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Investigation of the mechanism of ADR1 mediated transcriptional activation

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INVESTIGATION OF THE MECHANISM OF ADR1 MEDIATED TRANSCRIPTIONAL ACTIVATION

BY

PHILIP B. KOMARNITSKY

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DISSERTATION

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DEDICATION

To my beloved wife Svetlana and to my grandfather Alexander.

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I wish to thank my wife Svetlana for her support and love. I would also like to thank my parents, Boris and Evelina, for their unconditional love and support throughout my life. It was a pleasure to work with Brad Anderson, Vasudeo Badarinarayana and Julie Farrell - I am grateful to all these people for providing friendly atmosphere in the laboratory. I am also grateful to the other members of Dr. Denis laboratory for their advice and help. I would like to thank Dr. Clyde L. Denis for his fair and generous support and guidance. I would also like to thank Dr. Andy Laudano for his helpful comments and suggestions during the course of this work. I would like to thank Yatin Gokarn for indian style dinners provided by request.

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ABSTRACT

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ADR1 MEDIATED TRANSCRIPTIONAL ACTIVATION

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The purpose of these studies was to examine the mechanism of function of the transcriptional activator ADR1 of yeast *Saccharomyces cerevisiae*. ADR1 is required for the activation of the *ADH2* gene, as well as genes involved in glycerol metabolism and peroxisome biogenesis. ADR1 is a DNA-binding protein. It binds to a 22 bp palindromic upstream activating sequence 1 (UAS1) element located 215 bp 5' to the transcription start site of *ADH2*. Elements similar to UAS1 are found in promoters of all ADR1-dependent genes. ADR1 was shown previously to contain three transcriptional activation domains (TADs): TADI (amino acids 76-172), TADII (263-357) and TADIII (420-462).

A novel activation domain, TADIV (residues 642-704), was identified in ADR1. Analysis of different derivatives of TADIV indicated that its activation function is principally localised to residues 698-704. In contrast to deletion of other ADR1 activation domains, deletion of activation domain IV from ADR1 severely compromised its ability to activate *ADH2* transcription.

ADR1 activation domains have been shown to directly interact *in vitro* with ADA2 and GCN5 components of the ADA2 co-activator complex, and both ADA2 and GCN5 are also required for full *ADH2* derepression. In addition, direct interaction of ADR1 TADs with TFIIB was observed, in which TADI displayed the strongest binding. A point mutation in TFIIB that reduced *ADH2* derepression was found to result in decreased

in vitro interaction of TFIIB with TADI. These results suggest that TFIIB is a functional contact for ADR1 activation of transcription.

Defects in ADA2, GCN5 and TFIIB do not severely reduce ADR1 ability to activate transcription, suggesting that there may be additional contacts that ADR1 makes with the components of transcriptional machinery. In order to identify these proteins, I examined which known components of the general transcription machinery are retained by the ADR1 activation domains from yeast whole cell extracts. It was found that ADR1 transcription activation domain IV (TADIV) specifically retained core transcription factor IID. Moreover, ADR1 could be co-immunoprecipitated with the TAF90 component of the TFIID from yeast whole cell extracts. *ADH2* activation by ADR1 required the presence of intact TFIID *in vivo*, suggesting that the physical interactions that were observed had functional relevance.

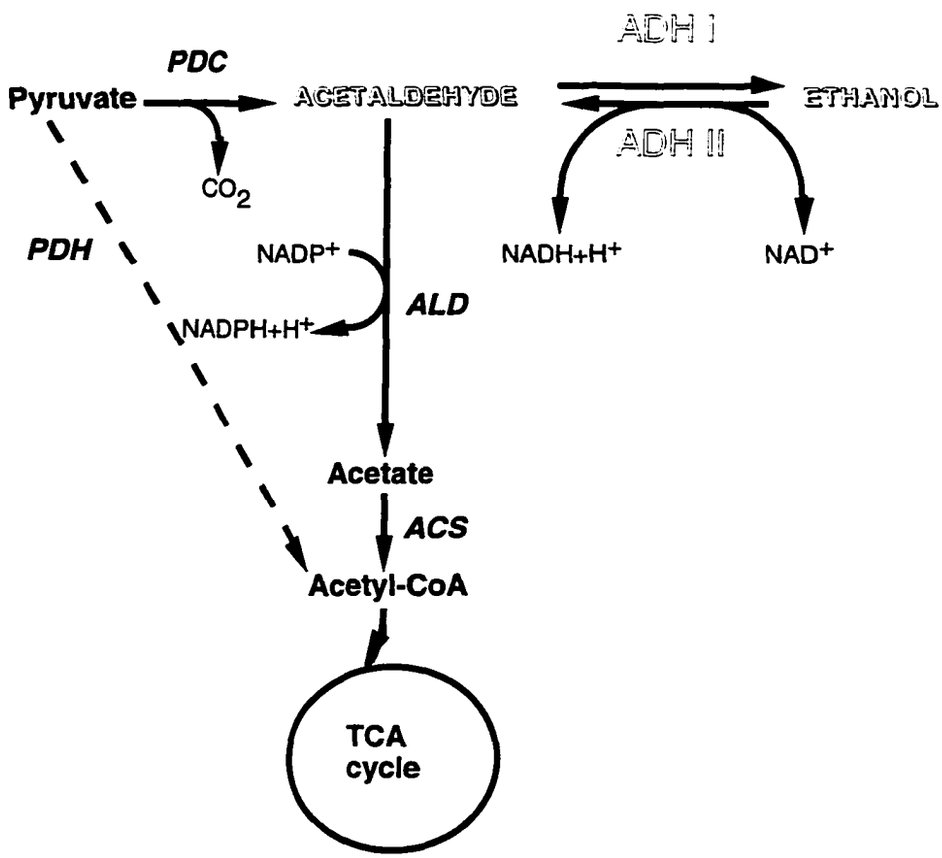
We propose that multiple contacts made by the ADR1 activation domains to different components of the general transcriptional machinery and to the ADA2 co-activator complex contribute synergistically to the activation of the *ADH2* gene. After facilitating the first rate limiting step - for example, TFIID recruitment, other steps in the preinitiation complex assembly become rate limiting, and even though transcription rate may have already become significant, it is further increased by ADR1 establishing new contacts to TFIIB and possibly other general transcription factors.

General Introduction.

The yeast *Saccharomyces cerevisiae*, when grown in the presence of glucose and a mixture of other carbon sources such as disaccharides, ethanol, or glycerol, are capable of selectively utilizing glucose only. This phenomenon is mediated by the repression of genes whose products are involved in carbon catabolic pathways other than glycolysis and is called "glucose repression". Under repressed conditions, glucose is fermented, and the end product is ethanol. Respiration in yeast is almost completely inhibited under these conditions. Yeast cells are believed to acquire a selective advantage by depriving other organisms of glucose [Ronne, 1995]. After glucose is depleted, yeasts can then utilize the previously secreted ethanol while most other organisms would not be able to use ethanol as a carbon source. The first enzyme in *Saccharomyces cerevisiae* ethanol utilization pathway is ADH II (alcohol dehydrogenase isozyme II) which catalyses formation of acetaldehyde from ethanol.

Yeasts contain three well characterized alcohol dehydrogenase isoforms - cytoplasmic ADH I and ADH II, and mitochondrial ADH III. ADH I isoform is most abundant when glucose is used as a carbon source. Its amount is reduced approximately 5-fold when nonfermentable carbon sources, such as ethanol or glycerol are used [Denis et al., 1983]. In contrast, ADH II isoform is undetectable under glucose (repressing) growth conditions and becomes a major isoform of ADH under nonfermenting growth conditions, i.e., it is subject to glucose repression. One of the biochemical reasons for the yeast cell switching from ADH I to ADH II is that ADH II has a lower K_m for ethanol [Wills, 1976]. ADH II therefore converts ethanol to acetaldehyde more efficiently than ADH I under the derepressing conditions, when acetaldehyde concentration in the yeast cell is very low (Figure 1).

Figure 1. Alcohol dehydrogenases in yeast metabolic pathways



Abbreviations:

ACS- acetyl-CoA synthase
ADH I- alcohol dehydrogenase isophorm I
ADH II- alcohol dehydrogenase isophorm II
ALD- acetaldehyde dehydrogenase
PDC- pyruvate decarboxylase
PDH- pyruvate dehydrogenase

Genes that are subject to glucose repression include gluconeogenic enzymes, Krebs cycle enzymes, components of the mitochondrial respiratory chain, peroxisomal proteins and ADH II. Glucose repression is achieved both at the posttranscriptional and transcriptional level. mRNA levels of some genes fluctuate by as much as 800-fold when yeast cells are shifted from a repressing carbon source, such as glucose, to derepressing conditions. In most cases this change is due to a dramatic increase in transcription, although some effects on mRNA stability have also been reported. For instance, a major determinant of the steady-state level of the mRNA encoding the iron protein (Ip) subunit of succinate dehydrogenase of yeast is its rate of turnover. This mRNA is significantly more stable in glycerol than in glucose media [Cereghino, 1996].

The molecular mechanisms underlying glucose repression are not completely understood, and the signal transduction pathway effecting glucose repression is largely unknown. The glucose itself or an early metabolite are not the triggers of the glucose repression pathway. It has been suggested that the repression is triggered by the glycolytic flux rather than by an early intermediate of glycolysis [Ronne, 1995].

The increase in the transcription rate of glucose-repressible genes occurs either by removal of negatively acting factors or by action of positive factors, or both. The mechanisms of transcriptional activation and repression in eucaryotes have been an area of intensive research for a number of years. A very large number of genes had been implicated in both processes. The genes participating directly in either process could be tentatively divided in two subgroups: one including genes whose products interact with or posttranslationally modify components of the general transcriptional apparatus and the other including genes involved in the maintenance or modification of chromatin.

The widely accepted model of DNA-binding transcriptional activator function proposes that these proteins, after binding to specific DNA sequences located in the

proximity of the transcription start site of the promoters, strongly interact with some components of the general transcriptional machinery and in turn bring them down to the promoter. This facilitates the assembly of a protein complex which is capable of transcription initiation. The minimal set of factors required for reconstituting accurate *in vitro* transcription initiation by RNA polymerase II includes TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and RNA pol II [Wang et al., 1992]. Since it had been established that the assembly of the initiation complex is an ordered process, i.e., every component joins the complex at a designated stage, it should be possible to identify the rate-limiting step in the process of the assembly of initiation complex on a given promoter. This step then should be the primary target of an activator, although considering that the formation of the initiation complex involves the interplay of many factors, other scenarios are also possible. *In vitro* studies with purified or partially purified components of general transcriptional apparatus have shown that TFIID binding to DNA is rate-limiting *in vitro* for at least some promoters, suggesting that this step represents a likely target for transcriptional activators. The observation that many activators work synergistically suggests that more than one step in the initiation complex assembly may be affected at once.

The sequential model of assembly of the initiation complex evolved in recent years with the discovery of the RNA polymerase II holoenzyme [Koleske and Young, 1989]. The largest subunit of RNA pol II, encoded in yeast by the gene *RPB1* has an unusual C-terminal structure, which consists of head-to-tail heptad repeats of the proline-rich sequence YSPTSPS. Truncation of a part of these repeats results in the inability of yeast carrying such a truncated *RPB1* to grow at 16^{0}C. This feature was used to select for mutations which would suppress temperature sensitivity and led to identification of a group of nine genes, called *SRB*s. SRB proteins turned out to be physically associated with RNA polymerase II in a protein complex which was named RNA polymerase II holoenzyme. Further analysis of the holoenzyme revealed that not only SRBs, but also such previously

identified general transcription factors as TFIIH, TFIIF, and TFIIB are associated in a large complex prior to the assembly on a promoter. The direct implication of this observation in the context of the activation of transcription is the substantial reduction of a number of steps required for assembly of the initiation complex. It also limits the number of steps that can be affected by an activator. It was suggested that the recruitment of the holoenzyme to the promoter is a one-step process [Carey, 1995].

DNA-binding transcriptional activators do not necessarily contact components of the general transcription machinery directly. A number of proteins have been found to be required for activated transcription but which are dispensable for basal transcription. Some of these proteins were shown to physically associate with transcriptional activators and were named co-activators or adaptors. These proteins are believed to mediate the activation of transcription by bridging DNA-bound transcriptional activators and the components of general transcriptional machinery. In yeast, co-activator function was attributed to the ADA2 complex and in mammals a number of co-activators had been described, for example CBP/p300, p/CIP, PCAF, TBP-associated factors (TAFs). Interestingly, it was found that many of the co-activators posess histone acetyltransferase activity, suggesting that they may mediate transcriptional activation in part by modifying chromatin structure at the target promoters.

Nucleosomes (chromatin) present a significant obstacle for the transcription factors in that it reduces the accessibility of DNA binding sites. A number of proteins have been identified in recent years that are capable of changing chromatin structure by modifying nucleosomes postranslationally or physically rearranging them. The influence of these factors on transcription can be both repressing or activating depending on what kind of modification they bring about. The specificity of the effect of chromatin modifying factors (i.e., how these factors locally modify chromatin at a particular promoter) is achieved via interaction with specific DNA-binding proteins or via incorporation of these factors into the general transcription apparatus. Protein complexes identified to date that are

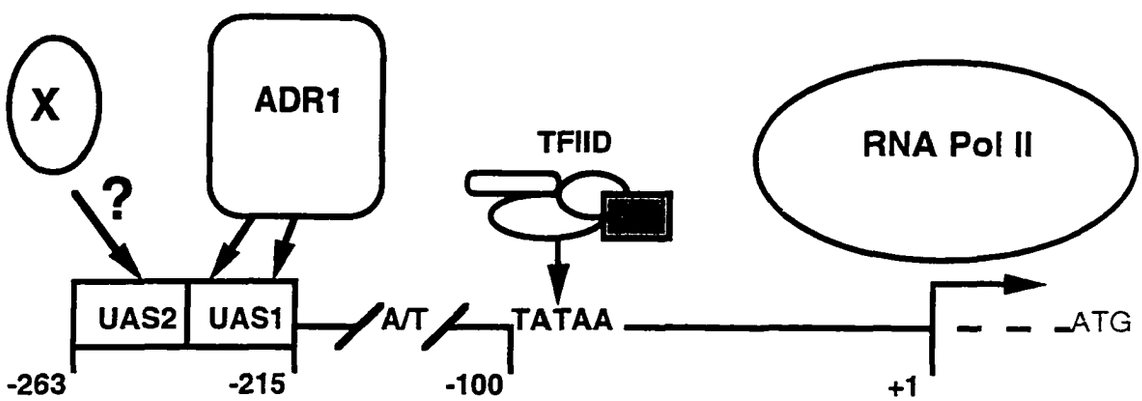
believed to be required for relieving the repressive structure of chromatin include the SNF/SWI complex, some of the SPT factors [Bortvin et al., 1996; Dollard et al., 1994], the RSC complex [Cairns et al., 1996], the ADA2/GCN5 complex *in Saccharomyces cerevisiae* , and a number of similar protein complexes in Drosophila, mice and humans. The nature of chromatin modification caused by SNF/SWI or RSC complex is unknown, and probably does not involve covalent modification of histones. The SNF/SWI complex was found to be tightly associated with the RNA polymerase II holoenzyme [Wilson et al., 1996]. The GCN5 component of multiprotein ADA2/GCN5 complex is a histone acetyltransferase, which is capable of acetylation of some of the lysine residues in the histone N-termini. GCN5 is required for activated transcription of some, but not all, yeast genes [Brownell at al., 1996, Candau et al., 1997]. Several other histone acetylases (HATs) playing a role in transcription have been identified to date, for example TAF250, CBP, pCIP [Mizzen et al. 1996, Yang et al., 1996]. Since the acetylation of histones results in relieving repression caused by chromatin at some promoters, the removal of acetyl residues from histones should result in the opposite effect. Indeed, a number of recent reports have appeared linking histone deacetylation with transcriptional repression. It was demonstrated that in yeast transcriptional repression by Ume6, a negative regulator of genes involved in meiosis, is mediated by recruitment of the Sin3/Rpd3 complex. Rpd3, a component of this complex and a histone deacetylase, can efficiently repress transcription by itself when brought to DNA by fusing to a heterologous DNA-binding domain [Kadosh, 1997]. Similarly, the mouse homolog of yeast Sin3, mSin3A, mediates transcriptional repression by Mad/Max heterodimer, and mSin3A is a component of large multiprotein complex with histone deacetylase activity essential for effecting repression [Hassig, 1997].

The research presented in this thesis is aimed at identifying the molecular mechanisms by which activation of the *ADH2* gene is achieved by its transcriptional activator ADR1.

When glucose is removed from the growth medium and yeast are forced to grow on a nonfermentable carbon source, *ADH2* transcription is derepressed and ADH II activity increases 500-fold [Ciriacy, 1975]. DNA sequences located -176 to -453 bp upstream of the *ADH2* transcription start site are required for regulated *ADH2* transcription [Beier and Young, 1982]. Two upstream activating sequences in this region, UAS1 and UAS2, were shown to be required for full derepression of the *ADH2* gene. The 22 bp palindromic UAS1 had been shown to bind transcriptional activator ADR1, and UAS2 is believed to bind an as yet unidentified factor which acts synergistically with ADR1 to activate *ADH2* transcription (Figure 2).

ADR1 had been cloned [Denis et al., 1983] and sequenced [Hartshorne et al, 1986]. It contains 1323 amino acids, and immunoprecipitation from yeast crude extracts reveals that the protein has a MW of 150 kDa [Vallari et al, 1992]. Residues 1-16 of ADR1 were found to be necessary for its nuclear localization [Thukral et al., 1989]. The DNA-binding domain of ADR1 is located in the N-terminus of the protein spanning residues 72-176. This domain contains two zinc fingers of C_2H_2 type (Figure 3), and mutation analysis had shown that this region is essential for ADR1 function and DNA binding [Thukral, 1989]. Zinc fingers of C_2H_2 type are also found in many DNA-binding proteins, for example TFIIIA [Miller et al., 1985], Sp1 [Kadonaga et al., 1987], Zif268 [Pavletich and Pabo, 1991], and Krox20 [Nardelli et al., 1991]. More detailed analysis of the 72-176 aa region suggested that besides being a DNA-binding domain this region also has a transcriptional activation function. First, expression of ADR1 fragment 72-176 or 1-220 in a yeast strain deleted for ADR1 resulted in *ADH2* derepression [Cook et al., 1994]. Second, some of the mutations in this region resulted in a loss of its ability to activate transcription from a heterologous promoter while apparently having no effect on DNA binding [Cook et al., 1994a]. Taken together, these observations designated amino acids 72-176 as transcriptional activation domain I (TADI).

Figure 2. *ADH2* promoter structure



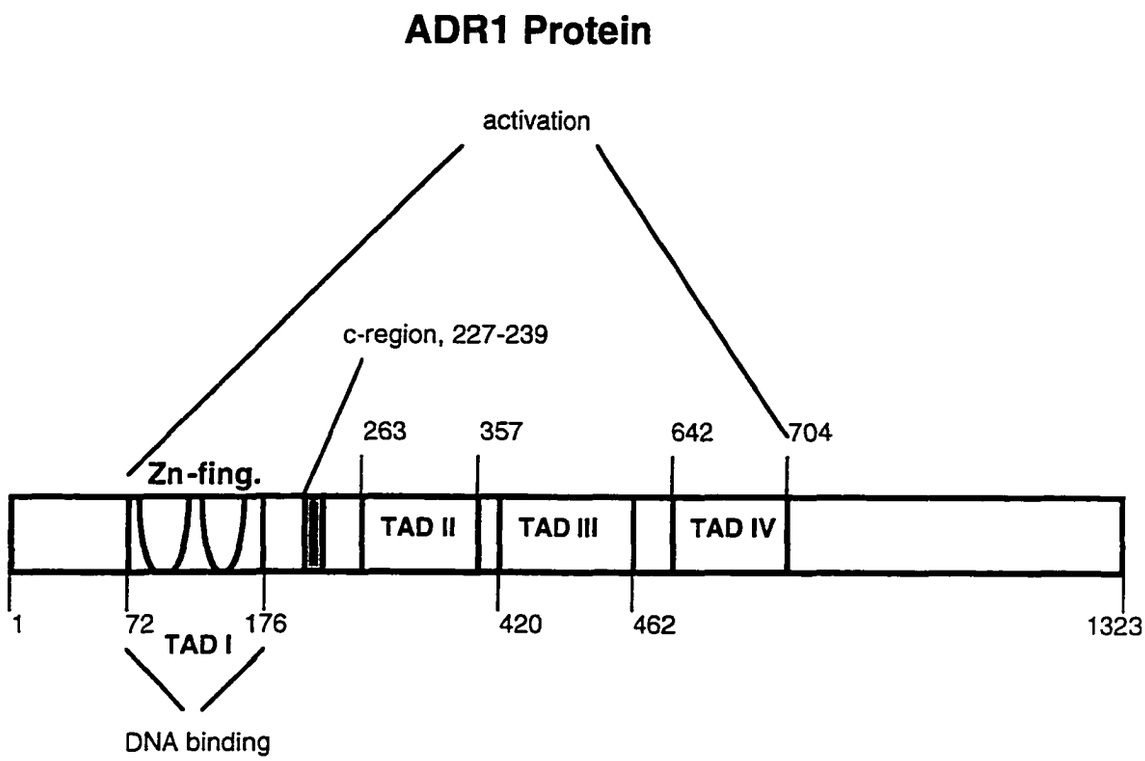
Sequence of ADH2 promoter:

5'-cgagacTGATCTCCTCTGCCGGAACACCGGgcaTCTCCAACTTATAAGTTGGAGaaa
taagagaatttcagattgagagaatgaaaaaaaaaaaaaaaaaaaggcagaggagagcatagaaatggggttcacttttggtaaagctatagcat
gcctatcacaTATAAatagagtgccagtagcgacttttttcacactcgaaatactcttactactgctctcttgttgtttttatcacttcttgtttcttcttggta
aatagaatatcaagctacaaaaagcatacaatcaactatcaactattaactatatcgtaatacacaATGTCTATTCCAGAAACTCAA
AAAGCCATT-3'

UAS2 and UAS1 region of the ADH2 promoter:

5'-acTGATCTCCTCTGCCGGAACACCGGgca**TCTCCAA**CTTATAAG**TTGGAG**a-3'

Figure 3. Structure of the ADR1 protein



Abbrevations:

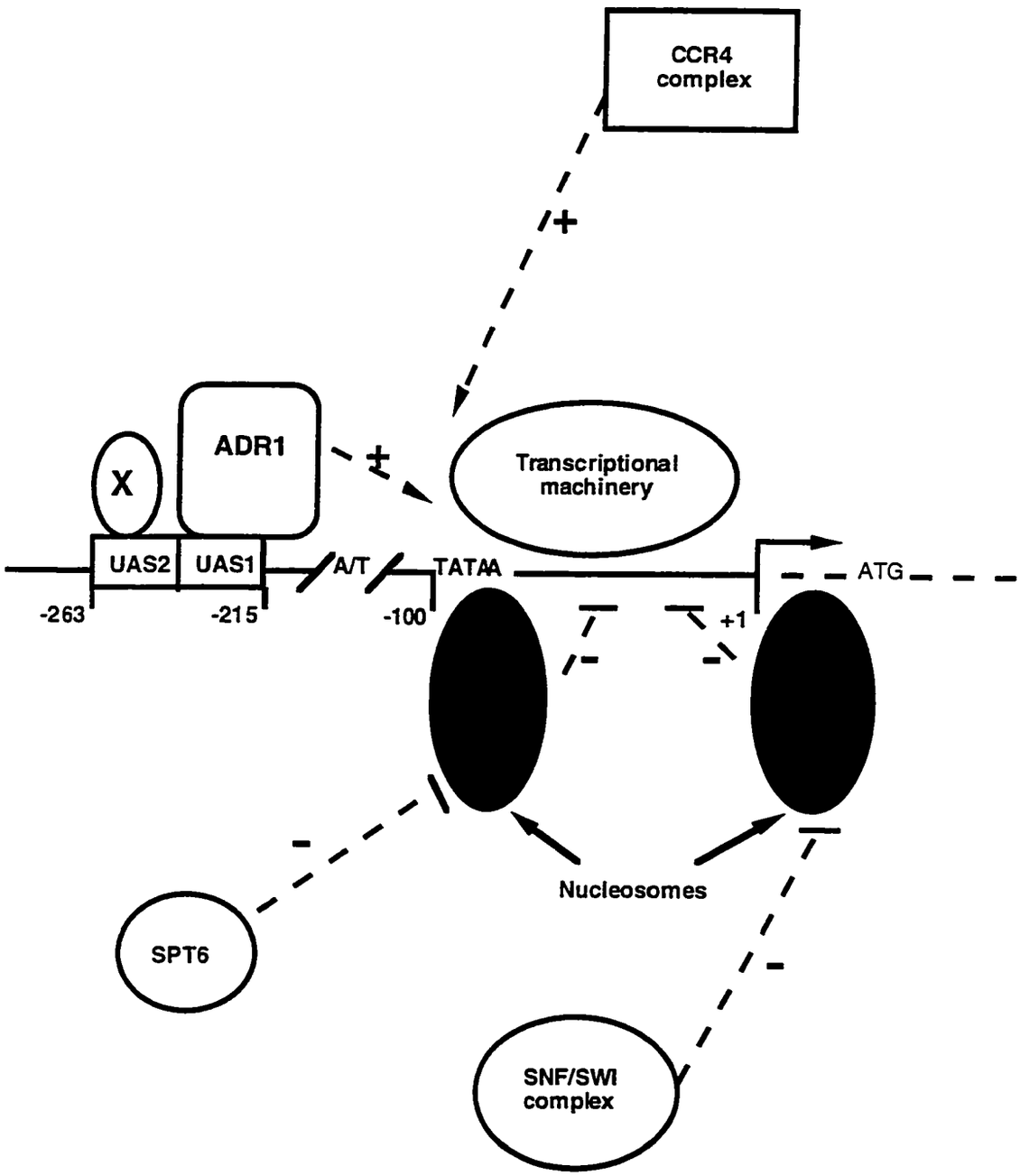
TAD- transcription activation domain
Zn-fing.- zinc finger DNA binding domain

Deletion analysis of the ADR1 protein revealed two additional regions important for activation of transcription that were localized to residues 262-288 (TADII) and 420-462 (TADIII) [Cook et al., 1994a, Young E.T., unpubl. observ.] (Figure 3). These fragments of ADR1 were also able to activate transcription when fused to a heterologous DNA-binding domain. Deleting both TADII and TADIII from the full length ADR1 protein resulted in the decrease of its activation potential. Deletion of any single one of these domains, however, had very little or no effect on ADR1-mediated activation. This result suggested that TADII and TADIII have partially redundant functions in transcriptional activation, presumably by affecting the same step in the activation process.

A substantial number of trans-acting factors other than ADR1 have been implicated in the regulation of *ADH2* gene expression (Figure 4). They can be divided into two groups based on the effect on *ADH2* expression. One is comprised of genes whose products are required for *ADH2* derepression to occur, and mutations in these genes result in complete or partial derepression defects on nonfermentable carbon sources. The second group includes genes required for glucose repression, and mutations in these genes result in improper expression of *ADH2* when glucose is present in the growth medium.

The first group includes ADR1, CCR1/SNF1, CCR4, CAF1, and components of the SNF/SWI complex. CCR1/SNF1 is a protein kinase whose activity is required for derepression of all glucose repressible genes, CCR4 is a component of a large protein complex whose other components are NOT1, NOT3 and a number of CAFs (CCR4 Associated Factors) and the precise role of this complex in transcription is not very well understood [H.-Y. Liu, pers. commun.]. Many of the components of this complex, including CCR4 itself, are well conserved among eucaryotes. SNF/SWI proteins are involved in nucleosome rearrangements, and mutations in those genes lead to mild defects in *ADH2* derepression. Also required for full *ADH2* derepression are some of the *SPT* genes, such as *SPT3* and *SPT7* [Denis C. L., unpubl. observ.], whose function is believed to be related to the TATAA box selection by TFIID.

Figure 4. Regulation of the *ADH2* promoter



The second group of genes, mutations in which allow partial escape from glucose repression, includes chromatin remodeling factor *SPT6* (*CRE2*), another member of the Spt group of proteins - *SPT10* (*CRE1*) [Denis, 1984], and the gene partly responsible for glucose repression at the *ADH2* locus, *REG1* [Dombek et al., 1993] . Surprisingly, a set of dominant mutations in the *ADR1* gene was isolated that allowed partial escape from glucose repression. All these mutations were amino acid substitutions in the narrow region of the ADR1 protein, amino acids 227-239. Deletion of this region from ADR1 also led to the partial escape from glucose repression. Since this region represented a consensus sequence for cAMP-dependent protein kinase (cAPK) phosphorylation, and some of the mutations leading to partial derepression of *ADH2* drastically reduced the ability of cAPK to phosphorylate this region, it was concluded that ADR1 activity is regulated posttranslationaly and that cAPK phosphorylates and inhibits ADR1 activity under fermentative growth conditions [Cherry et al., 1989]. However, additional experiments [Denis et al., 1992] proved this not to be the case: yeast strains with no detectable cAPK activity still were able to normally repress *ADH2* on glucose, and a number of mutations in the 227-239 region while allowing glucose-insensitive *ADH2* expression did not affect the ability of cAPK to phosphorylate this region. Based on these observations, as well as on the fact that the 227-239 region when fused to a synthetic activator reduces the synthetic activator ability to activate transcription [Cook et al., 1994], it was suggested that the 227-239 region binds a repressor protein. Yet another region of ADR1 which allowed partial escape from glucose repression as well as higher level of *ADH2* expression under derepressing conditions was mapped by deletion analysis to amino acids 282-330 [Cook et al., 1994a].

Since ADR1 plays a pivotal role in *ADH2* derepression, the transcriptional and translational effects of glucose on ADR1 were extensively investigated [Vallari et al., 1992, Cook, 1994a]. It was found that ADR1 protein level is reduced 5- to 8-fold in glucose

versus ethanol grown strains, and *ADR1* mRNA level is reduced approximately 2-fold. Two-fold reduction of *ADR1* mRNA level appears to result from the increased degradation rate of *ADR1* mRNA when yeast are grown in the presence of glucose, which is dependent on a long 5'-untranslated region found in *ADR1* mRNA. *ADR1* mRNA half life in a strain grown in glucose-containing medium is 45 min. compared a half life of 110 min. when ethanol is present as the carbon source. The much more significant reduction of ADR1 protein level in glucose-grown cells is due to the reduction of ADR1 protein synthesis, and this effect is mediated by the region of ADR1 encompassing amino acids 262-642 of the protein. A recent report [Dombek et al., 1997] indicates that the reduction of ADR1 protein level under fermentative growth conditions is mediated via the cAMP-dependent protein kinase pathway. An interesting observation made by Vallari et al., 1992 indicates that the reduction of ADR1 level on glucose plays a functional role in regulation of *ADH2* expression: an 8-fold dosage increase of *ADR1-5*^{c} allele, resulting in the ADR1-5^{c} protein level on glucose similar to the ADR1 protein level on ethanol in a strain carrying single copy of ADR1 gene, gives the level of *ADH2* expression on glucose identical to that for the single copy ADR1-containing strain on ethanol. This observation indicates that a combination of post-translational modification or interaction with repressor protein and reduction in the ADR1 protein level contributes to the glucose repression of the *ADH2* gene.

The analysis of sequence features of ADR1 TADs provided little clue as to the mechanism of their function. No significant similarity to the activation domains of other transcriptional activators from yeast or other eucaryotes was found. The comparison of transcriptional activation domains from a variety of activators allows one to outline only very general sequence features characteristic of transcriptional activation domains. One class of these domains contains acidic residues interspersed with bulky hydrophobic amino acids and is predicted to form amphipathic α-helices. However no conclusive experimental

proof that these sequences actually form amphipathic α-helices exist. Some experimental evidence [Sainz et al., 1997] indicates, that contrary to expectations, introduction of α-helix - incompatible prolines into transcription activation domain of maize activator C1 which is predicted to form an amphipathic α-helix, has no effect on C1 function. Recent experiments with the VP16 activatior showed that the activation domain is most likely an unstructured coil which is induced to undergo a significant structural alteration, presumably to an α-helix, upon binding to the human TAF31 [Uesugi et al., 1997]. Two hydrophobic residues and an aspartate were found to make direct contacts with hTAF31. The second class of the transcription activation domains is comprised of glutamine-rich activation domains. An example is the well described human transcriptional activator Sp1. Interestingly glutamine-rich transcriptional activators do not function in yeast, and the reason for this failure to activate transcription is believed to be the absence of the appropriate mediator of activation (co-activator) in yeast cells.

The focus of this dissertation is to elucidate the mechanisms of ADR1-mediated activation of *ADH2*. In order to address this problem, the approach was taken to identify proteins that physically interact with ADR1 or its individual activation domains and to use genetic techniques to study the effect of these proteins on *ADH2* transcription *in vivo*. It was found that ADR1 TADs make multiple contacts to components of basal transcriptional machinery and to coactivators. Using protein affinity chromatography and immunoprecipitation techniques specific interaction of ADR1 with coactivators GCN5 and ADA2 and with basal factors TFIIB and TFIID was demonstrated. Deletion of *GCN5* or *ADA2* genes negatively affected ADR1 mediated *ADH2* derepression. Targeted depletion of a TAF90 component of the TFIID was found to completely prevent *ADH2* derepression.

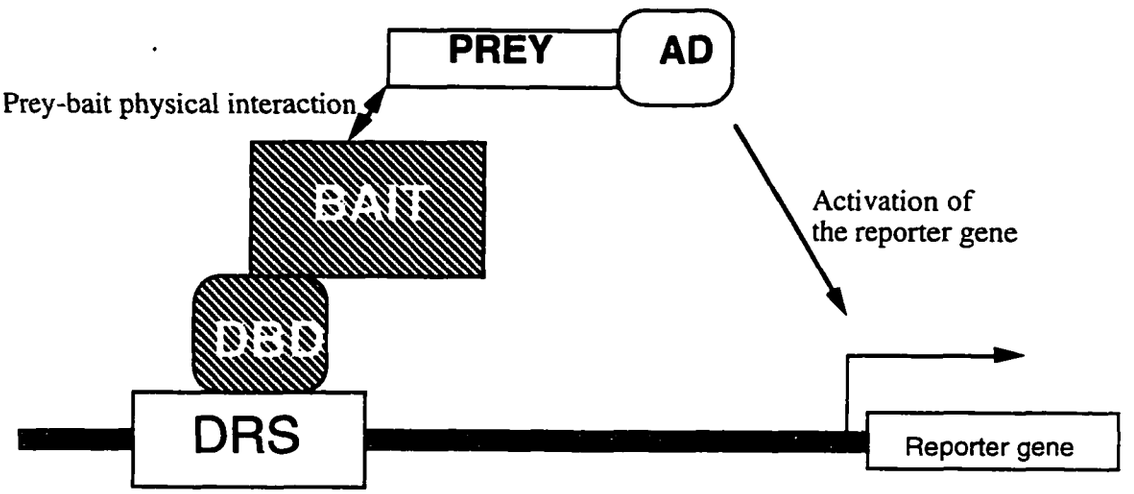
Chapter I.

The identification of factors, interacting with the ADR1 activation domains. Identification of Transcription Activation Domain IV.

Introduction.

Three activation domains had been previously identified in the ADR1 protein: transcription activation domain one (TADI; amino acids 72-176), which overlaps with the DNA-binding domain, TADII (amino acids 262-359), and TADIII (amino acids 420-462) (Figure 3). The sequence of these activation domains did not reveal significant similarity to any of the previously found transcriptional activators. TADIII amino acid composition is fairly acidic (10 acidic residues out of 30 in the 421-456 region), and the structure of this region partially agrees with the consensus sequence of the acidic activation domains of such *Saccharomyces cerevisiae* transcriptional activator as GAL4, and viral activator VP16 [Cook, 1994a]. A fourth region (642-1323) has been implicated for efficient peroxisomal gene expression [Simon et al., 1995] and was presumed to contain a cryptic activation domain. The precise function of the C-terminal region of ADR1 had been, however, unknown. In order to investigate the function of this region, we tried to identify other yeast proteins that may interact with it using the two-hybrid system [Fields and Song, 1989]. In our two-hybrid experiments we used the DNA-binding domain of the *Escherichia coli* LexA protein fused to residues 642-1323 or 1-262 of ADR1 and the GAL4 transcriptional activation domain or synthetic transcriptional activation domain B42 fused to the yeast genomic fragments (Figure 5).

Figure 5. Two-hybrid system



Abbreviations:

AD- activation domain
Prey- protein of interest fused to the activation domain
Bait- another protein of interest fused to the DNA binding domain
DBD- DNA binding domain
DRS- DNA recognition sequence

Materials and Methods.

Yeast and *Escherichia coli* strains, growth conditions and enzyme assays.

Yeast strains are listed in Table 1. Conditions for growth of yeast cultures on minimal medium or YEP medium, containing either 8% glucose or 2% ethanol have been described elsewhere [Cook et al., 1994]. YD8 solid medium contained YEP supplemented to 8% glucose and 2.5% agar.

All subcloning and DNA manipulations were conducted in *Escherichia coli* DH5α strain.

ß-galactosidase assays were conducted on yeast extracts as described elsewhere [Cook et al., 1994]. ADH II activity was assayed as described elsewhere [Denis, 1987].

Plasmids.

LexA202 plasmids are multicopy 2μ plasmids and have been previously described [Ruden et al., 1991]. The 34 "reporter" plasmid is a 2μ based plasmid containing eight LexA operator sites, controlling the *LacZ* gene [Brent and Ptashne, 1985]. The JG4-5 vector as well as yeast genomic library based on this vector have been described elsewhere [Zervos et al., 1993]. The pPC86 vector and the library with GAL4 transcriptional activation domain have been described elsewhere.

Plasmid pJK101 used in the transcription interference assay contains GAL1 promoter with LexA operator inserted 3' to the UAS_G and was constructed as described [Cook et al., 1994a and references therein].

The construction of plasmids $LexA_{202}$-ADR1-642-1323 and $LexA_{202}$-ADR1-1-220 is described elsewhere [Cook et al., 1994a].

Table 1. Yeast Strains

Strain	Relevant Genotype
EGY188	MATa ura3-52 his3 leu2 trp1 $LexA_{op}$-LEU2
938-9b	MATa ura3-52 his4 $LexA_{op}$-LEU2
YEK20	MATa ura3 his3 trp1 leu2 suc2 ade2 lys2 taf25::TRP1 pRS413-$HA1_3$-TAF25
yBY40-8'	MATa ura3 his3 trp1 leu2 suc2 ade2 lys2 taf90::TRP1 pRS413-$HA1_3$-TAF90
EGY188-aif1	MATa ura3-52 his3 trp1 $LexA_{op}$-LEU2 aif1::URA3
EGY188-g1	MATa ura3-52 his3 trp1 $LexA_{op}$-LEU2 gcn5::URA3
PSY316	MATα ade2-101 his3-Δ200 leu2-3, 112 lys2 ura3-52
PSY316-ada2	MATα ade2-101 his3-Δ200 leu2-3, 112 lys2 ura3-52 ada2::URA3
YMH130	MATa cyc1 his3Δ1 leu2 trp1 ura3-52 sua7::LEU2 ade1 pRS313-SUA7
GMY26	MATα ade2-101 his3-Δ200 leu2-3, 112 lys2 ura3-52 gcn5::URA3
CY26	MATα ade2-101o his3-Δ200 leu2-Δ1 trp1- Δ1 lys2-801a ura3-52
CY57	MATα ade2-101o his3-Δ200 leu2-Δ1 trp1- Δ1 lys2-801a ura3-52 swi2::HIS3
612-1d	MATa ura3 his3 leu2 trp1 adh1-11
612-1d-a1	MATa ura3 his3 leu2 trp1 adh1-11 ada2::URA3
612-1d-g1	MATa ura3 his3 leu2 trp1 adh1-11 gcn5::URA3
612-1d-6a	MATa ura3 his3 leu2 trp1 adh1-11 caf6::LEU2
1005-2-3b	MATα ura3 his3 leu2 trp1 spt10::TRP1 adh1-11
787-6b	MATα adh1-11 adr1-1::ADR1-5^c-TRP1 ura3 leu2 his3 trp1
40-1c	MATα ura3 his3 trp1 adh1-11 adr1-1::ADR1-TRP1

Plasmid construction

Deletion of amino acids 642-704 from the full length ADR1 was conducted as described [Appendix A]. The resulting pBR322-411BΔTADIV was cut with SstI and SnaBI and ligated to pAK52, cut with the same restriction enzymes. The resulting construct, pAK52ΔTADIV was then digested with BstEII, blunt ended with the large subunit of *Escherichia coli* DNA polymerase I (Klenow), and subsequently cut with NcoI. An ADR1 fragment containing residues 1-642/704-1323 was ligated into pLexA202-5, which was digested with XhoI, blunt ended with the Klenow enzyme, then cut with NcoI. This procedure yeilded $LexA_{202}$-ADR1 ΔTADIV. Since in this construction the junction between LexA and ADR1 moieties differed from that of the original $LexA_{202}$-ADR1, the new $LexA_{202}$-ADR1 with the identical sequence of the junction site was constructed. To construct $LexA_{202}$-ADR1 fusion deleted for residues 675-704, ADR1 from pAK52 was PCR amplified with two primer pairs. Amplification with pair one gave ADR1 fragment 1-675 with NcoI at 5' end and EcoRV site at 3' end. Amplification with pair two gave fragment 704-1323 with EcoRV site at the 5' end and SalI site at 3' end. Fragment one was digested with NcoI and EcoRV, and ligated into pGEM3Zf that had been digested with the same restriction enzymes. The resulting construct was digested with EcoRV and SalI and the PCR product corresponding to the fragment 704-1323 (digested with the same enzymes) was ligated into it. pGEM3Zf-ADR1Δ675-704 was cut with NcoI-SalI, and the ADR1 moiety was ligated into LexA202-5 digested with the same enzymes, resulting in $LexA_{202}$-ADR1Δ675-704.

The full length *AIF1* gene was isolated as described below, and subcloned into pUC19. The resulting construction was cut with NcoI and SalI, generating full length *AIF1* which was ligated into LexA202-5 vector digested with the same enzymes.

The $LexA_{202}$-AIF1 containing only the C-terminal fragment present in the library clone was constructed by cutting the library clone with EcoRI, and ligating the resulting AIF1 fragment into pLexA202-1 cut with the same restriction enzyme.

Plasmid rescue from yeast cultures

In order to obtain sufficient amount of plasmid DNA for cloning and sequencing purposes, plasmids need to be rescued from yeast and transformed into *E. coli*. The yeast strain containing the plasmid to be rescued was grown overnight in liquid culture in 2 ml of the appropriate selective medium supplemented with 2% glucose. Yeast cells were collected by centrifugation at 1000 g, and washed in 5 ml of TE. Washed cells were resuspended in 500 ml TE buffer, 200 units of glucilase were added to the tube and yeast cells were incubated with glucilase at 30°C for 1 hour in the shaker. Yeast cells were transferred to a microcentrifuge tube, collected by centrifugation at 500 g, and gently washed in 1 ml TE. Cell pellets were resuspended in 200 ml Cell Resuspension Buffer (Promega Corp., Magic Miniprep), 200 ul of Cell Lysis Buffer (Promega Corp., Magic Miniprep) and 120 ml 10% SDS was added and mixed thoroughly. The suspension was boiled for 5 minutes, then 300 ml Cell Neutralization Solution (Promega Corp., Magic Miniprep) was added and the mixture was kept at 4°C for 10 minutes. After centrifuging for 5 minutes at 6000 g in a tabletop microcentrifuge, 600 ml of the supernatant fluid were mixed with 1 ml of DNA Binding Resin Slurry (Promega Corp. Magic Miniprep), and the protocol for Promega Corp. Magic Miniprep was then followed. Five ml of rescued DNA solution was used to transform *Escherichia coli* DH5α strain.

Isolation of clones, sequencing and homology search

The $LexA_{202}$-ADR1-642-1323 truncations and AIF clones were rescued from yeast cells as described above and sequenced by using the Sanger dideoxy method or by

PCR-based cycle sequencing at the UNH Sequencing Facility. The full length clone of the *AIF1* gene was isolated from the yeast genome cosmid 9315 containing a fragment of yeast chromosome VIII.

Sequence comparisons were performed at the National Center for Biotechnology Information using the BLAST Web service [Altshul et al., 1990].

Western Blotting

Western blot anslysis of LexA and B42 fusion proteins was performed as describedby others [Draper et al., 1994]. To detect LexA fusions, rabbit polyclonal antibodies raised against the LexA 1-202 fragment were used. Commercially available mouse monoclonal 12CA5 anti-HA1 antibodies were used to detect HA1-tagged B42 fusion proteins.

Results.

Part I.

Identification of the Transcription Activation Domain IV of ADR1.

With the use of the two-hybrid system, we sought to identify proteins interacting with ADR1. The transcriptionally inactive ADR1 fragment spanning residues 642-1323 was fused to the LexA DNA binding domain [Cook et al., 1994a]. The vector expressing this fusion protein was introduced into yeast strain EGY188 containing two reporters: $LexA_{op}$-*LEU2* at the chromosomal locus and $LexA_{op}$-*lacZ* on a 2μ-based high copy vector. EGY188 with $LexA_{op}$-*LEU2*, $LexA_{op}$-*lacZ* and expressing LexA-ADR1-642-1323 was unable to grow on Leu^- selective plates and ß-galactosidase activity in this strain was <5 milliunits/mg protein. The strain was also white on plates containing X-gal, because of the absence of ß-galactosidase activity.

A yeast library, containing genomic fragments fused to the GAL4 transcription activation domain, under the control of the *ADH1* promoter, was transformed into this strain. The total number of transformants obtained was $1x10^6$. This number is sufficient to represent every gene in yeast genome with a 99% probability.

After the transformation, yeast were plated on $Ura^-His^-Trp^-Leu^-$ selective plates and incubated at 30^0C. The Ura^- selection is used to maintain plasmid borne $LexA_{op}$-*lacZ*, His^- selection maintains the $LexA_{202}$-ADR1-642-1323 expressing plasmid, Trp^- selection maintains the library plasmid, and the only yeast cells that were able to activate the $LexA_{op}$-*LEU2* reporter were able to form colonies on $Ura^-His^-Trp^-Leu^-$ selective plates. Such colonies were picked, plated on $Ura^-His^-Trp^-Leu^-$ selective plates containing X-gal to test

for the activation of the *LexA$_{op}$-lacZ* reporter. Finally, colonies that were able to activate both *LexA$_{op}$-lacZ* and *LexA$_{op}$-LEU2* reporters were tested for the dependency of the observed activation on the presence of library plasmid. The library plasmid containing the *TRP1* selectable marker was lost from the cells by repeatedly streaking them to single colonies on Ura$^-$His$^-$ plates that do not select for the maintenance of the *TRP1* gene. Colonies that lost *TRP1* were tested for the ability to activate *LexA$_{op}$-LEU2* and *LexA$_{op}$-lacZ* reporters. Surprisingly, in all colonies tested the activation of both reporters did not depend on the library plasmid. Further analysis indicated that the ability to activate the reporter genes was linked to the LexA$_{202}$-ADR1-642-1323 containing plasmid. Rescuing the LexA$_{202}$-ADR1-642-1323 from the colonies that were able to activate *LexA$_{op}$-lacZ* and *LexA$_{op}$-LEU2* reporters and re-transforming them into the original strain containing both reporters resulted in reporter activation.

Western analysis indicated that in all cases the truncation in the ADR1 moiety of the LexA$_{202}$-ADR1-642-1323 has occurred (Table 2). The 56 isolates analysed were subdivided into 9 groups according to the apparent size of the truncated derivative. The ß-galactosidase activities of the isolates were very close within each group, and the ß-galactosidase activity correlated with the truncation size, the longest truncation derivative having the highest transcriptional activity.

Sequencing of 2 representative clones from each truncation group revealed the premature stop codons corresponding to amino acids 675 (group 1), 679 (group 2), 687 (group 3), 692 (group 4), 694 (group 5), 698 (group 6), 701 (group 7), and 704 (group 8) of ADR1. The corresponding ß-galactosidase activities for each group are listed in Table 2. Since the 642-704 fragment of ADR1 was capable of activating transcription when fused to LexA DNA binding domain, we tested the effect of deletion of this small region on the transcriptional activation potential of the full length ADR1 protein. Deleting the amino acids 642-704 resulted in the 20-fold reduction of the LexA$_{202}$-ADR1-1-1323 fusion ability to

Table 2. Transcriptional activity of $LexA_{202}$-ADR1-(642-1323) truncation derivatives.

LexA-ADR1-TADIV deriva	ß-galactosidase activity *(units/mg)*
$LexA_{202}$-ADR1-(642-704)	190
$LexA_{202}$-ADR1-(642-701)	85
$LexA_{202}$-ADR1-(642-698)	70
$LexA_{202}$-ADR1-(642-694)	18
$LexA_{202}$-ADR1-(642-692)	30
$LexA_{202}$-ADR1-(642-687)	24
$LexA_{202}$-ADR1-(642-679)	8
$LexA_{202}$-ADR1-(642-675)	3
$LexA_{202}$ alone	1.5

β-Galactosidase activities were measured as described in Appendix A. Values represent averages of at least three separate transformants in the strain EGY188. S. E. values were less than 20%. The LexA reporter was p1840 in all cases.

activate transcription of the *LexA$_{op}$-lacZ* reporter gene (Table 3). To study the effect of amino acids 642-704 deletion on the ADR1 transactivation of its physiological target, the *ADH2* gene, this region was deleted from the chromosomal copy of the ADR1 gene by a one step intergation procedure [Rothstein, 1991]. The *ADR1* allele deleted for amino acids 642-704 had a 30-fold decreased ability to activate *ADH2* transcription. The ADR1 deleted for TADIV was also unable to support growth of *Saccharomyces cerevisiae* on oleic acid [C. L. Denis, pers. commun.] in agreement with the previously reported importance of C-terminal part of the protein for growth on this carbon source [Simon et al., 1995]. An *ADR1-5^c* allele deleted for residues 642-704 was also severely defective in *ADH2* gene activation. Adding back amino acids 642-675 did not restore the activity of LexA$_{202}$-ADR1 fusion to the wild type levels (Table 3).

The ability of amino acids 642-704 to activate transcription when fused to a heterologous DNA-binding domain as well as the importance of this region for the ADR1 function led us to designate the residues 642-704 as transcriptional activation domain IV (TADIV).

The BLAST homology search of the Genbank database did not reveal strong similarity of TADIV to any of the previously identified transcriptional activation domains. The TADIV region had weak similarity to a region of yeast transcriptional activator ZMS1 (37.1% identity in 35 residues overlap, Figure 6). ZMS1 contains other similarities as well: a highly homologous Zn-finger region with two C_2H_2 type fingers, and a region highly resembling the ADR1 227-239 region (Figure 6). No data are available on the function of the TADIV-like region in the ZMS1 protein.

The analysis of the TADIV sequence with the Protean program from DNA Star software package gives conflicting secondary structure predictions depending on the algorithm used. While residues 660 to 669 and 680 to 689 are predicted to form alpha helices by both Garnier-Robson and Chou-Fasman methods, the region of amino acids

Figure 6. Pairwise alignment of ADR1 and ZMS1.

```
Results of SIM with:

Sequence 1: adr1, (1323 residues)
Sequence 2: zms1, (1380 residues)

using the parameters:

Comparison matrix: BLOSUM62
Gap open penalty: 12
Gap extension penalty: 4


1. Zn-fingers of ADR1 and ZMS1

62.3% identity in 61 residues overlap; Score: 222.0; Gap frequency: 0.0%

adr1,        100 KLRSFVCEVCTRAFARQEHLKRHYRSHTNEKPYPCGLCNRCFTRRDLLIRHAQKIHSGNL
zms1,        147 KPRPFLCPTCTRGFVRQEHLKRHQHSHTREKPYLCIFCGRCFARRDLVLRHQQKLHAALV
                 * * * *  *** * ********  *** **** *  * *** ****  ** ** *


2. C-region of ADR1 and its homology to a region of ZMS1

55.6% identity in 18 residues overlap; Score: 46.0; Gap frequency: 0.0%

adr1,        222 LKKLTRRASFSAQSASSY
zms1,        336 MHKTKRHASFSASSAMTY
                   *  * ***** **  *


3. TADIV region of ADR1 and its homology to a region of ZMS1

37.1% identity in 35 residues overlap; Score: 68.0; Gap frequency: 0.0%

adr1,        632 PAVSELNEYLDLFKNNFLPHFPIIHPSLLDLDLDS
zms1,        786 PTTSQLNDYVTYYKEEFHPFFSFIHLPSIIPNMDS
                 * * ** *    *  * * *  **        **
```

The alignment shown was performed by the LANVIEW program. Only sequence fragments with homology to ADR1 Zn-fingers, C-region and TADIV are shown.

Table 3. The effect of deleting TADIV on ADR1 function.

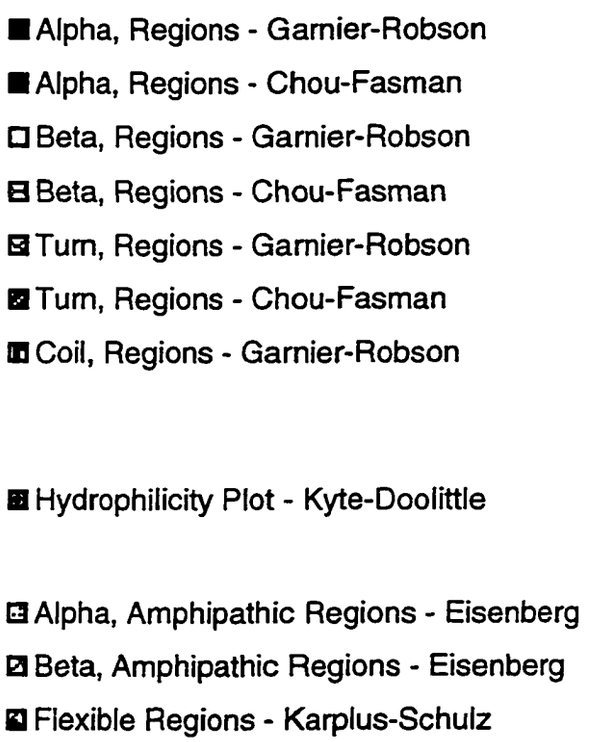
Relevant genotype	ß-galactosidase	ADHII	
		R	DR
	units/mg protein	*units/mg protein*	
$LexA_{202}$-ADR1	900		
$LexA_{202}$-ADR1- Δ642-704	48		
$LexA_{202}$-ADR1- Δ642-675	100		
ADR1		<5	2000
ADR1- Δ642-704		<5	60

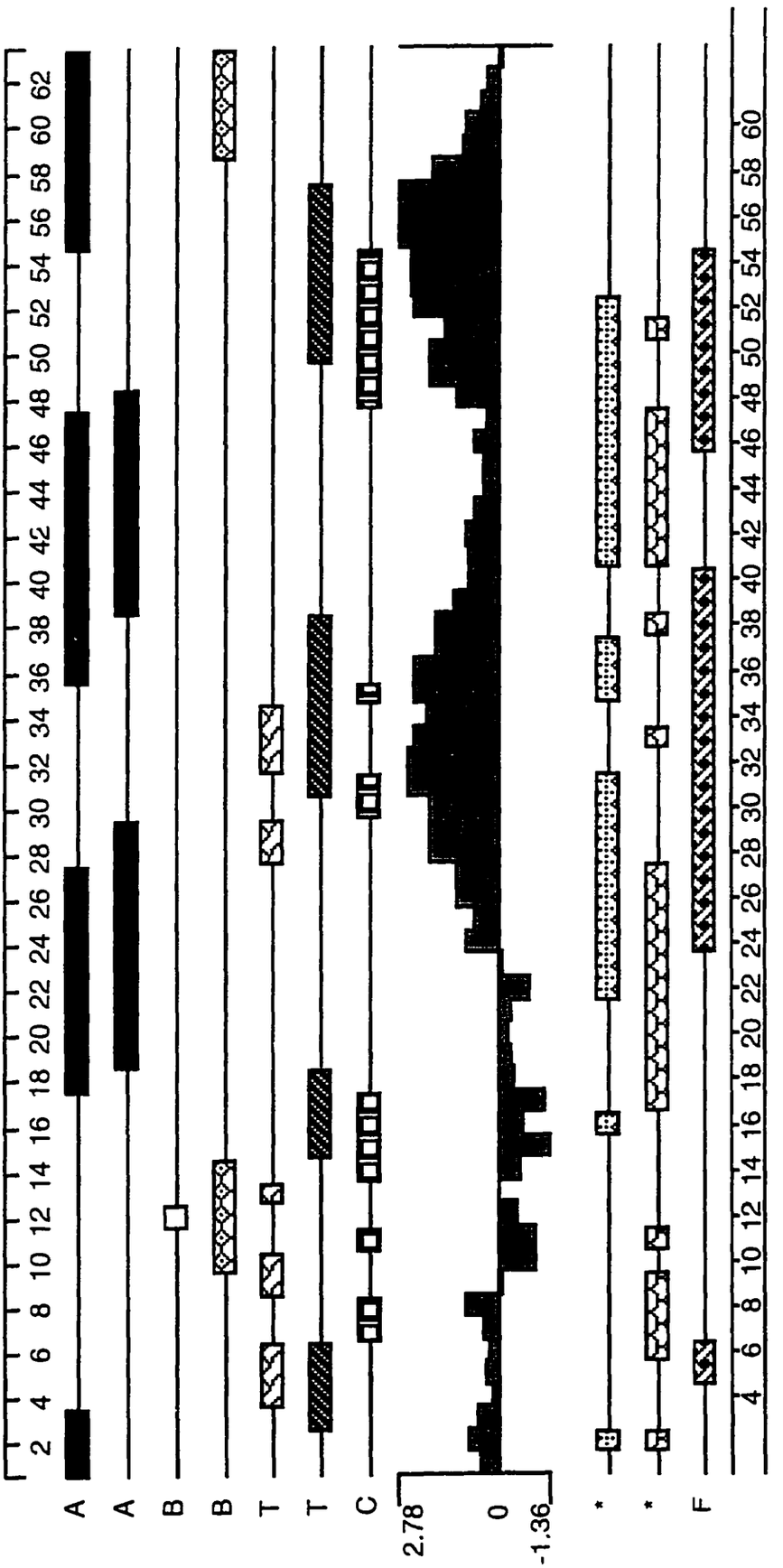
ADH II activities were conducted as described in Appendix A. β-Galactosidase activities were determined as described in Appendix A in strain EGY188. S. E. values were less than 15%. $LexA_{202}$-ADR1, $LexA_{202}$-ADR1-Δ642-704 and $LexA_{202}$-ADR1-Δ642-675 represent LexA-1-202 fused, respectively, to the full length ADR1 protein and to the full length ADR1 protein whose residues 642-704 or 642-675 have been deleted. All ADR1 proteins integrated at ADR1 chromosomal locus as well as all fusion proteins were expressed to comparable levels in vivo (data not shown). ADR1, strain 40-1c; ADR1-Δ642-704, same as 40-1c except adr1-1::ADR1-Δ642-704-TRP1.

698-704, contributing most of the activation potential to the TADIV is predicted to form alpha helix by Garnier-Robson algorithm and beta sheet by Chou- Fasman (Figure 7). The distinguishing feature of this region is the abundance of bulky hydrophobic tyrosine residues.

Figure 7. Secondary structure preduction of the ADR1 TADIV

Secondary structure prediction was performed wit the DNA Star software package module "Protean". ADR1 residues 642-704 were analyzed. Residue 1 in the figure corresponds to residue 642 of ADR1.





Part II.

Isolation of protein factors interacting with the ADR1 amino acids 1-262.

The two-hybrid screen for interacting proteins was also conducted with the $LexA_{202}$-ADR1-1-262 fusion. This fusion is transcriptionally inactive in spite of the fact that it includes TADI residues. The screen with JG4-5 vector-based yeast genomic DNA library was conducted by Dr. Yueh-Chin Chiang. The expression of B42-(genomic fragment) fusions in this vector is under the control of the galactose-inducible GAL1 promoter. This feature substantially alleviates the analysis of the dependency of activation of the reporter gene(s) on the B42 fusion, since its expression can be turned off if glucose is a carbon source and turned on if galactose is a carbon source.

Thirty seven yeast colonies were isolated from this screen whose ability to activate $LexA_{op}$-*LEU2* and $LexA_{op}$-*lacZ* reporter genes was dependent on the plasmids carrying

B42-fused yeast genomic fragments. These plasmid clones were rescued from yeast cells and organised into 4 groups based on the identity of the restriction digestion patterns. Western analysis indicated that each group of clones expressed a protein of larger size than the B42 alone, suggesting that an open reading frame fused to B42 is present in every case. Retransformation of the rescued library clones into parental strain restored its ability to activate reporter genes. The strength of interaction between $LexA_{202}$-ADR1-1-262 and different library clones varied substantially based on the measured β-galactosidase activity values (Table 4).

Library clones from each group were sequenced and BLAST search was conducted with every sequence obtained. The results of the BLAST search are summarized in the Table 4 . The genes were named *AIF1* through 4 (ADR1 Interacting

Table 4. Proteins that interact with ADR1-1-262.

Protein	Interaction with LexA-ADR1(1-262) ß-gal activity	Molecular Weight kDa	Homology	Interaction with LexA-AIF1 (blue/white)
AIF1	170	92	YNL144c	No
AIF2/MAF1	120	66	None	Yes
AIF3	160	45	None	No
AIF4	27	120	SNF2 family	Yes
JG4-5	2	N/A	N/A	No

The four proteins that were shown to interact specifically with LexA-ADR1-1-262 are shown on the left. JG4-5 is the B42 activation domain without yeast inserts. The ability of the B42-AIF fusions to interact with LexA-ADR1 or LexA is given as a function of their ability to activate $\underline{LexA_{op}\text{-}LacZ}$ (p34) reporter. Interaction with LexA-AIF1 was monitored by the ability to activate both $\underline{LexA_{op}\text{-}LEU2}$ and $\underline{LexA_{op}\text{-}LacZ}$ (p34) reporters. β-Galactosidase activities were determined as described in Appendix A in strain EGY188. S. E. values were less than 15%.

Factors 1 through 4). AIF1 is a highly acidic protein, AIF2 is identical to MAF1, a protein whose mutations are implicated in missorting MOD5 (tRNA modifying enzyme) to the nucleus [Murawski et al., 1994], and AIF4 is a SNF2 family member. I further concentrated on the analysis of AIF1, which gave the highest β-galactosidase activity values and thus quite likely was the strongest interactor with $LexA_{202}$-ADR1-1-262.

The AIF1 gene encodes an 800 residues long protein with a very acidic C-terminus. The library clone isolated in the screen contained only 400 C-terminal amino acids of the protein. The full length *AIF1* clone was isolated from the chromosome VIII 1-262, in contrast to the B42-AIF1-C-terminal interacted with this ADR1 fusion cosmid 9315 as described in "Materials and Methods". This clone was used to construct full length B42-AIF1 fusion. This fusion failed to interact detectably with $LexA_{202}$-ADR1-rather strongly.

Interestingly, the $LexA_{202}$-AIF1 fusion protein interacted in the two-hybrid system with AIF2 and AIF4 library clones in terms of a blue/white screen. In contrast, the four AIF proteins failed to interact with a number of other LexA fusions which we examined. It was further determined that other $LexA_{202}$-ADR1-1-262 constructs which differed solely in their sequences joined to LexA moiety or C-terminal to residue 262 were incapable of interacting with any of the AIF proteins [Y.- C. Chiang, pers. commun.]. These data suggest that non-ADR1 determinants of the original $LexA_{202}$-ADR1-1-262 fusion contributed significantly to its interaction with the AIFs.

The disruption of *AIF1* and *AIF2* resulted in no apparent *ADH2* phenotypes. The analysis of the yeast genome database revealed a presence of close homolog of AIF1 in yeast (Figure 8). It is possible that the functional redundancy masks the effect of *AIF1* deletion on the *ADH2* locus.

Figure 8. Similarity of AIF1 to YNL144c

The alignment shown was performed by the BLAST program at the National Center of Biotechnology Information.

```
 Score = 648 (228.1 bits), Expect = 3.8e-86, Sum P(3) = 3.8e-86
 Identities = 130/303 (42%), Positives = 190/303 (62%)

AIF1:       9 LKSPSSSSTCSMDEVLITSSNNSSSICLETMRQLPREGVSG----QINIIKETAASSSSH 64
              LK   ++S+ SM +  + SS  S+SI    + ++    +        NI+ E     S
YNL144c:   20 LKLSQTASSISMGDEFLCSSTTSNSILDSPLPKVTFNHIDSITDINTNIMNEIVEPQSGV 79

AIF1:      65 AALFIKQDLYEHIDPLPAYPPSYDLVNPNKEVRFPIFGDTAPCPKSSLPPLYAPAVYELT 124
                    +++   IDP P  PP YD  NP+K +R+PI+    PC  S  PP Y P+V   T
YNL144c:   80 DVDVADKNVLYCIDPYPVEPPCYDFANPSKVIRYPIYEHCRPCLTSVKPPSYTPSVEHYT 139

AIF1:     125 LISLKLERLSPYEISSNRSWRNFIIEINSTQLNFYHIDESLTKHIRNYSSGETKSEKEDR 184
              ++S+K+E+LSP+E +S+R W NFI++INSTQ+NFY ID+SLT+HI+NY  G+
YNL144c:  140 VVSMKMEKLSPFENASSRLWNNFILQINSTQINFYSIDDSLTRHIKNYRGGDMFDHSH-- 197

AIF1:     185 IHSDLVHRSDQSQHLHHRLFTLPTRSASEFKKADQERISYRVKRDRSRYLTDEALYKSFT 244
               HS     SD+       L    T+S  +F K D+ERI   + RD  ++L+DE L+KS++
YNL144c:  198 -HSKTA--SDRHHSARSLLNAFTTKSTYQFDKYDKERICGEIARDEHKFLSDERLFKSYS 254

AIF1:     245 LQNARFGIPTDYTKKSFVLRMSCESEQFLLRFSHIDDMIDWSMYLSIGISVSLDLEVREY 304
              LQ A+ G+P DY+ + FVLRM CE +QFL++FSH+D++I W+MYL++GIS+SLDLE+RE
YNL144c:  255 LQCAKVGLPIDYSSRDFVLRMRCEGQQFLVQFSHVDELIYWAMYLNMGISLSLDLELREM 314

AIF1:     305 PDYRIVP 311
              P YR VP
YNL144c:  315 PTYRSVP 321

 Score = 192 (67.6 bits), Expect = 3.8e-86, Sum P(3) = 3.8e-86
 Identities = 41/101 (40%), Positives = 61/101 (60%)

AIF1:     678 TLSCFSNIPYGTDDIKWKPAIKEISRRRYLRDSLKCIKPFLDSNDCLGKVIYIPVSGPTF 737
              +L C  N    ++D KW PA + +SR+RY++DSL+CIKP  + +  +GK+I+ P   P F
YNL144c:  626 SLKC-GNKNQSSNDSKWAPATQLVSRKRYIKDSLRCIKPLTEDHPWVGKIIFKPALPPAF 684

AIF1:     738 ETSNK-IH-FSNRQS--LQKQKNHFLKGFIVGPTALIELNCK 775
              ET+N  I  +S   S  L   KNH+LK +IVG    ++   K
YNL144c:  685 ETNNPPIRVYSGEDSTDLMHVKNHYLKPYIVGSCGFLKTGSK 726
```

Discussion.

The search for proteins interacting with ADR1 fragments 1-262 and 642-1323 resulted in the identification of 4 proteins capable of interaction with the 1-262 region, and revealed the fourth activation domain, TADIV, localized to the residues 642-704 of ADR1. No proteins interacting with the 642-1323 region were found.

The importance of the TADIV region for the function of the ADR1 is clear from the effect the deletion of residues 642-704 has on the activation potential of ADR1 both at the *ADH2* locus and at the heterologous promoter of $LexA_{op}$-*lacZ* reporter. Moreover, the 642-704 fragment of ADR1 by itself is capable of transcriptional activation when fused to a LexA protein DNA binding domain. The deletion of this region from the full length protein has a substantially stronger effect on ADR1 ability to activate transcription than the deletion of any one of the previously identified activation domains [Cook et al., 1994]. It is important to note, however, that the ADR1 fragment comprising only amino acids 1-642 is capable of strong activation of transcription. In fact this fragment is a better activator than full length ADR1Δ642-704. This observation suggests that C-terminal part of ADR1, residues 704 to 1323, may partially block the activation by the three N-terminal activation domains in the full length protein. Elements of sequence similarity of this region of ADR1 to self-repressive regions of some other yeast transcriptional activators [Poch, 1997] supports this suggestion. The sequence analysis of TADIV reveals that it contains a number of bulky hydrophobic residues in the 698-704 region, which by deletion studies is suggested to be especially important for TADIV function. The impotance of hydrophobic residues for function of other transcriptional activators has been described previously [Leuther at al., 1993, Drysdale et al., 1995, Jackson et al., 1996]. These residues are believed to participate in hydrophobic protein-protein interactions.

Four proteins were identified that contact a TADI-containing fragment of ADR1. The analysis of effects of disruption of these genes on the *ADH2* locus did not reveal clear

effects. The reason for the absence of effects may be that other yeast proteins perform redundant functions for those analyzed at the *ADH2* locus. The presence of a protein in yeast (Open Reading Frame YNL144C, chromosome XIV) with the sequence highly similar to that of AIF1 supports this notion. Further study of AIFs will be required to understand their function.

Chapter II.

ADR1 activation domains contact the histone acetyltransferase GCN5 and the core transcription factor TFIIB.

Introduction.

The presence of four activation domains in the ADR1 (TADI (76-172), TADII (263-357), TADIII (420-462), and TADIV (642-704)) suggests that ADR1 may interact with multiple transcriptional cofactors and/or components of general transcriptional machinery. The observation that TADII and III are functionally redundant [Cook et al., 1994] suggests that some of these interactions may be with the same protein.

There are a number of potential targets for ADR1 activation domains. Core transcriptional components including TBP, TFIIB, TFIIF, TFIIE, and TAFs have been implicated in mammalian system as being direct contacts for transcriptional activators [Choy and Green, 1993]. In addition to these general transcriptional machinery components, other cofactors or coactivators may mediate the action of activators. The ADA2 complex is one such coactivator complex. The ADA2 complex has been shown to bind activators like GCN4 and VP16 [Barlev et al., 1995, Silverman et al., 1994] and to be required for maximal transcriptional activity of several yeast activators [Berger et al., 1992]. The ADA2 complex has been also shown to bind TBP, and it was suggested that this complex acts as a direct mediator between activators and the general transcriptional machinery. However, some yeast activators like GAL4 or HAP4 [Berger et al., 1992, Pina et al., 1993] are only slightly or not at all affected by defects in the ADA2 complex.

Recent experiments indicate that the GCN5 component of the ADA2 complex is a histone acetyltransferase [Brownell et al., 1996] . Since chromatin structure at the promoter had been shown to have a strong effect on transcription and the acetylation of histone N-termini "loosens" chromatin and makes DNA more accessible, it was suggested that activator recruitment of the ADA2 complex results in targeted histone acetylation which allows for activated transcription to occur.

The analysis of chromatin structure at the *ADH2* promoter found that one nucleosome is positioned over the TATAA box and another on the transcription start site under repressing conditions, and they are removed in an ADR1-dependent fashion during *ADH2* derepression [Verdone et al., 1996]. The UAS1 sequence to which ADR1 binds is not covered by a nucleosome under either repressing or derepressing conditions which may explain why mutations in such chromatin remodeling proteins as components of SNF/SWI complex have little effect on *ADH2* derepression.

We found that mutations in ADA2 and GCN5 severely compromise the ability of ADR1 TADs to activate gene expression. In addition, we demonstrate that there is strong physical interaction between ADA2 and GCN5 and ADR1 TADs *in vitro*. TADIV binding to GCN5 was shown to directly correlate with TADIV activation function. TADs I and IV were also shown to make specific contacts to TFIIB. The interaction of TADI with the TFIIB is specifically impaired by a point mutation in TFIIB which *in vivo* leads to two-fold reduction of the *ADH2* derepression. These results suggest ADR1 TADs activate gene expression in yeast through direct physical contacts with multiple proteins including components of the ADA2 complex and the core transcriptional component TFIIB.

Materials and Methods.

Yeast Strains.

Yeast strains are listed in Table 1. Strain EGY188 was used for transformation with plasmids expressing $LexA_{202}$-ADR1 fusion proteins. The *ada2::URA3* and *gcn5::URA3* disruptions in strain PSY316 were a gift from L. Guarente, strains CY26 and CY57 were provided by C. Peterson, strain YMH130 and all isogenic strains containing mutant TFIIB alleles were provided by M. Hampsey.

Plasmid constructions.

Isolation of $LexA_{202}$-ADR1-642-704 and and its truncation derivatives, as well as construction of $LexA_{202}$-ADR1-D642-704 and $LexA_{202}$-ADR1-D675-704 is described in Chapter I of this thesis. All other $LexA_{202}$-ADR1 fusions are described elsewhere [Cook et al., 1994a, Appendix A]. The construction of all GST fusions is also described in Appendix A. Plasmid vectors used for one step gene replacements of *ADA2*, *GCN5*, and *ADH1* were described previously [Appendix A and references therein]. Construction of T7 *in vitro* expression vectors of different TFIIB alleles was conducted by PCR amplifying TFIIB from a pM plasmid series provided by M. Hampsey, using primers which introduce unique BglII and NdeI sites at 5' and 3' end of TFIIB open reading frame, respectively. PCR products were digested with BglII and NdeI, and digestion products were subsequently ligated into the T7 expression vector pET11a [BioGen], which was previously digested with BamHI and NdeI and treated with calf intestinal phosphatase. All other T7 *in vitro* expression constructions were constructed as described elsewhere [Appendix A].

Transformations, Enzyme assays and Growth Conditions.

All yeast transformations were conducted by using the lithium acetate method [Ito et al., 1983]. ADH II and ß-galactosidase assays were conducted as described [Cook et al., 1994]. Conditions for growth of cultures on minimal medium lacking uracil and histidine or YEP medium containing either 8% glucose, 2% ethanol and 2% glycerol, or 3% ethanol have been described elsewhere [Cook et al., 1994].

In vitro binding assay.

Conditions for *in vitro* binding assay are described in Appendix A.

Disruptions of *ADA2*, *GCN5* and *ADH1*.

All disruptions were conducted according to the one-step gene replacement protocol [Rothstein, 1991]. The disruption vector containing the *GCN5* gene replacement cassette was digested with XhoI and SalI and transformed into strain 612-1d, the disruption cassette for *ADA2* replacement was cut with BamHI and XhoI and transformed into strain 612-1d, the disruption of *ADH1* was done by transforming BamHI digested pHH20 disruption vector into strain YMH130.

Plasmid shuffle.

YMH130 yeast strain deleted for the chromosomal copy of the *SUA7* gene (TFIIB) and containing an *adh1::URA3* disruption and the plasmid-borne TFIIB allele on a *HIS3*-marked single copy plasmid vector was plated on 5-fluoroorotic acid plates to select for mutants in the *URA3* gene. The YMH130 strain with *adh2::ura3*^{mut} was subsequently transformed with the *URA3*-marked single copy vector containing *SUA7*. Transformants were subsequently allowed to lose the *HIS3*-marked *SUA7*-containing vector. The resulting

strain was transformed with a set of mutant *SUA7* alleles on *HIS3*-marked vectors. Resulting transformants containing wild type *SUA7* on a *URA3*-marked vector and mutant *sua7* alleles on *HIS3*-marked vectors were plated on 5-FOA plates to select for the loss of wild type *SUA7*.

Results.

In order to identify the factors through which the four individual ADR1 TADs act, the effect of deleting different general transcription cofactors or adaptors on LexA-ADR1-TAD transactivation has been analyzed. The results, described in detail in the Appendix A, can be briefly summarized as follows: ADR1 TADI, II, III and IV were capable of binding to the GCN5 and a GCN5 deletion resulted in 2 to 3-fold reduction of the *ADH2* gene derepression; ADR1 TADI and IV interacted with the core transcription factor TFIIB, and interaction of TFIIB with TADI was substantially stronger than with TADIV.

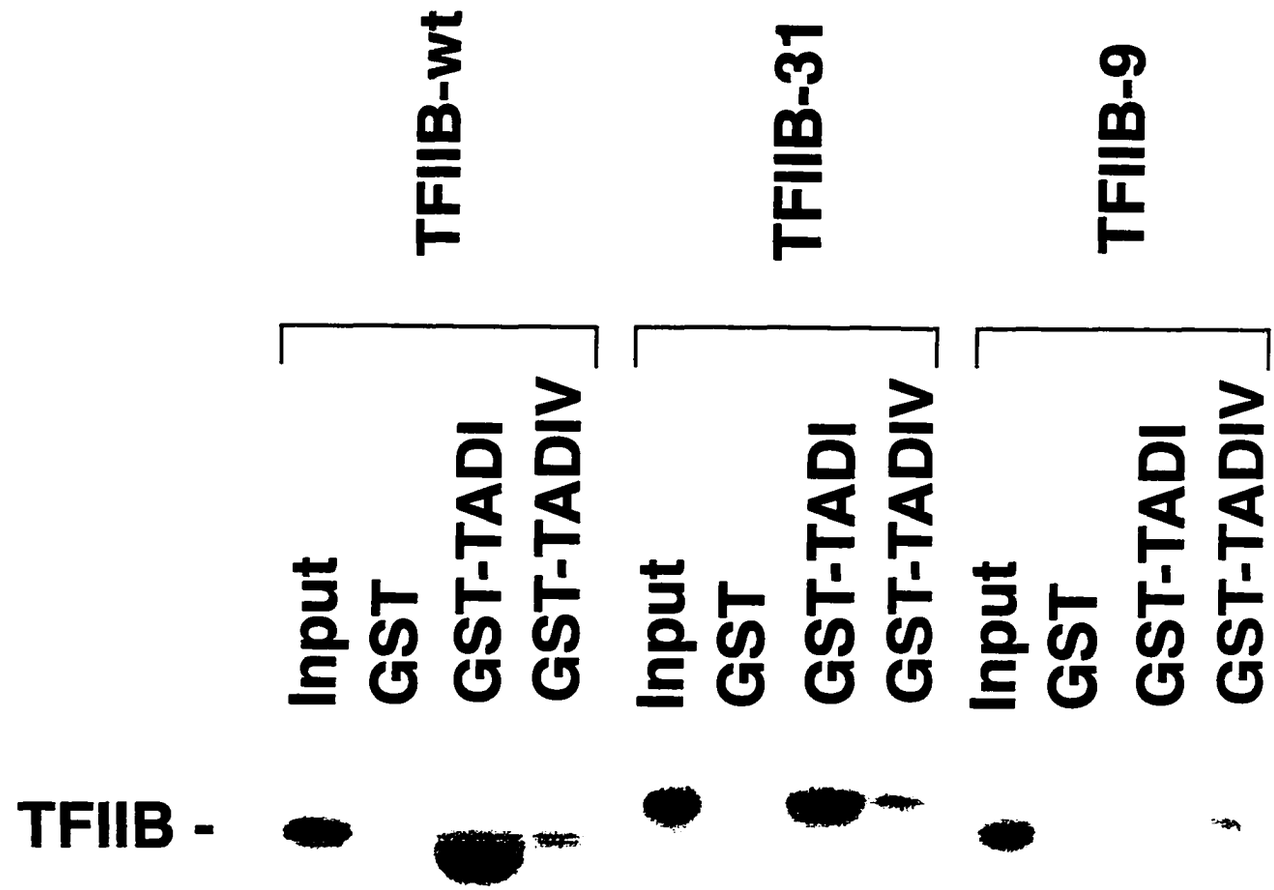
We further analyzed the TFIIB role in the *ADH2* derepression. Random PCR mutagenesis of *SUA7* gene conducted in Dr. Michael Hampsey laboratory yeilded a number of TFIIB derivatives containing single or multiple amino acid substitutions and defective in supporting growth at 37^{0}C or 16^{0}C. We have analyzed three of these mutant derivatives of TFIIB for their effect on *ADH2* transcription (Table 5). The *sua7-9* allele (encoding TFIIB-9), containing E202G and F289S amino acid substitutions, was found to reduce *ADH2* derepression by two-fold. The *in vitro* binding assay conducted with this mutant protein demonstrated (Figure 9) that its binding to TADI is severely diminished compared to the binding of wild type TFIIB or another mutant allele which has no effect on the *ADH2* derepression. In contrast, TFIIB-9 was unaffected in its binding to TADIV (Figure 9), suggesting that the observed decrease in the interaction with TADI is not due to the gross structural change in the TFIIB-9. The binding of other alleles of *sua7* , which conferred reduced levels of the *ADH2* derepression, to the ADR1 TADs is currently being investigated.

Table 5. Effect of point mutations in *SUA7* (TFIIB) on *ADH2* derepression

Yeast Strains	ADH II activity *units/mg protein*
WT	2400
TFIIB-9	1200
TFIIB-30	2200
TFIIB-31	2500

ADH II activities were conducted as described in Appendix A. S. E. values were less than 20%. Values shown represent averages of at least three separate measurements for each SUA7 allele. WT, strain YMH130; TFIIB-9 (mutations E202G, F289S), same as YMH130 except pRS313-sua7-9; TFIIB-30 (mutations T200I, K314M), same as YMH130 except pRS313-sua7-30; TFIIB-31 (mutation L284Q), same as YMH130 except pRS313-sua7-31

Figure 9. Binding of ADR1 TADI and TADIV to mutant variants of TFIIB.



Binding studies between GST fusion proteins indicated and radiolabeled wild type (TFIIB-wt) and mutant (TFIIB-9, -31) variants of TFIIB were conducted as described in the Appendix A. Amount of each GST fusion loaded on the gel is equivalent for all samples loaded.

Discussion.

ADR1 is the primary transcriptional activator of the *ADH2* gene and has been shown previously (Cook et al. 1994a) and herein to contain four separable domains capable of activating transcription. These individual activation domains were found to be highly dependent on a functional ADA2 complex for their ability as LexA fusions to activate a LexA reporter system. Full length ADR1 fused to LexA still displayed, however, a significant, albeit reduced, activation function when the ADA2 complex was inactive. The activation domains in full-length LexA-ADR1 must also be capable of binding targets in addition to the ADA2 complex [Appendix A].

Since we observed that *ada2* or *gcn5* deletions did not completely block ADR1 activation in all promoter contexts, it is likely that the ADR1 TADs make multiple contacts in promoter specific ways. It is known that the TADs play different roles at different promoters. TADI is solely required by ADR1 for maximal activation at the *FOX2* and *FOX3* promoter, but not at either *ADH2* or *CAT1*. TADIII, on the other hand, is required for genes expression involved in glycerol mutations [Bemis and Denis 1988; Cook et al. 1994] for which TADII is less important. TADIV alone plays a crucial role for activation of some unknown genes involved in peroxisomal biogenesis [Simon et al. 1995]. Therefore, in some promoter contexts a particular TADI interaction may be especially strong or essential, perhaps as modulated by chromatin structure and/or other promoter specific binding factors.

There are a number of potential targets for transcriptional activators. We showed that TFIIB could bind TADs I and IV of ADR1 but neither TADs II or III. TBP did not display any selective binding to any of the four TADs. These results are to be contrasted with that of GAL4 whose activation domain retains TBP but not TFIIB. The physiological relevance of these interactions is not certain. For TADIV of ADR1 and for the GAL4 activation domain, mutations or deletions which clearly decreased their functions also

directly reduced binding to TFIIB and TBP, respectively. Additionally, point mutation in TFIIB that reduced *ADH2* derepression had a stong negative effect on TADI interaction with TFIIB. This suports the biological importance of the interaction. It could also reflect that a particular region of TADIV or GAL4 is sticky and that TFIIB or TBP represent examples of proteins that could bind to the activator.

In any case, these results strengthen the case for examining a direct link between ADR1 and TFIIB using more genetic or *in vitro* experimental approaches.

Chapter III.

ADR1-mediated transcriptional activation requires the presence of an intact TFIID complex.

Introduction:

In the Chapter I of this thesis and in our earlier report [Appendix A] we had shown that individual activation domains of ADR1 contact TFIIB and histone acetyltransferase GCN5 *in vitro*. However, the deletion of *GCN5* and a point mutation in the TFIIB which dramatically reduced its binding to ADR1 had only moderate effects on the derepression of *ADH2* gene, suggesting the existence of additional activation mechanisms.

There are a number of potential targets for ADR1 activation domains among the core transcription factors. TFIID, TFIIF, TFIIB, RNA polymerase II, TFIIH and TFIIE have been implicated in mammalian and Drosophila systems as being direct contacts for various transcription activators. For example, glutamine-rich activation domain of Sp1 contacts TFIID component dTAF110 [Hoey et al., 1993], VP16 interacts with TFIIB, TBP and histone - like dTAF42/hTAF31, yeast GAL4 binds TBP and TFIIB [Gupta et al., 1996, Wu e al., 1996]. *In vitro* studies have shown that activators have both qualitative and quantitative effect on TFIID binding to the TATA box [Klemm et al., 1995]. It has been also demonstrated that the TFIID binding step is first and rate-limiting in the assembly of the initiation complex at many promoters [Shykind et al., 1997, Colgan et al., 1992], making it a likely target for transcriptional activators.

TFIID is a multimeric complex consisting of TATA box binding subunit TBP and TBP associated factors (TAFs) [Poon et al., 1995]. Both TBP and TAFs show significant degree of evolutionary conservation throughout the eucaryotic kingdom [Burley et al., 1996, Moqtaderi et al, 1996], suggesting that TFIID quaternary structure may also be conserved. Thirteen yeast TAFs have been cloned to date, with sizes ranging from 17 to 150 kDa [Tansey et al., 1997]. Most of these proteins are encoded by essential genes. The precise role of TAFs in transcription is largely unknown. *In vitro* data suggest that these proteins are required for activated transcription and that they can serve as direct targets *in vivo* for activation domains of DNA-binding transcriptional activators of higher eucaryotes [Hoey et al., 1993, Klemm et al., 1992, Sauer et al., 1996]. *In vivo* data, however, indicate that yeast TAFs are not universally required for pol II transcription and are dispensable for activated transcription of a number of yeast genes [Moqtaderi et al., 1996, Walker et al., 1996]. Although encoded by essential genes, temperature sensitive alleles of yTAF130/145 and yTAF90 affect expression of only a small fraction of yeast genes [Walker et al., 1996, Apone et al., 1996]. Similar results were observed when different TAFs were eliminated from the yeast cell using a double shut-off method [Moqtaderi et al., 1996].

We have found that ADR1 transcription activation domain IV can specifically retain TFIID from yeast whole cell extracts and that overexpressed ADR1 co-immunoprecipitates with yTAF90. Moreover, transcriptional activation of the *ADH2* gene does not occur *in vivo* if the yeast cell is depleted of TAF90. These results suggest that transcriptional activation of the *ADH2* gene by ADR1 is mediated through its contacts with the TFIID complex.

Materials and methods:

Strains and culture conditions:

Yeast strains are listed in Table 1. Strains 938-9b and yBY40-8' were used for transformation with plasmids expressing ADR1 from the G3PDH promoter. Strains ZMY60 and ZMY68 were a gift from Z. Moqtaderi and K. Struhl. For the TAF90 shut-off assay, ZMY60 and ZMY68 were grown in synthetic media lacking uracil (SC-Ura), supplemented with 5% glucose, until they reached $OD_{600} = 0.3$. At this time $CuSO_4$ was added to a final concentration of 500 μM, and incubation was continued for another 7 hours. Yeast cells were washed twice in SC-Ura supplemented with 3% ethanol, 500 μM $CuSO_4$, and incubated in this media for 6 hours. Aliquots were taken at 0, 1, 2 and 6 hour time points. For the preparation of yeast whole cell extracts yeast strains containing ADR1-overexpressing plasmid were grown in synthetic media lacking leucine (SC-Leu), supplemented with 3% ethanol, 3% glycerol or 5% glucose to the $OD_{600} = 0.7$. All other strains used in this work were grown in YEP supplemented with 3% ethanol, 3% glycerol, to the $OD_{600} = 0.7$.

All DNA manipulations and subcloning were done in E. coli DH5α strain. GST fusion proteins were expressed and purified from *E. coli* BL21 strain.

Plasmid constructions: The vector expressing full length ADR1 from G3PDH promoter was constructed by cutting pJC100 [Cherry, 1988] with BamHI and Hind III and isolating the fragment containing the entire G3PDH promoter and residues 1-649 of ADR1 and ligating it into pRS425 [Christianson, 1992] digested with BamHI and HindIII, creating pPK46. pAK52 containing the entire ADR1 gene with approximately 1.5 kb of 3' sequences, was cut with BstEII, blunt ended with Klenow enzyme, cut with Hind III and

the fragment containing residues 649-1323 of ADR1 was isolated from the agarose gel. pPK46 was cut with Sal I, blunt ended with Klenow enzyme, cut with Hind III and ligated to the amino acids 649-1323 ADR1 fragment , creating pPK47 containing full length ADR1 under the control of the G3PDH promoter. pPK50 containing full length ADR1 deleted for TADIV was constructed by cutting pPK47 with Nco I and Bsg I, isolating the vector backbone from the agarose gel, cutting pPK4 [Appendix A] with the same enzymes and ligating pPK4 fragment containing ADR1 residues 1-642-704-1200 into pPK47 backbone.

GST fusions of ADR1 TADs and Vpu were constructed as described in Appendix A. Isolation of LexA fusions of ADR1 TADIV is also described in Appendix A.

Binding assays and immunoprecipitations.

GST fusion proteins were expressed and bound to gluthatione-agarose beads as described in [Appendix A]. Yeast whole cell extracts were prepared from EGY188 cells grown to a density of $5x10^7$, collected by centrifugation and disrupted by bead beating in 2 volumes of A300 buffer (20 mM HEPES. pH 7.6, 1mM EDTA, 1 mM DTT, 300 mM potassium acetate, 1% Triton and protease inhibitors). Washed gluthatione-agarose beads were incubated with 1 mg of yeast whole cell extract protein in 250 ul of A300 buffer for 2 hours at 4°C on a rocking platform. Unbound proteins were removed by four rapid (<1 min) washes with 1 ml of A300 buffer and specifically bound proteins were resolved by SDS-PAGE after boiling beads directly in sample buffer. Proteins resolved by SDS-PAGE were transferred to PVDF membrane and analyzed by Western blotting as described [Draper et al., 1994]. Antibody to TAF90 was kindly provided by M. Green, antibody to RPB1 subunit of RNA pol II was a gift from J. Jaehning.

Preparation of yeast extracts for immunoprecipitation were made according to Woontner et al., 1991, with the following modification: DTT was omitted from the final dialysis step.

Immunoprecipitation.

Immunoprecipitation of triple HA1-tagged TAF90 from yeast WCE was conducted in immunoprecipitation (IP) incubation and washing buffer (25 mM KPO_4, pH 7.6, 150 mM KCl, 1 mM $NaPP_i$, 1 mM Na F, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40 and protease inhibitors cocktail). 20 microliters of packed protein A agarose beads were incubated with 2µl of monoclonal 12CA5 antibody for 1 hour at 4^0C and in 250 µl of IP incubation/wash buffer, washed once with the same buffer to remove excess antibody and mixed with 2 mg of yeast crude extract protein in 500 µl of IP incubation/wash buffer . Incubation was allowed to proceed for 2 hours at 4^0C with constant rocking. The beads were washed 4 times with 1 ml of the same buffer to remove unbound proteins from the yeast extract, mixed with SDS loading buffer, boiled for 5 min. and separated on a 8% polyacrylamide gel prior to Western analysis.

Northern analysis.

Total yeast RNA was isolated by the hot acidic phenol method [Ausubel, 1994], and quantified by spectrophotometry (A_{260}). Approximately 40 ug of total RNA per sample was denatured with glyoxal and dimethyl sulfoxide and mixed with gel loading buffer according to [Sambrook, 1989]. Denatured RNA samples were loaded in duplicate and resolved on a 1.2% agarose gel, half of which was stained with ethidium bromide in 0.1 M ammonium acetate to verify the equality of loading, and the other half was transferred to a GeneScreen™ nylon membrane and hybridized to the 3'-^{32}P-labelled *ADH2* oligonucleotide probe GTTGGTAGCCTTAACGACTGCGCTAAC [Di Mauro et al., 1993], according to the membrane manufacturer instructions, with the following modification: all posthybridization washes were for 1 minute each.

Results.

TADIV specifically contacts TFIID.

We used affinity chromatography to determine if components of general transcription factor IID interacted with transcription activation domains of ADR1. GST fusions of TADI, II, III, IV and Vpu bound to glutathione-S-transferase coupled beads were incubated with whole cell extracts obtained from yeast strains expressing triple HA1-tagged TAF90, TAF25 or BRF1. As shown in Figure 10, TADIV and to a lesser extent TADI were capable of specifically retaining both TAF90-$HA1_3$ and TAF25-$HA1_3$, but not BRF1-$HA1_3$. The GST-Vpu, -TADII, -TADIII fusions and GST alone bound none of the tagged proteins. We subsequently examined if other components of TFIID were being pulled down by TADIV and I. Yeast extracts were incubated with the same GST fusions described above and the proteins which were retained were probed with antibodies to different components of the TFIID complex: TAF130, TAF90, TAF60 and TBP (Figure 11). GST-TADIV fusion retained all of these components in amounts close to being stoichiometric (Figure 11, lane 4). The RPB1 subunit of Pol II, however, was not retained by GST-TADIV. The most likely interpretation of this result is that TADIV retains the entire TFIID complex. TADI displayed weak binding to TAF90 and did not retain any of the other components of TFIID (Figure 11, lane 2), including TBP. GST-TADII displayed weak binding to TBP as shown previously [Appendix A] and TAF130 (Figure 11, lane 6) while GST-TADIII did not bind any components of the TFIID complex (Figure 11, lane 7).

Figure 10. ADR1 TADI and TADIV interact with TAF90 and TAF25.

GST fusions were induced as described [Appendix A], bound to glutathione-agarose beads, and incubated with yeast crude extracts from strains yEK20 containing TAF25-$HA1_3$, yBY40-8' containing TAF90-$HA1_3$ and yKG1794 containing BRF1-$HA1_3$ as described in "Materials and Methods". Proteins were eluted from glutathione-agarose beads by boiling and separated by SDS-PAGE. They were subsequently transferred to the PVDF membrane and probed with 12CA5 mouse monoclonal antibody against the HA1 epitope. One hundred μg of GST-TAD fusion was loaded in each lane. GST-TADI contains residues 148-262 of ADR1; GST-TADII contains residues 262-359; GST-TADIII contains residues 420-462; and GST-TADIV contains residues 642-704.

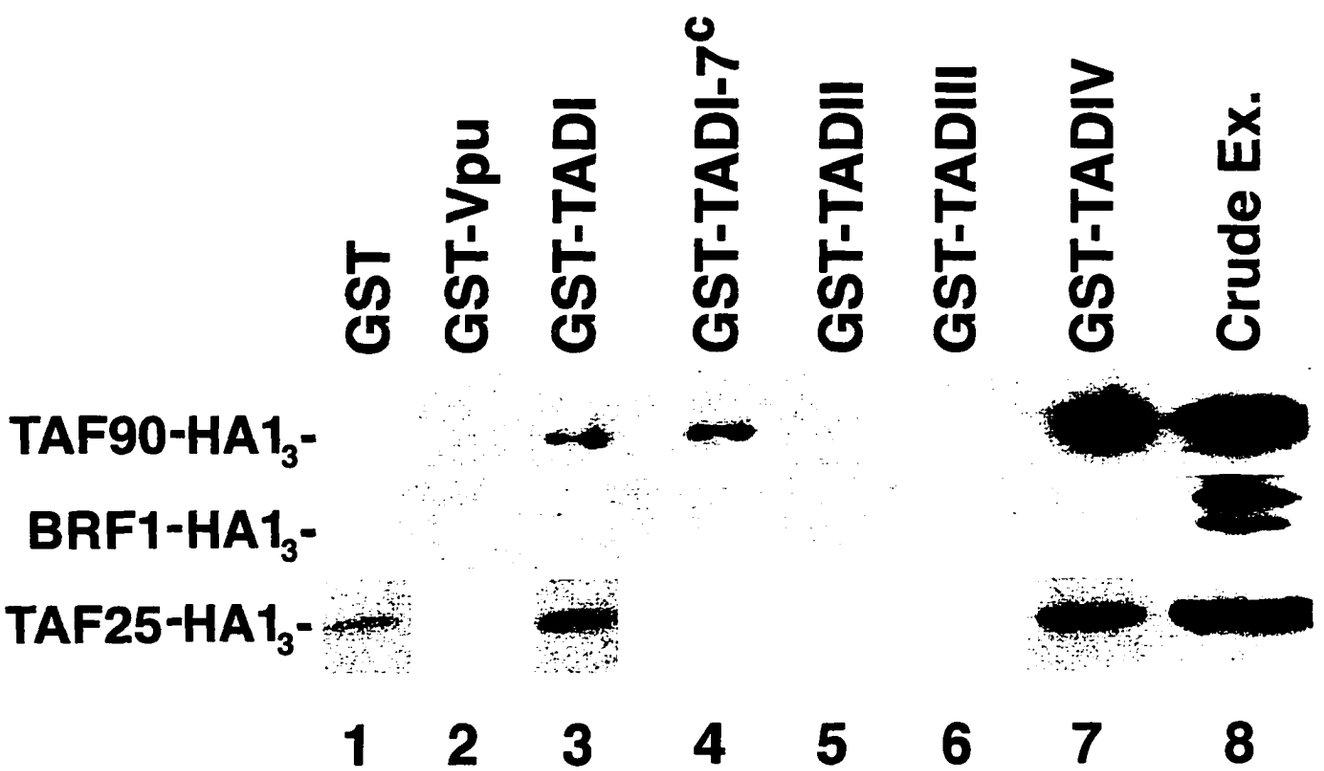
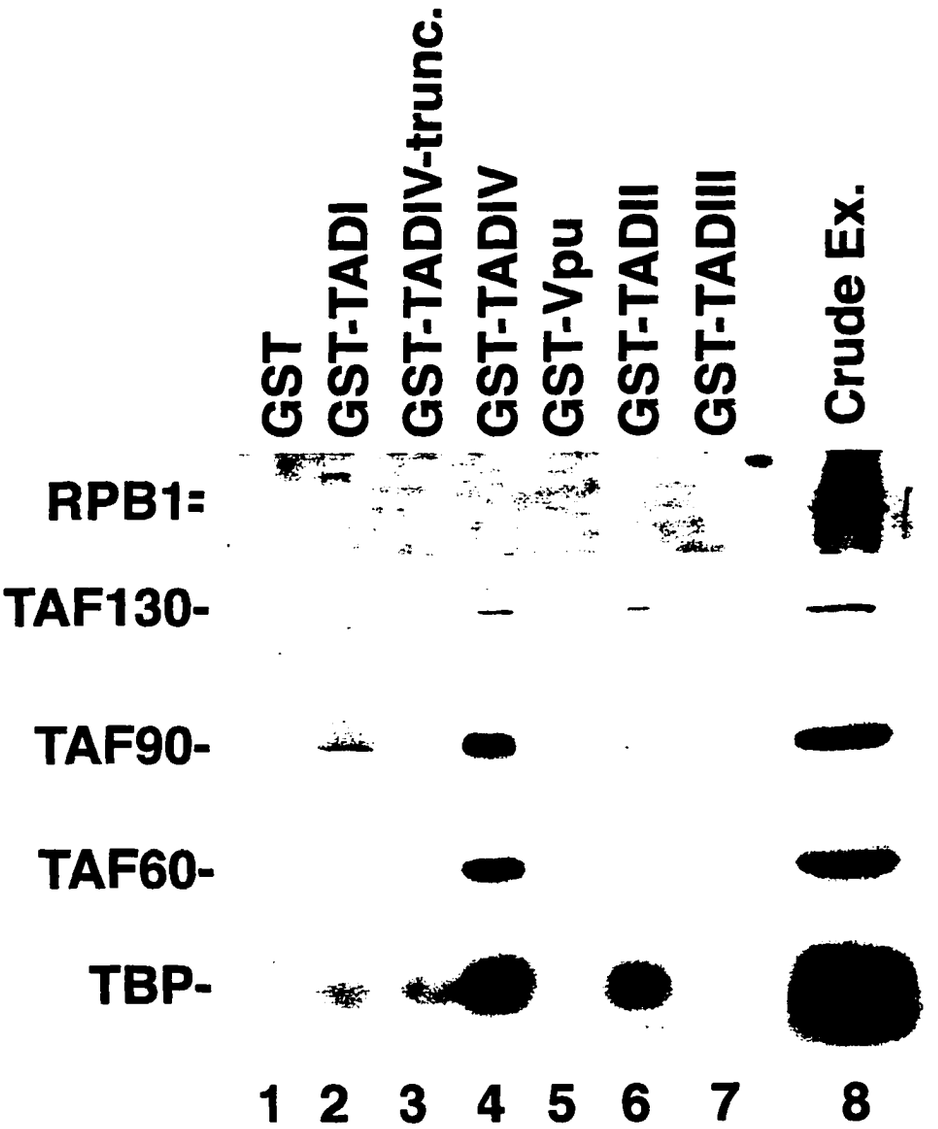


Figure 11. ADR1 TADIV retains TFIID from yeast crude extract.

GST fusions were induced as described [Appendix A], bound to glutathione-agarose beads, and incubated with yeast crude extract from strain EGY188 as described in "Materials and Methods". Proteins were eluted from glutathione-agarose beads by boiling and separated by SDS-PAGE. Proteins were then transferred to the PVDF membrane and probed with the appropriate antibodies. The amount of GST fusion per lane is the same as described in Figure 1.



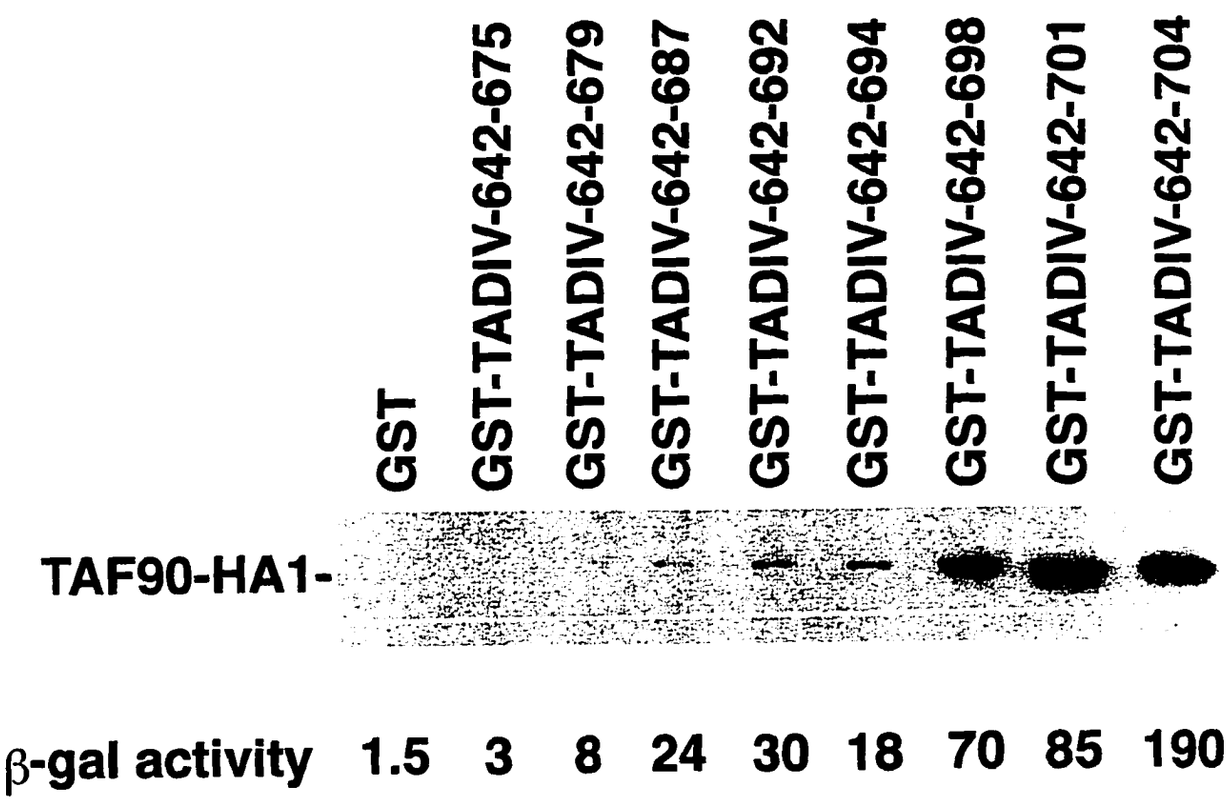
The ability of TADIV to bind TFIID correlates with the ability of LexA-TADIV fusions to activate transcription.

A set of GST-ADR1 truncation derivatives of TADIV that are modeled on LexA-TADIV derivatives which differ in their ability to activate transcription of a *$LexA_{op}$-lacZ* reporter construct was tested for their ability to retain TAF90-$HA1_3$ from yeast extracts. LexA-TADIV C-terminal deletions activated *$LexA_{op}$-lacZ* reporter to different extents depending on the length of the ADR1 moiety present in the fusion. LexA-ADR1-(642-704), -(642-701), and -(642-698) retained significant transcriptional activity, whereas shorter derivatives were less active (Table 2). As evident from Figure 12, the most active TADIV truncation derivatives bound TAF90-$HA1_3$ to the greatest extent, suggesting a functional relevance of the observed binding. Similarly, TAF130, TAF90, TAF60 and TBP were not retained by the transcriptionally inactive TADIV moiety represented in GST-TADIV-642-679 (Figure 11, lane 3).

We had shown previously that deletion of TADIV from the full length ADR1 protein drastically reduces its ability to activate transcription of *ADH2* and of a *$LexA_{op}$-lacZ* reporter gene. Adding back part of TADIV which does not bind any of the TFIID components does not restore the ability of LexA-ADR1 to activate transcription: the ß-galactosidase activities measured in strains expressing LexA-ADR1, LexA-ADR1-Δ642-704 and LexA-ADR1-Δ675-704 are 900, 50 and 105 Units/mg protein respectively. Each of these proteins was expressed to equivalent extents in the yeast cell (data not shown). This result indicates that it is the region capable of binding TFIID that is required for ADR1 function.

Figure 12. The ability of ADR1 TADIV to interact with TAF90 correlates with TADIV ability to activate transcription.

The effect of C-terminal deletions of LexA-TADIV on its ability to bind TAF90-$HA1_3$. GST-ADR1-TADIV derivatives of length as indicated in the Figure were expressed in E. coli, and bound to glutathione-agarose beads as described in "Materials and Methods", extracts from yeast strain EGY188 were bound to the GST fusions, and the resulting bound proteins were analyzed by Western analysis using 12CA5 antibody directed against the HA1 tag, as described in Figure 1. 100 µg of every GST fusion was loaded on the gel. Each GST fusion was expressed at comparable levels (data not shown; [Appendix A]).ß-galactosidase activities displayed are for LexA-TADIV derivatives in their activation of $LexA_{op}$-lacZ and are taken from [Appendix A].



ADR1 co-immunoprecipitates with TAF90-HA1$_3$.

To address the question of *in vivo* association of full length ADR1 with TFIID we tested if ADR1 can be co-immunoprecipitated with TAF90-HA1$_3$. As shown in Figure 13, ADR1 does co-immunoprecipitate with TAF90-HA1$_3$ from the yBY40-8' strain, but not from strain 938-9b, that lacks HA1-tagged components of TFIID. It is worth noting here that growth conditions do not have an effect on this observed interaction. ADR1 co-immunoprecipitated with TAF90-HA1$_3$ when yeast cells were grown under either repressing or derepressing conditions (data not shown). Although the ADR1 protein in both 938-9b and yBY40-8' was expressed from a multicopy vector, the pattern and nature of ADR1 activation of *ADH2* transcription remains the same when it is overexpressed [Denis et al., 1981, Cook et al., 1994] suggesting that the association of overexpressed ADR1 with TAF90 represents a physiologically relevant interaction.

TAF90 is required for *ADH2* derepression.

TAFs have been implicated in co-activator function in *in vitro* systems by a number of studies [Hoey et al., 1993, Wu et al., 1996, Sauer et al., 1996] . However, recent results [Moqtaderi et al., 1996, Walker et al., 1996] indicate that yeast TAFs are dispensable for transcription of a majority of yeast genes. Using a double shut-off system designed to eliminate TAF90 [Moqtaderi et al., 1996], we tested if TAF90 is required for *ADH2* activated transcription. Yeast cells were depleted of TAF90 (Figure 14b) and then tested for their ability to activate the *ADH2* gene upon depletion of glucose. As shown in Figure 14a, *ADH2* derepression does not occur in a strain depleted for TAF90, but does occur in an isogenic control strain. In contrast, the mRNA levels for ADR1 and CCR4,

Figure 13. ADR1 co-immunoprecipitates with TAF90.

Extracts from strain yBY40-8', containing triple HA1-tagged TAF90 and expressing ADR1 from multicopy vector, and strain 938-9b, containing no HA1-tagged TAF and also expressing ADR1 from the same vector, were incubated with 12CA5 antibody directed against the HA1 tag, and the resulting immunoprecipitates were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. ADR1 and TAF90-$HA1_3$ were detected by Western analysis: polyclonal antibody raised against ADR1 residues 208-231 was used for ADR1 detection, 12CA5 antibody was used for TAF90-$HA1_3$ detection. One hundred μg protein was loaded per each crude extract lane.

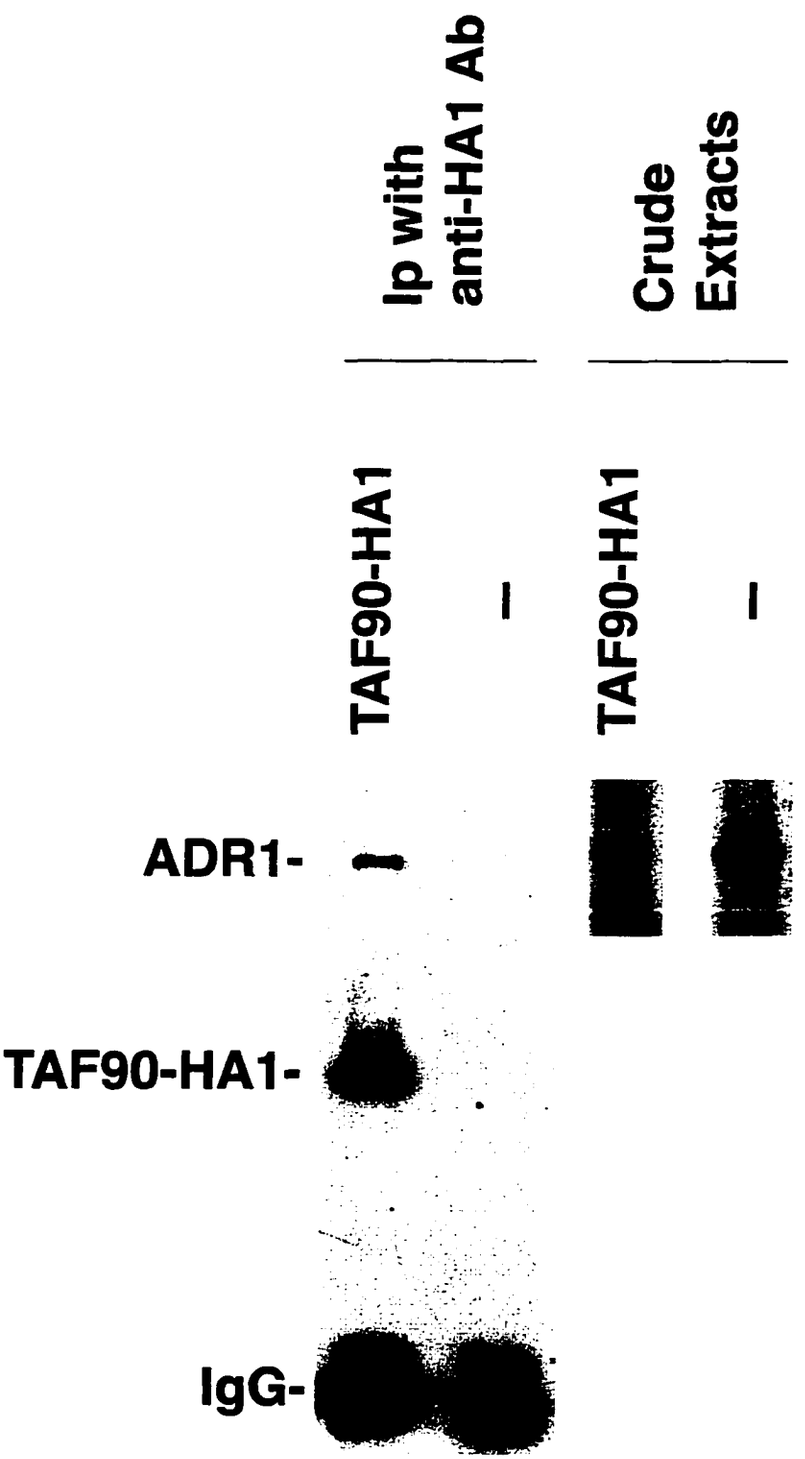


Figure 14. *ADH2* derepression does not occur in the absence of TAF90.

Total RNA from strains ZMY60 (control) and ZMY68 was prepared, separated in agarose gel and analyzed by Northern hybridization as described in the "Materials and Methods" section. Both strains were shifted to SC-Ura, 2% ethanol, 500 μM $CuSO_4$ after 7 hours incubation in SC-Ura, 4% glucose, 500 μM $CuSO_4$. Bottom panel: Identically loaded duplicate gel was stained with ethidium bromide for rRNA (23S and 16S) according to [Sambrook, 1989]. Previous experiments have determined that rRNA serves as a useful standard for quantitating mRNA loadings [Cook et al., 1993, Vallari et al., 1992].

another regulator of *ADH2* expression, were essentially unaffected by depletion of TAF90 (Figure 14a). This requirement for an intact TFIID complex *in vivo* for *ADH2* activated transcription confirms a role for TFIID in ADR1-dependent activated transcription.

Discussion.

The results reported herein demonstrate an association of ADR1 with the TFIID complex that appears important for ADR1 activation of *ADH2* . Five lines of evidence suggest that ADR1 contacts TFIID *in vivo*: 1) TADIV of ADR1 binds TFIID isolated from yeast crude extracts; 2) the ability of derivatives of TADIV to bind TFIID correlates with TADIV activation potential; 3) ADR1 co-immunoprecipitates with a known subunit of the TFIID complex TAF90; 4) intact TFIID is required for ADR1-dependent activation of *ADH2 in vivo*, as depletion of the yeast cell of TAF90 prevents *ADH2* derepression; and 5) deletion of TADIV from ADR1 severely compromises ADR1 function. This suggests a model where ADR1 either aids recruitment of TFIID to the promoter, induces structural reconfiguration of TFIID, or stimulates both processes, thereby allowing for transcription to occur. However, our attempts to show that TADIV contacts TAF90 directly have been unsuccessful to date (data not shown). There are several possible explanations for how depletion of TAF90 could affect ADH2 without ADR1 contacting TAF90 directly. Depletion of TAF90 may result in the release of other TAFs from the TFIID complex and one of those TAFs may be the one(s) that directly contact TADIV. Studies of the human yTAF90 homolog hTAF100 reveal that hTAF100 strongly interacts with a number of other TAFs [Tao et al., 1997 , Dubrovskaya et al., 1996] and this interaction could be necessary to keep these other TAFs in the TFIID complex. Alternatively, an as yet unidentified co-factor may mediate the interaction of TADIV with TAF90. It should be noted here that our previous results [Appendix A] indicate that TADIV does not contact TBP directly. It is also possible that TAF90 depletion may impose a new rate-limiting step on *ADH2* derepression unrelated to the ADR1-dependent recruitment of TFIID. The latter possibility seems quite unlikely though since TAF90 is dispensable for activated transcription of many yeast genes.

We found that ADR1 coimmunoprecipitates with TAF90 under both repressing and derepressing conditions. It needs to be taken into account, though, that in the strain in which the immunoprecipitation was done ADR1 was overexpressed from a multicopy vector and that this overexpression allowed partial derepression of the *ADH2* gene on glucose to approximately 10% of the fully derepressed level on ethanol. These results suggest that even in the presence of glucose, ADR1 can still make appropriate contacts at the *ADH2* promoter. It is possible that glucose repression affects not the ADR1-TFIID interaction, but some other aspect of ADR1-dependent *ADH2* promoter function. One such aspect could be ADR1-dependent chromatin remodeling that occurs only under derepressing conditions [Verdone et al., 1996]. On the other hand, glucose may negatively affect *ADH2* promoter function at least in part not through the ADR1, but via an ADR1-independent mechanism. The observation that LexA-ADR1 fusion protein functions as a potent activator outside the *ADH2* promoter context in the presence of glucose supports this notion [Cook et al., 1994a].

We had shown previously [Appendix A] that ADR1 TADs directly contact GCN5 and TFIIB and suggested that there should be additional targets for ADR1 activation domains. TFIID now seems to be a very likely target for ADR1 function. It should be noted, that TADIV of ADR1 is capable of strong binding of GCN5 and TFIID and that sequence determinants important for both interactions are located in residues 695-704 of TADIV.

The most widely accepted model describing how DNA-binding transcriptional activators function is that these proteins recruit components of the general transcription machinery to the promoter accelerating or allowing the formation of preinitiation complex (PIC) or stabilizing the PIC at the promoter [Ptashne, 1997]. The requirement imposed by this model on transcriptional activators is that they should physically interact with some of the general transcription factors either directly or via intermediate factors, which are called coactivators. Our results indicate that there may be several targets for ADR1 TADIV. A

number of targets for activation domains of various Drosophila, mammalian and yeast activators has been identified to date. They include a large number of core transcription factors, such as TFIIB, TFIID, TFIIF, TFIIA, TFIIH, RNA Pol II, TFIIE, etc. [Ptashne, 1997, Zawel, 1995, Burley, 1996 and references therein], as well as proteins dispensable for basal transcription *in vitro* (co-activators): for instance, CBP, SRC-1, or components of ADA complex [Heery et al., 1997, Barlev et al., 1995]. Some of the co-activators have histone acetyltransferase (HAT) activity, and the mechanism had been proposed [Grant et al., 1997] by which targeted recruitment of HATs to the promoters by activators results in removal or rearrangement of repressive nucleosomes, thus allowing formation of the initiation complex. In this regard, one of the Drosophila TAFs, dTAF 250, posesses HAT activity, and this HAT activity is conserved in its yeast homolog, yTAF130 [Bai et al., 1997; Gopal and Weil, unpublished observation]. Recently, the direct role of acetylation in modification of activity of some transcription factors had been suggested [Gu et al., 1997, Bannister et al., 1997]. Regardless of what the exact mechanism of the observed effect is, TAFs do seem to be required for *ADH2* derepression. This observation distinguishes *ADH2* from many tested yeast genes, whose basal and activated transcription does not seem to be dependent on TAFs [Moqtaderi et al., 1996, Walker et al., 1996].

General Discussion.

RNA polymerase II transcription is a very complex process and is influenced by a variety of regulatory stimuli. The mechanism of transduction of these diverse stimuli to the general transcription machinery involves post translational modifications of transcription factors as well as changes in their subcellular localization. The end result of the action of a given signal is a change in transcription rate of one or more genes. The aim of the above described research was to understand the mechanism of the ADR1-mediated increase in the transcription rate of the *ADH2* gene in response to glucose. In particular the focus of this research was understanding how the binding of the ADR1 to its cognate DNA recognition sequence brings about the activation of the downstream gene.

It was found that the activation domains of ADR1 are capable of physically contacting the components of general transcriptional machinery, namely TFIIB and TFIID, as well as the components of the ADA2 co-activator complex. Additionally, functional relevance of the observed physical interactions was supported by genetic methods. Point mutations in TFIIB that reduced its *in vitro* binding to ADR1 TADI decreased *ADH2* derepression *in vivo*. Targeted degradation of the TAF90 component of the TFIID abolished *ADH2* derepression. Deletion of *ada2* or *gcn5* also strongly affected ADR1-mediated transcription.

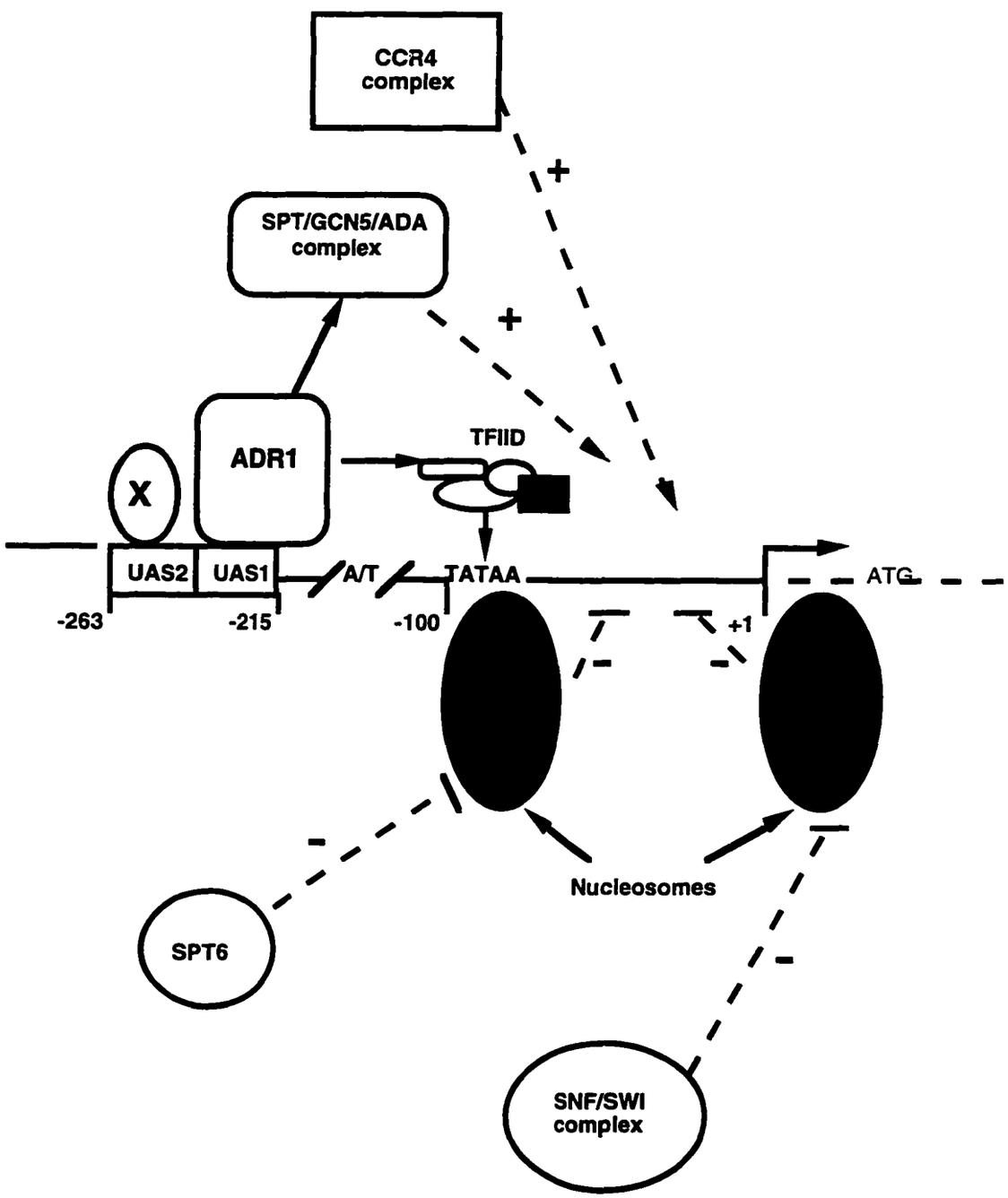
Interestingly, the extent of the effect of *ada2* or *gcn5* deletions or of a point mutation in the TFIIB on *ADH2* transcription was much less than the effect of TAF90 targeted degradation, even though the pattern of binding of these proteins to ADR1 appears to be similar. The possible reason for this may be that the TFIID binding step is strongly inhibited at the *ADH2* promoter and is the rate limiting step at this promoter in the absence of ADR1. The recruitment of the TFIID to the *ADH2* promoter by ADR1 requires TAF90,

and after TFIID is recruited, other steps, such as TFIIB entry into the preinitiation complex, become rate limiting. Even though these steps also can be influenced by ADR1, the "intrinsic" rate of TFIIB entry into the complex is higher than that of the TFIID. This hypothetically higher rate of TFIIB recruitment without ADR1 participation allows substantial transcription to occur even when ADR1-TFIIB interaction is disrupted.

The disruption of the ADA2 complex may have effects that are less severe than TFIID has on *ADH2* transcription due to the partial redundancy of function. Recent data [Brownell et al., 1996] indicate that the function of the ADA2 complex is to relieve repressive chromatin structure by histone acetylation. Yeast TFIID also contains a histone acetyltransferase (HAT) activity, namely the TAF130 component of TFIID is a histone acetyltransferase [Gopal and Weil, unpublished observations]. So, even though ADA2 function is required for maximal activity of the promoter, it seems likely that repressive nucleosomes can still be modified by some other HAT, one plausible example being TAF130. An alternative possibility is that the activation of the *ADH2* may occur without the ADA2 complex function simply because this function is not crucial for this particular promoter.

Interestingly, deletions of the *spt3* or *spt7* gene apparently results in a severe defect in *ADH2* derepression [V. Badarinarayana, C. L. Denis, pers. commun]. SPT3 had been shown to associate with TBP [Eisenmann et al., 1992], and both SPT3 and SPT7 were found to be a part of the SAGA (Spt/Ada/Gcn5) complex [Grant et al., 1997]. One possible model which may explain why some components of SAGA have a stronger effect on *ADH2* transcription than others is that the main function of SAGA at the *ADH2* promoter may be not to modify chromatin (this function would be completely abolished by the GCN5 disruption) but to function as an adaptor between ADR1 and TFIID. The SPT3 and SPT7 components of SAGA may be crucial for this adaptor function (Figure 15).

Figure 15. Mechanism of ADR1 function at the *ADH2* promoter



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Appendix A

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ADR1 Activation Domains Contact the Histone Acetyltransferase GCN5 and the Core Transcriptional Factor TFIIB*

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The yeast transcriptional activator ADR1, which is required for *ADH2* and peroxisomal gene expression, contains four separable and partially redundant activation domains (TADs). Mutations in *ADA2* or *GCN5*, encoding components of the ADA coactivator complex involved in histone acetylation, severely reduced LexA-ADR1-TAD activation of a *LexA-lacZ* reporter gene. Similarly, the ability of the wild-type *ADR1* gene to activate an *ADH2*-driven promoter was compromised in strains deleted for *ADA2* or *GCN5*. In contrast, defects in other general transcription cofactors such as CCR4, CAF1/POP2, and SNF/SWI displayed much less or no effect on LexA-ADR1-TAD activation. Using an *in vitro* protein binding assay, ADA2 and GCN5 were found to specifically contact individual ADR1 TADs. ADA2 could bind TAD II, and GCN5 physically interacted with all four TADs. Both TADs I and IV were also shown to make specific contacts to the C-terminal segment of TFIIB. In contrast, no significant binding to TBP was observed. TAD IV deletion analysis indicated that its ability to bind GCN5 and TFIIB was directly correlated with its ability to activate transcription *in vivo*. ADR1 TADs appear to make several contacts, which may help explain both their partial redundancy and their varying requirements at different promoters. The contact to and dependence on GCN5, a histone acetyltransferase, suggests that rearrangement of nucleosomes may be one important means by which ADR1 activates transcription.

In *Saccharomyces cerevisiae*, the transcriptional activator ADR1 is required for expression of the glucose-repressible alcohol dehydrogenase gene (*ADH2*) under nonfermentative conditions (1). It also regulates genes required in glycerol metabolism (2, 3) and peroxisome function and biogenesis (4, 5). ADR1 is a zinc finger, DNA-binding protein that is 153 kDa in size (6, 7). Its regulation of *ADH2* under nonfermentative growth conditions occurs by binding to UAS1, a palindromic site, located 110 bp[1] upstream of the *ADH2* TATAA sequence (7). Similar UAS1 elements are located upstream of other genes that ADR1 controls (4, 8). Three regions of ADR1 have been identified that are required for its efficient activation of *ADH2* transcription: transcription activation domain (TAD) I (76–172), TAD II (263–357), and TAD III (359–509) (2, 9, 10, 11). A fourth region (642–1323) has been implicated for efficient peroxisomal gene expression (5). The presence of four transactivation regions suggests that ADR1 may make multiple protein contacts to transcriptional cofactors and/or core transcriptional components. The observation that TADs II and III are functionally redundant (9) suggests that some of these contacts may be made to the same protein.

There are a number of potential targets for ADR1 activation domains. Core transcriptional components including TBP, TFIIB, TFIIF, TFIIE, and TAFs have been implicated in mammalians system as being direct contacts for transcriptional activators (12). In yeast, the GAL4 activation domain has been shown to bind TBP but not TFIIB *in vitro* (13). In addition to these core transcriptional factors, other cofactors or coactivators may mediate the action of activators. The ADA2 complex is one such coactivator complex. These proteins have been shown to bind activators like VP16 and GCN4 (14, 15) and to be required for maximal transcriptional activity of several yeast activators (16). However, some yeast activators like HAP4 and GAL4 (16, 17) are slightly affected or not affected by defects in the ADA2 complex. Because the ADA2 complex has been also been shown to bind TBP (14), it has been suggested that the ADA2 complex acts as a direct mediator between activators and core factors. Recent evidence indicates that GCN5 is a histone acetyltransferase (18). Activator recruitment of the ADA2 complex may result, therefore, in histone acetylation that would help relieve a repressive chromatin structure.

Genetic studies have implicated several general transcriptional factors as possibly mediating ADR1 activator function. The CCR4 and CAF1/POP2 proteins, components of a multisubunit transcriptional regulatory complex, are required for proper expression of a number of yeast genes including *ADH2* (19–22). In previous studies we have analyzed the dependence of ADR1 TADs on the CCR4 and CAF1 cofactors, but *ccr4* and *caf1* defects generally had only 2–3-fold effects on ADR1 TAD function (22, 23). Moreover, *ccr4* and *caf1* defects can affect *ADH2* expression under conditions when ADR1 is inactive (19, 22). The SNF/SWI factors involved in nucleosomal remodeling (24) are also known to be important to *ADH2* derepression (25–27). Consistent with the role of the SNF/SWI factors in *ADH2* expression are the presence of two repressing nucleosomes at the *ADH2* promoter that are removed in an ADR1-dependent manner during *ADH2* derepression (28). Neither of the repressing nucleosomes in the *ADH2* promoter occupies the

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This research is dedicated to the memory of Michael von Ciriacy, without whom it would not have begun.

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[1] The abbreviations used are: bp, base pair(s); TAD, transactivation domain; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; KAc, potassium acetate.

TABLE I
Yeast strains

Strain	Genotype
EGY188	*MATα ura3–52 his3 trp1 LexAop-LEU2*
cEGY188-g1	Same as EGY188 except *gcn5::URA3*
PSY316	*MATα ade2–101 his3-Δ200 leu2–3,112 lys2 ura3–52*
PSY316-ada2	Same as PSY316 except *ada2::URA3*
GMY26	Same as PSY316 except *gcn5::URA3*
CY26	*MATα ura3–52 his3-Δ200 trp1-Δ1 leu2-Δ1 lys2–801α ade2–101o*
CY57	Same as CY26 except *swi2::HIS3*
612–1d	*MATα ura3 his3 leu2 trp1 adh1–11*
612–1d-a1	Same as 612–1d except *ada2::URA3*
612–1d-g1	Same as 612–1d except *gcn5::URA3*
612–1d-6a	Same as 612–1d except *caf5::LEU2*
1006–2-3b	*MATα ura3 his3 leu2 trp1 spt10::TRP1 adh1–11*
787–6b	*MATα adh1–11 adr1–1::ADR1–5*-TRP1 ura3 leu2 his3 trp1*
40–1C	*MATα ura3 his3 trp1 adh1–11 adr1–1::ADR1-TRP1*

UAS1 site to which ADR1 binds (28), which may explain the limited effects of *snf/swi* mutations on *ADH2* derepression (25, 27).

In this paper we continue our investigation into the factors required for ADR1 function. We report that mutations in *ADA2* and *GCN5* severely compromise the ability of ADR1 TADs to activate gene expression *in vivo*. In addition, we demonstrate that there is physical interaction between ADA2 and GCN5 and the ADR1 TADs *in vitro*. TAD IV binding to GCN5 was shown to directly correlate with TAD IV activation function. TADs I and IV were also shown to make specific contacts to TFIIB but not to TBP. These results suggest that ADR1 TADs activate gene expression in yeast through direct physical contacts with multiple proteins.

EXPERIMENTAL PROCEDURES

Yeast Strains

Yeast strains are listed in Table I. Strain EGY188 was used for transformation with plasmids expressing LexA-ADR1 fusion proteins. The *ada2::URA3* and *gcn5::URA3* disruptions in strain PSY316 were a gift from L. Guarante, and strains CY26 and CY57 were provided by C. Peterson.

Plasmid Constructions

LexA-ADR1 Fusions—All LexA-containing plasmids encoded full-length LexA-(1–202) except for LexA-ADR1-(1–220), which contained only the DNA binding domain of LexA (residues 1–87). The LexA fusion proteins were expressed from an *ADH1* promoter and were expressed in yeast on a 2 μ plasmid containing the selectable marker *URA3* (9). Plasmids containing LexA-ADR1-(1–1323) (full-length), LexA-ADR1-(1–220)-TAD I, and ADR1-(262–359)-TAD II have been previously described (9). LexA-ADR1-(420–462)-TAD III was constructed following polymerase chain reaction using oligonucleotides designed to generate *NcoI* and *XhoI* sites at the ends of the 420–462 fragment of ADR1 (oligonucleotides: 5′-GTCATGCCATGGATCCTGATTTCGTCGAT-3′ and 5′-GCGTACTCGAGAAGCTTGACGTGGCGGTAG-3′). The resultant ADR1 polymerase chain reaction product after cleavage with *NcoI* and *XhoI* was ligated into the *NcoI-XhoI* sites of plasmid LexA-202-5 (9) to generate LexA-ADR1-(420–462). LexA-ADR1-(642–704)-TAD IV and its deletion derivatives were derived from plasmid LexA-ADR1-(642–1323) (9) by selecting for LexA-ADR1-(642–1323) derivatives that could activate the *LexAop-LEU2* reporter in strain EGY188. All Leu2$^+$ prototrophs analyzed were the result of alterations in the amino acid region 672–704 of ADR1 that created truncated LexA-ADR1-(642–704) derivative proteins. Each of these proteins was expressed to a comparable extent as analyzed by Western analysis. Sequencing of each of these resultant plasmids revealed stop codons in the DNA sequence that were in agreement with the relative size of each of the LexA-ADR1-(642–704) protein derivatives.

GST-ADR1 Fusions—The construction of GST-ADR1-(1–262)-TAD I was conducted by removing residues 1–262 from pJC100 (29) with *NcoI* and *XmnI* and ligating into pGEM-5ff(+) cut with *NcoI* and *EcoRV*. The resultant plasmid was then digested with *NcoI* and *SalI* and ligated into *NcoI* and *XhoI* sites of pGEX-KG (30) to create GST-ADR1-(1–262). GST-ADR1-(263–359)-TAD II was constructed by inserting a *BamHI-SalI* fragment of LexA-ADR1-TAD II into *BamHI-SalI* sites of pGEX-KG. GST-ADR1-(420–462)-TAD III was constructed by inserting a *NcoI-HindIII* fragment of LexA-ADR1-TAD III into the *NcoI-HindIII* sites of pGEX-KG. In the construction of GST-ADR1-TAD IV-(642–704), a DNA fragment from LexA-ADR1-TAD IV-(642–704) was synthesized by polymerase chain reaction with oligonucleotides 5′-GCTTCACCATTGAAGGGC-3′ and 5′-CCGAATTCACAAGATTTGATAGTGCTCG-3′ designed to create *SmaI-EcoRI* restriction sites at the ends. This fragment was ligated in *SmaI-EcoRI* sites of pGEX-KG that had been precut with *BamHI*, blunt ended with the large subunit of *Escherichia coli* DNA polymerase (Klenow), and religated. For all other GST-ADR1-TAD IV truncations, a *SmaI-EcoRI* fragment from the LexA-ADR1-TAD IV truncations was inserted into *SmaI-EcoRI* sites of pGEX-KG whose *BamHI* site had been filled in with Klenow to create the proper reading frame. GST-Vpu contains the hydrophilic segment of the HIV1 protein Vpu (residues 33–81) and was a gift of B. Kemp (St. Vincent's Institute of Medical Research, Melbourne, Australia). GST-CAF1 and GST-CCR4 were provided by J. Meegan and S. Fontaine (University of New Hampshire, Durham, NH), respectively.

Transformations, Enzyme Assays, and Growth Conditions

All yeast transformations were conducted by using the lithium acetate method (31). ADH II and β-galactosidase enzyme assays were conducted as described (9). Conditions for growth of cultures on minimal medium lacking uracil and histidine or YEP medium containing either 8% glucose, 3% ethanol or 2% ethanol, 2% glycerol have been described elsewhere (9).

In Vitro Binding Assay

GST fusion proteins were expressed and bound to glutathione-agarose beads (Sigma) in binding buffer (1 × phosphate-buffered saline, 1% Triton). Beads were washed 4 times with binding buffer and then incubated for 60 min at 4 °C in A300 buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 300 mM potassium acetate (KAc), 1% Triton) containing 1 mg/ml *E. coli* extract and 40–200 ng of [^{35}S]methionine-labeled *in vitro* translated proteins. *In vitro* translation of T7 fusion proteins was carried out by using the TNT-coupled transcription translation system (Promega). Unbound proteins were removed by four washes with A300 buffer, and specifically bound proteins were analyzed by SDS-PAGE after elution with 50 mM reduced glutathione in 50 mM Tris, pH 8.0, or by boiling beads directly in sample buffer. High salt washes of radiolabeled proteins bound to GST fusions were conducted as described above except that A300 buffer was changed to the appropriate KAc concentration (600, 900, or 1200 mM). The T7-GCN5, -ADA2, and -ADA3 plasmids were gifts of L. Guarente (MIT). T7-TFIIB was provided by M. Hampsey (University of New Hampshire), T7-TBP was a gift of S. Johnston (University of Texas Southwestern Medical Center), and T7-N-TFIIB and T7-C-TFIIB were provided by R. Pollock (Arias Pharmaceuticals).

Deletion of Residues 642–704 of ADR1 at Its Chromosomal Locus

The ADR1 moiety in pBR322–411B (32) was cut with *BglII* (bp +1,923) and *BstBI* (bp −2,119), and the overhangs were filled in with Klenow and religated with T4 DNA ligase. The resultant plasmid was digested with *SacI* (bp +1,713) and *BamHI* (bp −3,200), and the 1.5-kb ADR1 fragment containing the deletion was ligated to pCD10 (9) previously treated with *SacI* and *BamHI*. To improve the efficiency of integration, the *BamHI* fragment of pCD10 (bp 3,200–3,609) was added back to the pCD10 plasmid, which contained the *BglII-BstBI* deletion.

TABLE II

The effect of ada2, gcn5, and snf2 deletions on the ability of LexA-ADR1 fusions to activate a LexA-lacZ reporter

β-Galactosidase activities (units/mg) represent averages of at least three separate transformants measured in isogenic strains PSY316 (wild type), PSY316-ada2 (*ada2*), and GMY26 (*gcn5*) and the isogenic strains CY26 (*SNF2*), and CY57 (*snf2*) using the *LexA-lacZ* 1840 reporter gene as previously described (9). The 1840 reporter plasmid contains a single LexA operator site upstream of the *GAL1-lacZ* promoter. LexA-ADR1 fusions are described under "Experimental Procedures." TADs I and II were defined as activation domains previously (9, 10); TAD III previously localized to residues 359–506 (9) was more precisely located to 420–462 (J. Saario and T. Young, personal communication); and TAD IV was defined as shown in Fig. 3 and Table V. Strains were grown on minimal medium lacking uracil and histidine and supplemented with 8% glucose as previously described (9). LexA fusions were expressed to comparable levels as determined by Western analysis. The LexA-ADR1 fusion proteins were approximately 50-fold more abundant than single copy ADR1. S.E. values were less than 20% in each case.

LexA fusion	β-Galactosidase activity				
	wt	*ada2*	*gcn5*	*SNF2*	*snf2*
			units/mg		
ADR1-(1–1323) (full-length)	1900	100	58	1100	1300
ADR1-(1–220)-TAD I	23	<1	ND[a]	ND	ND
ADR1-(262–359)-TAD II	420	<1	7.5	280	220
ADR1-(420–462)-TAD III	46	1.3	1.8	41	22
ADR1-(642–704)-TAD IV	150	4.3	5.0	160	140
LexA alone	<2	<2	<2	<2	<2

The resulting construct was cut with *Sna*BI and transformed into strain 500-16 to site-specifically integrate at the *adr1-1* locus. Identification of single integrants and their subsequent analysis were conducted as described previously (33).

RESULTS

The ADA2 Complex Is Required for ADR1 Activation of Transcription—ADR1 contains three separate transcription activation domains, and a fourth activation region has been implicated in residues 642-1323 (5, 9). TAD IV was subsequently more precisely localized to residues 642–704 (see below). In order to identify the factors through which the four individual ADR1 TADs act, the effect of deleting different general transcription cofactors or adaptors on LexA-ADR1-TAD transactivation has been analyzed.

Deletion of the *ADA2* or *GCN5* gene dramatically reduced the ability of LexA-ADR1 (full-length) to activate the *LexA-lacZ* reporter gene (Table II). Moreover, the activation function of each LexA-ADR1-TAD was shown to be highly dependent on functional ADA2 complex components (Table II). The expression of the LexA-protein fusions was unaffected by deletions in *ADA2* or *GCN5* as analyzed by Western analysis (data not shown). An *ada2* or *gcn5* deletion does not uniformly affect all LexA transactivators (16, 17), and its reduction of ADR1 activation ability (20–50-fold effects) appeared to be one of the most severe of the several activators previously tested (17, 34). The observation that both an *ada2* and a *gcn5* deletion affected LexA-ADR1-TAD function is in agreement with previous results demonstrating that inactivation of any component of the ADA2 complex reduces the function of the complex (17, 35). In contrast to these results, the deletion of the *SNF2/SWI2* gene, which is known to severely reduce the activity of GAL4 and other LexA transactivators (36), displayed little or no effect on ADR1 TAD function (Table II). Similarly, deletion of the *SNF5* and *SWI1* genes, two other components of the SNF/SWI complex, resulted in little or no diminution of ADR1 TAD function (data not shown). In addition, we have previously shown that mutation of either *CCR4* or *CAF1*, two components of a multisubunit transcription complex required for maximal *ADH2* expression, had generally only 2–3-fold effects on LexA-ADR1-TAD activation of a *LexA-lacZ* reporter (22, 23).

In order to verify the physiological relevance of the requirement of the ADA2 complex for ADR1 activation, we examined whether the activation ability of the wild-type full-length ADR1 protein at its normal cellular concentration was also compromised. We found that deletion of the *ADA2* or *GCN5* gene dramatically reduced the ability of ADR1 to activate an *ADH2-lacZ* reporter under derepressed growth conditions (Table III). The derepression of the *ADH2-lacZ* reporter gene has been shown previously to be strictly dependent on ADR1 (7), and *ADH2-lacZ* contains all of the promoter sequences necessary for complete derepression of *ADH2* (37). In contrast, an *ada2* or *gcn5* deletion had at most 2-fold effects on the similar *CYC1-lacZ* reporter gene, which is under the control of the HAP1 and HAP2/3/4 activators (Table III). Relatedly, the LexA-HAP4 transactivation ability is not affected by *ada2* or *gcn5* deletions (16, 17).

We subsequently analyzed the effect of *ada2* and *gcn5* defects at the wild-type *ADH2* promoter. *ADH2* expression was reduced 2.5-fold by an *ada2* deletion under ethanol growth conditions (Table IV). Similarly, the ability of an *ADR1*^{c} allele to bypass glucose repression and allow increased *ADH2* expression was reduced about 3-fold by an *ada2* defect (Table IV). The *ada2* allele, in contrast, had no effect on *spt10*-enhanced *ADH2* expression under glucose-repressed conditions, which occurs in an ADR1-independent manner (Table IV) (19). A *gcn5* allele also reduced the ability of the *ADH2* gene to derepress (Table IV). These results indicate that the ADA2 complex is required for ADR1-dependent activation of transcription in different promoter contexts.

Components of the ADA2 Complex Physically Interact with Each of the TADs of ADR1—Since the activation ability of the ADR1 TADs was strongly ADA2 complex-dependent, we tested if this dependence was the result of ADA2, GCN5, and ADA3 proteins making direct contacts with ADR1 TADs. GST fusions to individual ADR1 TADs were constructed (Fig. 1*A*), and their ability to bind to [^{35}S]methionine-labeled *in vitro* translated ADA2, ADA3, and GCN5 proteins (Fig. 1*B*) was examined (Fig. 1*C*). As shown in Fig. 1*C*, *second row*, GST-ADR1-TAD II could bind the ADA2 protein. ADA2 did not bind control proteins GST and GST-Vpu, nor did it display significantly increased binding to GST-ADR1-TAD I, III, or IV (Fig. 1*C*). In control experiments, *in vitro* translated luciferase was incubated with each of the GST-ADR1-TADs and GST, and no binding to any of these fusions was observed (Fig. 1*C*). *In vitro* translated ADA3 did not bind to any of the four GST-ADR1-TADs (Fig. 1*C*, *third row*). In contrast, *in vitro* translated GCN5, while incapable of binding to GST alone, did bind to all four GST-ADR1-TADs (*bottom row*). GCN5, however, did not display binding to other GST-fusions such as GST-CAF1 or GST-Vpu (*bottom row*). For ADA2 binding to TAD II and GCN5 binding to each of the TADs, generally about 1–5% of the input radioactivity was retained by the GST-TADs (data not shown).

The stability of binding of ADA2 and GCN5 to ADR1 TADs was further tested by determining the effect of increasing salt concentrations on each of these interactions. As shown in Fig. 2, the binding between GST-ADR1-TAD II and ADA2 was

TABLE III

Effect of the ada2 and gcn5 alleles on ADR1 activation of the ADH2-lacZ gene

β-Galactosidase activities (units/mg) represent the average of at least three separate transformants measured in the isogenic strains PSY316 (wild type), PSY316-ada2 (*ada2*), and the strain GMY26 (*gcn5*) as described (9). Each of these strains carried either the ADCY4 reporter plasmid (*ADH2-lacZ*) or the LG699Z (*CYC1-lacZ*) reporter (7). ADCY4 and LG699Z are identical except for the upstream sequences controlling the CYC1 TATA region in front of the *LacZ* gene. Strains were grown in minimal medium lacking uracil and containing 2% each of glycerol and ethanol (DR) or 8% glucose (R). S.E. values were less than 15%. ND, not done.

Reporter gene	Strain	Wild type		*ada2*		*gcn5*	
		R	DR	R	DR	R	DR
		units/mg		*units/mg*		*units/mg*	
ADH2-lacZ	PSY316	1.8	290	1.5	6.3	ND	0.45
CYC1-lacZ	PSY316	12	640	2.5	290	ND	430

TABLE IV

The effect of ada2 and gcn5 alleles on ADH2 expression

ADH II enzyme activities were conducted as described under "Experimental Procedures." Strains were grown either on 8% glucose (R) or 3% ethanol (DR). Wild type, strain 612-1d; *ada2*, isogenic to 612-1d except *ada2*::*URA3*; *gcn5*, segregants from cross-EGY188-g1 (*gcn5*) and 612-1d-6a; *adr1*, data taken from Ref. 9. *ADR1-5*^{c} *ada2*, and *ADR1-5*^{c} segregants were from cross-612-1d-a1(*ada2*) and 787-6b (*ADR1-5*^{c}); *spt10 ada2*, and *spt10* segregants were from cross-612-1d-a1 (*ada2*) and 1005-2-3b (*spt10*). For the wild-type segregants from cross-EGY188-g1 and 612-1d-6a, ADH II enzyme activities ranged from 2000 to 2700 milliunits/mg. ADH II activities were the average of at least four separate determinations for individual strains or the average of at least three segregants. S.E. values were less than 20%.

Relevant genotype	ADH II	
	R	DR
	milliunits/mg	
Wild type	<5	2600
ada2	<5	970
gcn5	<5	310
adr1	<5	10
ADR1-5^c	150	ND
ADR1-5^c *ada2*	64	ND
spt10	85	ND
spt10 ada2	110	ND

stable to salt concentrations up to 1.2 M KAc. Similarly, the binding between GST-ADR1-TAD IV and GCN5 was relatively insensitive to increasing salt concentrations (Fig. 2). GCN5 binding to ADR1 TADs I, II, and III was also stable at high salt concentrations (Fig. 2). These results suggest that the dependence of ADR1 TAD activation on the ADA2 complex was the result of specific ADR1 TAD interactions with GCN5 and ADA2.

Binding of GCN5 to ADR1 TAD IV Deletion Derivatives Correlates with Their Ability to Activate Transcription—Deletion analysis of ADR1 suggested a fourth possible activation domain in the C-terminal 642-1323 region (9). We have subsequently shown that LexA-ADR1-(642-704) is capable of activating transcription of a *LexA-lacZ* reporter plasmid (Table II and Fig. 3*A*). This region was confirmed as important to full-length ADR1 function, since deletion of residues 642-704 reduced LexA-ADR1 activation of a *LexA-lacZ* reporter gene by 16-fold (Table V) without affecting LexA-ADR1 abundance (data not shown). Moreover, deletion of TAD IV from the wild-type *ADR1* at its chromosomal locus reduced ADR1 ability to activate *ADH2* by 20-fold (Table V). TAD IV has also been shown to be absolutely required for peroxisomal function in the utilization of oleate (5). Because TAD IV appears to be especially important for ADR1 function, we characterized TAD IV further as to its regions important for activation and binding to GCN5.

C-terminal deletions of LexA-TAD IV were assayed for their ability to activate a *LexA-lacZ* reporter gene (Fig. 3*A*). All deletion derivatives were expressed to comparable extents in yeast (data not shown), indicating that differences in protein stability were not the cause of the differences in activity. LexA-ADR1-(642-704), -(642-701), and -(642-698) retained significant transcriptional activity, whereas LexA-ADR1-(642-694) and smaller derivatives were less active.

To determine if these differences in LexA-TAD IV derivative activation abilities were a potential consequence of their ability to interact with GCN5, GST fusion proteins of the TAD IV deletion derivatives were constructed, and their ability to bind with radiolabeled GCN5 was analyzed. All of the GST-TAD IV derivatives were expressed to comparable extents (Fig. 3*B*, *bottom row*). The ADR1-(642-704), ADR1-(642-701), and ADR1-(642-698) moieties, which had the greatest activation abilities (Fig. 3*A*), displayed the strongest binding to GCN5 (Fig. 3*B*, *top row*). The remaining derivatives displayed reduced binding to GCN5 and reduced ability to activate. These data show that the activation strength of ADR1 TAD IV derivatives correlates directly with that of their binding to GCN5 (see Fig. 3*A*).

TADs I and IV of ADR1 Bind Specifically to TFIIB—The observation that a *gcn5* or *ada2* disruption resulted in only partial blockage of ADR1 activation of *ADH2* (Table IV) suggested that ADR1 was interacting with factors in addition to the ADA2 complex. Because it is known that transcriptional activators can make contacts to several different cofactors and core transcriptional components, we analyzed further whether ADR1 TADs could bind the transcriptional factor TFIIB. Incubation of *in vitro* translated yeast TFIIB with GST-ADR1-TAD fusions demonstrated that TAD I and to a lesser extent TAD IV could retain TFIIB (Fig. 4). *In vitro* translated TFIIB, in contrast, did not display significant binding to GST alone, GST-Vpu, GST-TAD II, or GST-TAD III (Fig. 4). TFIIB was able to bind about 5-fold better to GST-TAD II than to GST alone but only about 2-fold better than to GST-Vpu, suggesting that this interaction between TAD II and TFIIB may not be significant. The binding of TFIIB to TADs I and IV was also stable to high salt washes (1.2 M KAc) (data not shown). The region of TAD IV that was capable of binding TFIIB was analyzed using the series of GST-TAD IV variants described above. Deletions of TAD IV that reduced its activation function (Fig. 3*A*) similarly reduced its binding to TFIIB (Fig. 3*B*). As shown with GCN5 binding to these TAD IV deletion variants, the correlation between TAD IV activation ability and binding to TFIIB was nearly exact (Fig. 3*A*).

The region of TFIIB that interacted with TAD I and TAD IV was localized using the *in vitro* binding assay in order to further examine the specificity of this interaction. The C-terminal half of TFIIB (residues 135-345) was found to retain the ability to bind to both TADs I and IV, whereas the N-terminal segment of TFIIB (residues 1-134) did not bind GST-TADs I or IV (Fig. 5). In contrast to TFIIB, TBP displayed little or no increased binding to GST-ADR1-TADs I or IV as compared with the control GST-Vpu (Fig. 4). TBP also did not bind TAD III and interacted with TAD II only about 2-fold better than the GST-Vpu control. These data suggest that TFIIB may be an additional factor through which ADR1 acts.

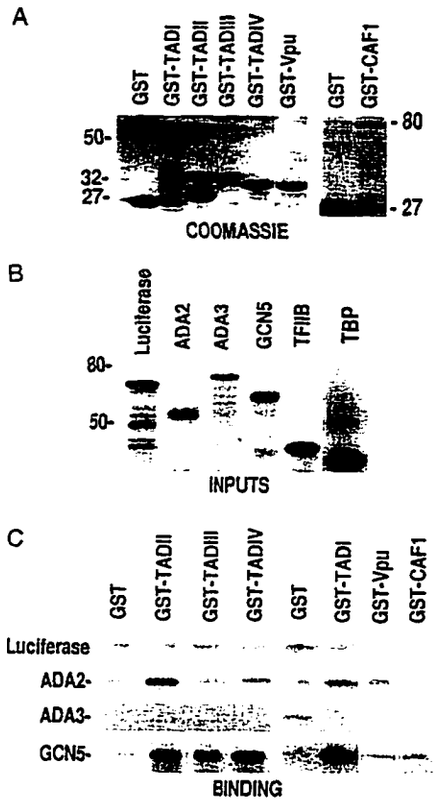

FIG. 1. **Binding of ADR1 TADs to ADA2 and GCN5.** *A*, Coomassie-stained GST-TAD fusions. GST fusions were induced as described (30), bound to glutathione-agarose beads, and eluted from glutathione-agarose beads by boiling and separation by SDS-PAGE. Fifty μg of GST-TAD fusion is represented in each lane. GST-TAD I contains residues 1–262 of ADR1; GST-TAD II contains residues 262–359; GST-TAD III contains residues 420–462; and GST-TAD IV contains residues 642–701. *B*, *in vitro* translated proteins. T7 fusion proteins were translated *in vitro* with [^{35}S]methionine as described under "Experimental Procedures." One μl of radioactive proteins was separated by SDS-PAGE and identified following fluorography. *C*, binding of *in vitro* translated proteins to GST-TAD fusions bound to glutathione-agarose beads. Forty μl of *in vitro* translated proteins were incubated with 50 μg of GST fusions and, after washing, eluted by boiling. Similar results were obtained when bound proteins were eluted with excess glutathione. In general, a protein was considered to bind specifically if at least 15–20-fold more radioactive protein was retained by the GST-TAD than by the GST-Vpu control and 20–40-fold more than for GST as observed in at least three separate *in vitro* binding analyses. Densitometric analysis using an EC-210 densitometer was used to quantitate relative intensities of bound proteins. *Top row*, binding of radioactive luciferase to GST and GST-TADs; *second row*, binding of ADA2 to GST, GST-Vpu, and GST-TADs; *third row*, binding of radioactive ADA3 to GST and GST-TADs; *bottom row*, binding of radioactive GCN5 to GST, GST-Vpu, GST-CAF1, and GST-TADs.

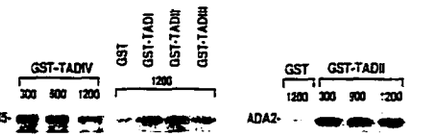

FIG. 2. **Stability of TAD binding to ADA2 and GCN5 to high salt washes.** After binding of radioactive proteins to GST-TADs as described in Fig. 1, different wash conditions were used. The *numbers* refer to the concentration (in mM) of KAc used in the wash buffer. Bound proteins were eluted with boiling and detected with fluorography following SDS-PAGE.

DISCUSSION

The ADA2 Complex Mediates ADR1 Activation—ADR1 is the primary transcriptional activator of the *ADH2* gene and has been shown previously (9) and herein to contain four separable domains capable of activating transcription. These individual activation domains were found to be highly dependent on a functional ADA2 complex for their ability as LexA fusions to activate a LexA reporter system. Individual LexA-TAD fusions were nearly completely blocked for activation when either the *ADA2* or *GCN5* gene was deleted. Other LexA-transactivators such as LexA-GCN4 have also been shown to function at reduced levels when components of the ADA2 complex are deleted (17, 34). In contrast, LexA fusions such as LexA-HAP4 are nearly fully transcriptionally active in the absence of components of the ADA2 complex (17). Full-length ADR1, when fused to LexA, also displayed a 20–30-fold reduction in activation function when components of the ADA2 complex were defective. The observation that LexA-ADR1 (full-length) retained a substantial, albeit reduced, function suggests that the activation domains in full-length LexA-ADR1 in this context must also be capable of binding targets in addition to the ADA2 complex.

Several other cofactors known to be required for *ADH2* derepression were similarly investigated in their requirement for LexA-ADR1-TAD function. Defects in components of the SNF/SWI complex had essentially no effect on ADR1 activation of the *LexA-lacZ* reporter in contrast to their sizable reduction in LexA-GAL4 activation of the same reporter gene (36). The CCR4 and CAF1 transcriptional regulatory factors that form a multisubunit complex reduced LexA-ADR1-TAD function generally about 2–3-fold (22, 23). This dependence on CCR4 and CAF1 appears, however, to represent an indirect requirement, since *in vitro* binding assays have been unable to substantiate any specific interaction between ADR1 TADs and CCR4 or CAF1 (23) and since *ccr4* and *caf1* can affect *ADH2* expression independent of ADR1 activity (19, 22). Core transcriptional factors may represent other potential targets for ADR1 (see below).

The ADR1 activation of transcription of the *ADH2-lacZ* reporter was also severely reduced by defects in the ADA2 complex. In addition, *ada2* and *gcn5* deletions reduced the ability of ADR1 to activate the *ADH2* gene at its chromosomal location. These results confirm the importance of the ADA2 complex in ADR1 activation. Yet, the fact that an *ada2* or *gcn5* disruption does not give the same *ADH2* phenotype as an *ADR1* deletion highlights the fact that at the *ADH2* locus ADR1 must also be capable of making additional contacts to activate transcription. It should be noted that the effect of *ada2* or *gcn5* deletions on ADR1 activation ability was more severe when the reporter gene (either *LexA-lacZ* or *ADH2-lacZ*) was on a high copy plasmid than when it was positioned in the chromosome (the *ADH2* locus). The cause of this difference is unclear, although the plasmid-borne promoters and the chromosomal promoter may differ in terms of chromatin structure or in assembly of ADA2 complex-dependent transcription complexes.

Physical Interaction of ADR1 TADs with Components of the ADA2 Complex—Each of the ADR1 TADs could selectively bind components of the ADA2 complex. ADA2 interacted with TAD II and GCN5 with each of the TADs. These interactions appear specific for several reasons. First, for the interactions of GCN5 and ADA2 to the ADR1 TADs to be considered significant, at

A

LexA-ADR1-TADIV derivatives	β-galactosidase activity (U/mg)	Relative binding to GCN5	Relative binding to TFIIB
LexA-ADR1 642-704	190	34	17
LexA-ADR1 642-701	85	19	12
LexA-ADR1 642-698	70	24	10
LexA-ADR1 642-694	18	4.1	1
LexA-ADR1 642-692	30	4.0	1
LexA-ADR1 642-687	24	1.6	1
LexA-ADR1 642-679	8	1.3	1
LexA-ADR1 642-675	3	1.7	1
LexA	1.5	1	1

B

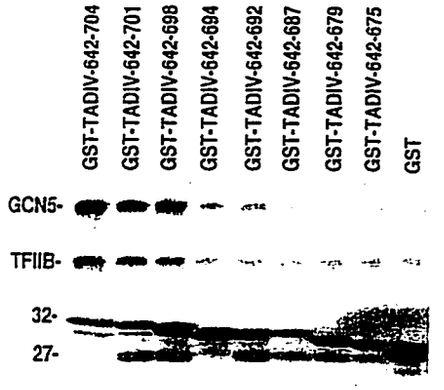

FIG. 3. **TAD IV activation ability correlates with binding to GCN5 and TFIIB.** *A*, effect on LexA-ADR1-(642–704) transcriptional activity by C-terminal deletions. LexA-ADR1-(642–704) and its deletion derivatives were derived as described under "Experimental Procedures." Each derivative was expressed to a comparable extent in yeast strain EGY188 as analyzed by Western analysis (data not shown). β-Galactosidase activities represent averages of at least three separate transformants and were assayed as described in Table II. S.E. values were less than 20%. The LexA reporter was p1840 in all cases. Relative binding to GCN5 or TFIIB compares the amount of radiolabeled GCN5 or TFIIB bound to the GST-ADR1-TAD IV derivative with the amount bound to GST alone. Data for relative binding was obtained following densitometric analysis of the data displayed in *B*. *B*, the effect of C-terminal deletions of LexA-ADR1-(642–704) on its ability to bind GCN5 or TFIIB. GST-ADR1-TAD IV derivatives of length as indicated in the *figure* were expressed in *E. coli* and bound to glutathione-agarose beads as described under "Experimental Procedures." The *bottom row* is a Coomassie Blue-stained SDS-PAGE separation of 50 μg of each of these proteins and illustrates the relative abundance of each of the GST-derivatives used for binding. The *top row* displays the binding of GCN5 to each of the GST-derivatives as analyzed by SDS-PAGE and fluorography, whereas the *middle row* displays the binding of TFIIB to each of the GST- derivatives.

least 20-fold more GCN5 or ADA2 had to be retained by the GST-ADR1-TAD constructs than by the GST and GST-Vpu controls (See legend to Fig. 1). In many cases, up to 30–40-fold more GCN5 was bound to the target than to the controls: *e.g.* GCN5 binding to GST-ADR1-TAD IV and GST-ADR1-TAD III. Second, GCN5 failed to bind other GST fusions such as GST-Vpu and GST-CAF1. ADA2 also failed to bind well to TAD I, III, or IV. Each of the significant bindings of an ADR1 TAD to ADA2 or GCN5 was, moreover, shown to be stable even under high salt wash conditions. This result suggests that these contacts are not solely dependent on ionic interactions. Finally, the GST-ADR1-TADs, whereas they could bind ADA2 or GCN5, failed to bind a number of radioactively labeled proteins including luciferase, ADA3, CAF1, CCR4, TBP, and N-TFIIB.

TABLE V
The effect of a TAD IV deletion on ADR1 activation

ADH II activities were conducted as described in Table IV. β-Galactosidase activities were determined as described in Table II in strain EGY188. S.E. values were less than 15%. LexA-ADR1 and LexA-ADR1-Δ642/704 represent LexA-1–202 fused, respectively, to the full-length ADR1 protein and the full-length protein whose residues 642–704 have been deleted. ADR1 and ADR1-Δ642/704 were expressed to comparable extents *in vivo* (data not shown). *ADR1*, strain 40–1C; *ADR1-Δ642–704*, same as 40–1C except *adr1–1*; *ADR1-Δ642–704-TRP1*.

Relevant genotype	β-Galactosidase	ADH II	
		R	DR
	units/mg	*milliunits/mg*	
LexA-ADR1	900		
LexA-ADR1-Δ642/704	48		
ADR1		<5	2000
ADR1-Δ642/704		<5	60

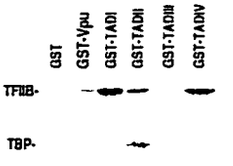

FIG. 4. **TFIIB and TBP binding to GST-TADs.** Binding of *in vitro* translated and radiolabeled TFIIB and TBP to GST-fusion proteins was conducted as described in Fig. 1.

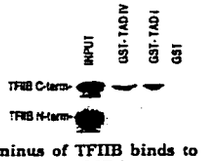

FIG. 5. **The C terminus of TFIIB binds to TADs I and IV of ADR1.** Binding studies between GST fusion proteins and radiolabeled TFIIB fragments were conducted as described in Fig. 1. *Input* represents [1/13] of C- or N-TFIIB used in the binding reaction. C-TFIIB contains residues 135–345 of TFIIB, and N-TFIIB contains residues 1–135 fused to GST, respectively.

The observation that each of the ADR1 TADs and the full-length ADR1 are dependent on a functional ADA2 complex for maximal activation suggests that the physical interaction observed between the individual ADR1 TADs and the ADA2 and GCN5 proteins represents a physiologically significant interaction. This implication is further supported by the TAD IV deletion studies. Progressive C-terminal deletions of TAD IV reduced TAD IV ability to activate and to directly interact with GCN5. The correlation between TAD IV activation and its ability to bind GCN5 was nearly exact, suggesting biological relevance for the GCN5-TAD IV interaction.

The C-terminal region of TAD IV (residues 695–704, EYDYEHYQIL), which was required for TAD IV function and binding to GCN5, is rich in acidic and bulky hydrophobic amino acids. The residues important for transcriptional activation have been analyzed for several other activators, and hydrophobic residues have increasingly been shown to be crucial for activation function (38–40). The hydrophobic residues in the C terminus of TAD IV may be similarly used for providing contacts or a particular secondary structure that interacts with transcriptional factors. However, the actual region of contact may be complex, since even derivative LexA-ADR1-(642–679) retained some transcriptional activity (Fig. 3*A*).

In view of the recent identification of GCN5 as a histone acetyltransferase, our results suggest that ADR1 recruits the ADA2 complex, resulting in increased histone acetylation. Such

acetylation would presumably relax the nucleosomal structure at the promoter and facilitate nucleosomal rearrangement. Since, upon derepression, ADR1 is required at the *ADH2* promoter for nucleosomal removal from the TATA and mRNA start site regions (28), ADR1 binding to GCN5 and ADA2 may be one means by which ADR1 accomplishes this. This model does not exclude, however, a role for other nucleosomal rearrangement factors such as the SNF/SWI factors. At *ADH2*, *snf/swi* defects also result in reduced expression. In many strains this reduction is only 1.5-fold (25, 27, 41), although at least in one strain a more drastic dependence on SNF/SWI was observed (26). A combination of ADR1 recruitment of GCN5 and ADA2 and SNF/SWI-facilitated nucleosomal remodeling may, therefore, contribute to ADR1-dependent *ADH2* expression.

TFIIB Interacts with TADs I and IV—In addition to observing that GCN5 and ADA2 bind to the ADR1 TADs, we showed that TFIIB could bind TADs I and IV of ADR1. Again these interactions appear specific in that they were stable to high salt washes and that TFIIB could not bind TADs II or III, GST, or GST-Vpu. Moreover, the C-terminal region of TFIIB, but not its N terminus, could bind TADs I and IV. In addition, none of the TADs displayed strong binding to TBP. The physiological relevance of these interactions, however, is not certain. For TAD IV of ADR1, deletions that clearly decreased its function also directly reduced binding to TFIIB (Fig. 3). This finding supports the biological importance of the interaction between TAD IV and TFIIB but does not confirm that TFIIB is a real target for TAD IV.

It is possible that the ability of a TAD to activate represents some overall property of "stickiness," *i.e.* the ability to interact in the cell with various target factors: GCN5, TFIIB, etc. Many target factors for VP16 have been identified (42, 43) as well as for the yeast GAL4 activation domain (13). It has recently been suggested that this pleiotropic ability of activators to bind many core transcriptional factors and coactivators may represent a physiological method for an activator in recruiting the transcriptional machinery to a promoter (44).

The multiplicity of ADR1 activation domains argues similarly that these activation domains could make contacts to different factors: GCN5 by one or more domains, TFIIB by others. The sum total of these interactions and recruitments would constitute the ability to promote transcriptional initiation. ADR1 activation of *ADH2* and other genes, therefore, appears to result from contributions from various factors and interactions. Nucleosomal rearrangement promoted by ADR1 recruitment of the ADA2 complex and aided by SNF/SWI may be one determinant. ADR1 binding to transcription factors might also play a critical role, and ADR1 contacts to TFIIB represent one possible example of this interaction.

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