression from the 1840 reporter plasmid (Brent and Ptashne 1985, Figure 12). LexA-ADR1 fusion proteins were visualized by Western analysis using a LexA antibody as described (Vallari et al. 1992).

Transformations. All yeast transformations were conducted using the lithium acetate method as described (Ito et al. 1983). Linearized ADR1 and ADR1^c deletion plasmids were site-specifically targeted for integration at either the adr1-1 or trp1 locus. The methods used for identifying integration events and for determining the number of ADR1 or

ADR1^c genes integrated were as described (Denis 1987).

Growth conditions and assays. Growth conditions and ADH II assays are described (Chapter 2; Denis and Young 1983). ADH II activity values represent an average of at least three independent measurements. For individual strains, the standard error of the mean (SEM) was less than 20% of the average ADH II value except when the ADH II activity value was less than 20 mU/mg. For the determination of the maximal ADH II activity and the slope of the line relating ADH II activity to ADR1 gene dosage under glucose growth conditions, the computer program Enzfitter (Elsevier-Biosoft) was used. Enzfitter determined maximal ADH II values by fitting the plot of ADH II activity versus ADR1 gene dosage under ethanol growth conditions to the Michaelis-Menten equation, and calculating the Vmax value. For the determination of the slope of the line relating ADH II activity as a function of ADR1 gene dosage under glucose conditions, Enzfitter fit the plot to the linear regression equation and calculated the gradient of this linear line.

β-galactosidase assays were conducted as described (Brent and Ptashne 1985) except values were measured per milligram total protein. ADH II activities were conducted in the same yeast extracts that were assayed for β-galactosidase activity. as described (Denis 1987).

Results

Carboxy-terminal deletions of ADR1 and ADR1^c

In a previous study, carboxyl-terminal deletions of the ADR1 protein were used to localize functional regions in ADR1 (Bemis and Denis 1988). This analysis was expanded by constructing additional C-terminal deletions in ADR1. New C-terminal deletions constructed included ADR1-1068 (with a different C-terminus than Bemis and Denis [1988]), ADR1-709, ADR1-571, ADR1-397, and ADR1-329. All C-terminal truncations were also constructed with either an ADR1-5^c (R229K) or an ADR1-7^c (S230L) mutation. ADR1-5^c results in the same phenotype as ADR1-7^c (Cherry at al. 1989; Denis et al. 1992)

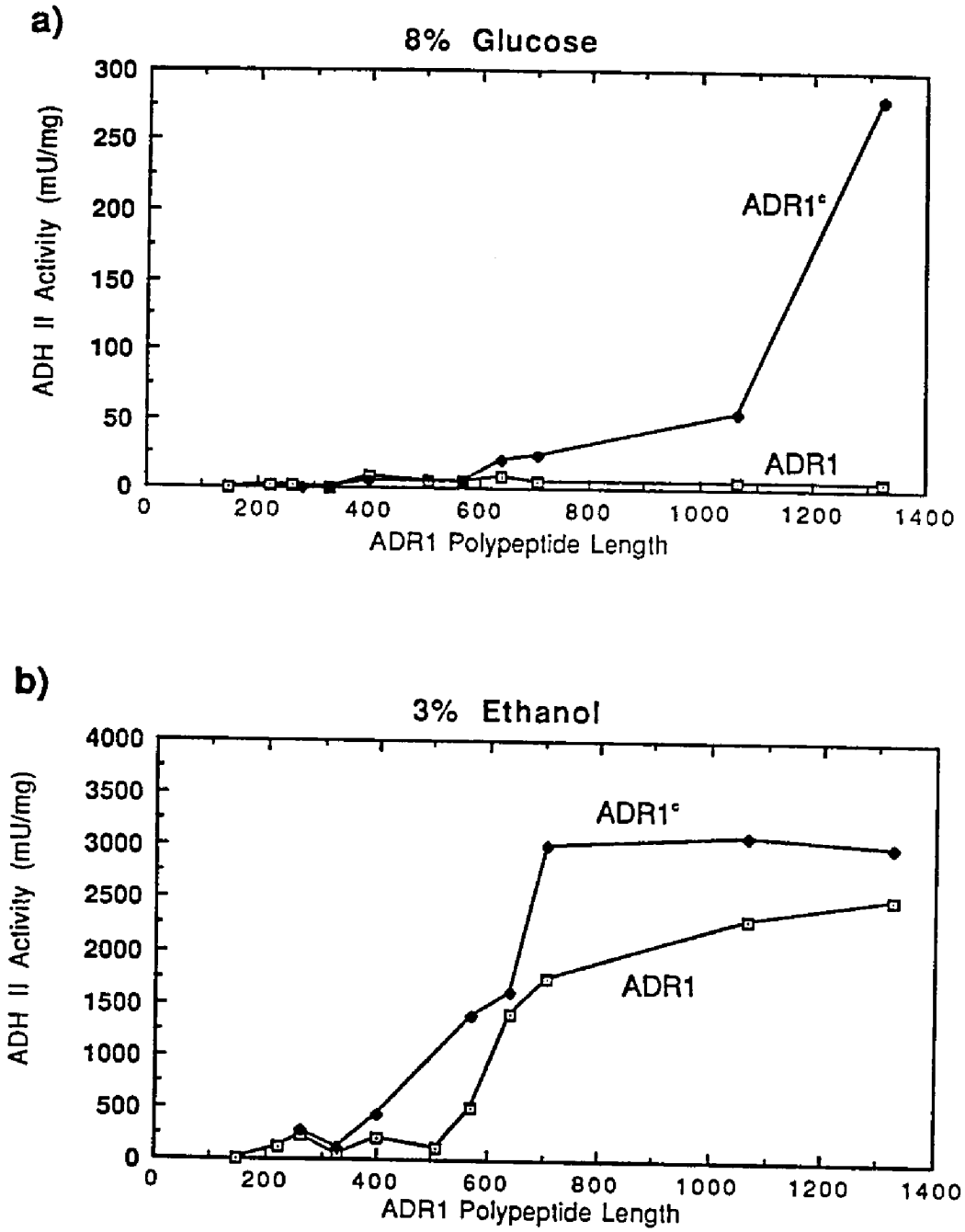
To compare the activating ability between different ADR1 truncated proteins, and to compare activating ability between various ADR1 and ADR1^c truncated proteins, several methods were employed. First, ADR1 and ADR1^c genes containing deleted 3′ sequences were integrated at a single gene copy at the trp1 locus in an adr1-1 strain. Following growth in either glucose- or ethanol-containing medium, the activating ability of these various ADR1 C-terminal truncated proteins was measured by assaying ADH II activity. Figure 13 shows the results of this analysis in which ADH II activity was plotted as a function of ADR1 polypeptide length following growth in medium containing either 8% glucose (Figure 13a) or 3% ethanol (Figure 13b).

Inspection of ADH II activity versus ADR1 length in Figure 13a indicated that ADR1 remained suppressed by glucose (i.e. ADH II activity remained less than 10 mU/mg) irrespective of ADR1 length. In contrast, no ADR1^c C-terminal deletion protein was as active as the full-length. ADH II activity dropped rapidly as C-terminal residues were removed from ADR1^c. Only ADR1^c proteins containing 642 or more N-terminal residues allowed glucose-insensitive ADH II activity suggesting that residues C-terminal to amino acid 571 are required for the ADR1^c effect under glucose growth conditions. However, at this single gene dosage it was not possible to quantify ADR1 or ADR1^c protein levels. Therefore, the drop in activation ability as residues were removed from the C-terminus of ADR1^c may have been due to decreases in protein stability. When some ADR1 truncations, including ADR1-506 and ADR1-329, were overexpressed they still could not be detected by Western and immunoprecipitation analysis (R. Vallari and S. Fontaine pers. comm.). To compensate for variations in protein stability, strains containing multiple copies of integrated ADR1 and ADR1^c deletion derivatives were analyzed as described below.

In Figure 13b, ADH II activity as a function of ADR1 and ADR1^c length are compared following growth in ethanol-containing medium. As C-terminal residues were removed from the ADR1 protein, the ability to activate ADH2 expression

Figure 13.

Effects of varying ADR1 and ADR1^c polypeptide length on ADH II activity.



ADH II activity is given as a function of ADR1 polypeptide length. Strains isogenic to 500-16 contained single gene copies of ADR1 and ADR1^c genes with 3' deletions. Growth conditions and ADH II assays were as described in Table 1.

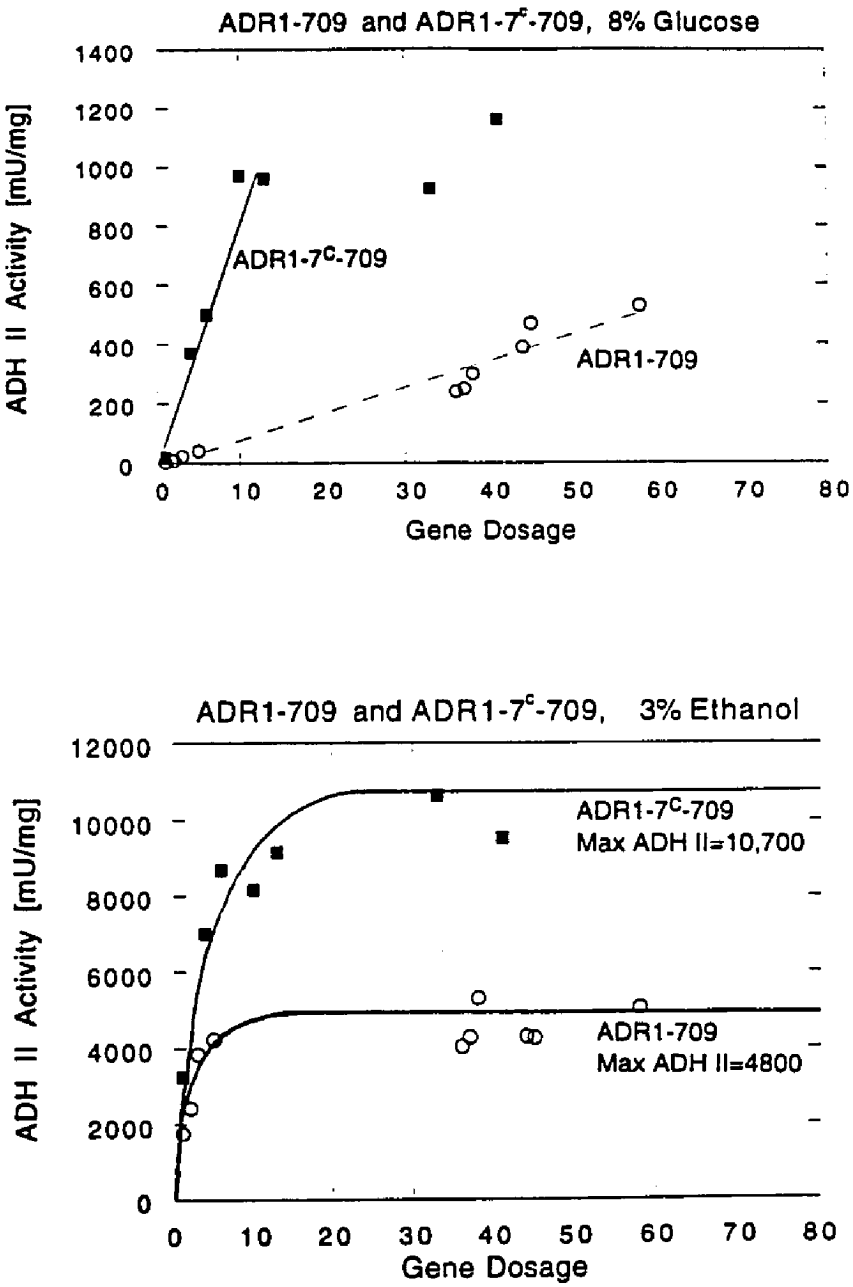
steadily decreased. However, this decrease in activating ability was not completely proportional to the decrease in ADR1 length. ADH II activity dropped most precipitously when ADR1 amino acids between 709 and 506 were removed, suggesting that this region is important for activation. A comparison between ADR1 and ADR1^c showed that for every C-terminal deletion containing residues of 397 or more, the ADR1^c protein was more active than ADR1. In contrast, when ADR1 or ADR1^c proteins containing amino acids 329 or less were compared, ADH II activities were equal. These results suggest that ADR1^c proteins require residues C-terminal to amino acid 329 in order to allow heightened ADH II activity. However, as mentioned above, ADR1 protein levels were not quantified in these strains containing single ADR1 genes and, therefore, such comparisons may have been compromised due to differences in ADR1 deletion protein levels.

To compensate for differences in mRNA stability, protein stability, or differences in conformational effects that might have influenced binding affinities, an approach similar to the approach described in Bemis and Denis (1988) was taken. This method entails calculating a maximal ADH II activity for strains expressing ADR1 or ADR1^c deletions. The maximal activation ability of ADR1 is based on observations that high ADR1 dosages allow for the saturation of ADH2 expression during nonfermentative growth when ADH2 copy number is limiting (Denis 1987). At this level of ADH2

saturation it was theorized that all binding sites for ADR1 were occupied and, therefore, maximal ADH II activity is a direct measure of the activating potential of the ADR1 protein. This approach was expanded by calculating the maximal ADH II activities in strains expressing N-terminal ADR1 polypeptides of 1068, 709, 571, and 329 amino acids to add to results from Bemis and Denis (1988). In addition, the maximal ADH II activities of ADR1-5^{c}-1323, ADR1-5^{c}-1068, ADR1-7^{c}-709, ADR1-7^{c}-571, and ADR1-7^{c}-329 were calculated. This was accomplished by obtaining a range of dosages for the truncated ADR1 genes integrated at the trp1 locus and determining the resulting ADH II activities as described (Bemis and Denis 1988).

An example of how ADH II activity increases as a function of ADR1 gene dosage is shown in Figure 14 using the ADR1-709 and ADR1-7^{c}-709 deletions. Figure 14a shows a comparison between activating ability of the ADR1-709 and ADR1-7^{c}-709 deletions made by plotting ADH II activity as a function of ADR1 gene dosage following growth in glucose-containing medium. As observed in other studies using full-length ADR1 (Denis 1987) or ADR1 C-terminal truncations (Bemis and Denis 1988), ADH II activity increased linearly as ADR1-709 and ADR1-7^{c}-709 gene dosage was increased. However, for ADR1-7^{c}-709, and other highly active ADR1^{c} deletions (data not shown), this linear relationship was only observed at low gene dosages (i.e. less than 25

Figure 14. Effects of *ADR1-709* and *ADR1-7^{C}-709* gene dosage on ADH II activity.



ADH II activity is given as a function of gene dosage in strains containing different numbers of integrated *ADR1-709* or *ADR1-7^{C}-709* genes. All strains are isogenic to 500-16.

copies), while at high dosages the ADH II activity appeared to level off indicating saturation of some component in ADH2 activation.

While an approximate comparison between the slopes generated in strains containing either ADR1-709 or ADR1-7^c-709 alleles could be determined graphically as shown in Figure 14a, a precise comparison was made by using the computer program Enzfitter. Enzfitter fit the plotted points to linear regression, and precisely calculated the slopes of these linear regression lines. For highly active ADR1^c deletions such as ADR1-7^c-709, only strains containing less than 25 copies of ADR1 deletion alleles were used since above this copy number the slope of the line relating ADH II activity to gene dosage was not linear. Enzfitter was used to calculate slopes for other C-terminal deletion derivatives, and a comparison between different ADR1 and ADR1^c truncations is shown in Table 4. In all cases analyzed, the increased slope measured for the ADR1^c deletions indicated that the ADR1^c deletion proteins were better activators than the ADR1 counterpart under glucose-repressed conditions. This result indicates that ADR1 residues C-terminal to amino acid 262 are not required for the ADR1^c effect. However, no difference between the different slopes was as great as that between the full-length ADR1 and ADR1^c proteins. This finding was consistent with results in Figure 13a which showed that when

Table 4. Slopes of the curve relating ADH II activity to gene dosage for C-terminal truncations of ADR1 and ADR1^c.

C-terminal truncation	Relative Slope
ADR1-1323	1
ADR1-5^c-1323	49.0
ADR1-1068	1
ADR1-5^c-1068	3.3
ADR1-709	1
ADR1-7^c-709	2.8
ADR1-571	1
ADR1-7^c-571	6.3
ADR1-329	1
ADR1-7^c-329	2.0
ADR1-262	1
ADR1-7^c-262	4.1

Yeast strains isogenic to 500-16 were grown in YEP medium supplemented with 8% glucose. Slopes were determined by using the Enzfitter computer program to plot ADH II activity as a function of ADR1 gene dosage and fitting these points to a linear regression equation as described in Materials and Methods. An example of the graphical data is shown in Figure 14a.

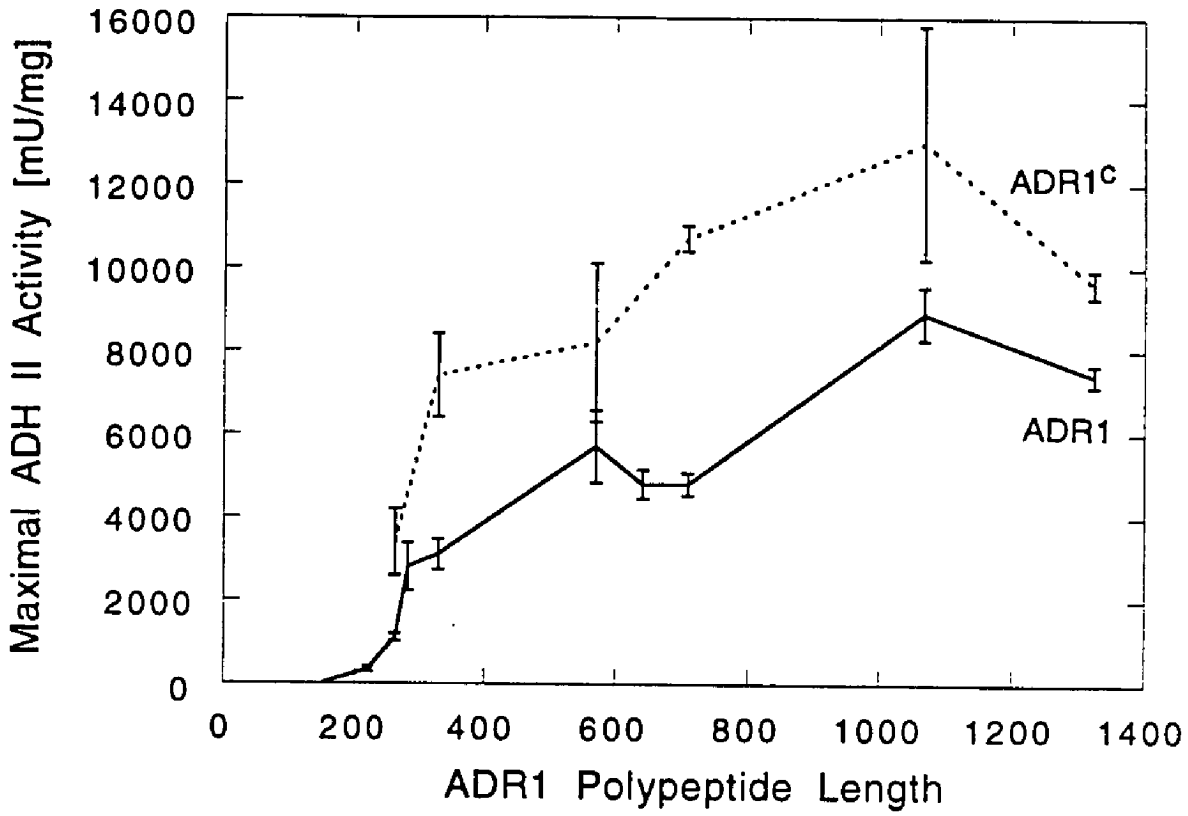
ADR1^c and ADR1 C-terminal deletion proteins were expressed from a single gene copy under glucose growth conditions, the greatest difference in activation was observed between full-length ADR1^c and full-length ADR1 proteins.

While the comparison between strains containing multiple gene copies of either ADR1 or ADR1^c C-terminal truncations under glucose conditions gave a reliable measure of activating differences, I was also interested in conducting this analysis under ethanol growth conditions as this allowed a comparison of the maximal ADH II activities. In Figure 14b, an example of this analysis is shown graphically for the ADR1-709 and ADR1-7^c-709 deletions. When ADH II activity was plotted as a function of gene dosage under ethanol growth conditions, ADH II activity initially rose in a linear fashion until a saturation level was reached. The level where the curve plateaus corresponds to the maximal ADH II activity. While an approximate maximal ADH II activity could be measured for ADR1 deletion derivatives on plots as shown in Figure 14b, a more exact measure of this value was obtained using the Enzfitter program. Because ADH II activity under ethanol conditions increased as a function of ADR1 gene dosage according to Michaelis-Menten kinetics, Enzfitter fit these plotted points to the Michaelis-Menten equation. A Vmax value which corresponded to the maximal ADH II activity was calculated for each ADR1 and ADR1^c truncation protein.

In Figure 15, maximal ADH II activity is plotted as a function of ADR1 and ADR1^c length. Values calculated for the new C-terminal deletions and the ADR1^c C-terminal deletions were added to values taken from Bemis and Denis (1988). An analysis of the figure addressed two questions: (i) What regions of the ADR1 protein are important for activation? and, (ii) What regions of the ADR1^c protein are required for the ability of ADR1^c to enhance ADH2 expression?

In Bemis and Denis (1988), maximal ADH II activity was shown to slightly but gradually decrease as residues were removed from the C-terminus of ADR1 back to residue 282. When ADR1 residues between 282 and 262 were removed, a dramatic decrease in maximal ADH II activity was observed. In the expanded graph shown in Figure 15, the drop in maximal ADH II activity as C-terminal residues were deleted from ADR1 was not consistently linear. Statistically significant decreases in maximal ADH II activity were observed when ADR1 residues were removed between amino acids 1068 and 709, 571 and 329, 282 and 262, 262 and 220, and 220 and 151. This result suggests that ADR1 contains at least three separate regions important for activation: residues 709 to 1068, 329 to 571, and 151 to 282. While results based on maximal ADH II activity values were not in complete agreement with results obtained with strains harboring single gene copies of ADR1 truncation alleles (Figure 13b),

Figure 15. Maximal ADH II activity as a function of ADR1 and ADR1^{C} polypeptide length.



Maximal ADH II activity was calculated by using the computer program Enzfitter to fit plots of ADH II activity as a function of *ADR1* and *ADR1^{C}* gene dosage to the Michaelis-Menten equation as described in Materials and Methods. Error bars represent standard deviation from the Michaelis-Menten equation as determined by Enzfitter.

both analyses indicated that ADR1 amino acids 329-709 are important for ADR1 activity.

In addition, maximal ADH II activity as a function of ADR1^c polypeptide length was compared to maximal ADH II activity as a function of ADR1 length in Figure 15. In all cases analyzed the ADR1^c truncations were better activators. This result confirmed that residues C-terminal to 262 are not absolutely required for the increased activity of the ADR1^c proteins.

Internal deletions of ADR1 and ADR1^c amino acids 263-572

Since the results shown in Figure 15 suggested that ADR1 amino acids 262-to-571 are important for activating ADH2 transcription, internal deletions spanning this region were made. Deletional analysis of this region was used to address the following questions: (i) What are the residues in the 262-571 region that are important for activation? (ii) Does the 262-571 region contain inhibitory elements? and (iii) Does this region contain residues that are required for the enhanced activity of the ADR1^c protein. Since two general questions were being addressed, those being what are the functional regions in the ADR1 protein and what are the regions in the ADR1^c proteins required for enhanced activation, data from the internal deletion analysis is split into two sets of tables for ease of inspection. Tables 5 and 6 compare the effects of internal

deletions within the ADR1 protein, and Tables 7 and 8 compare the same internal deletions between the ADR1 and ADR1-7^c proteins.

Integration of ADR1 genes with in-frame internal deletions was site-directed to the adr1-1 locus to create a full-length ADR1 gene containing internal deletions. The activation abilities of these deletions, when present as a single copy in the genome, were assayed by ADH II activity following growth in either glucose- or ethanol-containing medium. Table 5 shows that only when we deleted ADR1 residues in the 330-to-574 region did we observe a drop in activation ability. On the contrary, many internal deletions produced an ADR1 protein that had better activation ability than full-length ADR1. A previous study (Denis et al. 1992) showed that deletion of the 220/262 region, which contains the site of the ADR1^c mutations, allowed elevated ADH II activity compared to wild-type ADR1 indicating that the 220-262 region has inhibitory function. In the present study, deletion of amino acids 262/330 and 262/399 also allowed elevated ADH II activities under glucose conditions (75 mU/mg and 45 mU/mg respectively for the deleted proteins compared to 5 mU/mg for full-length ADR1). The 262/330 and 262/399 deletions also caused elevated ADH II activity under ethanol growth conditions.

As results reported in Vallari et al. (1992) indicated that the glucose control of ADR1 protein synthesis is

Table 5. ADH II activity in strains containing single gene copies of ADR1 alleles with internal deletions.

	ADH II Activity (mU/mg)	
	Glucose	Ethanol
[a]ADR1	5	2400
[b]ADR1-220/262	160	5900
ADR1-262/288	5	2600
[c]ADR1-282/288	5	3100
ADR1-262/330	75	4300
ADR1-262/399	45	3900
ADR1-330/507	0	1600
ADR1-399/507	5	1700
ADR1-506/574	0	1400
ADR1-262/288,399/507	0	700
[a]adr1-1	2	10

All strains are isogenic to 500-16. Growth conditions were as described in Table 1. ADH II activity values represent the average of at least three independent determinations. SEM values are less than 20% except where ADH II activity is less than 20 mU/mg.

[a] Data taken from Bemis and Denis (1988).
[b] Data taken from Denis et al. (1992).
[c] Unpublished data taken from A. Rovelli and C. L. Denis.

mediated by codons 262-to-642 in the ADR1 mRNA, I sought to determine if the 262/330 or the 262/399 deletions were affecting ADR1 protein levels. Western analysis indicated that this was not the case. The levels of ADR1-262/330 and ADR1-262/399 deletion proteins measured in strains containing single copy alleles were compared to the full-length ADR1 protein expressed in a strain harboring a single gene copy of ADR1, and to full-length ADR1 in a strain containing 16 copies of ADR1. ADH II activity under glucose conditions in the strain containing 16 copies of ADR1 is similar to ADH II activity under the same growth conditions for the ADR1-262/330 and ADR1-262/399 deletions: 50 mU/mg, 75 mU/mg, and 45 mU/mg respectively. While a quantitative comparison between ADR1 protein levels in the strains containing single gene copies was not possible due to the very low levels of ADR1 expression, results clearly showed that the deleted proteins expressed from single gene copies were expressed at levels similar to the full-length protein also expressed from a single gene. These levels were more than 10-fold lower than ADR1 levels observed in the 16X ADR1 strain (data not shown). These results indicate that in addition to the 220-262 inhibitory region a second inhibitory region located between amino acids 287 and 330 is present in the N-terminal 571 amino acids of ADR1.

Results shown in Table 5 indicate that internal deletions of ADR1 amino acids 330/507, 399/507, and 506/574

all reduced ADH II activity under ethanol conditions by approximately 40%. Deletion of residues 506-574 caused a similar decrease in ADR1 activation suggesting that either the activating residues cover a broad region between amino acids 330-574, or the region immediately surrounding residue 506 is the important activating region. In Figure 15, the severe drop in maximal ADH II activity observed when ADR1 residues between 262 and 282 were removed suggested that the 262-282 region is important for activation. Deletion of 262/288 had no effect on activation as shown in Table 5; however, when ADR1 contained both 262/288 and 399/507 deletions, ADH II activity decreased 70% under ethanol conditions. This suggests that both the 262-288 and 399-507 regions contain activating residues, but the 262-288 region is not required when other activation domains are present in ADR1.

Because it was not possible to accurately quantitate ADR1 protein levels in strains containing single copies of full-length or deleted ADR1 we could not definitively conclude from these results that the 262-288 and the 330-574 regions contain activating sequences. Therefore, the same approach as described in Figure 15 was taken to calculate a maximal ADH II activity in strains expressing the internal deletions. Since obtaining strains with multiple integrated copies of full-length ADR1 alleles is difficult, deletions in ADR1-571 were used to conduct this study. Table 6 shows

a comparison between the ADH II activities measured in strains that contained either ADR1-571 or ADR1-571 with internal deletions in the 262 to 507 region. Two measures of activation ability were determined: i) The ADH II activities in strains containing single copies of undeleted or deleted alleles, and ii) maximal ADH II activities measured in strains containing multiple gene copies integrated at the trp1 locus.

Results shown in Table 6 confirmed that the 262/330 region contains inhibitory elements. Both ADR1-1-262/330-571 and ADR1-1-262/399-571 allowed higher ADH II activity than undeleted ADR1-571 when expressed from single genes (under ethanol conditions), and when maximal ADH II activities were compared. The requirement of residues in the 330-507 region for activation was also confirmed since both the 330/507 and 399/507 deletions resulted in significantly reduced activation when either single gene copies were compared (under ethanol conditions), or when maximal ADH II activities were compared. Deletion of the 262-288 region had only a slight effect on ADR1-571 activity. This result confirms that deletion of the 262-288 region has little significance when other activation domains are present in ADR1. The role of the 262-288 region in activation is addressed further below.

Protein levels in strains containing 20-25 copies of the ADR1-571 alleles were quantitated using Western analysis

Table 6. Internal deletions in ADR1-571.

	ADH II Activity (mU/mg)[a]		Max. ADHII[b] Activity	Rel.[c] Abund.
	Glucose	Ethanol		
ADR1-571	2	390	5700 ± 870	1.0
ADR1-1-262/288-571	0	240	ND	1.0
ADR1-1-262/330-571	0	1200	10,500 ± 1000	1.5
ADR1-1-262/399-571	0	710	10,700 ± 1840	1.5
ADR1-1-330/507-571	0	40	1800 ± 50	2.0
ADR1-1-399/507-571	0	80	3600 ± 710	0.5

[a] ADH II activity was measured in strains containing single gene copies of ADR1-571 deletion alleles.

[b] Maximal ADH II activity (± standard deviation) was calculated using Enzfitter as described in Figure 15.

[c] Relative protein abundance was maesured using Western analysis on strains containing 15-25 gene copies of ADR1-571 deletion alleles, and is given relative to the undeleted ADR1-571 protein. An affinity-purified N-terminal antibody raised to ADR1 amino acids 2-21 was used as described (Vallari et al. 1992, Appendix).

(Table 6). The protein concentrations, given in relative amounts based on the undeleted ADR1-571 protein, showed slight fluctuations. However, it was concluded that the slight differences in steady state protein levels had minimal influence on the ability of these internally deleted ADR1 proteins to activate ADH2 transcription.

Internal deletions in ADR1^c

The analysis of internal deletions in the 262-574 region was expanded to include the ADR1^c proteins. In Table 7, internal deletions in full-length ADR1 and ADR1-7^c proteins expressed from single gene copies are compared. Except for the weakly activating 330/507 deletion under glucose conditions, all ADR1-7^c deletion proteins were more active than their ADR1 counterpart. This result confirmed that residues between 262 and 574 are not absolutely required for the enhanced activity of the ADR1^c proteins.

As mentioned above, protein levels were not quantitated in strains containing single gene copies of ADR1 deletion alleles. However, earlier studies indicated that the levels of ADR1^c protein were not elevated relative to ADR1 (Vallari et al. 1992, Taylor and Young 1990). Therefore, it was assumed that differences in protein abundance were not responsible for the ADH II activity differences seen in Table 7.

Table 7. ADH II activity in strains containing single gene copies of ADR1 and ADR1-7^c deletion alleles.

	ADH II Activity (mU/mg)	
	Glucose	Ethanol
ADR1	5	2500
ADR1-7^c	280	3000
ADR1-262/288	7	2650
ADR1-7^c-262/288	85	3250
ADR1-262/330	75	4290
ADR1-7^c-262/330	380	5010
ADR1-262/399	45	3930
ADR1-7^c-262/399	170	4470
ADR1-330/507	0	1560
ADR1-7^c-330/507	0	2610
ADR1-399/507	6	1710
ADR1-7^c-399/507	25	2900
ADR1-506/574	0	1400
ADR1-7^c-506/574	9	2460
ADR1-262/288, 399/507	0	710
ADR1-7^c-262/288, 399/507	6	1690

ADH II values measured in strains containing single gene copies of ADR1-7^c deletion alleles are compared to values taken from Table 5. SEM values are less than 20% except where ADH II activity is less than 20 mU/mg.

To confirm that residues in the 262/507 region were not required for the enhanced activity of ADR1^c, internal deletions were made in the ADR1-7^c-571 protein to allow a comparison to values shown in Table 6. In Table 8, the activation abilities of strains containing internal deletions in ADR1-7^c-571 were compared to ADR1-571 in three ways. First, strains containing single gene copies of either ADR1-571 or ADR1-7^c-571 with or without internal deletions were compared for their ability to activate ADH2. Inspection of ADH II activity under ethanol conditions in these single copy strains indicated that except for the very weakly activating 330/507 and 399/507 deletions, the ADR1-7^c-571 deletion derivatives were better activators than ADR1-571 deletions.

Second, following isolation of strains containing multiple gene copies of ADR1-7^c-571 deletion alleles, the slope of ADH II activity versus gene dosage under glucose growth conditions was compared between ADR1-7^c-571 and ADR1-571 deletion derivatives. For every deletion derivative analyzed, the ADR1-7^c-571 deletions all displayed a steeper slope than the ADR1-571 deletions. However, for the 262/330 deletion, the slight increase in the slope for the ADR1-7^c-1-262/330-571 as compared to the ADR1-1-262/330-571 was not significantly different as determined by Enzfitter. This result is discussed below.

Table 8. Internal deletions in ADR1-571 and ADR1-7^c-571.

	ADH II Activity (mU/mg)[a]			Max ADH II[c]
	Gluc	Etoh	Slope[b]	Activity
ADR1-571	2	390	1	5700 ± 870
ADR1-7^c-571	5	1480	6.3	8200 ± 1900
ADR1-1-262/288-571	0	240	1	ND[d]
ADR1-7^c-1-262/288-571	0	610	2.0	ND
ADR1-1-262/330-571	0	1210	1	10,500 ± 1000
ADR1-7^c-1-262/330-571	3	2140	1.2	15,300 ± 4570
ADR1-1-262/399-571	0	710	1	10,700 ± 1840
ADR1-7^c-1-262/399-571	0	900	1.7	10.700 ± 2280
ADR1-1-330/507-571	0	40	1	1800 ± 50
ADR1-7^c-1-330/507-571	0	30	4.9	ND
ADR1-1-399/507-571	0	80	1	3600 ± 710
ADR1-7^c-1-399/507-571	4	70	2.1	ND

[a] ADH II activity was measured in strains containing single gene copies of ADR1-571 and ADR1-7^c-571 deletion alleles. Values measured for ADR1-7^c-571 strains are compared to values taken from Table 6.

[b] Slopes were measured by plotting ADH II activity as a function of gene dosage under glucose conditions as described in Table 4.

[c] Maximal ADH II activity was calculated as described in Figure 15. Values measured for ADR1-7^c-571 deletion derivatives are compared to values taken from Table 6.

[d] ND- not determined. The maximal ADH II activities were not determined due an inability to isolate a sufficient number of strains containing multiple gene copies.

Finally, maximal ADH II activities were compared for some deletion deletions. While this analysis was incomplete due to difficulties in isolating strains containing a sufficiently broad range of integrated ADR1-7^c-571 deletion gene copies, a comparison was made between the two deletions in which the 288/330 inhibitory region was removed (deletions 262/330 and 262/399). ADR1-7^c-571 proteins containing either of these two deletions did not produce significantly higher maximal ADH II activities than their ADR1-571 counterparts. These results suggested that ADR1-7^c proteins which contained the 262/330 and 262/399 deletions were not better activators under both glucose conditions (indicated by the slopes) and ethanol conditions (indicated by maximal ADH II activities) than their ADR1-571 counterparts. If the 227-239 and the 288-330 inhibitory regions acted through separate mechanisms, we would expect the increase in activity caused when these two disruptions are combined would be multiplicative. Therefore, it is possible that the two inhibitory regions act through a common mechanism. Further experiments are needed to resolve the relationship between these two inhibitory regions.

To summarize the results obtained with the deletion analysis of ADR1 and ADR1^c, and to combine these results with previous findings, the following functional regions in ADR1 have been identified: activation domains appear to be present in the N-terminal 220 amino acids (Bemis and Denis

1989), most likely centered around residues 76-172 (Thukral et al. 1989), and also in residues 330-574. Results shown in Figure 15 and Table 5 suggested, albeit weakly, that the 262-288 region contains activating residues. However, deletion of this region only affected ADR1 activity when the activating residues between 330-574 were not present. Inhibitory regions in ADR1 have been localized to the 227-239 region (Denis and Gallo 1986; Cherry et al. 1989; Denis et al. 1992) and residues 288-330.

LexA-ADR1 fusion proteins

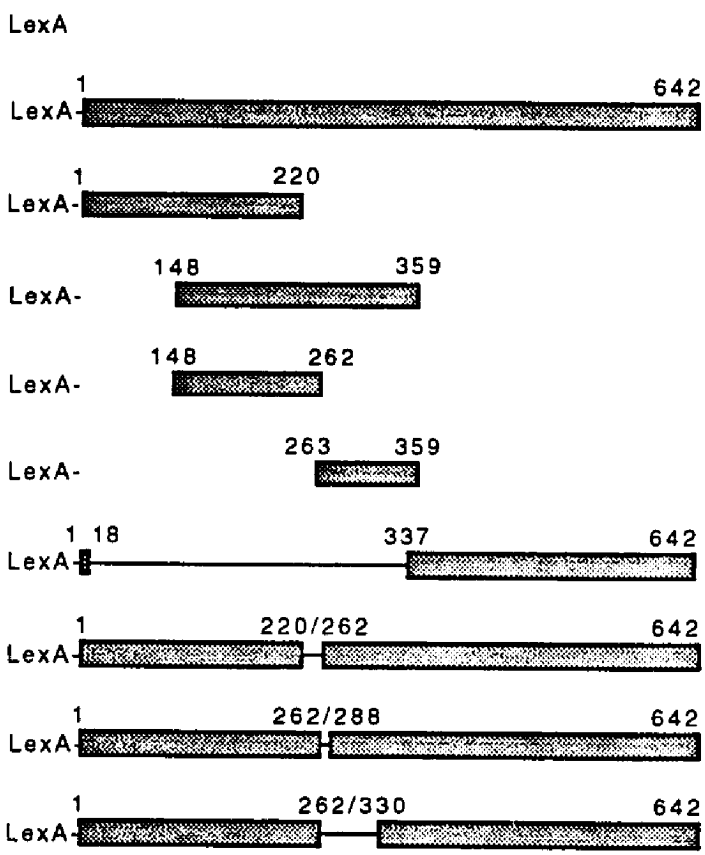
To better define the regions of ADR1 involved in activation and inhibition of transcription, lab colleagues D. Chase, D. Audino and I incorporated the use of hybrid proteins containing the complete LexA protein fused to different regions of ADR1 (Figure 16). Fusion proteins were expressed from 2μ plasmids in strains containing a LacZ reporter plasmid with one LexA operator (Brent and Ptashne 1985). Expression of the LacZ reporter gene was assayed by measuring β-galactosidase activity in yeast cell extracts. This transcriptional assay system allows the positive measurement of the activation ability of specific ADR1 fragments independent of DNA binding. LexA-ADR1 fusion proteins containing the ADR1 zinc-finger DNA-binding domain were also able to activate ADH II activity as shown in Figure 16 thus allowing a second measure of the activating

ability of ADR1 fragments. The expression of all LexA-ADR1 fusion proteins was verified by Western analysis (data not shown). However, some fluctuations in relative levels between the differently sized fusion proteins were detected. As other reports (Golemis and Brent 1992; Bruhn et al 1992) have attributed some differences in fusion protein activity to differences in protein stability or changes in conformation that may affect binding, data were interpreted in a semiquantitative manner.

Inspection of Figure 16 indicated that LexA-ADR1-1-642 was a strong activator of both β-galactosidase and ADH II expression, confirming that this fragment of ADR1 contains important activating regions. The activating regions in ADR1-1-642 were localized by analyzing smaller fragments fused to the LexA binding domain. Three separate regions of the ADR1-1-642 protein were able to activate transcription: ADR1-1-220, ADR1-263-359, and ADR1-337-642. This result is consistent with findings presented in earlier sections that suggested the presence of three activating regions in ADR1. However, the boundaries of the activating region contained by ADR1-263-359 remained unclear. Deletion of amino acids 262-330 and 262-399 produced a more active ADR1 protein suggesting that an inhibitory region is also located in these sequences (Table 5 and Table 6). The dual nature of the 263-359 region is discussed below.

To better define the inhibitory residues in ADR1 amino

Figure 16. Transcriptional activation by LexA-ADR1 fusion proteins.



ß-galactosidase Activity (U/mg)	ADH II Activity (mU/mg)
2	-
1300	410
12	40
33	-
2	-
210	-
900	-
2000	780
840	360
1100	550

The solid bars represent ADR1 protein residues fused to LexA-1-202, and numbers refer to ADR1 amino acids. Strains isogenic to 237-1b-10 were grown in minimal medium supplemented with 8% glucose and lacking uracil and histidine to select for maintenance of the LexA-ADR1 expression plasmid and the 1840 reporter plasmid. Activity values represent an average of at least three separate determinations, and SEMs were less than 20% except when activity values were less than 20. This study was a combined effort by D. Audino, D. Chase and myself.

acids 1-642, LexA-ADR1-1-642 fusion proteins were made that contained internal deletions of the 220-330 region.Deletion of the 220/262 region allowed for increased activation relative to the undeleted LexA-ADR1-1-642 fusion protein. In contrast, deletion of the 262/330 inhibitory region did not allow increased activation of the LacZ reporter gene. However, this deleted fusion protein did allow an increase in ADH II activity suggesting that the function of this inhibitory region is specific to ADH2 transcription. Deletion of the 262/288 region reduced the ability of the LexA-ADR1-1-642 fusion protein to activate confirming that this region is important for activation. Western analysis indicated that protein levels of LexA-ADR1-1-642 and LexA-ADR1-1-642 with internal deletions were equal (not shown).

Results obtained using the LexA fusion protein system confirmed many of the findings from the deletion analysis. The N-terminal 642 amino acids of ADR1 appeared to contain three activating regions. These regions were localized to amino acids 1-220, 262-288, and 337-642. This analysis verified the inhibitory function of the 227-239 region, yet did not show the 287-330 region to inhibit transcription.

LexA-ADR1^c fusion proteins

The results presented in the deletion analysis identified the N-terminal 262 amino acids of ADR1^c as being minimally required for enhanced activation. These results

indicated that the ADR1^c effect is not dependent on either the 262-288 or the 330-507 activating regions. To better define how the ADR1-5^c protein enhances activation, LexA-ADR1-5^c fusion proteins were expressed and assayed for their ability to activate transcription of a LacZ reporter gene (Table 9). Under glucose growth conditions, when expression of the LexA-ADR1 fusion proteins was high due to the ADH1 promoter, the LexA-ADR1-5^c-1-642 and the LexA-ADR1-5^c-148-359 fusion proteins were better activators than the same fusion proteins containing wild-type ADR1 residues (Table 9). The enhanced activation was approximately two-and four-fold respectively. The increased activity of the LexA-ADR1-5^c-148-359 fusion protein indicated that an ADR1^c mutation could enhance ADR1 activity when the 262-288 activation domain was present. Therefore, an ADR1^c mutation can enhance ADR1 activity when either of two separate activation domains are present: the 262-288 region and the 76-172 activation region (as evidenced by ADR1-7^c-262 in Figure 15).

When the same fusion proteins were assayed for LacZ activation following growth in ethanol-containing medium, the LexA-ADR1-5^c-1-642 and LexA-ADR1-5^c-148-359 fusion proteins enhanced activation 25-and 8-fold respectively when compared to wild-type LexA-ADR1 counterparts. Under ethanol growth conditions, LexA-ADR1 fusion proteins were expressed at low levels (i.e. 5- to 10-fold less than under glucose

Table 9. Transcriptional activation by LexA-ADR1 and LexA-ADR1-5[c] fusion proteins.

Fusion protein	β-Galactosidase Act (U/mg) Glucose[a]	Ethanol[b]	Gluc/Etoh Ratio	Fusion prot Abund[c] Gluc	Etoh
LexA-ADR1-1-642	1780	110	16.2	1.0	0.1
LexA-ADR1-5[c]-1-642	4110	2490	1.6	1.0	0.1
[d]LexA-ADR1-148-359	26	3	8.7	1.0	0.2
[d]LexA-ADR1-5[c]-148-359	106	23	4.6	1.0	0.2
[c]LexA	2	2	1.0	1.0	0.5

All strains are isogenic to 237-1b-10. SEM values are less than 20% except where activity is less than 20U/mg.

[a] Yeast were grown in minimal medium lacking uracil and histidine and supplemented with 8% glucose.

[b] Growth medium was the same as in [a] except supplemented with 3% ethanol.

[c] The relative abundance of LexA-ADR1 fusion proteins was measured by Western analysis using a LexA antibody as described in the Materials and Methods section.

[d] The construction of plasmids encoding these fusion proteins and the subsequent analyses of these proteins was primarily conducted by D. Chase, M. Liu, and D. Audino.

[e] LexA protein was expressed from a LexA(1-202)+PL plasmid that lacked ADR1 sequences.

conditions). Therefore, these results suggest that the ADR1-5^c protein has the most dramatic effect on enhancing activation when ADR1-5^c and ADR1 protein levels are very low. This result is consistent with data shown in Figure 13a for the full length ADR1^c and ADR1 proteins. Figure 13a showed that under glucose conditions, when ADR1 protein levels are very low, the ADR1^c protein allows an approximate 60-fold increase in activation. Figure 13b showed that ADH II activity under ethanol growth conditions, when full-length ADR1 protein levels are 10- to 20-fold higher than under glucose conditions, was enhanced by the ADR1^c mutations only 25%.

Discussion

To better define the functional regions in the wild-type ADR1 protein, and to identify regions in the ADR1^c proteins that are required for enhanced activation, a deletion analysis was conducted on the ADR1 and ADR1^c proteins. Findings reported here indicate the following: (i) ADR1 contains multiple activating regions, (ii) in addition to the 227-239 inhibitory region, ADR1 contains another region located between amino acids 262 and 330 that inhibits activation, and (iii) while no specific region was identified that was required for the ADR1^c effect, an ADR1^c mutation was able to enhance ADR1 activity when either of

two separate activation domains were present.

Initial deletion analysis suggested that two regions of ADR1 contain activation domains, the ADR1-1-220 and -262-642 regions (Thukral et al. 1989, Bemis and Denis 1988). Results presented here verified that both these regions contain activating residues. The activating regions within amino acids 262-642 were localized, however, to two regions. Residues 262-288 and 330-574 appeared to have activating ability. Taken together, these results demonstrate that three activating regions are present in ADR1, these localized to amino acids 76-172, 262-288, and 330-574.

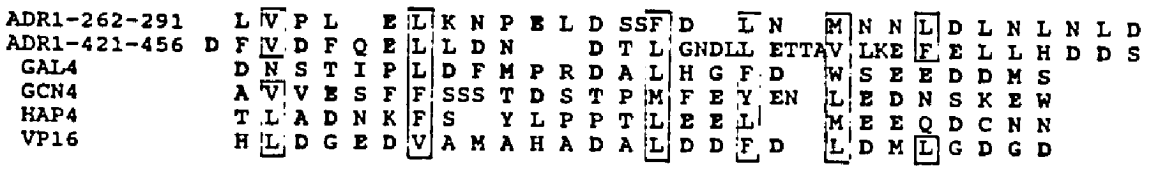
As discussed in the General Introduction, activation domains in other yeast transcriptional activators have been characterized as containing an excess of acidic residues and as having a propensity to form predicted amphipathic alpha-helical structures (Mitchell and Tjian 1989; Cress and Triezenberg 1991). Using the Chou-Fasman rules contained in the GCG program, the putative activation domains of ADR1 were analyzed for these characteristics. The N-terminal activation domain within residues 76-172 does not contain an excess of acidic residues, however, many sub-regions within this segment can be predicted to form amphipathic alpha-helical structures according to Chou-Fasman rules. The alpha-helical nature of this region is not surprising since zinc-fingers form alpha-helices (Pavletich and Pabo 1991). Residues 68-103 were predicted to form an amphipathic alpha-

helix in an earlier study (Thukral et al. 1989). Six of 29 residues in the 262-291 region are acidic, and this region shows alpha-helical characteristics according to Chou-Fasman rules (data not shown). One region within amino acids 399-574, residues 421-456, contains 10 out of 30 amino acids that are acidic and can also be predicted to form alpha-helical structure.

The acidic nature of these putative activation regions agrees with other well characterized acidic activation regions including Gal4 (8 of 27 acidic), GCN4 (7 of 29), HAP4 (6 of 25), and the herpes virus VP16 (10 of 27) (Cress and Triezenberg 1991). Cress and Triezenberg (1991) presented an alignment of these acidic activation domains to allow a comparison of the spacing of bulky hydrophobic residues. The alignment of bulky hydrophobic residues is indicative of the ability of these domains to form amphipathic alpha-helices. I have attempted to align ADR1 residues 262-291 and 421-456 with Cress and Triezenberg's alignment in Figure 17. While the gaps and insertions required to align bulky hydrophobic residues is extensive, the acidic activation domains in ADR1 do exhibit features common to acidic activation domains.

Other transcriptional activators also contain multiple activation domains (Mitchell and Tjian 1989). It has been proposed that multiple activation regions allows synergistic activation (Pascal and Tjian 1991). Synergism between the

Figure 17. Comparison of acidic activation domains.



ADR1 acidic regions 262-291 and 421-456 are compared to other acidic activation domains that function in yeast. The ADR1 262-291 and 421-456 regions are fitted to an alignment presented in Cress and Triezenberg (1991) that used six bulky hydrophobic residues (boxed). Acidic residues (bold) are not aligned. A direct inference to similarity is not intended, but rather the alignment is for comparative purposes.

multiple activation domains allows amplification effectswhich may be important for the high levels of induction observed in many gene activation systems. The multiple activation domains in ADR1 might also be important for ADR1 to control multiple gene targets such as genes involved in glycerol metabolism (Bemis and Denis 1988), mitochondrial function (Cherry and Denis 1989), and peroxisomal function (Simon et al. 1991).

ADR1 appears to contain multiple inhibitory regions. The 227-239 region displayed strong inhibitory function which was removed by mutations in single amino acids or by deleting this region (Denis et al. 1992). Based on deletion analysis, residues 288-330 also appear to inhibit ADR1 function. Numerous models suggest themselves as to how a specific region of ADR1 might function to inhibit activation. For example, a protein could bind to an inhibitory region and repress ADR1 activity as observed with the GAL80-GAL4 interaction (Lue et al. 1987) or the c-jun inhibitor (Baichwal et al. 1991). In the GAL80-GAL4 system, GAL80 binds to GAL4 in the absence of galactose to form a complex which is unable to activate transcription. The repressive effect of GAL80 is removed when cells are grown in galactose-containing medium. A similar mechanism, however, does not appear to function in the control of ADR1. The evidence against a repressor-ADR1 interaction includes the fact that no gene encoding a repressor protein has been

identified in numerous genetic selections (C. L. Denis pers. comm.), and no titration of a repressor function was achieved in other studies. The repressor titration experiments involved overexpressing both full-length adr1 proteins, which failed to bind UAS1 due to mutations in the zinc-fingers (Cook et al. 1993), and different adr1 polypeptides, which contained both the 227-239 and the 288-330 inhibitory regions (D. Chase unpublished data). The overexpressed adr1 polypeptides failed to relieve glucose repression of ADH2 in a wild-type ADR1 strain, suggesting that there is no repressor of ADR1 function.

Another possibility is that the inhibitory regions are involved in conformational changes in the ADR1 protein that affect ADR1 function. These conformational changes might be direct; for example, the inhibitory regions might directly mask an activation domain (Denis et al. 1992). Alternatively, an inhibitory region may function by causing an allosteric effect whereby conformation of a distant region of ADR1 is altered. It should be noted also that we could not rule out the possibility that alteration of the spacing between activation domains (e.g. by deleting the 263-330 region) might merely bring two activating regions into closer proximity allowing enhanced activation.

As mentioned in the Results section, the ADR1-263-359 region appears to have dual function. Deletion of these residues in both full-length ADR1 (Table 5) and in ADR1-571

(Table 6) allowed enhanced ADH II activity under both glucose and ethanol conditions. However, a LexA-ADR1-263-359 fusion protein was a strong activator. The residues in ADR1-263-359 that are responsible for activation appeared to be amino acids 262-288. This identification is based on the severe drop in maximal ADH II activity when ADR1 residues between 282 and 262 were removed in a C-terminal deletion analysis (Figure 15), and on the effects of a 262/288 deletion on LexA-ADR1 fusion protein activity. Deletion of 262/288 reduced the activity of LexA-ADR1-1-642 by 40% (Figure 16), and a LexA-ADR1-147-358 fusion protein with a 262/288 deletion was completely inactive (D. Audino unpublished data). While it is possible that activating residues in the 263-359 fragment reside in residues 330-359 instead of 262-288, this is not consistent with the internal deletion study that showed deletion of 330/507 had a suppressive effect similar to a 399/507 deletion (Tables 5 and 6). These results together suggest that residues 262-288 contain activating residues, while amino acids 288-330 function in an inhibitory manner. Further experiments are in progress to better define the boundary between these two functional regions (D. Audino and D. Chase pers. comm.).

The observation that the 263-359 region contains juxtaposed activating and inhibitory domains raises the possibility that this arrangement of positive and negative functional elements may be common to transcriptional

activators. A recent study has shown that a region of c-jun that was previously identified as one of the two activation domains can be dissected into separate activation and inhibition domains (Baichwal et al. 1992). This bipartite and molecular organization may also be present in the ADR1 263-359 region. It will be interesting to see if juxtaposed activating and inhibitory domains are present in other activators.

Deletion analyses presented in this chapter did not identify a region of the ADR1^c protein required for enhanced activation. ADR1^c proteins that lacked residues C-terminal to amino acid 262, or that contained internal deletions in the 262-574 region were better activators than their ADR1 counterparts under both glucose and ethanol growth conditions. In addition, a LexA-ADR1-5^c-148-359 fusion protein was also a better activator than LexA-ADR1-148-359. These results indicate that ADR1^c mutations enhance ADR1 activity when either of the 76-172 or the 262-288 activation domains are present. Therefore, the 227-239 inhibitory region must not work simply by interfering with a single activation domain. It should be noted that ADR1 amino acids 148-220 have yet to be analyzed for their requirement in the ADR1^c effect. Deletions are being made in the 148-220 region to determine if this region is required (D. Audino pers. comm.).

The ADR1^c effect appeared to be most dramatic when ADR1^c and ADR1 proteins were expressed at very low levels. This was most apparent when comparing ADR1^c to ADR1 when both were expressed from single genes under glucose conditions (Figure 13a), and when LexA-ADR1^c and LexA-ADR1 fusion proteins were assayed following growth under ethanol condition (Table 9). This observation, and other results from related studies using ADR1^c proteins, is discussed in the General Discussion section below.

GENERAL DISCUSSION

Glucose repression of the ADH2 gene involves the suppression of the transcriptional activator ADR1. ADR1 activity appears to be regulated at the level of ADR1 protein expression and by a posttranslational modification. The objectives of this dissertation research were to characterize the mechanisms by which glucose regulates ADR1 activity. To characterize the glucose control of ADR1 protein expression, experiments were conducted to identify regions in the ADR1 mRNA that are responsible for the control of ADR1 protein expression. To better identify the posttranslational mechanisms, experiments were conducted to identify regions in the ADR1 protein that are involved in the control of ADR1 activity. Genetic experiments were also conducted in an effort to identify other genes involved in the regulation of ADR1. Finally, in order to determine the mechanisms of ADR1 regulation, a better understanding of the functional regions in ADR1 was required. To this end, the functional regions in the ADR1 protein were more precisely mapped and characterized.

Early reports suggested that in many yeast strains including strains employed in our lab, glucose had very little if any influence on the steady state levels of ADR1 mRNA. However, in some laboratory strains, ADR1 mRNA levels

were reduced 10- to 20-fold under glucose conditions as opposed to ethanol conditions. Results presented in Vallari et al. (1992; Appendix) indicated that glucose reduced ADR1 mRNA levels two-fold in our lab's strains. A genetic analysis of a strain in which glucose represses ADR1 mRNA expression revealed that this strain lacks a factor or factors normally responsible for ADR1 mRNA accumulation under glucose conditions (Chapter 2). This result suggests that strains in which glucose represses ADR1 mRNA expression do not represent the typical yeast strain in the control of ADR1 mRNA.

Steady state levels of mRNA result from a balance between transcription and mRNA degradation. Therefore, it is possible that while glucose exerts little effect on the steady state levels of ADR1 mRNA, carbon source may actually have very different influences on these two processes of transcription and degradation. Glucose may drastically reduce ADR1 transcription yet the low amounts of ADR1 mRNA produced under glucose conditions may be more stable than under ethanol conditions. Experiments measuring ADR1 mRNA stability under different growth conditions showed this to not be the case. ADR1 mRNA was approximately two-fold less stable under glucose conditions than under ethanol conditions. It would appear that this two-fold decrease in ADR1 mRNA stability is responsible for the two-fold lower steady state levels of ADR1 mRNA under glucose conditions.

The role of a two-fold decrease in the steady state levels of ADR1 mRNA in the overall glucose control of ADR1 expression is unclear. However, the important conclusion to be drawn from these results is that glucose does not exert control on ADR1 expression at the transcriptional level. This is consistent with other studies that showed glucose typically does not control transcriptional activators at the level of mRNA synthesis (Gancedo 1992).

In contrast to the slight effect of glucose on ADR1 mRNA expression, glucose was found to decrease the rate of ADR1 translation by 5- to 8-fold (Vallari et al. 1992, Appendix). When cells were shifted from glucose- to ethanol-containing medium, ADR1 protein and ADH2 mRNA expression appeared to commence within the same time frame: 40 to 60 minutes. This result supports the importance of ADR1 protein synthesis in the glucose repression of ADH2. ADR1 mRNA sequences between nt 786 to 1926 (codons 262 to 642) were found to be involved in this glucose control of translation (Vallari et al. 1992, Appendix). The unusually long 5′ UTR of ADR1 was found to not be involved in this translational control but rather these mRNA sequences were involved in the glucose control of ADR1 mRNA stability.

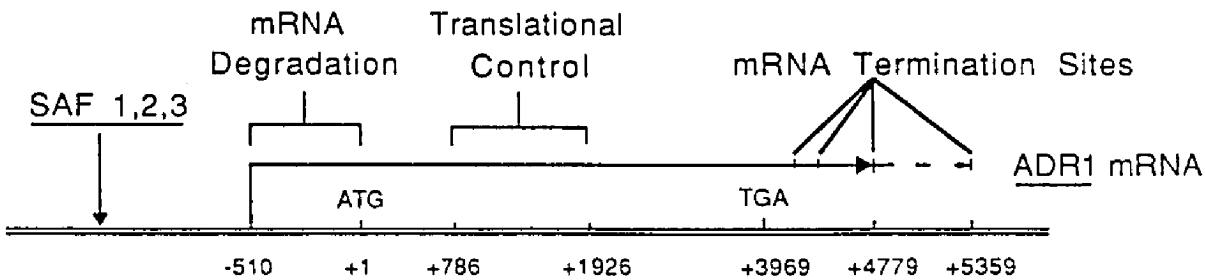
Taken together, glucose appears to control ADR1 protein expression primarily by inhibiting the rate of ADR1 translation and to a lessor extent by promoting ADR1 mRNA degradation. A summary of important structural and

regulatory features of the ADR1 gene and mRNA is shown in Figure 18.

The regulation of some other transcription factors in yeast occurs by translational control. For example, GCN4 is controlled at the level of translational initiation (Thireos et al. 1984). In contrast to this control mechanism, ADR1 appears to be controlled at the level of translational elongation. Regulation at the level of mRNA degradation has been observed for genes encoding the glucose-repressed metabolic enzyme SDH Ip (Lombardo et al. 1992) and CYC1 (Zitomer and Nichols 1978). However, no other examples of yeast transcription factors regulated at the level of mRNA degradation have been reported.

Gene regulation by posttranscriptional mechanisms would at first appear to be inefficient. The large energetic investment in mRNA synthesis, and in the case of ADR1, in the initiation of protein synthesis would appear to be wasteful particularly when considering that single-celled yeast must have efficient growth processes to allow short replication times. To understand how these control mechanisms are efficient for ADR1 activation of the ADH2 gene, we must consider the role of ADH II in yeast. ADH II is essential for yeast to grow under nonfermentative conditions when alternate carbon sources such as ethanol are metabolized. We can envision that glucose supplies might be rapidly depleted and a switch to growth using the

Figure 18. Sites at which glucose controls ADR1 RNA and protein metabolism.



Regions involved in the function and regulation of the ADR1 gene and mRNA are dissected based on findings reported here and previously (Vallari et al. 1992). Numbers listed below the ADR1 gene (double lines) correspond to base-pairs numbered in relation to the translational start site (+1). The transcriptional start site and three of the transcriptional termination sites were reported in Blumberg et al. (1988). The transcriptional termination site that is furthest downstream (dashed line) corresponds to the alternate transcriptional termination described in Figure 6 and in the text (Chapter 2).

fermentative byproduct ethanol is suddenly required. Rapid expression of ADH II is dependent on rapid expression of ADR1. Therefore, the most efficient mechanism for rapid ADR1 expression requires ADR1 mRNA to be constantly present, thereby saving the time required for ADR1 transcription. We might also envision that glucose does not inhibit the initiation of ADR1 translation in order to further save time. In this way, control of ADR1 protein synthesis at the level of elongation might be the most practical control mechanism to allow yeast to quickly acclimate to changes in carbon source utilization. Control of ADR1 at the level of protein expression might also be the most efficient means of allowing a prolonged response to nonfermentative growth conditions.

ADR1 also controls the expression of other yeast genes. Therefore, control of ADR1 at the level of translational elongation might be the most efficient way to control these various genes. Since overexpression of ADR1 has a deleterious effect on yeast due to disruptions of mitochondrial genes (Cherry and Denis 1989), we would predict that ADR1 protein levels need be strictly controlled to minimize this effect.

ADR1 also appears to be controlled by posttranslational mechanisms (Denis and Gallo 1986; Blumberg et al. 1988; Cherry et al. 1989; Denis and Audino 1991). The posttranslational control of ADR1 activity is evidenced by

the fact that cAPK inhibits ADH2 expression in an ADR1-dependent manner, and that mutations in a cAPK recognition sequence, designated ADR1^c, allow 50- to 75-fold increases in ADH II activity under glucose growth conditions. Posttranslational modification might be used to immediately activate the very low levels of ADR1 protein that are present under glucose growth conditions and thus allow for the immediate activation of ADH2 transcription. In addition, posttranslational modification might be the mechanism by which ADR1 is inactivated upon the addition of glucose to a nonfermentative growth environment and serves as a means of quickly halting ADH2 transcription when ADH II is not required. Alternatively, posttranslational modification of ADR1 might be important in modulating the ADR1-dependent activation of other genes.

To characterize the posttranslational control of ADR1, the mechanisms by which the ADR1^c proteins function to enhance ADH2 transcription were analyzed. Three approaches were taken: (i) mutations that suppressed the enhanced activity of the ADR1^c alleles were characterized, (ii) deletion analysis was conducted on the ADR1^c proteins, and (iii) LexA-ADR1^c fusion protein activity was analyzed in an *in vivo* assay.

Both extragenic and intragenic suppressor mutations of ADR1^c have been analyzed. In this report, the extragenic *saf* mutations were found to affect ADR1-5^c and ADR1

transcription. Since glucose does not appear to regulate ADR1 at the transcriptional level, the saf mutations did not reveal significant information regarding glucose control of ADR1. Various reasons can explain why genes required for the ADR1^c effect were not identified by this genetic screen. First, the mechanism by which the ADR1-5^c alleles allow glucose-insensitive ADR1 activity may not require other factors. It is also possible that other genes required for the ADR1^c effect are essential for viability. A final possibility is that the mutant screen employed simply missed identifying the genes in question. Gancedo (1992) describes how bias introduced by selection is a common problem in mutant screens.

Using EMS mutagenesis, intragenic suppressor mutations had been previously identified that caused single amino acid changes in the zinc-finger region of ADR1-5^c (D. Mullaney, M.S. Thesis). However, these zinc-finger mutations had a similar effect on the ADR1 protein indicating that the zinc-finger mutations did not specifically suppress the ADR1-5^c protein. While it is possible that no intragenic mutations have the ability to specifically suppress ADR1-5^c, an alternate explanation is that only massive mutations such as deletions would destroy function in the regions of the ADR1^c protein required for the ADR1^c effect.

Continuing the approach of identifying regions in the ADR1^c protein that are required for enhanced activity,

deletion analysis was conducted on the ADR1^c protein (Chapter 3). No deletion derivative was identified that specifically suppressed ADR1^c activity. Similar results were observed with LexA-ADR1^c fusion proteins analyzed in the LexA transcriptional assay system. All LexA-ADR1^c fusion proteins analyzed exhibited enhanced activity when compared to their LexA-ADR1 counterparts. Taken together, these results indicate that the ADR1^c effect is not mediated through specific ADR1 residues other than the 227-239 inhibitory region. Since results showed that ADR1^c mutations could enhance ADR1 activity when either the 76-172 or the 262-288 activation domains were present, the ADR1^c effect is not dependent on a single activation domain. It should be noted, however, that a small region between amino acids 147-220 has yet to be thoroughly analyzed for being required for the ADR1^c effect. A deletion analysis of the 147-220 region is in process (D. Audino pers. comm.).

How then do the ADR1^c proteins function to allow enhanced activation? One clue to this function comes from findings which indicated that the ADR1^c effect is most pronounced when ADR1^c and ADR1 were compared for activating ability when each protein was expressed at very low concentrations. These conditions were met when ADR1^c and ADR1 proteins were expressed from single gene copies under glucose growth conditions (Figure 13a), or when LexA-ADR1^c and LexA-ADR1 fusion proteins, whose expression was driven

by the glucose-induced ADH1 promoter, were analyzed under ethanol growth conditions (Table 9). Interpretations of this observation include the following:

The ADR1^c mutations might stabilize weak or transient interactions. These interactions might be between ADR1 and another transcription factor, a coadapter (Berger et al. 1990), factors belonging to the general transcriptional machinery, or another ADR1 protein. This model implies that the wild-type ADR1-227-239 region is in a conformation under glucose-repressed conditions that inhibits or disrupts an interaction essential to ADR1 transcriptional activation function. Mutations or deletions of the 227-239 region relieve this inhibitory function.

The model in which the ADR1^c mutations promote positive interactions between ADR1 proteins at the UAS1 binding site is particularly attractive. As mentioned in the General Introduction, ADR1 appears to bind to UAS1 as two independent monomers. A single ADR1 monomer was able to bind to a half UAS1 site but was unable to activate transcription, implying that two ADR1 proteins bound at UAS1 are required for ADR1 activity (Thukral et al. 1991). In a study analyzing diploid strains produced by crosses between various ADR1, adr1, and ADR1^c strains, results suggested that ADR1 monomers interact with one another to activate transcription (C. L. Denis pers. comm.). Further evidence of ADR1-ADR1 interaction was observed in the LexA-ADR1

study. LexA amino acids 1-87 contain only a DNA-binding motif, while the complete LexA protein consisting of amino acids 1-202 contains both DNA-binding and dimerization domains. Since efficient binding of LexA or LexA fusion proteins to the LexA Operater site is dependent on dimer formation (Kim and Little 1992), dimerization function of LexA(1-87)-ADR1 fusion proteins must be provided by ADR1 residues. LexA(1-87)-ADR1-1-642 and LexA(1-202)-ADR1-1-642 both activated transcription to similarly high levels (data not shown), indicating that ADR1-1-642 contains residues that allow dimerization. Similar results were obtained with other LexA-ADR1 fusion proteins.

Assuming that ADR1-ADR1 interaction at the UAS1 site is involved in activation, it is unclear if dimerization (or oligomerization) is required or is inhibitory to ADR1 activation. The role of the 227-239 region in ADR1-ADR1 interaction remains similarly unclear. Experiments are in progress to determine if ADR1 self-associates and how glucose influences this self-association. Examples of dimerization and oligomerization controlling the activity of transcription factors show self-association can have both positive and negative effects. Transcriptional activators of the basic-leucine zipper family (B-zip), which includes GCN4, fos, jun, and CREB, contain the leucine zipper motif which mediates the dimerization of these factors (Landshultz et al. 1988). B-zip proteins must dimerize in order to

transactivate. The transcription factor MyoD appears to oligomerize to form inactive mycils (T. Laue pers. comm.). In these examples, the regulatory mechanisms controlling self-association and dissociation are poorly understood. Phosphorylation may play a role since it has been shown to influence the polymerization state and the activity of the enzymes glycogen phosphorylase (Wingebder-Drissen and Becker 1983) and acetyl-CoA Carboxylase (Lane et al. 1974).

An alternate model explaining the low protein concentration effect on ADR1^c activity may be that the ADR1^c proteins bind DNA more stably. An early study indicated that ADR1^c and ADR1 bound equally well to UAS1 *in vitro* (Taylor and Young 1990). However, *in vivo* this may not be the case. Glucose may weaken the transient binding of two ADR1 monomers to UAS1; the ADR1^c proteins may promote the stabilization of this weak DNA binding. Stabilization of ADR1^c binding might involve other factors (e.g. the stabilizing factors SWI1-3 [Peterson and Herskowitz 1992]) or may solely involve ADR1^c-ADR1^c interaction. Experiments using *in vivo* footprinting of the *ADH2* promoter are in progress which might expose subtle changes in ADR1^c and ADR1 binding (D. Chase pers. comm.).

Another clue as to how the ADR1^c proteins enhance activation comes from a collaborative study conducted with M. Simon and H.Ruis (University of Vienna). The Ruis lab has shown that ADR1 controls the expression of the glucose-

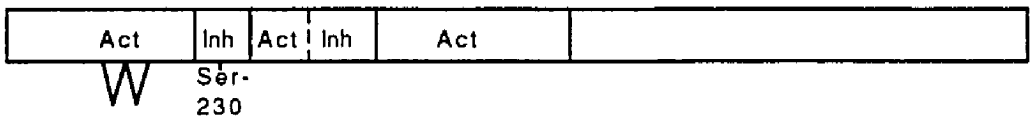
repressible CTA1 gene which encodes the peroxisomal catalase (Simon et al. 1991). ADR1 binds to a DNA element in the CTA1 promoter that weakly resembles UAS1 of ADH2 but is not a perfect palindrome. Using Northern analysis, we found that ADR1^c does not enhance CTA1 expression under glucose conditions while multiple copies of ADR1 do cause increased CTA1 expression. This result suggests that the dyad symmetry of the UAS1 palindrome may be required for the ADR1^c effect. Alternately, other DNA elements in the ADH2 promoter (e.g. UAS2) or other protein factors that act specifically at the ADH2 promoter may be required for this function. It should be noted that the ADR1^c mutations also enhanced the activity of LexA-ADR1 fusion proteins that act at the LacZ reporter gene. The effect of the ADR1^c mutations on LexA fusion protein activity may be completely due to enhanced dimerization of LexA-ADR1^c proteins as described above. This result, coupled with the fact that the element in the CTA1 promoter that binds ADR1 lacks dyad symmetry, argues for the model in which the ADR1^c mutations act at the level of ADR1 self-association. Further experiments are needed to resolve this question.

The present study also further characterized the functional regions in ADR1. Results using both deletion analysis and the LexA transcriptional assay system showed that ADR1 contains multiple activation domains. These activation domains are separated by regions that inhibit

activation. In Figure 19, the activating and inhibiting regions are identified in the ADR1 protein. In the Chapter 3 Discussion, the properties of these activating and inhibiting regions are discussed. An intriguing possibility that presents itself upon inspection of Figure 19 is that modulations between the different activating and inhibiting regions of ADR1 might be important in controlling ADR1 activity. When ADR1 is in an inactive form, the inhibitory regions may mask or in some other way inactivate the activating regions. Glucose may promote such an inhibitory conformation in ADR1. Removal of glucose may cause an ADR1 conformational change in which activating regions are exposed to interact with the transcriptional machinery. Experiments are needed to characterize the conformational changes that occur in the ADR1 protein upon shifts in carbon source, and to decipher the mechanisms by which glucose might induce conformational changes in ADR1.

The object of this study was to characterize the glucose regulation of ADR1. Glucose controls ADR1 activity by multiple mechanisms including control of ADR1 mRNA turnover, control of ADR1 protein synthesis, and by a posttranslational mechanism that probably includes conformational changes in the ADR1 protein. Another well characterized transcriptional activator in yeast, GAL4, has been shown to also be regulated by multiple mechanisms including control of protein expression and

Figure 19. ADR1 contains alternating activating (Act) and inhibitory (Inh) domains.



The most N-terminal (left side) activation domain overlaps the zinc-fingers (represented by inverted triangles). The border between the middle activation domain (which corresponds to residues 262-288) and the C-terminal inhibitory region (amino acids 287-330) is represented by a hatched line due to the undefined boundary between these juxtaposed domains.

posttranslational effects (Lampier and Ptashne 1992). The most common explanation for redundant regulatory mechanisms controlling transcriptional activators is that multiple regulatory mechanisms provide yeast with the ability to rapidly adapt to changing growth conditions by directing the flow of metabolites into alternate pathways (Wills 1990). For ADR1, this appears to be especially true in light of the multiple genes controlled by this transcriptional activator.

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APPENDIX

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Glucose Repression of the Yeast *ADH2* Gene Occurs through Multiple Mechanisms, Including Control of the Protein Synthesis of Its Transcriptional Activator, ADR1†

ROBERT C. VALLARI,‡ W. JAMES COOK, DEBORAH C. AUDINO, MICHAEL J. MORGAN,§ DONNA E. JENSEN,‖ ANDREW P. LAUDANO, AND CLYDE L. DENIS*

Department of Biochemistry and Molecular Biology, University of New Hampshire, Durham, New Hampshire 03824

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The rate of *ADH2* transcription increases dramatically when *Saccharomyces cerevisiae* cells are shifted from glucose to ethanol growth conditions. Since *ADH2* expression under glucose growth conditions is strictly dependent on the dosage of the transcriptional activator ADR1, we investigated the possibility that regulation of the rate of ADR1 protein synthesis plays a role in controlling ADR1 activation of *ADH2* transcription. We found that the rate of ADR1 protein synthesis increased 10- to 16-fold within 40 to 60 min after glucose depletion, coterminous with initiation of *ADH2* transcription. Changes in *ADR1* mRNA levels contributed only a twofold effect on ADR1 protein synthetic differences. The 510-nt untranslated *ADR1* mRNA leader sequence was found to have no involvement in regulating the rate of ADR1 protein synthesis. In contrast, sequences internal to *ADR1* coding region were determined to be necessary for controlling ADR1 translation. The *ADR1*^{c} mutations which enhance ADR1 activity under glucose growth conditions did not affect ADR1 protein translation. ADR1 was also shown to be multiply phosphorylated in vivo under both ethanol and glucose growth conditions. Our results indicate that derepression of *ADH2* occurs through multiple mechanisms involving the ADR1 regulatory protein.

In the yeast *Saccharomyces cerevisiae*, glucose represses the transcription of numerous genes, including those required for ethanol and alternate sugar metabolism, the glyoxylate shunt, the tricarboxylic acid cycle, respiration, gluconeogenesis, and mitochondrial function. Several regulatory genes which, when mutated, either allow glucose-insensitive transcription or inhibit derepression upon glucose removal have been identified (34). The mechanisms by which glucose repression occurs, however, remain largely obscure. We have previously implicated the glucose-induced adenyl cyclase signalling system in the inhibition of alcohol dehydrogenase II (ADH II; *ADH2* gene) expression (7, 13, 16). This mechanism, though, would account for only a small portion of the total glucose repression of *ADH2* (7, 14). We show in the present study that glucose-dependent control of ADR1 protein synthesis contributes significantly to the overall glucose regulation of *ADH2* expression.

The ADR1 regulatory protein is a transcriptional activator of the *ADH2* gene (14, 33) and is required for the dramatic 500-fold increase in ADH II activity, which is initiated upon depletion or removal of glucose from the growth medium (8). Activation of *ADH2* transcription requires the binding of ADR1 protein to a 22-bp dyad upstream activation sequence in the noncoding region of the *ADH2* gene (35). Evidence indicates that such binding occurs through two zinc fingers located in the N-terminal region of ADR1 (35) and that the ability of ADR1 to bind *ADH2* appears to be carbon source independent (31). In addition to its control of *ADH2*, ADR1 is required for transcription of genes involved in peroxisome function (28) and for undefined factors required for nonfermentative growth (2).

Glucose regulation of ADR1 function appears to occur principally at the posttranscriptional level since *ADR1* mRNA levels do not differ significantly between glucose- and ethanol-grown cells (4, 12, 17). Dominant mutations in *ADR1* (designated *ADR1*^{c}), which allow glucose-insensitive *ADH2* transcription (14), have been identified as point mutations causing single amino acid substitutions between amino acids 227 and 239 of ADR1 (7, 16, 17). While it was originally postulated that these mutations affected the cyclic AMP-dependent protein kinase phosphorylation site at ser-230 of ADR1 (7, 17), more recent evidence indicates that this protein kinase inhibits ADR1 function by a mechanism that is independent of effects on ser-230 (16). How ADR1^c mutations activate ADR1 under glucose growth conditions remains unclear. It is apparent, however, that additional mechanisms controlling ADR1 function must be operative, since *ADR1*^{c}-containing strains remain partly subject to the effects of glucose repression (10, 14).

We have reported previously that *ADH2* expression under glucose growth conditions increased linearly in response to increased *ADR1* dosage (12). This observation raises the possibility that changes in the rate of ADR1 protein synthesis may play a role in the control of ADR1 activation of *ADH2* transcription. We report here that the rate of ADR1 protein synthesis increased dramatically within the first 40 min of shifting cells from glucose to ethanol growth medium.

* Corresponding author.

† New Hampshire Agriculture Experiment Station publication 1726.

‡ Present address: Department of Biology, St. Anselm College, Manchester, NH 03555.

§ Present address: Department of Biochemistry, Brandeis University, Waltham, MA 02254.

‖ Present address: Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118.

MATERIALS AND METHODS

Yeast strains. All yeast strains used in this study are isogenic to strain 500-16, *MATa adh1-11 adh3 ura1 his4 trp1*, and have been described previously (2, 12).

Northern analysis. Cells were grown overnight at 30°C in YEP medium consisting of 2% Bacto Peptone, 1% yeast extract, 20 mg of adenine and uracil per liter, and either 8% glucose or 3% ethanol. Cultures were inoculated from YEP plates containing 2% agar and either 8% (YD8 plate) or 2% (YD plate) glucose.

Total yeast RNA was extracted as previously described (15). Northern (RNA) analysis was conducted according to the manufacturer's specifications as described in the New England Nuclear GeneScreen Instruction Manual. The *ADR1* hybridization probe, a 3.4-kb *Hin*dIII fragment from plasmid YRp7-ADR1-23A (17), was radiolabeled by using a random priming kit (Boehringer-Mannheim). *ADR1* RNA levels were quantitated by densitometric analysis with an EC610 densitometer and normalized to the amount of rRNA present when comparing samples grown under glucose and ethanol growth conditions (15, 18). rRNA has been found to be a useful standard for comparing *ADR1* mRNA extracted from cells harvested from the different growth conditions (3, 12, 15, 17). The wild-type strain used in this study, which lacks ADH I activity, grows at the same rate in medium containing glucose as in medium containing ethanol, obviating a carbon source variation in rRNA levels (23). Moreover, other experiments have indicated that normalization to *CCR4* mRNA levels gives results in agreement with those obtained by using rRNA as a standard. Normalization to the level of *UR4.3* RNA was used as another standard when comparing samples from cells grown under the same growth conditions, yielding results in agreement with those obtained from the use of rRNA as standard.

Plasmid construction. Strain WC1 was derived from 500-16 by site-directed integration of the *G-ADR1* allele at the *adr1-1* locus. Plasmid G-ADR1 was constructed from plasmid JS119, which contains the complete *ADR1* gene under the control of the *G3PDH* promoter (7), by ligating a *Bam*HI fragment containing the *G3PDH* promoter and the first 3,200 bp of *ADR1* into YRp7 at its *Bam*HI site. The resultant G-ADR1 plasmid was cut in the interior of the *ADR1* gene with *Sst*I (at bp 1715) prior to transformation of strain 500-16.

In vivo labelling of yeast proteins with ^{35}S-amino acids. In vivo labelling of yeast cell proteins with ^{35}S-amino acids was conducted by the method essentially as described by Reid (27). Cells labelled under glucose growth conditions were prepared by inoculation from a YD8 plate into liquid growth medium made with sulfate-free salts and containing 8% glucose. Cells labelled under ethanol growth conditions were prepared by inoculation from a YD plate into the same sulfur-deficient medium but with the substitution of 3% ethanol for the glucose. Following overnight growth (17 to 20 h) at 30°C in a shaker-incubator, the yeast cells were harvested by centrifugation. Cells were resuspended in fresh media of the same composition used for overnight growth but containing an added specified amount (typically 200 to 250 μCi ml of culture) of Tran [^{35}S] label (ICN Corp.). Tran [^{35}S] label contains ca. 70% [^{35}S]Met and ca. 15% [^{35}S]Cys. Cultures were returned to the shaker-incubator for labelling during further growth at 30°C until harvesting or further treatment. The time allowed for pulse-labelling varied from 15 min to 2 h, as indicated in the text. Control experiments demonstrated incorporation of ^{35}S label into total yeast proteins to be linear as a function of time and the rates of incorporation to be nearly identical under glucose and ethanol growth conditions. The levels of ADH II activity were determined (11) for each strain grown under the conditions described above to be unchanged from the activity levels determined for cells grown in the typical YEP growth medium.

In vivo labelling of yeast proteins with [^{32}P]phosphate and [^{35}S]thiophosphate. In vivo labelling of yeast cell proteins was accomplished by incubating cells, pregrown in YEP medium and then washed once with low-phosphate medium (UMD medium [5] containing 0.3 mg of KH_2PO_4 per ml and the appropriate carbon source (3% [vol/vol] ethanol or 8% [wt/vol] glucose), at 30°C with shaking in low-phosphate medium containing either 3% (vol/vol) ethanol or 8% (wt/vol) glucose and 0.5 mCi of [^{35}S]thiophosphate (New England Nuclear) or [^{32}P]orthophosphate (New England Nuclear) per ml of culture. In control experiments, ADR1 was phospholabeled in vivo to the same extent by using either [^{32}P]phosphate or [^{32}S]thiophosphate. Incubation times varied from 15 min to 3 h according to each experiment.

Pulse-chase and growth-shift experiments. For the pulse-chase experiments, total yeast proteins were radiolabeled for 2 h with ^{35}S-amino acids as described above. The chase was accomplished by adding sufficient unlabelled methionine to give a final concentration of 2 mM in the culture. Extracts were prepared from portions of the culture harvested at selected times following addition of the unlabelled methionine. In experiments in which cells were shifted from glucose- to ethanol-containing medium, cells were rapidly washed twice with distilled water before being resuspended in growth medium containing 3% ethanol. Shifted cultures were pulsed as described above for 20 min with ^{35}S-amino acids at the times indicated in Fig. 9.

Preparation of yeast cell extracts. Pulse-labelled cells, harvested after centrifugation and removal of the labelling medium, were suspended in 0.4 ml of cold lysis buffer (1.0 mM EDTA, 0.50% [wt/vol] sodium dodecyl sulfate [SDS], 0.01 mg of leupeptin per ml [Sigma], 0.01 mg of pepstatin per ml [Sigma], 1 mM phenylmethylsulfonyl fluoride [PMSF] [Sigma], 1% aprotinin [Sigma], 1 mM dithiothreitol, 10 mM Tris-Cl, pH 7.4) and vortexed in the cold for 45 s in glass tubes containing sterile glass beads to accomplish cell lysis. Cell extracts were removed from the glass beads into 1.5-ml microfuge tubes, using an additional 0.6 ml of lysis buffer to rinse the beads and to assure a quantitative transfer of the extract. The resulting extracts (ca. 1.0- to 1.1-ml final volumes) were boiled in capped microfuge tubes for 5 min, cooled to room temperature, and centrifuged for 10 min to remove cell debris and other insolubles. Nine hundred microliters of each supernatant (the clarified whole yeast cell extract) was removed to fresh microfuge tubes and mixed with 100 μl of 10% (vol/vol) Nonidet P-40 (Sigma). Fresh PMSF and aprotinin were added to concentrations of 1 mM and 1%, respectively, before the mixture was frozen at −20°C.

Immunoprecipitation of ADR1 from SDS-denatured yeast cell extracts. ADR1 protein labelled in vivo with ^{35}S-amino acids was immunoprecipitated from SDS-denatured yeast cell extracts (prepared as described above) by using the protein A-agarose method (22) with slight modifications. Nonidet P-40-neutralized SDS-denatured extracts were precleared by being mixed end over end for 15 min at 4°C with 0.4-ml (packed volume) of protein A-agarose-nonimmune IgG (prepared by mixing equal volumes of protein A-agarose [Boehringer Mannheim] and nonimmune serum end over end

for 30 min at 4°C followed by being washed with 3 15-ml volumes of Triton wash buffer [0.05% (vol/vol) Triton X-100, 0.14 M NaCl, 1 mM EDTA, 20 mM potassium phosphate, pH 7.0]). Following a brief centrifugation to pellet the protein A-agarose-nonimmune IgG and nonspecifically bound yeast proteins, the precleared extracts were removed to fresh microfuge tubes. Portions (5 μl) of each precleared extract were transferred in triplicate to separate Whatman 3MM filter paper disks. Each disk was treated under suction in a Millipore rinsing manifold with 3 15-ml volumes of ice-cold 10% (wt/vol) trichloroacetic acid (TCA) and 1 15-ml volume of ice-cold 95% ethanol. Disks were air dried and transferred to scintillation vials containing 5 ml of Aquasol before the radioactivity was counted in a Beckman liquid scintillation counter.

Appropriate volumes of precleared yeast extracts (enough to give 5.0×10^6 TCA-precipitable cpm, unless otherwise indicated) were incubated for 2 h on ice with 10 μl of immune sera, preimmune sera, or immune sera pretreated for 10 min with an excess of the antigenic ADR1 peptide. In some experiments, unlabelled yeast extract lacking the ADR1 protein was added to the incubations to reduce nonspecific binding of labelled yeast proteins to the antibodies and protein A-agarose. Immune complexes were precipitated by the addition of 50 μl of a 50% (vol/vol) suspension of protein A-agarose in Triton wash buffer and then mixed end over end for 20 min at 4°C. After centrifugation for 30 s in a microfuge, the pellets were washed five times with 1.5-ml volumes of ice-cold RIPA buffer (10 mM sodium phosphate [pH 7.0] containing 175 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.1% [wt/vol] SDS, and 1% sodium deoxycholate). The washed pellets were resuspended in 0.1 ml of SDS sample buffer (0.1 M Tris [pH 6.8], 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 1% [wt/vol] SDS) and heated in a boiling water bath for 3 min. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by fluorography. The intensities and patterns of bands corresponding to nonspecifically precipitated proteins were found to vary from one experiment to the next. In contrast, specific precipitations of *ADR1*, as determined by comparison to immunoprecipitates preincubated with excess antigenic peptide, were found to be highly reproducible. Among the likely possible sources producing variable background levels of non-ADR1 proteins were (i) different batches of protein A-agarose having different capacities for nonspecific binding of yeast proteins, (ii) the presence of different levels of nonlabelled yeast proteins competing for nonspecific binding sites, (iii) variable effectiveness of the precleaning step, (iv) variable effectiveness of the immunoprecipitated-pellet wash step, and (v) the use of different preparations of antisera. It was observed, however, that for immunoprecipitations conducted on a given day with different yeast extracts, differences in background between immunoprecipitations were minimal (e.g., see Fig. 5 and 7 to 9).

Western blot (immunoblot) analysis of ADR1 protein in whole yeast cell extracts. Polyacrylamide gels were soaked in transfer buffer (20% methanol, 0.025 M Tris [pH 8.3], 0.192 M glycine) for 20 min and then blotted to Immobilon transfer membrane at 60 V for 2 h. The membrane was subsequently soaked in 100 ml of Blotto (50 mM Tris [pH 8.0], 2 mM $CaCl_2$, 80 mM NaCl, 5% nonfat milk, 0.2% NP40) for 1 h at room temperature, washed three times for 5 min each in phosphate-buffered saline (PBS)-Tween (0.14 M NaCl, 1 M KCl, 15 mM KH_2PO_4, 0.15 mM K_2HPO_4, 0.1% Tween 20), and then incubated for an additional hour in Blotto with 30 μl of anti-ADR1 208-231 peptide antibody. The membrane was rewashed three times in PBS-Tween, for 5 min each wash, and reincubated for 1 h at room temperature in Blotto with 60 μl of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham). The membrane was washed with PBS-Tween three times and then with PBS for 5 min each time. The blot was developed with 32 mg of diaminobenzimine in 100 ml of PBS with 60 μl 30% H_2O_2 as a substrate.

Peptide synthesis and antibody production. ADR1 peptides comprising amino acid residues 2 to 21 and 208 to 231 were synthesized by the method of Merrifield as previously described (29). Conjugation of peptides to bovine serum albumin (BSA) and production of antibodies have been described previously (24, 25, 32). Preimmune and immune sera were used without further purification. Anti-ADR1-208-231 peptide antibody immunoprecipitation of ADR1 polypeptide was found to be unaffected by phosphorylation of Ser-230 or mutation of the Arg-228 to lysine (*ADR1-5*° allele) or Ser-230 to leucine (*ADR1-7*° allele) (data not shown).

Alkaline phosphatase treatment of ADR1. Treatment with alkaline phosphatase of immunoprecipitated radiolabeled ADR1 was accomplished by the method essentially as described by Barber and Verma (1). Radiolabeled ADR1 was liberated from the washed protein A-agarose immunoprecipitates by incubation for 2 min in a boiling-water bath following the addition of 100 μl of 50 mM Tris (pH 8.0) containing 2 mM PMSF, 5 mM dithiothreitol, and 0.3% (wt/vol) SDS. Following a brief spin (5 to 7 s) in the microfuge, the supernatant containing the labelled ADR1 was transferred to fresh microfuge tubes and adjusted to contain 1% (vol/vol) Nonidet P-40, 1% (wt/vol) sodium deoxycholate (Sigma), and 150 mM NaCl. Neutralized ADR1 extracts were treated with 25 U of unweened-calf alkaline phosphatase (Sigma) at 37°C. Reactions were terminated by the addition of SDS sample buffer and incubation for 3 min in a boiling-water bath. The resulting samples were analyzed by using the SDS-PAGE and fluorography system described above.

RESULTS

ADR1 protein identification. The ADR1 protein was immunoprecipitated from SDS-denatured extracts of yeast cells labelled in vivo with ^{35}S-amino acids. ADR1, immunoprecipitated with antibodies directed against a synthetic peptide corresponding to amino acid residues 208 to 231 of the ADR1 protein, migrated on SDS-polyacrylamide gels as a single species corresponding to a polypeptide of ca. 152 kDa (Fig. 1, lane 2). The identification of the polypeptide migrating at 152 kDa as ADR1 is supported by the following results: (i) the actual calculated molecular mass of unmodified ADR1 is 150,185 Da (20), (ii) the 152-kDa polypeptide did not immunoprecipitate from incubations in which preimmune sera were substituted for immune sera (Fig. 1, lane 1), (iii) the 152-kDa polypeptide did not immunoprecipitate from incubations containing immune sera preincubated with the antigenic peptide (Fig. 1, lane 3), (iv) the 152-kDa band was not detected in immunoprecipitates of cell extracts of a strain deficient in *ADR1* expression (nonsense mutation in the 11th codon of the *adr1-1* gene [3]) (Fig. 1, lane 4 (*adr1-1*) compared with lane 6 [multicopy *ADR1*]), (v) the intensity of the 152-kDa band increased in proportion to the number of integrated *ADR1* gene copies contained in strains overexpressing the *ADR1* gene (see Fig. 3 and 5), and (vi) the 152-kDa species was not detected in strains producing truncated versions of ADR1 protein, in which shorter versions of ADR1 were detected (e.g., see Fig. 10, lane 3). We have

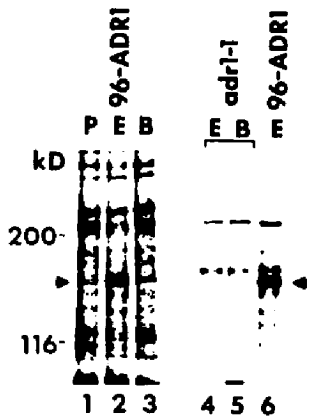

FIG. 1. Identification of ADR1 by immunoprecipitation. Cultures (4 ml) of strain 411-1 (lanes 1 to 3) (96 copies of *ADR1*) were radiolabeled for 15 min with 210 μCi of ^{35}S-amino acids per ml, as described in Materials and Methods. Two hundred microliters of precleared extract was treated with 15 μl of preimmune sera (lane P), anti-ADR1-208-231 peptide antibody (lane E), or anti-ADR1-208-231 peptide antibody pretreated with excess ADR1 peptide (lane B). Equal volumes of the immunoprecipitates were separated on a 5% polyacrylamide gel. Cultures of strain 500-16 (*adr1-1*) (lanes 4 and 5) and strain 411-1 (lane 6) were radiolabeled as described for lanes 1 to 3 except 310 μCi of ^{35}S-amino acids per ml was used. To enhance detection of immunoprecipitated species, precleared extracts were first immunoprecipitated with anti-ADR1-208-231 antibody and then reimmunoprecipitated as described for lanes 2 and 3, respectively. Control experiments indicated that reimmunoprecipitation did not impair ADR1 detection. This figure is a composite of two different autoradiograms depicting the results of two different immunoprecipitation experiments (lanes 1 to 3 and lanes 4 to 6) and, as a result, shows variation in the patterns and intensities of nonspecific background bands.

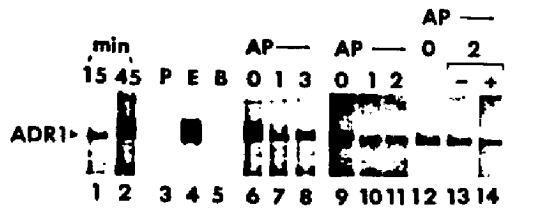

FIG. 2. ADR1 is multiply phosphorylated. Lanes 1 and 2 compare the ADR1 signals in immunoprecipitates obtained by incubating anti-ADR1-208-231 peptide antiserum with extracts of yeast strain 411-1 (96 integrated *ADR1* gene copies) labelled under glucose growth conditions for 15 and 45 min, respectively, with ^{35}S-amino acids. Lanes 3, 4, and 5 compare immunoprecipitates obtained by incubating the extract of yeast strain 411-1 labelled in vivo for 3 h with [^{35}S]thiophosphate (350 μCi ml) under ethanol growth conditions with preimmune serum, anti-ADR1-208-231 peptide antiserum, and anti-ADR1-208-231 peptide antiserum, that was blocked by preincubation with the antigenic peptide, respectively. The effect of in vitro alkaline phosphatase (AP) treatment on the breadth of the ADR1 signal from yeast strain 411-1 labelled in vivo either for 45 min with ^{35}S-amino acids under glucose growth conditions (lanes 6 to 8) or for 1 h with [^{35}S]thiophosphate (0.4 mCi ml) under ethanol growth conditions (lanes 9 to 11). ADR1 was either not treated with alkaline phosphatase (control) (lanes 6 and 9) or treated with alkaline phosphatase for 1 (lanes 7 and 10), 2 (lane 11), or 3 (lane 8) h. Lanes: 12, strain 411-1 labeled under glucose growth conditions as described for lane 9; 13, same as lane 12 except incubated for 2 h without alkaline phosphatase; 14, same as lane 13 except incubated with alkaline phosphatase.

obtained these results by using antisera raised against a peptide corresponding to amino acid residues 208 to 231 of ADR1 (Fig. 1 to 4) or a second synthetic peptide corresponding to residues 2 to 21 of the ADR1 protein (see Fig. 3 to 5 and 7 to 10). Taken together, our results provide conclusive evidence that the identity of the polypeptide migrating at 152 kDa in lane 2 of Fig. 1 is the ADR1 protein.

ADR1 protein is multiply phosphorylated in both glucose- and ethanol-grown cells. We observed during the course of the above immunoprecipitation experiments that the ADR1 signal broadened in the direction of higher-molecular-weight species as a function of the length of the labelling time (compare lanes 1 [15 min] and 2 [45 min] of Fig. 2). Such broadening suggests the likelihood that newly synthesized ADR1 protein is subject to rapid posttranslational modification. Thus, to ensure our accuracy in the identification and quantitation of all forms of newly synthesized ADR1 protein, we sought to identify the mechanism responsible for creating the observed ADR1 signal pattern.

Although direct biochemical evidence that ADR1 occurs in vivo as a phosphoprotein has not been reported, phosphorylation appeared to be a likely mechanism responsible for the observed broadening of the ADR1 signal. To investigate this hypothesis, we applied the ADR1 immunoprecipitation protocol described above to determine the in vivo phosphorylation state of ADR1 under glucose and ethanol growth conditions. ADR1 is shown in lane 4 of Fig. 2 after its immunoprecipitation from yeast labelled in vivo with [^{35}S]thiophosphate under ethanol growth conditions. Phospholabelled ADR1 did not precipitate with preimmune sera (lane 3) or with immune sera that were preincubated with ADR1 peptide (lane 5). ADR1 was also shown to be a phosphoprotein under glucose growth conditions (data not shown; see also Fig. 2, lanes 6 to 8 and 12, and Fig. 4).

We observed phospholabelled ADR1 to be immunoprecipitated specifically by two different antisera raised against two different regions of the ADR1 protein (residues 2 to 21 and 208 to 231) (Fig. 4) and to comigrate on SDS-polyacrylamide gels with immunoprecipitated ADR1 labelled with [^{35}S]methionine (Fig. 2, lane 6) (data not shown), confirming the identity of this species as phosphorylated ADR1. In addition, a strain lacking the full-length ADR1 protein but containing a truncated *ADR1* gene (*ADR1-262*) lacks the phosphorylated species migrating at 152 kDa and instead contains peptide-blockable phosphorylated proteins that comigrate with ^{35}S-amino-acid-labeled ADR1-262 (see Fig. 10, lanes 9 and 10 compared with lanes 5 and 6) (data not shown).

Alkaline phosphatase treatment of ADR1 immunoprecipitated from cells pulse-labelled for 2 h with ^{35}S-amino acids caused the size of the ADR1 signal to be dramatically reduced and to resemble the thin band produced at 152 kDa by ADR1 from cells pulse-labelled for 15 min (Fig. 2, lanes 6 to 8 compared with lane 1). Treatment with alkaline phosphatase of ADR1 immunoprecipitated from cells labelled with [^{35}S]thiophosphate caused the ADR1 signal to be reduced in breadth but not to disappear completely (lanes 9 to 11), suggesting that one or more of the phosphorylated residues may be resistant to the action of alkaline phosphatase. In contrast, incubation of ADR1 immunoprecipitated from cells labelled with [^{35}S]thiophosphate in the

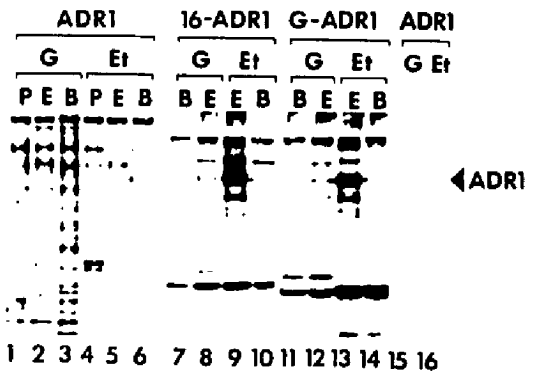

FIG. 3. Glucose control of ADR1 protein synthesis. Yeast cells were labeled with ^{35}S-amino acids and immunoprecipitated as described in the legend to Fig. 1 and Materials and Methods (lanes 1 to 14). Anti-ADR1-208-231 peptide antibody (lanes 1 to 6) and anti-ADR1-2-21 peptide antibody (lanes 7 to 14) were used. For glucose (lanes G) and ethanol (lanes Et) comparisons of the same strain, equivalent amounts of precleared extracts based on TCA-precipitable counts were incubated with 15 μl of immune sera (lane E), immune sera pretreated with excess ADR1 peptide (lane B), or preimmune sera (lane P). For lanes 1 to 6, precleared extracts from glucose-grown cultures (lanes G) contained twice the number of TCA-precipitable counts as that of ethanol-containing cultures (lanes Et). Black dots beside the ADR1 polypeptide indicate its position. The strains 411-40 (*ADR1*), 411-12 (16-ADR1 [16 copies of ADR1]), and WC1 (G-ADR1) which contains one copy of the *ADR1* gene under the control of the weak *GAPDH* promoter (Materials and Methods) are indicated above the lanes. Samples were separated on a 5% (lanes 1 to 6), 6% (lanes 15 and 16), or 7.5% (lane 7 to 14) polyacrylamide gel. Radioactive labelling was done for 15 (lanes 1 to 6), 90 (lanes 7 to 10) or 60 min (lanes 11 to 14). (Lanes 15 and 16 depict a Western blot in which equivalent amounts of total yeast extracts grown on YEP medium containing either 8% glucose (lanes G) or 3% ethanol (lanes Et) were blotted to Immobilon paper (Millipore) and treated with anti-ADR1-208-231 peptide antibody, as described in the Materials and Methods.) This figure is a composite of several different autoradiograms as described in the legend to Fig. 1.

absence of alkaline phosphatase did not affect the breadth of the ADR1 signal (lane 12, no incubation; lane 13, no alkaline phosphatase treatment; and lane 14, alkaline phosphatase treatment). These results clearly define the forms of newly

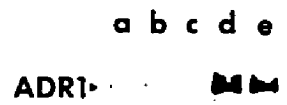

FIG. 4. ADR1 was immunoprecipitated equally well by both anti-ADR1 peptide antibodies. Yeast strain G-ADR1 was labeled with [^{35}S]thiophosphate under glucose and ethanol growth conditions as described in the legend to Fig. 2. Immunoprecipitations and blocks were conducted as described in the legend to Fig. 2. Anti-ADR1-208-231 peptide antibody (lanes a, c, d, and f) and anti-ADR1-2-21 peptide antibody (lanes b and e) were used. ADR1-208-231 peptide was added to lanes c and f as described in the legend to Fig. 2. Lanes: a to c, glucose-grown cells; d to f, ethanol-grown cells. It should be noted that the apparent difference in immunoprecipitated ADR1 between lanes d and e was the result of a nonspecific immunoprecipitable band (migrating slightly slower than ADR1 in lane d) that was not blocked by incubation with excess antigenic peptide (lane f).

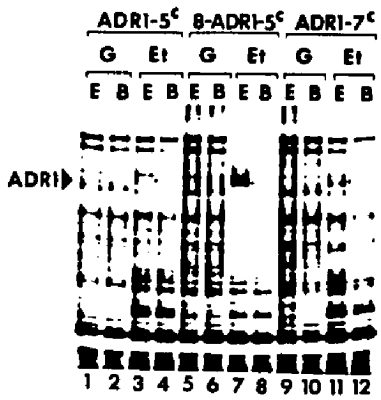

FIG. 5. Effect of *ADR1^c* mutations on ADR1 protein synthesis regulation. Labelling of yeast extracts, immunoprecipitation of ADR1, and analysis of samples was conducted as described in the legend to Fig. 3 for lanes 7 to 14. Lanes: ADR1-5^c, strain 6-60 (one copy of *ADR1-5^c*); 8-ADR1-5^c, strain 6-2 (eight copies of *ADR1-5^c*); ADR1-7^c, strain 7^c-b (one copy of *ADR1-7^c*).

synthesized ADR1 and indicate that ADR1 is multiply phosphorylated in vivo under both glucose and ethanol growth conditions during or soon after its synthesis (within 45 min).

The rate of ADR1 protein synthesis is reduced in glucose-grown cells. We observed the amount of ADR1 immunoprecipitated from extracts of ethanol-grown cells to be consistently and significantly greater than the amount of ADR1 immunoprecipitated from extracts of cells grown on glucose, suggesting that ADR1 activity might be regulated through a carbon source-linked control of ADR1 protein synthesis. Figure 3 illustrates the results of an ADR1 immunoprecipitation experiment using SDS-denatured extracts of yeast strain 411-40 (containing a single copy of *ADR1*) pulse-labelled for 15 min with ^{35}S-amino acids under glucose (lane 2) and ethanol (lane 5) growth conditions. As shown in the Fig. 3, we were able to detect ADR1 under ethanol growth conditions but not under glucose growth conditions. We obtained similar results by Western analysis of whole-cell (strain 411-40) extracts (Fig. 3, lanes 15 and 16, respectively), indicating that in addition to the rate of ADR1 protein synthesis, the steady-state level of ADR1 protein accumulated in the cell is also affected by the carbon source.

We subsequently analyzed yeast strains 411-26, 411-3, and 411-12 carrying 4, 9, and 16 copies, respectively, of the *ADR1* gene integrated into the genome to estimate the minimum *ADR1* dosage at which ADR1 protein could be detected in glucose-grown cells. *ADH2* regulation in strains 411-26, 411-3, and 411-12 is identical to that of isogenic strain 411-40 carrying a single copy of the *ADR1* gene except for commensurate dosage-dependent elevations in ADH II activity (2, 12). We were unable to detect ADR1 protein in strains 411-26 (four copies of *ADR1*) and 411-3 (nine copies of *ADR1*) grown under glucose growth conditions (data not shown). We obtained an ADR1 protein signal from each of these strains, however, that was clearly both visible and present in the expected relative proportions from extracts of ethanol-grown cells (data not shown). We determined that an *ADR1* dosage of 16 (strain 411-12) yielded a reproducibly detectable ADR1 protein signal under glucose growth conditions that was roughly equivalent to that obtained under

ethanol growth conditions in the single copy strain (compare lanes 5 and 8 of Fig. 3). Densitometric analysis indicated that ADR1 protein accumulated in strain 411-12 (16 *ADR1* copies) at about an 11-fold faster rate under ethanol growth conditions compared with glucose growth conditions. This result is in good agreement with our observation above that a dosage of 16 *ADR1* gene copies is the approximate minimal dosage required for detection of ADR1 in glucose-grown cells.

We observed the same ADR1 abundance patterns, using two different antisera raised against peptides corresponding to two different regions (amino acids 208 to 231 and 2 to 21) of the ADR1 protein (compare lanes 1 to 6 with 7 to 18 of Fig. 3). This result provides evidence that the amounts of ADR1 protein observed in our immunoprecipitation experiments are accurate reflections of the quantities of newly synthesized ADR1 protein present in the cell extracts (and therefore, ADR1 protein synthetic rates) and are not attributable to carbon source-related bias in ADR1-antibody interaction. Such a bias might be envisioned to exist between the antisera raised against the ADR1-208-231 peptide (which contains a putative regulatable phosphorylation site at Ser-230) and the actual in vivo forms of the ADR1 protein. Previous experiments indicated, however, that the anti-ADR1-208-231 peptide antibody identifies ADR1 regardless of the phosphorylation state at Ser-230 or whether the serine site is altered to an amino acid that cannot be phosphorylated (7). In order to further confirm that the anti-ADR1-208-231 peptide antibody precipitates all phosphorylated ADR1 species, we compared the ability of the two antipeptide antibodies to precipitate ADR1 phosphorylated under glucose versus ethanol growth conditions. We observed no difference in either the intensity or banding pattern of ADR1 signals immunoprecipitated with each antiserum (Fig. 4, compare lanes a and d [anti-ADR1-208-231 antibody] with lanes b and c [anti-ADR1-2-21 antibody]).

ADR1^c-type mutations do not affect ADR1 protein synthesis. Our observation that the rate of ADR1 protein synthesis is regulated by the carbon source suggests the possibility that the effect of *ADR1^c*-type mutations might be to increase the rate of synthesis of the ADR1 protein. We therefore applied the labelling and immunoprecipitation techniques described above to investigate the potential effect of *ADR1^c*-type mutations on ADR1 protein synthesis. Yeast strain 6-60 expresses a single copy of the *ADR1-5^c* allele (R228K; contains a lysine at residue 228 instead of an arginine) and results in ADH II activities of ca. 300 mU mg on glucose and ca. 5,000 mU mg on ethanol (12). The isogenic strain 411-40 carrying the wild-type *ADR1* gene by comparison yields ADH II activities of only 5 and 2,000 mU mg on glucose and ethanol, respectively (12). Figure 5 demonstrates the disparity between the rates of ADR1 synthesis with the two carbon sources by comparing the amounts of the ADR1 protein immunoprecipitated from extracts of strain 6-60 following growth on glucose and on ethanol. As illustrated in lane 1 of Fig. 5, the immunoprecipitates corresponding to glucose-grown cells did not produce a discernible ADR1 signal. We repeated this experiment with strains 6-60 and 411-40 numerous occasions, each time attempting to adjust conditions (e.g., labelling time, amount of labelled extract in the immune reactions, gel conditions, and double immunoprecipitations) so as to maximize the likelihood of ADR1 protein detection. We were unable, however, to detect a band representing the ADR1 protein, either at the usual 152-kDa position or at any other region of the gel. The immunoprecipitates of ethanol-grown cells, in contrast, always produced a clearly detectable ADR1 signal (Fig. 5, lane 3) which was peptide blockable (Fig. 5, lane 4). Similar results were obtained with a strain carrying eight copies of the *ADR1-5^c* gene and a strain expressing the *ADR1-7^c* allele (S230L) (Fig. 5, lanes 5 to 8 and 9 to 12). Our results indicate that the enhanced ADR1 activity conferred by the *ADR1^c* mutations is not attributable to an increase in ADR1 protein synthesis.

The conclusion that *ADR1^c* mutations cause increased ADR1 activity by a mechanism which does not rely upon increased ADR1 protein translation is supported further by the results of our experiments with strain 411-12. Strain 411-12 contains 16 integrated *ADR1* gene copies and produces ca. 80 to 90 mU of ADH II activity per ml when grown on glucose (12). Although cells from strain 411-12 produce only one-fourth of the amount of ADH II activity produced by the *ADR1-5^c* single-gene-copy-containing strain (above), immunoprecipitates of glucose-grown 411-12 cells routinely produce a clearly visible ADR1 signal (Fig. 3, lane 16). These results indicate that the *ADR1^c* mutations cause enhanced ADR1 activity by a mechanism independent of ADR1 protein synthesis control.

Differences in *ADR1* mRNA abundance contribute slightly to the regulation of the rate of ADR1 protein synthesis. We next sought to identify the step, or steps, leading to the production of the ADR1 protein which are responsible for the observed increase in the rate of ADR1 protein synthesis. To distinguish between *ADR1* mRNA availability and other translational control mechanisms, we compared the levels of *ADR1* mRNA in glucose- and ethanol-grown cells. Previous investigations of strain 411-40 (single *ADR1* gene copy) and strains isogenic to 411-40 carrying multiple copies of the *ADR1* gene indicated the level of *ADR1* mRNA in glucose-grown cells to be comparable to or slightly less than that found in ethanol-grown cells (3, 12). Although these strains are identical or comparable to the ones used in the present investigation, to ensure consistency between all phases of our analysis we measured *ADR1* mRNA levels by using the strains and growth conditions employed throughout our current investigation of the ADR1 protein abundance effect.

Figure 6 illustrates the results obtained with a Northern blot analysis of *ADR1* mRNA for strains 411-40 (1 *ADR1* gene copy) and 411-12 (16 *ADR1* gene copies) grown on glucose (lanes G) and on ethanol (lanes Et). As shown, for each of the strains, the levels of *ADR1* mRNA were found to be only slightly elevated in the ethanol-grown cells. Densitometric analysis indicates that *ADR1* mRNA, when normalized to rRNA, is twice as abundant under ethanol growth conditions as under glucose growth conditions. These results are essentially the same as those previously obtained by us (3, 12, 17) and others (4). It, therefore, appears that control mechanisms other than those controlling *ADR1* mRNA abundance must be responsible for the greater part of regulating the rate of ADR1 translation.

The rate of ADR1 protein degradation does not contribute to differences in ADR1 protein synthesis. The greater part of the increase in ADR1 protein synthesis rate under ethanol growth conditions must result from either an elevated rate of ADR1 translation or a decreased rate of ADR1 protein degradation, or possibly both. To distinguish between these possibilities we examined rates of ADR1 protein degradation in glucose grown cells by pulse-labelling the cells in vivo with ^{35}S-amino acids for 2 h and chasing the cells with nonradioactive methionine. Cells were removed at selected time points during the chase and analyzed for their content of labelled ADR1 and total yeast proteins. Samplings of cells taken just before and at times following the addition of

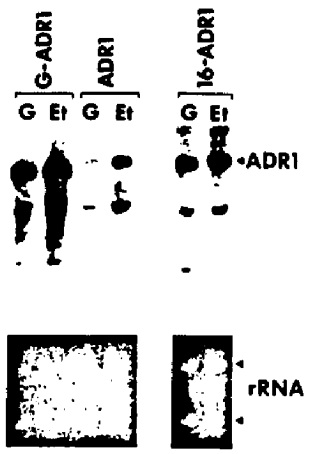

FIG. 6. Effect of carbon source on *ADR1* mRNA levels. Total RNA extracted from cells grown on glucose- or ethanol-containing medium was subjected to Northern analysis as described in Materials and Methods. The RNA size for *G-ADR1* is smaller than that for *ADR1* because of the deletion of the 510-nt untranslated 5′ leader sequence of *ADR1*. A duplicate gel stained with ethidium bromide is displayed in the bottom panels and shows the amount of RNA (25S, top bands; 18S, bottom bands) present in each lane. Lanes: G-ADR1, 15.5 μg of RNA extracted from strain WC1 which carries a single copy of the *G-ADR1* gene integrated at the *adr1-1* locus; ADR1, 31 μg of RNA extracted from strain 411-40 carrying a single copy of *ADR1*; 16-ADR1, 7.5 μg of RNA extracted from strain 411-12 which carries 16 copies of *ADR1* tandemly integrated at *adr1-1* (12). G, glucose growth conditions; Et, ethanol growth conditions.

unlabelled methionine showed identical levels of ^{35}S label incorporated into proteins, indicating that the chase with nonradioactive methionine efficiently blocked all further incorporation of ^{35}S-amino acids into total yeast proteins (Fig. 7b). Minimal ADR1 protein degradation was observed during the first 2 to 3 h of the chase under glucose growth conditions (Fig. 7a). (The apparent decreased abundance of ADR1 at time zero in Fig. 7a was due to the antigenic ADR1 peptide being added during the immunoprecipitation; other experiments showed that ADR1 abundance did not significantly change between 0 and 1 h after the chase.) The data presented in Fig. 7 indicate a half-life of 3 to 4 h for ADR1 under glucose growth conditions. Significant differences in the rates of ADR1 protein synthesis were observed, however, in cell extracts pulse-labelled for only 15 min. An accelerated rate of ADR1 protein degradation, therefore, cannot account for the relatively low level of ADR1 protein present under glucose growth conditions. Indeed, the half-life of the ADR1 protein was actually found to be slightly shorter (about 2- to 3-h half-life) (Fig. 8) under ethanol growth conditions than under glucose growth conditions. This result confirms that differences in ADR1 degradation cannot significantly contribute to the differences in ADR1 protein abundance and indicates instead that differences in the rates of ADR1 protein translation must account for the higher rate of ADR1 protein accumulation observed for ethanol-grown cells.

Rate of ADR1 protein synthesis increases within 40 to 60 min after shifting cells to ethanol-containing medium. ADR1-dependent *ADH2* transcription is known to be initiated

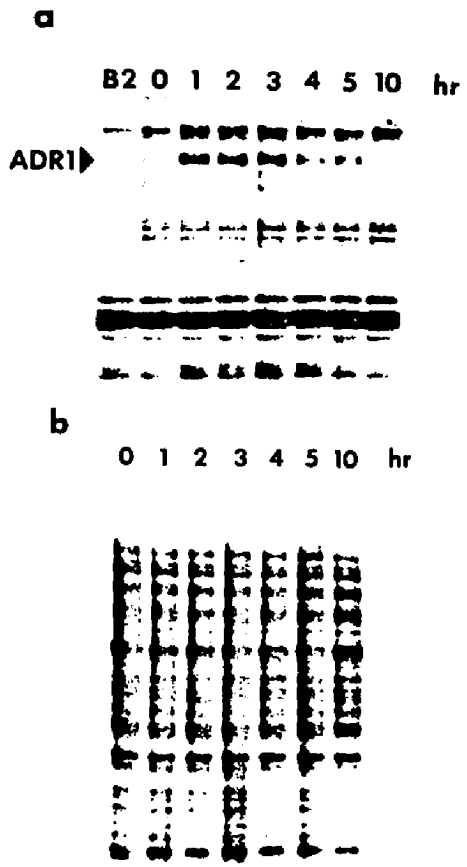

FIG. 7. ADR1 protein turnover under glucose growth conditions. Cultures (15 ml) of strain 411-12 (16 copies of *ADR1*) were radiolabeled to steady-state levels under glucose growth conditions, as described in Materials and Methods, and chased with unlabeled methionine. At the times indicated above the lanes, 2-ml portions were removed and extracts were prepared. (a) Precleared extracts normalized on the basis of TCA-precipitable counts (observed to be nearly equivalent volumes) immunoprecipitated with anti-ADR1-2-21 peptide antibody. The resultant immunoprecipitates were separated on a 10% polyacrylamide gel. (b) Equal volumes of total precleared extracts (essentially equivalent to equal amounts of precleared extracts based on TCA-precipitable counts). The immunoprecipitated ADR1 polypeptide is indicated by the arrow. Lane B2 contains precleared extracts from the 2-h time point treated with excess antigenic peptide and antibody. The rate of ADR1 protein turnover was determined after first calculating the ratio of the amount of radioactivity in the ADR1 protein immunoprecipitated band to the total TCA-precipitable counts for each time point.

within 1 h of depletion of glucose from the medium (14). We, therefore, examined the rate of ADR1 protein synthesis during the time period immediately following removal of glucose from the medium to determine whether the increased ADR1 protein translation rate is correlated with the time at which ADR1 activation is presumed to occur. Figure 9 illustrates that the rate of ADR1 protein synthesis increased dramatically in strain 411-3 (9 *ADR1* gene copies)

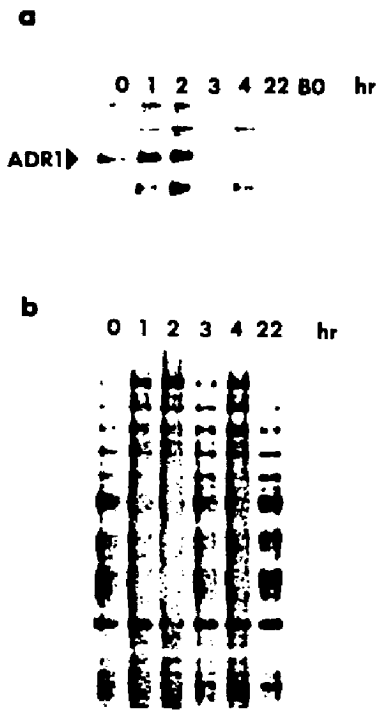

FIG. 8. ADR1 protein turnover under ethanol growth conditions. Conditions for determining ADR1 protein turnover were the same as those described in the legend to Fig. 7, except growth of cells occurred in ethanol-containing medium. At the times indicated above the lanes, 2-ml portions were removed and extracts were prepared. (a) Equal volumes of precleared extracts were immunoprecipitated with anti-ADR1-2-21 peptide antisera, and the resultant immunoprecipitates were separated on 10% polyacrylamide gel; (b) equal volumes of precleared extracts (in this case not equivalent to equal amounts of TCA-precipitable counts) were subjected to SDS-PAGE. Lane B contains precleared extracts from the zero time point treated with antibody and excess antigenic peptide.

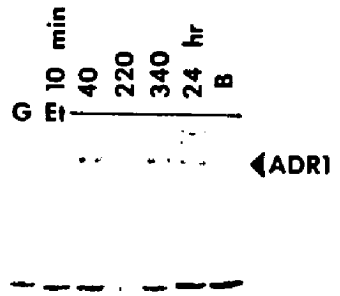

FIG. 9. ADR1 protein synthesis as a function of growth on ethanol-containing medium. Cells from strain 411-3 (9 *ADR1* genes) were pulse-labelled for 20 min with ^{35}S-amino acids either on glucose-containing medium (lane G) or at the times indicated above the lanes following transfer of cells to ethanol-containing medium. Equivalent amounts of precleared extracts as determined by TCA-precipitable counts were immunoprecipitated and analyzed as described in the legend to Fig. 6a. Lane B represents the blocked experiment in which the addition of excess antigenic peptide blocked the immunoprecipitation of ADR1 from extracts at the 24-h time point.

within 40 to 60 min after shifting cells from glucose to ethanol growth conditions. At time points beyond the first 40 to 60 min (i.e., 220 min, 340 min, and 24 h) the rate of ADR1 synthesis remained relatively constant (Fig. 9). Similar results were obtained for a strain (6-2) containing eight integrated copies of the *ADR1*-5′ gene (data not shown). These results provide strong evidence that the glucose regulation of *ADH2* expression by the control of ADR1 protein synthesis is a physiologically significant control mechanism since *ADH2* expression is known to be directly responsive to the dosage of *ADR1* (12).

The 510-nt 5′ untranslated region of *ADR1* mRNA is not involved in ADR1 translational control. We investigated the potential role of the 510-nucleotide (nt) 5′ untranslated region of *ADR1* mRNA in the control of ADR1 translation. While this region in *ADR1* lacks short open reading frames found to be important in controlling translation of the yeast *GCN4* mRNA (26), we considered the possibility that other features contained within the region might mediate carbon source-dependent translation. To investigate this possibility, we replaced the 510-nt untranslated region of *ADR1*, including the upstream promoter sequences, with a truncated promoter derived from the glyceraldehyde 3-phosphate dehydrogenase (*GAP*) gene. The truncated *GAP* promoter was used because it is a low-expression promoter that would be expected to produce *ADR1* mRNA at levels equivalent to or only slightly higher than those normally expressed by the cell and because its efficiency is not regulated by the carbon source (27a).

Northern analysis indicated the level of *G-ADR1* mRNA in a yeast strain containing a single integrated dose of *G-ADR1* to be approximately 15-fold higher than that in the isogenic strain carrying a single *ADR1* gene copy (Fig. 6, first two lanes compared with the third and fourth lanes). Thus, *G-ADR1* produced *ADR1* mRNA in amounts which would be expected for a strain carrying about 15 copies of *ADR1* (12). We determined ADH II enzyme activity in the *G-ADR1*-carrying strain for glucose and ethanol growth conditions to be 50 and 5,000 mU/mg, respectively. These values are consistent with those expected for a strain carrying 10 to 12 copies of *ADR1* (12) and are in good agreement with the elevated levels of *G-ADR1* mRNA production illustrated in Fig. 6. The amount of *G-ADR1* mRNA in ethanol-grown cells was determined to be twice that found under glucose growth conditions. In contrast, we determined the amount of ADR1 protein immunoprecipitated from ^{35}S-amino acid extracts from the *G-ADR1*-carrying strain to be 10- to 15-fold greater under ethanol growth conditions than under glucose growth conditions (Fig. 3, compare lanes 12 and 13), consistent with our above analysis of strains containing one or more copies of the complete *ADR1* gene. These surprising results indicate that the ADR1 translation rate differences under the glucose and ethanol growth conditions are retained when the 510-nt 5′ untranslated region of *ADR1* mRNA is replaced with a different sequence and that this region is, therefore, not involved in the glucose regulation of ADR1 translation.

ADR1 coding sequences corresponding to amino acid residues 262 through 642 are involved in ADR1 translational control. Having determined that the 5′ untranslated region of *ADR1* mRNA does not control the ADR1 translation rate,

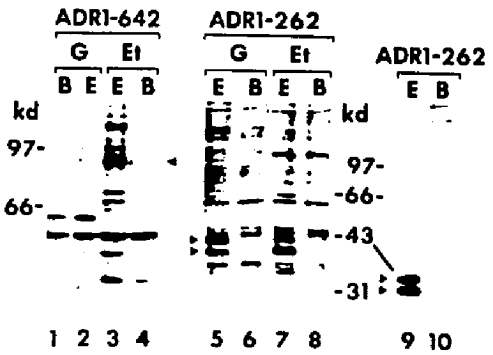

FIG. 10. Identification of the regions controlling ADR1 translation. Cells were grown and radiolabelled as described in the legend to Fig. 4 and Materials and Methods except lanes 9 and 10 were radiolabelled during glucose growth conditions with [^{35}S]thiophosphate for 1 h as indicated in the legend to Fig. 2. Lanes: E, immunoprecipitation with anti-ADR1-2-21 peptide antibody; B, immunoprecipitation in the presence of excess ADR1-2-21 peptide. The ADR1-642 and ADR1-262 species are indicated. Equivalent amounts of radiolabelled extract were analyzed for each strain grown under glucose (lanes G) and ethanol (lanes Et) growth conditions. ADR1-642 immunoprecipitates were subjected to electrophoresis on a 7.5% polyacrylamide gel, while the other samples were separated on a 10% polyacrylamide gel. Bands corresponding to ADR1 species are indicated by arrowheads and the filled dot (interior lanes only).

we next investigated the potential involvement of the coding sequences of *ADR1*. Two strains, isogefic to each other and to the above strains, carrying *ADR1* alleles with sequential C-terminal deletions of *ADR1* coding sequences, were subjected to ^{35}S-amino acid labelling and analyzed for newly synthesized ADR1 protein. Strain B19 carries 21 copies of *ADR1-642*, while strain 35 carries 21 copies of *ADR1-262* (2). (The numbers 642 and 262 correspond to the numbers of N-terminal ADR1 amino acids in the truncated ADR1 protein products expressed in each strain.) The ADR1-642 protein from strain B19 gave a boldly visible signal following growth on ethanol-containing medium but was not detected in glucose-grown cells (Fig. 10, lanes 3 and 2, respectively). These results are similar to those observed for the full-length ADR1 protein and would be expected if the translational control mechanism does not become disrupted as a result of removal of over half of the ADR1 coding sequence (681 C-terminal residues). Similar results were obtained with a yeast strain containing only nine copies of *ADR1-642* (data not shown). Also, the amounts of *ADR1-642* mRNA, when normalized to rRNA levels, were found to be only slightly elevated in ethanol-grown cells compared with glucose-grown cells, data consistent with our observations for strains containing the full-length *ADR1* gene.

The ADR1-262 protein, in contrast to ADR1-642 and ADR1-1323, was observed to yield boldly visible signals under both glucose and ethanol growth conditions. Densitometric analysis indicated ADR1-262 protein, though present in two forms, to be equally abundant in glucose- and ethanol-grown cells (Fig. 10, lanes 5 and 7). We presume the two forms of ADR1-262, which migrate in the 40-kDa region of the SDS-polyacrylamide gel, to be due to the occurrence of multiple phosphorylated species (both species are phosphoproteins: Fig. 10, lanes 9 and 10) or possibly to proteolysis. Antibodies directed against ADR1 peptides corresponding to regions 208 to 231 and 2 to 21 immunoprecipitated both forms with equal efficiencies (data not shown), indicating that the majority of the 262 amino acids were intact. Although the observed abundance differences of the individual forms of ADR1-262 between glucose and ethanol growth conditions were not reproducible, the total amount of ADR1 protein (both forms together) always remained equivalent under the two growth conditions. The *ADR1-262* mRNA levels were found to be similar under ethanol and glucose growth conditions (data not shown), results which are consistent with the ADR1 protein synthetic rates determined for ADR1-262 and the *ADR1* mRNA patterns observed for all of the strains investigated in this study. The translational control of ADR1 appears mediated, therefore, by sequences localized within the coding region of the transcript corresponding to amino acid residues 262 through 642 of the ADR1 protein.

DISCUSSION

The results of our investigation demonstrate that the rate of protein synthesis for the transcriptional activator ADR1 is 10- to 16-fold greater under ethanol growth conditions than under glucose growth conditions. This derepression in ADR1 protein translation was found to occur within 40 to 60 min of depleting cells of glucose, the same time frame defined previously for the commencement of ADR1-dependent *ADH2* transcription (14). Our results indicate that glucose represses *ADH2* expression by reducing the rate of ADR1 protein synthesis, a conclusion supported by our previous observation that linear increases in *ADR1* dosage result in corresponding linear increases in the amount of *ADR1* mRNA and in *ADH2* expression under glucose growth conditions (12). Strains containing *ADR1^c* alleles display reduced levels of ADR1 protein synthesis under repressed conditions in a manner similar to that observed for strains expressing the wild-type *ADR1* allele. We, therefore, conclude that the mechanism by which *ADR1^c* alleles cause increased *ADH2* transcription must be distinct from that responsible for effecting changes in the rate of ADR1 protein translation.

Our previous studies indicate that yeast strains expressing a single copy of the *ADR1^c* allele show an eightfold increase in *ADH2* transcription within 1 h of shifting cells from glucose to ethanol growth conditions (14). Our current data suggest that this increase is a direct result of increased ADR1 protein synthesis signalled by glucose depletion. Interestingly, strains carrying eight copies of an *ADR1^c* allele display glucose-repressed ADH II levels that are nearly equivalent to the level of ADH II found under ethanol growth conditions in an isogenic strain containing a single wild-type *ADR1* allele (12). We interpret these results to suggest that the overall derepression of *ADH2* results from the combined effects of increased ADR1 protein translation rates and some other activation of the already synthesized ADR1 protein (equivalent to an *ADR1^c* allele). The posttranslational activation may, as previously suggested, be triggered by a dephosphorylation event (7, 16). It remains possible, however, that as yet undefined mechanisms contribute to the regulation of *ADH2* transcription in addition to control of ADR1 protein translation.

The half-life of ADR1 protein was found to be roughly equivalent under glucose and ethanol growth conditions, indicating that the observed regulation of ADR1 is independent of ADR1 degradation rates. This point is further sup-

ported by our observation that the ADR1 half-life (3-4 h on glucose) is much longer than the time required to visualize ADR1 protein differences in the amounts of newly synthesized protein during a pulse-labelling experiment (15 min). We have also demonstrated that the long untranslated 5' leader sequence of *ADR1* mRNA is not involved in controlling ADR1 protein synthesis. Instead, coding sequences corresponding to amino acid residues 262 through 642 were found to be required for regulating ADR1 protein synthesis. The potential role of the 5' untranslated leader sequence of *ADR1* mRNA in controlling other processes, such as mRNA stability, remains to be investigated.

Our previous deletion analysis of the *ADR1* gene (2) did not reveal internal regions involved in glucose regulation of ADR1 protein synthesis due to the diminished capacity of the truncated ADR1 forms to activate *ADH2*. ADR1-262 protein, for example, would be expected to produce 10- to 16-fold more ADH II activity than ADR1-642 under glucose growth conditions on the basis of their respective rates of protein synthesis. However, one copy of *ADR1-642* is about four- to sixfold more active than *ADR1-262*. An increase in ADR1-262 protein abundance relative to ADR1-642 due to increased translation would, therefore, have been masked by its diminished intrinsic activity with respect to *ADH2* activation.

The molecular mechanism by which glucose reduces the rate of ADR1 translation, other than the small effect of *ADR1* mRNA levels, remains unclear. None of the genes known to affect *ADH2* expression, including *CCR1*, *ADR6*, *CCR4*, *CRE1*, and *CRE2* (9, 11, 30) appear likely to be involved in controlling *ADR1* mRNA translation. Previous searches for *trans*-acting genes that affect *ADH2* expression under glucose growth conditions have identified only *ADR1*^{c} mutations or mutations in the *CRE* genes, the latter of which act independently of ADR1 in controlling *ADH2* expression (11). Interestingly, the sequence in *ADR1* between nt 840 and 869 (corresponding to amino acids 281 and 290) predicts a perfect 13-nt stem and 4-nt loop structure. It remains possible that such a stem-loop might operate to impede translation directly or to serve as a binding site for a protein that, in turn, functions to regulate ADR1 translation rate. Deletion of this region, however, appears to have no effect on ADR1-dependent *ADH2* expression (unpublished observations). Other sequences that could potentially control ADR1 translation have not been identified.

Our results indicate that the overall repression of *ADH2* by glucose results from a combination of factors, including effects on *ADR1* RNA abundance, protein translation, and posttranslational activity. The existence of a single on-off switch controlling glucose repression in yeast, therefore, is unlikely. Instead, contributions from several mechanisms accrue to produce the 500-fold difference in ADH II enzyme levels observed between glucose and ethanol growth conditions. The occurrence of such a multicomponent system suggests an accretion of regulatory mechanisms during the evolution of *ADH2* regulation. The various components may represent historical additions that are mechanistically and perhaps evolutionarily unrelated to each other. The only comparably studied system with respect to the ADR1 *ADH2* system in yeast cells involving glucose repression is that of the galactose-metabolizing genes. In this system, at least several different control mechanisms have been identified to be responsible for the differences amounting to the 1,000-fold change in *GAL* gene expression observed under contrasting growth conditions (20).

A recent report (31) has suggested that ADR1 protein levels are equivalent under glucose and ethanol growth conditions. This study for the most part, however, utilized strains containing plasmid-borne copies of the *ADR1* gene. The possibility therefore exists that glucose-induced increases in *ADR1* plasmid dosage (in order to maintain the high ADH II activity required for fermentative growth) (12) and ethanol-induced reductions in *ADR1* plasmid dosages (in order to reduce *ADR1*-induced petite formation) (6) might have influenced the results. Because in this other study (31) neither the relative levels of *ADR1* mRNA nor the rates of ADR1 protein degradation or synthesis were analyzed for the one strain not containing plasmid-borne copies of ADR1, it is not possible to fully interpret the significance of these results with respect to ours. The regulation of ADR1 protein synthesis could be strain dependent since it has been observed that a couple of yeast strains, in contrast to the vast majority of strains analyzed (3, 4, 12, 17) (Fig. 6), display much reduced *ADR1* mRNA levels under glucose growth conditions (4). We have analyzed one of these strains (4) and have found that the greatly reduced *ADR1* mRNA levels are the result of at least several different factors not normally present in the yeast strains we commonly use (unpublished observations). To ensure consistency in all of our results and to avoid possible strain-dependent differences, our analysis of glucose regulation of *ADH2* (2, 7, 12, 17) has utilized isogenic or very closely related strains.

Our results indicate that ADR1 turns over with a half-life of about 3 to 4 h under glucose growth conditions. Other yeast proteins, as observed in Fig. 6a and b, appear to be significantly more stable than ADR1. ADH I and ADH II also have relatively long half-lifes in comparison to ADR1, on the order of at least 20 hours (10a, 19). We have also observed that the transcriptional activator CCR4 displays a half-life of 7 to 9 h (unpublished observations). The relatively short half-life of ADR1 protein suggests a mechanism for controlling cellular levels of ADR1 and for removing altered or damaged forms of ADR1 which may potentially decrease the efficiency of *ADH2* regulation. It is unclear whether the relatively rapid degradation observed for ADR1 is generally true for other transcriptional factors or whether such a mechanism contributes to the low cellular levels observed for such proteins. Such a mechanism might minimize the deleterious effects known to result from their overproduction in yeast cells (6, 21).

We also have demonstrated that ADR1 is a phosphoprotein. Phosphorylation of ADR1 occurred at multiple sites to generate multiple forms of the ADR1 protein that were distinguishable on the basis of their SDS-PAGE migration patterns and in their sensitivity to in vitro dephosphorylation by alkaline phosphatase. Phosphorylation of ADR1 was found to occur in cells pulse-labelled for only 15 min (data not shown), indicating that it occurred either during ADR1 translation or soon after translation was completed. The pattern of ADR1 phosphorylation also appeared to be the same in ethanol- and glucose-grown cells on the basis of SDS-PAGE analysis.

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