

Characterization of the ADA/GCN5 Transcriptional Adaptor Complex

by

Junjiro Horiuchi

B. S. Biology
Stanford University

Submitted to the Department of Biology
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in Biology

at the
Massachusetts Institute of Technology
June 1997

© 1997 by Junjiro Horiuchi. All rights reserved.

The author hereby grants to MIT permission to reproduce and to distribute publicly copies of
this thesis document in whole or in part.

Signature of Author

Department of Biology
May 30, 1997

Certified by

Dr. Leonard Guarente
Professor, Department of Biology
Thesis Supervisor

Accepted by

Dr. Richard A. Young
Chairman, Biology Graduate Committee

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
Science

JUN 24 1997

Characterization of the ADA/GCN5 Transcriptional Adaptor Complex

by

Junjiro Horiuchi

Submitted to the Department of Biology on May 30, 1997
in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy in Biology

Abstract

In order for transcriptional activators to fully stimulate transcription, auxiliary factors, termed adaptors, coactivators, or mediators, are necessary. The *ADA* genes (*ADA1*, *ADA2*, *ADA3*, *GCN5*, and *ADA5*) were isolated in a genetic selection for genes encoding adaptors for the activator GAL4-VP16. As may be expected for adaptor mutants, strains deleted for the *ADAs* are defective for activation by GAL4-VP16 and certain other activators, including *GCN4*.

The *ADA* genes can be separated into two classes. Mutants in the *GCN5* class, which include strains with mutations in *ADA2*, *ADA3*, and *GCN5*, have less severe growth defects than mutants in the *ADA1/ADA5* class. *ADA2*, *ADA3*, and *GCN5* have other characteristics in common. Fusing the LexA DNA binding domain to either *ADA2* or *ADA3* creates a chimeric protein that is able to activate transcription from promoters containing LexA binding sites. This activation is dependent on the other two *ADAs*. Furthermore, LexA-*ADA2* activity can be hyperstimulated by overexpressing *ADA3* and LexA-*ADA3* activity can be hyperstimulated by overexpressing *ADA2*. These data suggest that *ADA2*, *ADA3*, and *GCN5* interact with each other and may bind to one another *in vivo*. In further support of this model, we have determined that the three proteins can form a trimeric complex *in vitro*. This complex is specific, requiring *ADA2* to serve as a lynchpin holding *ADA3* and *GCN5* together. *ADA3* can be separated into two domains, both of which are required for transcription. Only one of these domains, the carboxyl terminal one, binds to the *ADA2/GCN5* complex *in vitro*.

The *ADA1/ADA5* class of *ADAs* is distinct from the *GCN5* class because mutations in members of the *ADA1/ADA5* class affect a larger range of promoters (ie. the *ADH1* promoter), are auxotrophic for inositol, display *Spt⁻* phenotypes, and have more severe growth defects. However, the two classes of *ADAs* have similarities as well. The promoters affected by mutations in the *GCN5* class are not unrelated, but are a subset of those affected by mutations in the *ADA1/ADA5* class. Furthermore, strains deleted for both a member of the *GCN5* class and a member of the *ADA1/ADA5* class

do not display phenotypes any more severe than strains disrupted of only a member of the ADA1/ADA5 class. Finally, the five ADA gene products have all been partially purified from yeast extract in an approximately 2 MDa complex. The interaction between all five known components of the complex have been verified by immunoprecipitation experiments. These data are consistent with a model in which the ADA gene products form a large transcriptional adaptor complex.

Recently, the identification of GCN5 as a histone acetyltransferase has suggested that the ADA/GCN5 complex functions by removing the repressive effects of nucleosomes. However, the complex must have an additional function since there are two classes of ADAs and mutations in the ADA1/ADA5 class lead to more severe defects than mutations in the GCN5 class. With the observations that mutations in *SPT15* (which encodes the TATA-binding protein) and *SPT7* can cause *Ada*⁻ phenotypes, we propose two possible models for the function of the ADA/GCN5 complex.

Thesis Supervisor: Dr. Leonard Guarente
Title: Professor of Biology

This thesis is dedicated to my parents, Kensuke and Atsuko Horiuchi, for their love, encouragement, and support.

When I was a child, my father once brought home some ultra-centrifuge tubes for me to play with. That event may have ultimately led to this thesis. My father, who studies DNA replication, once said, "Replication is the most fundamental process of life. It's not something peripheral, like transcription." Hopefully, this thesis will convince him that transcription is cool, too.

My mother has always worried about me. I hope now, she can worry a little less.

Acknowledgements

I would first like to thank Lenny Guarente. The combination of his insights and guidance in scientific matters, his ability to know when to let a graduate student work on his own and when to direct him, and his ability to maintain a friendly and cohesive laboratory have made him an ideal advisor. I thank Lenny for teaching me and for imparting to me his enthusiasm for science. Now, I am excited and confident about going to Cold Spring Harbor, rather than apprehensive as I would have been before.

I am grateful to my thesis committee, Phil Sharp, Chris Kaiser, Robert Kingston, and Rick Young. I am especially grateful to Phil and Chris for their interest in and helpful advice for my project over the years.

It was great fun working on the ADA project with Neal Silverman and Greg Marcus. I had an incredible time with them discussing models and planning and performing experiments. I would also like to thank them as well as Rainer Knaus ("This trailguide is for old women..."), James Claus ("We've got enough floatation in the canoe..."), Paul Sugiono, Ken Keiler, Sarah Ades, Julie Agapite, Rachel Kindt, and Ed Rebar ("It's better than bare wall") for dragging me out of the lab sometimes and trying to instill some semblance of coolness in me.

I would like to thank Benjamin Pina for getting me started on the ADA project, and other former members of the lab, Nick Austriaco, Brian Kennedy, Roy Pollock, Myrna Helfenstein, Jennifer Zamanian, Thomas Oehler, Sheri Treadway, Francesca Cole, Mike Stanbrough, Maryann Haldi, Shelley Berger, Bernard Turcotte, and John Fikes.

I am grateful to the current members of the lab, Sally Pak, Tod Smeal, Brad (nutmeg) Johnson, Mitch McVey, Stan Mah (and Sue, for the 500 lessons), Dave Sinclair, Dave Lombard, Chris ("You guys are pickin' on me") Armstrong, Kevin Mills (take good care of the Pits), Peter Park, Pierre Antonine Defossez, Bob Marciniak, Alix Van Geel, and Christine Ahern. A special thanks to the Anti-suck, Dave McNabb for all his help reading drafts and discussing experiments. Every lab should have an anti-suck and every post-doc should strive to be one.

Thanks to the kitchen and custodial staff, especially Josie Caloruso, Ruth Cumminsky, and Mario Roccuza for taking such good care of me.

I thank my previous mentors, Elaine Diacumakos, Joe Nevins, Paul Greengard, Tatsuya Kurihara, Yoshi Nagamine, Bruce Stillman, and Toshiki Tsurimoto for encouraging my developing interest in research.

Finally, I would like to thank my parents, and Kenta, Wendy, Kenji, Kody, Yozo, and Malani. I was able to make it through the tough times because I knew you were there to support me.

Table of Contents

Title	1
Abstract	2
Dedication	4
Acknowledgements	5
Table of Contents	7
Chapter 1:	9
Introduction	
I. The General Transcription Factors	11
II. Gene Specific Transcriptional Activators	14
III. Coactivators, Mediators, and Adaptors	17
IV. Summary and Perspectives	29
V. References	33
VI. Figures	47
Chapter 2:	50
ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex	
Summary	51
Introduction	52
Materials and Methods	53
Results	57
Discussion	62
References	67
Tables	71
Figures	74

Chapter 3:	87
ADA1, a novel component of the ADA/GCN5 complex has broader effects than GCN5, ADA2, or ADA3	
Summary	88
Introduction	89
Materials and Methods	91
Results	97
Discussion	103
References	108
Tables	115
Figures	118
Appendix 1:	131
ADA5/SPT20 links the ADA and SPT genes involved in yeast transcription	
Summary	132
Introduction	133
Materials and Methods	135
Results	142
Discussion	149
References	153
Tables	160
Figures	162
Appendix 2:	176
Further interaction studies of the ADAs	
Figures	180

Chapter 1

Introduction

Transcription is the first step in the synthesis of proteins from DNA and is one of the most fundamental of biological processes. Activation of many signal transduction pathways result in the specific activation of various genes and one of the hallmarks of differentiated cells is the activation and repression of specific genes. In a simple example of gene regulation in yeast, when cells are grown in media containing glucose, the genes required for the metabolism of galactose are repressed, whereas, when yeast are grown in galactose containing media, lacking glucose, these genes are activated. These genes, and all genes that are transcribed into mRNA, are transcribed by RNA polymerase II (pol II). Thus, there must be at least two classes of pol II transcription factors: general transcription factors such as pol II itself that are required for the transcription of all mRNAs and regulatory factors that determine which genes should be transcribed at which times.

In parallel with these two different types of factors, the transcriptional regulatory regions of pol II transcribed genes can be separated into two distinct regions (86). The first of these is the core promoter region which is located 5' to the structural gene and consists of an initiator motif (Inr) encompassing the transcription start site and a TATA-box located upstream from the Inr. The TATA and the Inr motifs are recognized by components of the general transcription factors (126). The second element, the enhancer, consists of the binding sites for regulatory factors, or gene specific transcriptional activators (39). In higher eukaryotes, enhancers can be tens of kilobase-pairs upstream or downstream from the start site of transcription. In yeast, enhancers are referred to as upstream activating sequences (UASs) and can be found hundreds of base-pairs upstream from the start site.

This chapter is organized into three sections. The first section reviews the general transcription factors and the steps involved in transcriptional initiation. The second section describes gene specific transcriptional activators and discusses various models by which activators may stimulate transcription. The final section describes a

third class of factors which have been termed coactivators, adaptors, or mediators. These factors are neither activators nor general factors but may mediate the stimulatory signal from activators to general factors.

I. The General Transcription Factors

The general transcription factors include TFIIA, TFIIB, the TATA binding protein (TBP), TFIIE, TFIIIF, TFIIH, and pol II (15, 16, 86). *In vitro*, transcription in the absence of gene specific activators (termed basal transcription), can occur in the presence of the general transcription factors from templates containing core promoter elements.

The first step in this *in vitro* transcription is the recognition and binding of the TATA box by TBP (16). The genes encoding TBP have been cloned from a variety of organisms and have divergent amino-terminal domains but highly homologous 180 amino acid carboxyl-terminal domains (117). The carboxyl-terminal domain consists of two direct repeats of 60 amino acids separated by a basic region and it is responsible for DNA binding by recognizing the minor groove of the TATA box. Its binding results in a dramatic distortion of the DNA, inducing a sharp bend in the DNA and a widening of the minor groove (59, 60). The binding of TBP to the TATA box nucleates the assembly of the other basal factors at the promoter.

The TBP/TATA box complex is next bound by TFIIB (16, 40). In yeast, TFIIB is a 38 kDa protein and similar to the TFIIB proteins from other eukaryotes, consists of an amino terminal cysteine rich region which can coordinate zinc and a direct repeat at the carboxyl terminal region (92). TFIIB binds beneath TBP within the bend caused by TBP binding to the TATA box, allowing it to make protein-protein contacts with TBP as well as contacts with the DNA both upstream and downstream of the TATA box (83). The gene encoding TFIIB in yeast, *SUA7*, was cloned as a suppressor of a *cyc1-5000* mutant (92). This mutation contains an aberrant ATG codon upstream and out of frame with the wild type start codon. The method of suppression of the *cyc1-*

5000 allele by *sua7* mutants was determined to be an alteration of transcriptional start sites to sites downstream of the aberrant ATG, suggesting that TFIIB plays a role in positioning the start site of transcription.

Once TFIIB is bound at a promoter, the pol II/TFIIF complex is recruited. Pol II is a twelve subunit enzyme that, in the absence of the other general transcription factors, is able to incorporate rNTPs into RNA in a template, but not promoter, dependent manner (125). Mammalian TFIIF consists of a heterotetramer of two subunits, RAP30 and RAP74 (86). It stabilizes the interaction between pol II and the TATA-TBP-TFIIB complex, increases the rate of transcriptional elongation, and prevents the binding of pol II to nonspecific sites on DNA. TFIIF and pol II have also been implicated, along with TFIIB, in start site selection. *RPB1*, the gene encoding the largest subunit of pol II has been isolated as *SUA8* and thus is similar to *SUA7* in altering transcriptional start sites (10). Also, a gene encoding the yeast homolog of RAP74 has been isolated as a suppressor of the alternate start sites resulting from an *sua7* mutation (104). The three largest subunits of pol II, RPB1, RPB2, and RPB3 have extensive homologies with the β' , β , and α subunits of *E. coli* RNA polymerase while the RAP30 subunit of TFIIF has sequence similarities with σ factors (125).

RPB1 has a particularly intriguing carboxyl terminal domain (CTD) consisting, in yeast, of 26 or 27 tandem repeats of a highly conserved heptapeptide sequence, Tyr-Ser-Pro-Thr-Ser-Pro-Ser (125). This domain is not found in the subunits of RNA polymerase I or III, or in prokaryotic polymerases. It has been found in all eukaryotic pol IIs examined with the general observation that the more complex the organism, the more repeats present. A deletion of the yeast CTD such that only 10 to 12 repeats remain leads to temperature sensitive and cold sensitive phenotypes as well as inositol auxotrophy (64). Deletions leaving fewer repeats are lethal. It is likely that the CTD plays a role in the initiation of transcription (73). Progressive deletions of the CTD lead to decreases in GAL4 and GCN4 dependent transcription *in vitro*. Basal

transcription is also affected although to a lesser extent, while elongation and termination are unaffected.

Phosphorylation has been implicated in the regulation of both TFIIF and pol II. *In vitro* experiments suggest that the phosphorylation of RAP74 stimulates both the initiation and elongation activities of TFIIF. A possible RAP74 kinase, the TBP associated factor (TAF) 250, has been identified *in vitro* (29). The CTD has also been shown to be phosphorylated. Usheva et al. have demonstrated that TBP binds specifically to the nonphosphorylated form of pol II and not to the phosphorylated form, supporting a model where TBP may function to recruit nonphosphorylated pol II into the initiation complex (112). Once bound, the tethered polymerase may be released to form an elongation complex through the phosphorylation of the CTD. The basal factor TFIIF has been postulated to be responsible for CTD phosphorylation (102).

After the pol II/TFIIF complex has been recruited to the promoter, TFIIE and TFIIF can bind to complete the formation of the preinitiation complex (37, 86). TFIIE is thought to play a role in promoter melting while TFIIF is a multisubunit complex shown to have helicase, kinase, and DNA repair activities. It has been demonstrated that the immunoglobulin heavy chain promoter, when highly negatively supercoiled, does not require TFIIE, TFIIF, and TFIIF for accurate initiation (87). However, when linear, these factors are required, suggesting that they may function by stimulating formation of an open complex. Open complex formation refers to the localized separation of the DNA strands in preparation for the synthesis of the first phosphodiester bond of the mRNA. The potential energy stored in negatively supercoiled templates could stimulate or stabilize the formation of an open complex, bypassing the need for TFIIE, TFIIF, and TFIIF (87).

Following the synthesis of the first bond of the mRNA, pol II is released and extends along the DNA leading to elongation of the mRNA transcript. TFIIA has not

been included in the above ordered assembly of factors since its binding is not restricted to a particular step (55). Rather, it seems to be able to bind at any stage between TBP-TATA box binding and preinitiation complex formation and functions to stabilize the existing complex. In highly purified *in vitro* systems, the presence of TFIIA is not essential for transcription suggesting that the role of TFIIA may be to relieve the effects of certain negative regulators that exert their effects by preventing the general transcription factors from binding to each other or to DNA.

Whether the ordered assembly of factors occurs *in vivo* as well as *in vitro* is not clear. Pol II can be purified from cell extracts in a complex referred to as the pol II holoenzyme (65). The holoenzyme contains pol II, TFIIF, TFIIB, TFIIH and additional proteins known to play a role in transcription (see SRB section below). Thus, *in vivo*, TBP binding may be followed simply by the binding of the large holoenzyme complex and TFIIE. Other forms of pol II holoenzyme have also been isolated lacking various members of the general transcription factors (61). The physiological significance of these alternate holoenzymes is as of yet, unclear.

II. Gene Specific Transcriptional Activators

Besides the general factors, transcriptional activators are required for transcription *in vivo*. As mentioned earlier, these bind to enhancers or UASs and serve a regulatory role. Since there are a large number of activators with different binding specificities and since different genes are under the control of different enhancers or UASs, regulating the activities of specific activators regulates the transcription of specific genes. Genes may be under the control of combinations of different UASs and the activators that bind to these UASs may act in synergy. Also, many activators contain dimerization domains and can form homodimers and heterodimers with different binding specificities. These combinations add further complexity to the regulation of gene expression and allow a relatively small number

of gene specific activators to regulate the complex patterns of gene expression in eukaryotes.

Activators consist of at least two distinct domains: a DNA binding domain and an activation domain (50). Various DNA binding domains have been characterized including homeodomains, various types of zinc fingers, basic leucine zipper domains, and basic helix loop helix domains. Activation domains have also been characterized and include acidic, proline rich and glutamine rich domains (109). This characterization by amino acid type seems to have functional relevance since proline rich and glutamine rich activation domains do not function in yeast while acidic domains do. This suggests that acidic domains function differently than proline or glutamine rich regions and only the mechanism by which acidic domains function is conserved in yeast (38). On the other hand, mutagenesis studies have demonstrated that the acidic, proline or glutamine residues are not necessarily the critical residues for activity in these various. Rather, hydrophobic residues interspersed within these domains appear to be important (109). Structural studies have not revealed evidence of defined secondary structure to activation domains and it is proposed that these domains only form a defined structure upon binding to a specific target protein.

How do activators stimulate transcription? As mentioned previously, the first step in *in vitro* transcription is the binding of general transcription factors to form a preinitiation complex at promoters. Thus, one role of transcriptional activators may be to recruit general transcription factors (94). In support of this model, it has been demonstrated that transcription can be stimulated by increasing the affinity of basal factors for promoters. Artificially tethering TBP to a promoter results in an increased level of transcription (23, 63, 121). Furthermore, a mutation has been isolated in a component of the pol II holoenzyme, GAL11, creating an interaction between GAL11 (and presumably the holoenzyme itself), and the dimerization domain of the

activator GAL4 (46). This mutation bypasses the need for the activation domain of GAL4 verifying that activation can occur via recruitment of general factors. Finally, yeast containing components of the pol II holoenzyme fused to heterologous DNA binding domains display activated transcription of genes whose promoters contain binding sites for the fused domains (6).

There is evidence that naturally occurring activators may also function by recruiting general factors. TBP (103) and TFIIB (75, 76, 98) bind to the activation domains of the viral VP16 protein, as well as other activators, *in vitro*. Mutations that inactivate VP16 *in vivo* have been shown to prevent binding of these general factors *in vitro*, suggesting that these interactions may be physiologically relevant (54). On the other hand, some mutations in TBP which abolish the binding of VP16 do not abolish VP16 dependent activation (106), suggesting that either recruitment of TBP by VP16 is not important for activation or that VP16 has other mechanisms of activation that work in parallel but not synergistically with TBP recruitment. Some of these other mechanisms may include inducing conformational changes in general transcription factors to stimulate preinitiation complex formation (97), inducing covalent modifications to general factors, or stimulating promoter clearance and elongation (86).

A final method by which activators may function is via the removal of repressors of transcription. DNA *in vivo* is packaged into nucleosomes and higher order chromatin structures. This packaging has been shown to inhibit each step in transcription including the binding of activators, the binding of general factors to form a preinitiation complex, and the initiation and elongation by pol II (62).

An early experiment demonstrating repression by chromatin in yeast involved constructing a strain where the gene expressing histone H4 was expressed from the *GAL1* promoter (41, 42). When the strain was grown in galactose, the *PHO5* promoter was bound by nucleosomes. When the strain was grown in glucose, the

number of nucleosomes bound was decreased and the *PHO5* gene was highly expressed, even when under normally repressing conditions. Besides *PHO5*, when histone H4 is depleted, transcription from the *CYC1* and *GAL1* promoters are also activated in a UAS independent manner (41). In another case, the *BUR* genes were isolated as suppressors of a crippled *SUC2* promoter deleted of its UAS. *BUR5* is identical to *HHT1*, which encodes histone H3, again implicating histones in repressing transcription (93).

Domains in the amino terminal tails of all four core histones are required for repression of basal transcription in yeast (72). The amino terminal tail of histone H4 has also been shown to be necessary for activation of the *GAL1*, *GAL10*, *GAL7*, and *PHO5* promoters (30). These results support a model in which activators may function by interacting with the amino-terminal tail of histones, resulting in a removal of the repressive effects of nucleosomal structure.

III. Coactivators, Mediators, and Adaptors

The mechanisms of transcriptional activation mentioned above can occur through either direct or indirect means. Some data, including those that demonstrate direct binding between general factors and activators, suggest that for recruitment, auxiliary factors are not necessary. On the other hand, there is much evidence that activators and general factors alone are not sufficient to activate transcription and that another class of factors, termed coactivators, mediators or adaptors, are necessary (8, 58, 95). The following sections describe representative examples of eukaryotic coactivators.

1) TAFs and the TFIID complex

In higher eukaryotes, TBP is found tightly associated with eight or more TBP associated factors (TAFs) in a complex known as TFIID (31). TBP in the absence of

the TAFs is sufficient for basal transcription *in vitro* but the TFIID complex is required for activated transcription (95). This led to the proposal that TAFs may function as coactivators bridging the interactions between the general transcription factors and activators. In support of this model, it was determined that various activation domains formed specific contacts with various TAFs. For example, TAF110 was shown to interact with the glutamine rich activation domain of Sp1 (49) and TAF40 was shown to interact with the acidic activation domain of VP16 (36). Furthermore, TAFs were demonstrated to interact with other members of the general transcription factors such as TFIIA and TFIIB (36).

The architecture of the TBP/TAF complex has been studied extensively *in vitro* (24). A subset of TAFs, including TAF250, TAF150, and TAF30 α bind directly to TBP while the others seem to interact indirectly by TAF-TAF interactions. The largest TAF, TAF250 seems to have a function as a scaffold protein. TAF150, TAF110, TAF60, TAF30 α and TAF30 β all interact efficiently with TAF250. TAF110 can then connect TAF80 and TAF60 can connect TAF40 to the complex.

A TFIID complex consisting of recombinant TAFs and TBP is able to support activated transcription *in vitro* from a variety of activators (24). In addition, subcomplexes consisting of TBP and a subset of TAFs is sufficient for *in vitro* activation by certain activators. For example, activation by Sp1 can be reconstituted in the presence of TBP, TAF250, TAF150, and TAF110 (24). Sp1 has been demonstrated to bind directly to TAF110 (49) which seems to be tethered to TBP through an interaction with TAF250, while TAF150 may recognize specific promoter sequences (see below). Activation by another activator, NTF1 has been shown to require either of two subcomplexes. These consist of TBP, TAF250 and either TAF150 or TAF60 (24). The hydrophobic activation domain of NTF1 has been demonstrated to interact specifically with both TAF150 and TAF60. These results suggest that different TAFs mediate interactions with different classes of activators.

Although the importance of TAFs for activation *in vitro* has been demonstrated, the role of TAFs *in vivo* is more controversial. In *Drosophila*, mutations in TAF60 and TAF110 reduce transcription of bicoid dependent genes (101). However, in yeast, transcription from a large number of genes is not affected by removal of TAFs (82, 115). Although most of the genes encoding TAFs are essential in yeast, removal of specific TAFs leads to arrest at specific stages in the cell cycle suggesting that only a specific subset of genes may be dependent on TAFs (1). These results may reflect differences in the importance of TAFs in yeast compared to higher organisms. Alternatively, they may indicate that only a subset of genes require TAFs for activation or that *in vivo*, various redundant pathways may exist for transcriptional activation.

Besides bridging interactions between general factors and activators, TAFs have been implicated in affecting the promoter selectivity of TFIID binding. TAF150 recognizes and binds to sequences overlapping initiator elements (Inr) (113). It also is a component of human CIF (cofactor of Inr function) which stimulates *in vitro* basal transcription specifically from promoters containing intact Inr sequences (57). Furthermore, a TFIID subcomplex containing TBP, TAF250 and TAF150 binds preferentially to promoters containing intact Inrs (113).

TAF250, besides being proposed to function as a protein scaffold, also has two other functions. It is a bipartite protein kinase which can both phosphorylate itself and bind to and phosphorylate the RAP74 subunit of TFIIF (29). Although there is no evidence for a functional role for phosphorylation by TAF250, it is tempting to speculate that TAF250 may first play a role in recruiting the pol II/TFIIF complex to initiation complexes and then helping in the transition from initiation to elongation.

The second function attributed to TAF250 is histone acetylation (81). Histone acetylation appears to be an important mechanism by which DNA bound up in chromatin is made accessible for transcription (13). Presumably, the acetylation of

lysine residues in histones neutralizes the positive charges of the residues, decreasing histone-DNA interactions and allowing transcription factors to bind. Certain TAFs have been demonstrated to interact in histone octamer like structure suggesting the possibility that TFIID may interact with promoter DNA in a nucleosome like manner (122). The presence of a histone acetyltransferase activity in TAF250 may allow TFIID to compete effectively with nucleosomes for binding at promoters.

2) SRBs and the RNA polymerase II holoenzyme

As mentioned above, the largest subunit of pol II, RPB1, has a CTD which when partially truncated, leads to conditional phenotypes (125). A selection for suppressors of the cold sensitive phenotype of a partial truncation yielded mutations in nine *SRB* genes (suppressors of RNA polymerase B) (44, 74, 84, 107). Four of the *SRBs*, *SRB2*, 4, 5, and 6, yielded dominant gain of function mutations (64, 107). Deletions of *SRB2* and 5 cause conditional phenotypes similar to CTD partial truncations while deletions of *SRB4* and 6 are lethal. It is thought that these *SRBs* function by stimulating transcription through interactions with the CTD. Both basal and activated transcription are severely reduced in extracts from Δ *srb2* and Δ *srb5* mutants. These defects can be complemented by adding recombinant *SRB2* or *SRB2* and *SRB5* respectively.

The other five *SRB* genes, *SRB7*, 8, 9, 10, and 11 were isolated as recessive suppressors, and deletions in them are viable and suppress CTD partial truncations (44, 74). This suggests that they are responsible for negatively regulating the activity of polymerase through their interactions with the CTD. *SRB10* and 11 have extensive homologies to known cyclin and cyclin dependent kinase pairs (74). Extracts from strains in which *SRB10* has been mutated show decreased levels of CTD phosphorylation suggesting that *SRB10* and 11 may play a role in the transition from initiation to elongation of transcription.

Purification of the *SRB* gene products demonstrated that they cofractionate in a large complex with RNA polymerase (61, 66, 107). This complex was termed the RNA polymerase II holoenzyme and contains not only the above mentioned proteins but also the basal factors TFIID, TFIIF, and in some cases TFIIB. Other factors that are necessary for the activation and repression of transcription, including GAL11, SIN4, RGR1, and the SWI/SNF complex are also part of the holoenzyme (65, 118). The holoenzyme is capable of supporting basal transcription *in vitro* with the addition of TBP and TFIIE. Interestingly, it is also capable of supporting GAL4 dependent activated transcription with the further addition of GAL4. Activated transcription is about five fold higher than basal in this case compared to about ten fold higher in crude yeast extracts, perhaps reflecting the absence of TAFs in the holoenzyme. The holoenzyme binds to wild type VP16 activation domains but not to mutated variants that do not activate transcription (44), suggesting that one mechanism of transcriptional activation may occur through recruitment of the large holoenzyme complex.

3) SWI/SNF complex

The *SNF* genes (sucrose non-fermenting) were originally isolated as genes required for the expression of the *SUC2* gene which is required for growth on sucrose and raffinose (22). It was later determined that *SNF2*, *SNF5*, and *SNF6* were required for expression of a wide variety of genes including glucose repressible genes, *PHO5*, Ty elements, cell type specific genes and protease B. Similarly, the *SWI* genes were identified as positive regulators of *HO* gene expression and *SWI1*, *SWI2*, and *SWI3* were determined to have more pleiotrophic effects, being important for the transcription of many other genes (45). The discovery that *SWI2* is identical to *SNF2*, served to link the five *SWI* and *SNF* genes (71, 90).

Several lines of genetic data suggested that the five *SWI/SNF* gene products function in a complex. This was verified by biochemical purification of an approximately 2 MDa complex from yeast containing all five *SWI/SNFs* as well as at least six other proteins (18, 89). Three of these other proteins have been identified. One, *SNF11*, was isolated from two hybrid interaction studies with *SWI2/SNF2* (108), and the other two, *SWP73* and *TFG3/TAF30/ANC1*, were identified biochemically as members of the complex (17, 19). *TFG3/TAF30/ANC1* is a particularly intriguing transcription factor since it was also identified as a component of both *TFIID* and of yeast *TFIIF*. The *SWI/SNF* complex is evolutionarily well conserved since homologs of *SWI2/SNF2* have been identified in both *Drosophila* and humans, and human homologs of the yeast complex has been purified (53, 70, 116).

Clues to the function of the complex were first obtained from suppressors to the defects of *swi/snf* mutants. These were initially isolated in histone genes and genes related to non-histone chromatin structural components (67). Deletion of the *hta1-htb1* locus, one of two gene pairs that encode histones H2A and H2B, also suppress *Swi/Snf* phenotypes (47), suggesting that the *SWI/SNF* gene products function by counteracting the repressive effects of chromatin. Studies on the *SUC2* gene demonstrated that in *snf5* or *swi2/snf2* strains, the chromatin structure of the promoter region was modified relative to wild type strains as measured by micrococcal nuclease sensitivity (47). In the *snf* strains, new micrococcal nuclease resistant sites appear between the structural gene and the UAS, one directly over the TATA box. These new resistant sites are not a consequence of the reduced transcription of *SUC2* in the mutants since *SNF* strains with a defective TATA box do not have these resistant sites. Deleting the *hta1-htb1* locus both partially suppresses the *SUC2* transcription defect and the formation of the nuclease resistant sites. These studies suggest that the *SWI/SNF* complex directly alters chromatin structure at certain promoters.

In further support of this model, it has been determined that the purified yeast SWI/SNF complex interacts with nucleosomal DNA *in vitro*, and stimulates the binding of GAL4 to nucleosomal GAL4 binding sites (27). The human complex has also been demonstrated to disrupt nucleosomal structure and facilitate the binding of GAL4 and TBP to nucleosomal sites (53, 70).

SWI2/SNF2 contains motifs characteristic of nucleic acid-stimulated ATPases (88). Mutational studies in yeast indicate that these motifs are essential for SWI/SNF activity. Furthermore, the purified SWI/SNF complex contains a DNA stimulated ATPase activity and its ability to affect chromatin structure and stimulate activator binding to nucleosomal DNA *in vitro* is dependent on hydrolyzable ATP (27, 53, 70). Several other proteins with homology to the SWI2/SNF2 ATPase motifs have been identified. STH1 was isolated from yeast based on its homology to SWI2/SNF2 and is a subunit of the RSC (remodel the structure of chromatin) complex along with 14 other polypeptides of which at least two others have homologies to members of the SWI/SNF complex (20). Similar to the SWI/SNF complex, the RSC complex also has DNA dependent ATPase activity and can perturb nucleosomal structure. In *Drosophila*, an ATP dependent nucleosome remodeling factor (NURF) consists of at least four polypeptides of which one, ISWI, is highly homologous to SWI2/SNF2 in the ATPase domain (110, 111). The NURF complex, acts together with the GAGA transcriptional activator to alter chromatin structure at the hsp70 promoter. *MOT1* was identified as an essential gene which when mutated, increased basal transcription of many genes (28). It too has homologies to the ATPase domains of SWI2/SNF2 and encodes an ATP-dependent inhibitor of TBP binding to DNA (4). Recent studies suggest that *MOT1* may also function to stimulate transcription from several promoters (78), indicating that further studies are necessary to determine its actual role in transcription.

Several questions remain about the SWI/SNF and related complexes. Although a DNA or nucleosome dependent ATPase activity has been demonstrated in these nucleosome remodeling complexes, it is unclear exactly how this ATPase activity results in altering chromatin DNA interactions. It has been suggested that these complexes translocate along DNA utilizing the energy derived from ATP hydrolysis (88). As they traverse DNA bound in nucleosomes, the DNA may be temporarily removed from the nucleosome in the vicinity of the complex facilitating the binding of transcription factors to their sites. Further experimentation is required to support this hypothesis. It is also unclear how the SWI/SNF complex is recruited to particular promoters. It has been proposed that the complex is recruited through interactions with transcriptional activators. However, although SWI3 has been demonstrated to interact with the glucocorticoid receptor (124), interactions between the complex and other activators have not been observed. Recently, the observation that the SWI/SNF complex is a component of the pol II holoenzyme has led to an alternate proposal that the SWI/SNF complex may be recruited to promoters along with the holoenzyme (118).

4) **SPT genes**

The *SPT* genes were isolated in yeast as suppressors of transposon insertions in the promoters of biosynthetic genes (119). Ty transposons and Ty long terminal repeats (δ elements) can abolish or alter transcription of neighboring genes. This is thought to occur as a result of the δ element promoter competing with and inhibiting transcription from the promoter of the neighboring gene (48). *spt* mutants suppress these effects by decreasing transcription from the δ element and restoring transcription of the adjacent gene (119).

The *SPT* genes have been separated into two classes based upon the transposon insertions that they suppress (119). One class, the TBP class, includes

SPT15 which encodes yeast TBP (35). Another member of this class, *SPT3*, has been demonstrated, using genetic and biochemical methods, to encode a protein which binds to TBP (33). Furthermore, *SPT3* interacts genetically with *TOA1* (which encodes one of the yeast TFIIA subunits) and *MOT1* (78). Both TFIIA and MOT1 bind TBP and both *toa1* and *mot1* mutants display Spt⁻ phenotypes. *SPT8*, another member of the TBP class interacts with *SPT3* genetically (34) and other members, *SPT7* and *SPT20* share Ada phenotypes (see below) with *SPT15* (79, 99). Thus, although the exact role of several of the SPTs of the TBP class are not known, they all seem to be linked to TBP by several criteria and may exert their effects by affecting TBP binding to TATA elements or to other general factors.

The second class of *SPT* genes, the histone class, includes *SPT11* and *SPT12* which encode histones H2A and H2B (119). Yeast have four divergently transcribed pairs of histone genes, *HTA1-HTB1*, *HTA2-HTB2*, *HHT1-HHF1* and *HHT2-HHF2*. Overexpression or deletion of any one of these loci yields Spt⁻ phenotypes (26), while overexpression of all four histones eliminates this phenotype, suggesting that it is the altered ratio of histones relative to one another that causes the Spt⁻ phenotypes.

Besides the histone genes themselves, the histone class of *SPT* genes includes *SPT4*, *SPT5*, and *SPT6*. Genetic and biochemical data suggest that their gene products form a transcriptional repressor complex which functions by interacting with histones and modifying chromatin structure (96, 105, 120). In support of this model, an *spt6* mutation has been demonstrated to suppress the transcription defect at the *SUC2* promoter caused by a *snf5Δ* deletion (see SWI/SNF complex) (11). Suppression by *spt6* coincides with the removal of the nuclease resistant sites caused by the *snf5Δ* deletion. Furthermore, there is data indicating that SPT6 binds to histone H3 and may be capable of assembling nucleosomes on plasmid DNA *in vitro* (11).

5) CBP/p300 and PCAF

CBP and p300 (sometimes referred to as p300/CBP) are highly homologous mammalian coactivators (2). CBP was isolated as a coactivator for the transcription factor CREB (25) and binds specifically to the transcriptionally active phosphorylated form of CREB (3, 69), while p300 was isolated as an adenoviral E1A associated protein (32). CBP and p300 are functionally similar (77) and act as coactivators for a large number of transcription factors including Fos, Myb, Jun, MyoD, YY1 as well as CREB. p300/CREB are also believed to inhibit exit from the G0/G1 phases of the cell cycle and to stimulate cell differentiation. Binding of p300/CREB by E1A is thought to inhibit p300/CREB function, a mechanism which may contribute to E1A mediated cell transformation.

It has recently been determined that p300/CBP forms a complex with another protein P/CAF (p300/CBP associated factor) (123). It has also been shown that all three proteins can acetylate histones *in vitro* suggesting that this coactivator complex may stimulate transcription by weakening nucleosomal structure at promoters (5, 85, 123). It is not clear why two proteins, both with separate acetyl transferase activities need to be bound together in a complex. P/CAF in the absence of p300/CBP is able to acetylate histones *in vitro* as are p300/CBP in the absence of P/CAF. However, E1A seems to inhibit coactivator activity by binding to p300/CBP and preventing P/CAF from doing so (123). At least in one case, E1A does not prevent binding of p300 to an activator possibly suggesting that recruiting only one of the two acetyl transferases to a promoter is not sufficient for activation. It should be mentioned that the two histone acetyl transferase activities are not similar and there is no sequence homology between the transferase domains of P/CAF and p300/CBP (85). p300 can acetylate all four histone subunits whether they are added as free histones or as a mononucleosome (85), while P/CAF is only able to acetylate histones H3 and H4 as free histones and only H4 in a mononucleosome (123). Also, the subset of lysine

residues acetylated in histones seems to be different between p300 and P/CAF. Thus, it is possible that both acetylases together are required for optimum acetylation of nucleosomes.

6) The ADA complex

Members of the ADA transcriptional adaptor complex were first identified genetically in yeast (9, 80). When a strong transcriptional activator is highly overexpressed in yeast, it is toxic to them. This toxicity is likely to occur because the overexpressed activator outcompetes endogenous activators for binding to general transcription factors. Titrating away general transcription factors leads to toxicity. The ADA genes were isolated as suppressors of GAL4-VP16 mediated toxicity and are thus proposed to encode adaptors that mediate the interaction between that activator and the general transcription factors.

Five *ADA* genes were isolated in the selection (9, 52, 79, 80, 91). As expected, transcriptional activation by GAL4-VP16 is reduced in *ada* mutants. Also, activation by several other activators is reduced in these mutants, demonstrating that the ADAs may function as adaptors for a subset of activators. Characterization of the *ada* mutants have divided them into two classes (52, 79). One includes *ADA2*, *ADA3*, and *GCN5* while the other includes *ADA1* and *ADA5*. Mutants in the *ADA1/ADA5* class of ADAs have more severe growth defects, display Spt⁻ phenotypes (see above) and have more severe transcription defects than strains deleted of the *GCN5* class (52, 79, 99).

Despite these differences, both classes also share similarities. The promoters affected by mutations in the *GCN5* class of ADAs are a subset of those affected by mutations in the *ADA1/ADA5* class. Furthermore, strains deleted for members of both classes of ADAs do not show additive defects as might be expected if the two classes were unrelated (79). Finally, biochemical methods have demonstrated that all

five ADA gene products bind in a complex (52). Although members of the GCN5 class can bind in a trimeric complex in the absence of ADA1 and ADA5 (21, 51, 100), in wildtype cell extracts, the five ADA proteins associate in an approximately 2 MDa complex (52).

Recently, a function for the ADA complex has been proposed. A *Tetrahymena* nuclear histone acetyltransferase activity was purified (12) and demonstrated to be a homolog of GCN5 (14). Yeast GCN5 has also been shown to function as a histone acetyltransferase. With the subsequent findings (see above) that TAF250, P/CAF, p300, and CBP can all acetylate histones as well (81, 85, 123), it seems that a large number of transcriptional coactivators may function in this manner. Yeast GCN5 is able to acetylate free histones H3 and H4 (68) but is unable to acetylate mononucleosomes *in vitro*, suggesting that the function of the other ADAs of the GCN5 class may be to allow access for GCN5 to acetylate histones in intact nucleosomes.

Although one function for the ADA complex has been proposed, GCN5 mediated histone acetylation cannot be the sole function of the ADA complex. The observation that disrupting *ADA1* or *ADA5* results in a more severe phenotype than disrupting *GCN5* indicates that the *ADA1/ADA5* class of ADAs must have an additional function (52, 79). The role of this second function is unknown but there is some data that suggests some possibilities. As mentioned above, *ADA1* and *ADA5* are *SPTs* (52, 99). *ADA5* is identical to *SPT20*, a member of the TBP class of *SPTs* (99). Testing a panel of *spt* mutants has shown that some *spts* of the TBP class but not of the histone class have *Ada*⁻ phenotypes (79). Specifically, some *spt15* and *spt7* mutants are resistant to GAL4-VP16 toxicity. This suggests that these *SPTs* may also be part of the ADA complex. *SPT7* is a large acidic protein that contains a conserved domain of unknown function, the bromodomain. It is interesting to note that GCN5, TAF250, P/CAF, p300, CBP, SWI2/SNF2, and Brahma all contain bromodomains.

All of these factors are either histone acetyl transferases or ATPases that are proposed to destabilize nucleosomes. Thus, although the bromodomain has been shown to be dispensable for function for most of these proteins, the presence of this domain in a member of the ADA1/ADA5 class of ADAs suggests that the function of this class of ADAs may also be to interact with nucleosomes. P/CAF is highly homologous to GCN5 and has been shown to interact with p300/CBP. Therefore, it is possible that GCN5 also associates with another histone acetyltransferase activity (Fig. 1A).

A second possibility is that the ADA1/ADA5 class of ADAs may function to recruit the general transcription factors to promoters. As mentioned, some mutations in *SPT15*, which encodes yeast TBP, display Ada^- phenotypes (79). Furthermore, interactions between ADA2 and TBP have been demonstrated (7). Thus it is possible that the ADA complex activates transcription through two mechanisms; one class of factors antagonizes the repressive effects of chromatin, and the other class actively recruits general factors to promoters (Fig. 1B). With the observation that TAF250 functions as a histone acetyltransferase as well, it is possible that the TAFs mediate transcription in a similar two step manner.

III. Summary and perspectives

The general transcription factors are sufficient to induce a basal level of transcription from promoters *in vitro*. However, *in vivo*, it is necessary to differentially regulate the transcription of different genes. Thus, basal transcription must be repressed *in vivo*. The mechanism by which this occurs involves chromatin structure, which not only serves to efficiently package DNA in the nucleus but also to inhibit transcription factors from binding (62). Transcription can therefore be thought of as a competitive process in which the general factors and chromatin components act antagonistically and compete for binding to promoters.

Transcriptional activators can be thought of as factors which may either increase the

affinity for general factors to bind to promoters or decrease the affinity of chromatin components to bind promoters. On the other hand, repressors should decrease the affinity of general factors to promoters or increase the affinity of chromatin components for promoters.

It has become clear that sequence specific transcription factors require auxiliary factors to mediate their effects on transcription. Recent studies have shown that these cofactors utilize all four of the above mentioned mechanisms for stimulating or repressing transcription. Examples of coactivators that function by stimulating general factor binding include the TAFs, the TBP class of SPTs, the holoenzyme complex, and possibly the ADAs. Elegant *in vitro* experiments have demonstrated that the TAFs bridge interactions between activators and general factors (24). Also, the holoenzyme complex has been shown to interact with activators (44). Some members of the TBP class of SPTs such as SPT3 and TOA1 have been demonstrated to bind to TBP and presumably help TBP bind to TATA boxes (79). Links between the ADAs and TBP have also been demonstrated (79).

The second method that coactivators use to stimulate transcription, weakening interactions between chromatin components and DNA, has also been demonstrated for many factors. Currently, there are two mechanisms that have been demonstrated. The first involves the presence of DNA or nucleosome dependent ATPases of the SWI2/SNF2 family of proteins (88). Several complexes, including the SWI/SNF complex, the RSC complex and NURF contain these ATPases and have been demonstrated to alter nucleosomal structure. The second mechanism involves histone acetyltransferases (13). GCN5, TAF250, p300, CBP, and P/CAF have all been shown to be able to acetylate histones *in vitro*. Furthermore, there is a correlation between hyperacetylated DNA and transcribed regions and a correlation between silenced DNA and hypoacetylated regions, supporting the idea that acetylation may weaken DNA-nucleosome contacts.

Although this introduction has not mentioned repressors, it is evident that repressors may function by similar mechanisms. MOT1 has been proposed to function by binding to and removing TBP from TATA boxes, thus weakening general factor-promoter binding (4). Also, several repressor cofactors have been demonstrated to function as histone deacetylases, which should function by strengthening nucleosome-DNA contacts (43, 56, 127).

It is interesting to note that there are two classes of SPTs and two classes of ADAs which seem to correspond to the two mechanisms that activators may use to stimulate transcription. In the case of the ADAs, it is clear that both classes of ADAs interact in a single complex. Thus, individual coactivator complexes can utilize both methods of activating transcription. The observation that the TFIID complex, which is proposed to function by recruiting TBP to TATA boxes, contains a histone acetyltransferase activity indicates that this combined method may be a general feature of coactivators.

Although tremendous progress has been made in recent years on the function of transcriptional coactivators, many questions still remain. It will be fascinating to find out what changes actually occur in nucleosome-DNA contacts after nucleosomes are subjected to ATPase activity or to acetylation. Do these coactivator complexes translocate along DNA? If so, what is their range? Does GCN5 only acetylate nucleosomes near UASs while TAF250 acetylates nucleosomes at the TATA box? The different types of coactivators will also allow certain questions of transcriptional synergy to be analyzed. In cellular transformation, for example, multiple mutations are necessary. However, not all combinations of mutations in oncogenes leads to transformation demonstrating the existence of different complementary classes of oncogenes (114). It will be interesting to determine whether transcriptional synergy may function in a similar manner. If two coactivators that function through the same mechanism, ie. ATPase activity, are targeted to a promoter, will they act in synergy

or is it necessary to target two different types of coactivators, ie. ATPase activity and histone acetyltransferase, or acetyltransferase and recruitment factor, for transcriptional synergy to occur?

In the study of the ADAs in particular, many specific questions remain. Five specific components of the complex have been identified but the estimated size of the complex is much larger than the sum of the individual molecular masses of the components. Purification of the complex and identification and characterization of other subunits is necessary. Biochemical studies of the purified complex in *in vitro* transcription experiments (preferably on nucleosomal templates) are necessary to demonstrate a direct role of the ADAs in transcription. Also, genetic suppressors of *ada* mutants need to be isolated. Although it has been demonstrated that GCN5, and the other histone acetyltransferases, can acetylate histones *in vitro*, it has not been rigorously demonstrated that histones really are the physiological substrates for all of these factors. Suppressor analysis of *ada* mutants, similar to the isolation of the *sin* mutants as suppressors to *swi/snf* mutants, may be helpful in this respect. *In vivo* footprinting of nucleosomes at specific promoters in *ada* mutants as well as analysis of overall histone acetylation in *ada* mutants will also be helpful. Finally, elucidating the role of the ADA1/ADA5 class of ADAs by genetic and biochemical means will be extremely exciting.

References

1. **Apone, L. M., C. A. Virbasius, J. C. Reese, and M. R. Green.** 1996. Yeast TAF(II)90 is required for cell-cycle progression through G2/M but not for general transcription activation. *Genes Dev.* **10**:2368-2380.
2. **Arany, Z., W. Sellers, D. Livingston, and R. Eckner.** 1994. E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators [letter]. *Cell* **77**:799-800.
3. **Arias, J., A. S. Alberts, P. Brindle, F. X. Claret, T. Smeal, M. Karin, J. Feramisco, and M. Montminy.** 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**:226-229.
4. **Auble, D. T., K. E. Hansen, C. Mueller, W. S. Lane, J. Thorner, and S. Hahn.** 1994. Mot1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism. *Genes Dev.* **8**:1920-1934.
5. **Bannister, A. J., and T. Kouzarides.** 1996. The CBP co-activator is a histone acetyltransferase. *Nature* **384**:641-643.
6. **Barberis, A., J. Pearlberg, N. Simkovich, S. Farrell, P. Reinagel, C. Bamdad, G. Sigal, and M. Ptashne.** 1995. Contact with a component of the polymerase holoenzyme suffices for gene activation. *Cell* **81**:359-368.
7. **Barlev, N. A., R. Candau, L. Wang, P. Darpino, N. Silverman, and S. L. Berger.** 1995. Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein. *J. Biol. Chem.* **270**:19337-19344.
8. **Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg, and L. Guarente.** 1990. Selective inhibition of activated but not basal transcription by the acidic

- activation domain of VP16: evidence for transcriptional adaptors. *Cell* **61**:1199-1208.
9. **Berger, S. L., B. Pina, N. Silverman, G. A. Marcus, J. Agapite, J. L. Regier, S. J. Triezenberg, and L. Guarente.** 1992. Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell* **70**:251-265.
 10. **Berroteran, R. W., D. E. Ware, and M. Hampsey.** 1994. The *sua8* suppressors of *Saccharomyces cerevisiae* encode replacements of conserved residues within the largest subunit of RNA polymerase II and affect transcription start site selection similarly to *sua7* (TFIIB) mutations. *Mol. Cell. Biol.* **14**:226-237.
 11. **Bortvin, A., and F. Winston.** 1996. Evidence that Spt6p controls chromatin structure by a direct interaction with histones. *Science* **272**:1473-1476.
 12. **Brownell, J. E., and C. D. Allis.** 1995. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in *Tetrahymena* macronuclei. *Proc. Natl. Acad. Sci. USA* **92**:6364-6368.
 13. **Brownell, J. E., and C. D. Allis.** 1996. Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr. Opin. Genet. Dev.* **6**:176-184.
 14. **Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth, and C. D. Allis.** 1996. *Tetrahymena* histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* **84**:843-851.
 15. **Buratowski, S.** 1994. The basics of basal transcription by RNA polymerase II. *Cell* **77**:1-3.
 16. **Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp.** 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56**:549-561.

17. **Cairns, B. R., N. L. Henry, and R. D. Kornberg.** 1996. TFG/TAF30/ANC1, a component of the yeast SWI/SNF complex that is similar to the leukemogenic proteins ENL and AF-9. *Mol. Cell. Biol.* **16**:3308-3316.
18. **Cairns, B. R., Y.-J. Kim, M. H. Sayre, B. C. Laurent, and R. D. Kornberg.** 1994. A multisubunit complex containing the *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* gene products isolated from yeast. *Proc. Natl. Acad. Sci. USA* **91**:1950-1954.
19. **Cairns, B. R., R. S. Levinson, K. R. Yamamoto, and R. D. Kornberg.** 1996. Essential role of Swp73p in the function of yeast Swi/Snf complex. *Genes Dev.* **10**:2131-2144.
20. **Cairns, B. R., Y. Lorch, Y. Li, M. Zhang, L. Lacomis, H. Erdjument-Bromage, P. Tempst, J. Du, B. Laurent, and R. D. Kornberg.** 1996. RSC, an essential, abundant chromatin-remodeling complex. *Cell* **87**:1249-1260.
21. **Candau, R., and S. L. Berger.** 1996. Structural and functional analysis of yeast putative adaptors. Evidence for an adaptor complex in vivo. *J. Biol. Chem.* **271**:5237-5245.
22. **Carlson, M.** 1987. Regulation of sugar utilization in *Saccharomyces* species. *J. Bact.* **169**:4873-4877.
23. **Chatterjee, S., and K. Struhl.** 1995. Connecting a promoter-bound protein to TBP bypasses the need for a transcriptional activation domain. *Nature* **374**:820-822.
24. **Chen, J. L., L. D. Attardi, C. P. Verrijzer, K. Yokomori, and R. Tjian.** 1994. Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. *Cell* **79**:93-105.
25. **Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman.** 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**:855-859.
26. **Clark-Adams, C. D., and F. Winston.** 1988. Changes in histone gene dosage alter transcription in yeast. *Genes Dev.* **2**:150-159.

27. **Cote, J., J. Quinn, J. L. Workman, and C. L. Peterson.** 1994. Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science* **265**:53-60.
28. **Davis, J. L., R. Kunisawa, and J. Thorner.** 1992. A presumptive helicase (*MOT1* gene product) affects gene expression and is required for viability in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **12**:413-421.
29. **Dikstein, R., S. Ruppert, and R. Tjian.** 1996. TAFII250 is a bipartite protein kinase that phosphorylates the base transcription factor RAP74. *Cell* **84**:781-790.
30. **Durrin, L. K., R. K. Mann, P. S. Kayne, and M. Grunstein.** 1991. Yeast histone H4 N-terminal sequence is required for promoter activation *in vivo*. *Cell* **65**:1023-1031.
31. **Dynlacht, B. D., T. Hoey, and R. Tjian.** 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* **66**:563-576.
32. **Eckner, R., M. E. Ewen, D. Newsome, M. Gerdes, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.* **8**:869-884.
33. **Eisenmann, D. M., K. M. Arndt, S. L. Ricupero, J. W. Rooney, and F. Winston.** 1992. SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae*. *Genes Dev.* **6**:1319-1331.
34. **Eisenmann, D. M., C. Chapon, S. M. Roberts, C. Dollard, and F. Winston.** 1994. The *Saccharomyces cerevisiae* SPT8 gene encodes a very acidic protein that is functionally related to SPT3 and TATA-binding protein. *Genetics* **137**:647-657.
35. **Eisenmann, D. M., C. Dollard, and F. Winston.** 1989. *SPT15*, the gene encoding the yeast TATA binding factor TFIID, is required for normal transcription initiation *in vivo*. *Cell* **58**:1183-1191.

36. **Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon, and R. Tjian.** 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* 75:519-530.
37. **Goodrich, J. A., and R. Tjian.** 1994. Transcription factors IIE and IIIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. *Cell* 77:145-156.
38. **Guarente, L.** 1992. Mechanism and regulation of transcriptional activation in eukaryotes: conserved features from yeast to humans, p. 1007-1036. *In* S. L. McKnight, and K. R. Yamamoto (ed.), *Transcriptional Regulation*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
39. **Guarente, L.** 1988. UASs and Enhancers: Common Mechanism of Transcriptional Activation in Yeast and Mammals. *Cell* 52:303-305.
40. **Ha, I., W. S. Lane, and D. Reinberg.** 1991. Cloning of a human gene encoding the general transcription initiation factor IIB. *Nature* 352:689-695.
41. **Han, M., and Grunstein, M.** 1989. Nucleosome loss activates yeast downstream promoters *in vivo*. *Cell* 55:1137-1145.
42. **Han, M., U. J. Kim, P. Kayne, and M. Grunstein.** 1988. Depletion of histone H4 and nucleosomes activates the *PHO5* gene in *Saccharomyces cerevisiae*. *EMBO J.* 7:2221-2228.
43. **Hassig, C. A., T. C. Fleischer, A. N. Billin, S. L. Schreiber, and D. E. Ayer.** 1997. Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell* 89:341-347.
44. **Hengartner, C. J., C. M. Thompson, J. Zhang, D. M. Chao, S. M. Liao, A. J. Koleske, S. Okamura, and R. A. Young.** 1995. Association of an activator with an RNA polymerase II holoenzyme. *Genes Dev.* 9:897-910.
45. **Herskowitz, I., B. Andrews, W. Kruger, J. Ogas, A. Sil, C. Coburn, and C. Peterson.** 1992. Integration of multiple regulatory inputs in the control of *HO* expression in yeast, p. 949-974. *In* S. L. McKnight, and K. R. Yamamoto (ed.),

Transcriptional Regulation, vol. 2. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

46. **Himmelfarb, H. J., J. Pearlberg, D. H. Last, and M. Ptashne.** 1990. GAL11P: A yeast mutation that potentiates the effect of weak GAL4-derived activators. *Cell* **63**:1299-1309.
47. **Hirschhorn, J. N., S. A. Brown, C. D. Clark, and F. Winston.** 1992. Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev.* **6**:2288-2298.
48. **Hirschman, J. E., K. J. Durbin, and F. Winston.** 1988. Genetic evidence for promoter competition in *Saccharomyces cerevisiae*. *Genetics* **8**:4608-4615.
49. **Hoey, T., R. O. Weinzierl, G. Gill, J. L. Chen, B. D. Dynlacht, and R. Tjian.** 1993. Molecular cloning and functional analysis of *Drosophila* TAF110 reveal properties expected of coactivators. *Cell* **72**:247-60.
50. **Hope, I., and K. Struhl.** 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* **46**:885-894.
51. **Horiuchi, J., N. Silverman, G. A. Marcus, and L. Guarente.** 1995. ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. *Mol. Cell. Biol.* **15**:1203-1209.
52. **Horiuchi, J., N. Silverman, B. Pina, G. A. Marcus, and L. Guarente.** 1997. ADA1, a novel component of the ADA/GCN5 complex has broader effects than GCN5, ADA2, or ADA3. *Mol. Cell. Biol.* **17**:3220-3228.
53. **Imbalzano, A. N., H. Kwon, M. R. Green, and R. E. Kingston.** 1994. Facilitated binding of TATA-binding protein to nucleosomal DNA. *Nature* **370**:481-485.
54. **Ingles, C. J., M. Shales, W. D. Cress, S. J. Triezenberg, and J. Greenblatt.** 1991. Reduced binding of TFIID to transcriptionally compromised mutants of VP16. *Nature* **351**:588-590.

55. **Jacobson, R. H., and R. Tjian.** 1996. Transcription factor IIA: A structure with multiple functions. *Science* **272**:827-828.
56. **Kadosh, D., and K. Struhl.** 1997. Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. *Cell* **89**:365-371.
57. **Kaufmann, J., C. P. Verrijzer, J. Shao, and S. T. Smale.** 1996. CIF, an essential cofactor for TFIID-dependent initiator function. *Genes Dev.* **10**:873-886.
58. **Kelleher, R. J. I., P. M. Flanagan, and R. D. Kornberg.** 1990. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell* **61**:1209-1215.
59. **Kim, J. L., D. B. Nikolov, and S. K. Burley.** 1993. Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* **365**:520-527.
60. **Kim, Y., J. H. Geiger, S. Hahn, and P. B. Sigler.** 1993. Crystal structure of a yeast TBP/TATA-box complex. *Nature* **365**:512-520.
61. **Kim, Y. J., S. Björklund, Y. Li, M. H. Sayre, and R. D. Kornberg.** 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**:599-608.
62. **Kingston, R. E., C. A. Bunker, and A. N. Imbalzano.** 1996. Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev.* **10**:905-920.
63. **Klages, N., and M. Strubin.** 1995. Stimulation of RNA polymerase II transcription initiation by recruitment of TBP *in vivo*. *Nature* **374**:822-823.
64. **Koleske, A. J., S. Buratowski, M. Nonet, and R. A. Young.** 1992. A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell* **69**:883-894.
65. **Koleske, A. J., and R. A. Young.** 1995. The RNA polymerase II holoenzyme and its implications for gene regulation. *Trends Bioch. Sci.* **20**:113-116.

66. **Koleske, A. J., and R. A. Young.** 1994. An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**:466-469.
67. **Kruger, W., and Herskowitz.** 1991. A negative regulator of *HO* transcription, *SIN1 (SPT2)*, is a nonspecific DNA-binding protein. *Mol. Cell. Biol.* **11**:4135-4156.
68. **Kuo, M. H., J. E. Brownell, R. E. Sobel, T. A. Ranalli, R. G. Cook, D. G. Edmondson, S. Y. Roth, and C. D. Allis.** 1996. Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. *Nature* **383**:269-272.
69. **Kwok, R. P. S., J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. E. Roberts, M. R. Green, and R. H. Goodman.** 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370**:223-226.
70. **Kwon, H., A. N. Imbalzano, P. A. Khavari, R. E. Kingston, and M. R. Green.** 1994. Nucleosome disruption and enhancement of activator binding by a human SWI/SNF complex. *Nature* **370**:477-481.
71. **Laurent, B. C., and M. Carlson.** 1992. Yeast SNF2/SWI2, SNF5, and SNF6 proteins function coordinately with the gene-specific transcriptional activators GAL4 and Bicoid. *Genes Dev.* **6**:1707-1715.
72. **Lenfant, F., R. K. Mann, B. Thomsen, X. Ling, and M. Grunstein.** 1996. All four core histone N-termini contain sequences required for the repression of basal transcription in yeast. *EMBO J.* **15**:3974-3985.
73. **Liao, S. M., I. C. A. Taylor, R. E. Kingston, and R. A. Young.** 1991. RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators *in vitro*. *Genes Dev.* **5**:2431-2440.
74. **Liao, S. M., J. Zhang, D. A. Jeffrey, A. J. Koleske, C. M. Thompson, D. M. Chao, M. Viljoen, H. J. van Vuuren, and R. A. Young.** 1995. A kinase-cyclin pair in the RNA polymerase II holoenzyme. *Nature* **374**:193-196.

75. **Lin, Y. S., and M. R. Green.** 1991. Mechanism of action of an acidic transcriptional activator *in vitro*. *Cell* **64**:971-981.
76. **Lin, Y. S., E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activating region. *Nature* **353**:569-571.
77. **Lundblad, J. R., P. S. Kwok, M. E. Lurance, M. L. Harter, and R. H. Goodman.** 1995. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature* **374**:85-88.
78. **Madison, J. M., and F. Winston.** 1997. Evidence that Spt3 functionally interacts with Mot1, TFIIA, and TATA-binding protein to confer promoter-specific transcriptional control in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **17**:287-295.
79. **Marcus, G. A., J. Horiuchi, N. Silverman, and L. Guarente.** 1996. *ADA5/SPT20* links the *ADA* and *SPT* genes, which are involved in yeast transcription. *Mol. Cell. Biol.* **16**:3197-3205.
80. **Marcus, G. A., N. Silverman, S. L. Berger, J. Horiuchi, and L. Guarente.** 1994. Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. *EMBO J.* **13**:4807-4815.
81. **Mizzen, C. A., X. J. Yang, T. Kokubo, J. E. Brownell, A. J. Bannister, T. Owen-Hughes, J. Workman, L. Wang, S. L. Berger, T. Kouzarides, Y. Nakatani, and C. D. Allis.** 1996. The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* **87**:1261-1270.
82. **Moqtaderi, Z., Y. Bai, D. Poon, P. A. Weil, and K. Struhl.** 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* **383**:188-191.
83. **Nikolov, D. B., H. Chen, E. D. Halay, A. A. Usheva, K. Hisatake, D. K. Lee, R. G. Roeder, and S. K. Burley.** 1995. Crystal structure of a TFIIB-TBP-TATA-element ternary complex. *Nature* **377**:119-128.

84. **Nonet, M. L., and R. A. Young.** 1989. Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. *Genetics* **123**:715-724.
85. **Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani.** 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**:953-959.
86. **Orphanides, G., T. Lagrange, and D. Reinberg.** 1996. The general transcription factors of RNA polymerase II. *Genes Dev.* **10**:2657-2683.
87. **Parvin, J. D., and P. A. Sharp.** 1993. DNA topology and a minimal set of basal factors for transcription by RNA polymerase II. *Cell* **73**:533-540.
88. **Pazin, M. J., and J. T. Kadonaga.** 1997. SWI2/SNF2 and related proteins: ATP-driven motors that disrupt protein-DNA interactions? *Cell* **88**:737-740.
89. **Peterson, C. L., A. Dingwall, and M. P. Scott.** 1994. Five SWI/SNF gene products are components of a large multisubunit complex required for transcriptional enhancement. *Proc. Natl. Acad. Sci. USA* **91**:2905-2908.
90. **Peterson, C. L., and I. Herskowitz.** 1992. Characterization of the yeast *SWI1*, *SWI2*, and *SWI3* genes, which encode a global activator of transcription. *Cell* **68**:573-584.
91. **Pina, B., S. Berger, G. A. Marcus, N. Silverman, J. Agapite, and L. Guarente.** 1993. *ADA3*: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of *ADA2*. *Mol. Cell. Biol.* **13**:5981-5989.
92. **Pinto, I., D. E. Ware, and M. Hampsey.** 1992. The yeast *SUA7* gene encodes a homolog of human transcription factor TFIIB and is required for normal start site selection *in vivo*. *Cell* **68**:977-988.
93. **Prelich, G., and F. Winston.** 1993. Mutations that suppress the deletion of an upstream activating sequence in yeast: involvement of a protein kinase and histone H3 in repressing transcription *in vivo*. *Genetics* **135**:665-676.

94. **Ptashne, M., and A. Gann.** 1997. Transcriptional activation by recruitment. *Nature* **386**:569-577.
95. **Pugh, B. F., and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* **61**:1187-1197.
96. **Recht, J., B. Dunn, A. Raff, and M. A. Osley.** 1996. Functional analysis of histones H2A and H2B in transcriptional repression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**:2545-2553.
97. **Roberts, S. G. E., and M. R. Green.** 1994. Activator-induced conformational change in general transcription factor TFIIB. *Nature* **371**:717-720.
98. **Roberts, S. G. E., I. Ha, E. Maldonada, D. Reinberg, and M. R. Green.** 1993. Interaction between an acidic activator and transcription factor IIB is required for transcriptional activation. *Nature* **363**:741-744.
99. **Roberts, S. M., and F. Winston.** 1996. *SPT20/ADA5* encodes a novel protein functionally related to the TATA-binding protein and important for transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**:3206-3213.
100. **Saleh, A., V. Lang, R. Cook, and C. J. Brandl.** 1997. Identification of native complexes containing the yeast coactivator/repressor proteins NGG1/ADA3 and ADA2. *J. Biol. Chem.* **272**:5571-5578.
101. **Sauer, F., D. A. Wassarman, G. M. Rubin, and R. Tjian.** 1996. TAF(II)s mediate activation of transcription in the *Drosophila* embryo. *Cell* **87**:1271-1284.
102. **Serizawa, H., J. W. Conaway, and R. C. Conaway.** 1994. An oligomeric form of the large subunit of transcription factor (TF) IIE activates phosphorylation of the RNA polymerase II carboxyl-terminal domain by TFIIF. *J. Biol. Chem.* **269**:20750-20756.
103. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature* **345**:783-786.

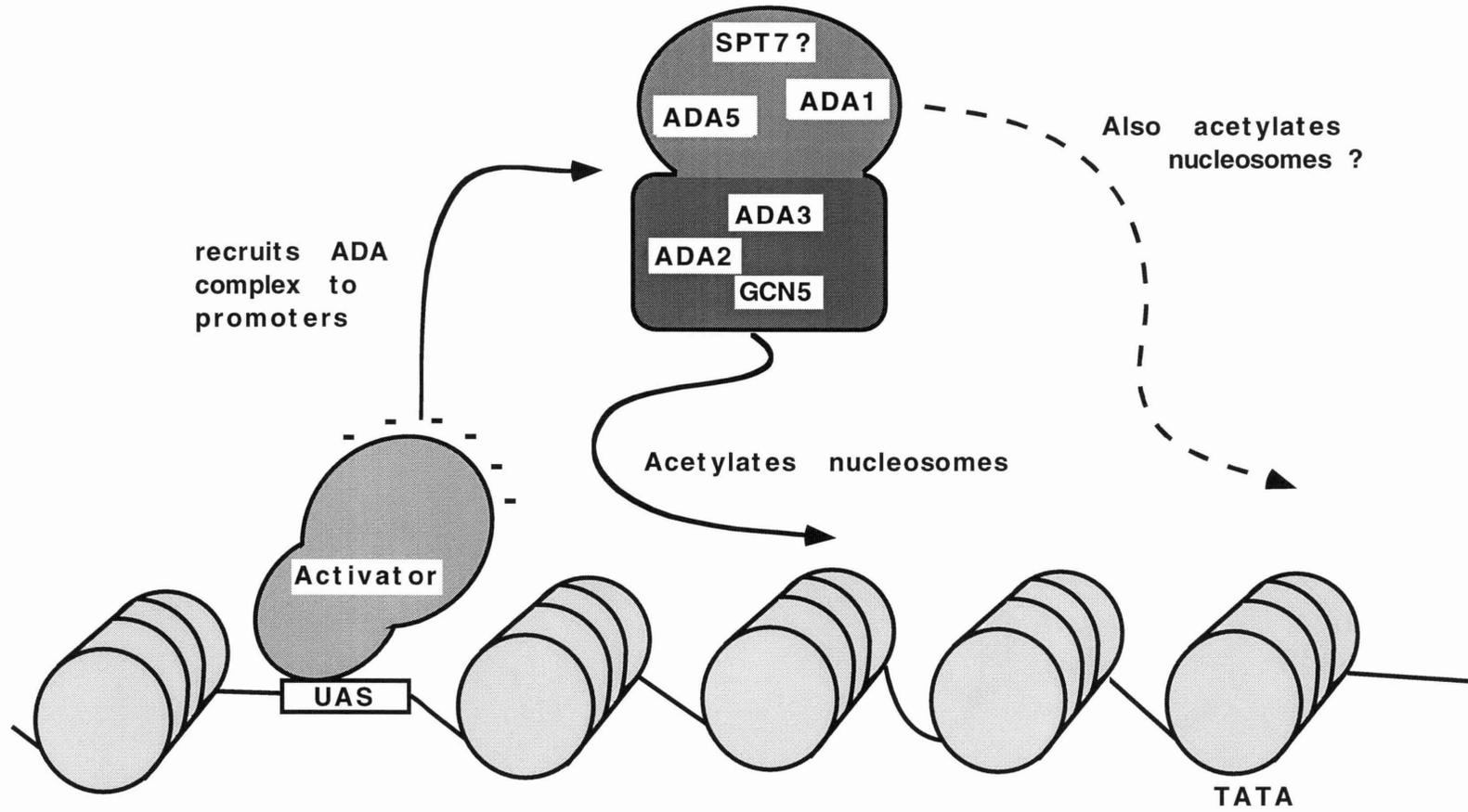
104. **Sun, Z. W., and M. Hampsey.** 1995. Identification of the gene (*SSU71/TFG1*) encoding the largest subunit of transcription factor TFIIIF as a suppressor of a TFIIIB mutation in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **92**:3127-3131.
105. **Swanson, M. S., and F. Winston.** 1992. SPT4, SPT5 and SPT6 interactions: effects on transcription and viability in *Saccharomyces cerevisiae*. Genetics **132**:325-336.
106. **Tansey, W. P., and W. Herr.** 1995. The ability to associate with activation domains *in vitro* is not required for the TATA box-binding protein to support activated transcription *in vivo*. Proc. Natl. Acad. Sci. USA **92**:10550-10554.
107. **Thompson, C. M., A. J. Koleske, D. M. Chao, and R. A. Young.** 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73**:1361-1375.
108. **Treich, I., B. R. Cairns, T. de los Santos, E. Brewster, and M. Carlson.** 1995. SNF11, a new component of the yeast SNF/SWI complex that interacts with a conserved region of SNF2. Mol. Cell. Biol. **8**:4240-4248.
109. **Triezenberg, S. J.** 1995. Structure and function of transcriptional activation domains. Curr. Op. Gen. Dev **5**:190-196.
110. **Tsukiyama, T., C. Daniel, J. Tamkun, and C. Wu.** 1995. *ISWI*, a member of the *SWI2/SNF2* ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. Cell **83**:1021-1026.
111. **Tsukiyama, T., and C. Wu.** 1995. Purification and properties of an ATP-dependent nucleosome remodeling factor. Cell **83**:1011-1020.
112. **Usheva, A., E. Maldonado, A. Goldring, H. Lu, C. Houbavi, D. Reinberg, and Y. Aloni.** 1992. Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. Cell **69**:871-881.

113. **Verrijzer, C. P., J. L. Chen, K. Yokomori, and R. Tjian.** 1995. Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II. *Cell* **81**:1115-1125.
114. **Vogelstein, B., and K. W. Kinzler.** 1993. The multistep nature of cancer. *Trends Genet.* **9**:138-141.
115. **Walker, S. S., J. C. Reese, L. M. Apone, and M. R. Green.** 1996. Transcription activation in cells lacking TAFII. *Nature* **383**:185-188.
116. **Wang, W., Y. Xue, S. Zhou, A. Kuo, B. R. Cairns, and G. R. Crabtree.** 1996. Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev.* **10**:2117-2130.
117. **White, R. J., and S. P. Jackson.** 1992. The TATA-binding protein: a central role in transcription by RNA polymerases I, II and III. *Trends Genet.* **8**:284-288.
118. **Wilson, C. J., D. M. Chao, A. N. Imbalzano, G. R. Schnitzler, R. E. Kingston, and R. A. Young.** 1996. RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell* **84**:235-244.
119. **Winston, F.** 1992. Analysis of SPT genes: a genetic approach toward analysis of TFIID, histones, and other transcription factors of yeast, p. 1271-1293. *In* S. L. McKnight, and K. R. Yamamoto (ed.), *Transcriptional Regulation*, 1 ed, vol. 2. Cold Spring Harbor Laboratory Press, United States.
120. **Winston, F., and M. Carlson.** 1992. Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. *Trends Genet.* **8**:387-91.
121. **Xiao, H., J. D. Friesen, and J. T. Lis.** 1995. Recruiting TATA-Binding Protein to a promoter: transcriptional activation without an upstream activator. *Mol. Cell. Biol.* **15**:5757-5761.
122. **Xie, X., T. Kokubo, S. L. Cohen, U. A. Mirza, A. Hoffmann, B. T. Chait, R. G. Roeder, Y. Nakatani, and S. K. Burley.** 1996. Structural similarity between TAFs and the heterotetrameric core of the histone octamer. *Nature* **380**:316-322.

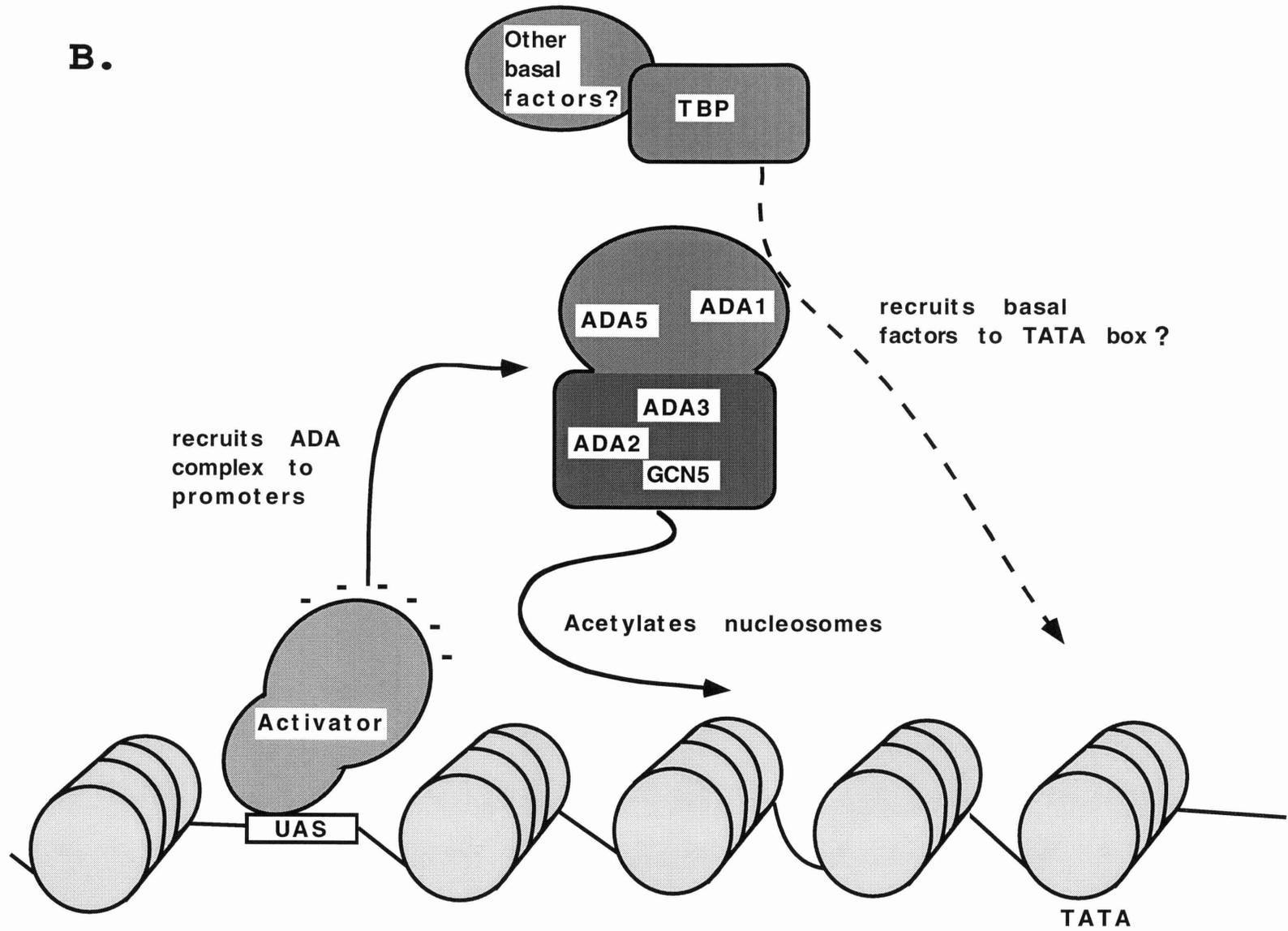
123. **Yang, X. J., V. V. Ogryzko, J. Nishikawa, B. H. Howard, and Y. Nakatani.** 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**:319-324.
124. **Yoshinaga, S. K., C. L. Peterson, I. Herskowitz, and K. R. Yamamoto.** 1992. Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. *Science* **258**:1598-1604.
125. **Young, R.** 1991. RNA polymerase II. *Annu. Rev. Biochem.* **60**:689-715.
126. **Zawel, L., and D. Reinberg.** 1993. Initiation of transcription by RNA polymerase II: a multi-step process. *Progress in Nucleic Acid Research and Molecular Biology* **44**:67-108.
127. **Zhang, Y., R. Iratni, E. Hediye, P. Tempst, and D. Reinberg.** 1997. Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. *Cell* **89**:357-364.

Figure 1. Two possible models for the role for the ADA/GCN5 complex in transcriptional activation. The ADA/GCN5 complex is proposed to be recruited to promoters through interactions with the acidic activation domains of transcriptional activators. Once there, the GCN5 class of ADAs may acetylate nucleosomes, countering the repressive effects of the nucleosomes on transcription. A) In one model, another component of the ADA1/ADA5 class of ADAs is SPT7. Similarly to the case of p300/CBP interacting with P/CAF, where two bromodomain containing histone acetyltransferases bind together in a complex, it is possible that the ADA1/ADA5 class also functions by acetylating histones. B) In the second model, the ADA1/ADA5 class, which may or may not contain SPT7, functions by recruiting TBP and/or other basal factors to the TATA region of the promoter. Thus, the ADA/GCN5 complex may function by both removing repressive effects and actively recruiting basal factors.

A.



B.



Chapter II

ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex

This chapter was previously published in *Molecular and Cellular Biology*, Vol. 15 pages 1203-1209, March 1995. The authors were Junjiro Horiuchi, Neal Silverman, Gregory A. Marcus, and Leonard Guarente.

Summary

Mutations in yeast *ADA2*, *ADA3*, and *GCN5* weaken the activation potential of a subset of acidic activation domains. In this report, we show that their gene products form a heterotrimeric complex *in vitro*, with *ADA2* as the linchpin holding *ADA3* and *GCN5* together. Further, activation by *lexA-ADA3* fusions *in vivo* are regulated by the levels of *ADA2*. Combined with a prior observation that *lexA-ADA2* fusions are regulated by the levels of *ADA3* (N. Silverman, J. Agapite, L. Guarente, Proc. Natl. Acad. Sci. USA, 91:11665-11668, 1994), this finding suggests that these proteins also form a complex in cells. *ADA3* can be separated into two non-overlapping domains, an amino-terminal domain and a carboxyl-terminal domain, which do not separately complement the slow growth phenotype or transcriptional defect of a $\Delta ada3$ strain but together supply full complementation. The carboxyl-terminal domain of *ADA3* alone suffices for heterotrimeric complex formation *in vitro* and activation of *lexA-ADA2* *in vivo*. We present a model depicting the *ADA* complex as a coactivator in which the *ADA3* amino-terminal domain mediates an interaction between activation domains and the *ADA* complex.

Introduction

In eukaryotes, several factors that are important in the activation of transcription by RNA polymerase II are in large, heteromeric complexes. For example, the yeast SWI2/SNF2, SWI1, SWI3, SNF5 and SNF6 proteins form a large multisubunit complex, which apparently counters repression by chromatin (4, 5, 7, 23). Mutations in *SWI2* and *SNF5* result in decreased transcription and altered chromatin structure at certain promoters (19). These phenotypes can be suppressed by mutations in histone genes. In another case, the yeast, SRB2, SRB4, SRB5, and SRB6 proteins form a holoenzyme complex with RNA polymerase II and certain basal transcription factors (21). The SRB proteins interact with the carboxyl-terminal domain of the largest subunit of RNA polymerase II and are important for both basal and activated transcription *in vitro* (33). In higher eukaryotes, the TFIID complex is composed of TBP and TAFs (TBP associated factors) (9). While TBP with the other basal factors is sufficient for basal transcription, the TAFs are required for activated transcription (12, 13). Thus the TAFs are proposed to be coactivators or adaptors required to mediate the stimulatory signal from activators to basal factors. There is also evidence that a family of factors interact with TBP in yeast (10, 25, 34).

In addition to the TAFs, other factors, such as the yeast *ADA2*, *ADA3*, and *GCN5* gene products, have been proposed to be coactivators (3, 22, 24). Mutations in *ADA2*, *ADA3* and *GCN5* were isolated in a selection for mutants which confer resistance to toxicity from overexpressed GAL4-VP16. This toxicity is postulated to occur by titration of basal transcription factors away from productive transcription complexes by the strong acidic activation domain of GAL4-VP16 (2). If this titration by GAL4-VP16 requires proteins with coactivator/adaptor function, alterations in these proteins should cause resistance to toxicity. Interestingly, *GCN5* had been

isolated previously as a transcription factor necessary for full activity of the activator GCN4 (11).

ADA2, *ADA3*, and *GCN5* mutants share several phenotypes (3, 22, 24). Strains disrupted for any of the three genes display temperature sensitive growth as well as a severe growth defect on minimal media. Double mutants between any two of the three genes do not have a more severe slow growth phenotype than the single mutants. Also, all three genes are required for full transcriptional activity of a similar subset of activators. *GAL4-VP16* and *GCN4* are dependent on *ADA2*, *ADA3*, and *GCN5* activity whereas other activators such as *HAP4* and *GAL4* are independent or only slightly dependent.

Because of the similar phenotypes between mutations in *ADA3*, *ADA2* and *GCN5*, we wanted to determine whether their gene products interacted in a complex. In this study, we demonstrate that a trimeric complex is indeed formed by *ADA3*, *ADA2*, and *GCN5*. We further characterize *ADA3* and show that it consists of two separable functional domains, both of which are required for function and one of which interacts in the adaptor complex. A model for the structure and function of the ADA complex is proposed.

Materials and methods

Strains and plasmids. Assays were carried out with *Saccharomyces cerevisiae* BWG1-7a (*MATa*, *leu2-2*, 112, *his4-519*, *ade1-100*, *ura2-52*), BWG1-7a Δ *trp1* and derivatives disrupted for *ADA3* or *ADA2*. BWG1-7a Δ *trp1* is BWG1-7a with the *trp1* gene disrupted using pNKY1009 (1). *ADA3* was disrupted using the *Bam*HI/*Sal*I fragment of plasmid p Δ A3-i (24). BWG1-7a Δ *ada2* has been previously described (3).

Plasmids expressing size variants of *ADA3* were constructed by first generating various *ADA3* fragments using PCR. Oligonucleotides hybridizing to

various portions of *ADA3* were synthesized and used to amplify the appropriate regions of *ADA3*. The numbers in the parentheses after each *ADA3* construct correspond to the amino acids encoded by the various *ADA3* fragments. In all constructs, six histidines were fused to the C-terminus of *ADA3* by encoding them on the 3' primers except when HA follows the parentheses, in which case the 12CA5 hemagglutinin tag (HA) was fused instead. The *ADA3* plasmids carrying the *LEU2* marker were generated by cloning the *ADA3* fragments into the yeast expression plasmid pDB20L (3), while plasmids carrying the *URA3* and *TRP1* markers were generated by cloning the *ADA3* fragments into the plasmid pJH1 and pJH2 respectively. pJH1 was generated by subcloning the *ADH1* promoter/terminator fragment from pDB20L into the *Bam*HI site of pRS316 (28) and pJH2 was generated by subcloning the same fragment into pRS314 (28). Templates used for *in vitro* transcription/translation of *ADA3* fragments were generated by cloning the above *ADA3* fragments behind T7 promoters in the plasmids pT7plink (8) or pCITE2A (Novagen).

The construction of plexA-*ADA2* and plexA-*ADA3* which express LexA-*ADA2* and LexA-*ADA3* respectively, have been previously described (22). The pT7*ADA2* and pT7*GCN5* constructs used for *in vitro* transcription/translation of *ADA2* and *GCN5* are described by Marcus et al. (22). pLexA-*GCN4* (24), the lexA β -galactosidase reporter, pRBHis (22), and p*ADA2*-6HisL (29), which was used to overexpress *ADA2*, have also been described previously. p14x2His, a *lacZ* reporter plasmid with two synthetic *GCN4* binding sites upstream of a minimal *CYC1* promoter, was constructed from HIS(2)14x2 (18). The *URA3* marker was removed from HIS(2)14x2 by digesting with *Stu*I. *Not*I linkers were ligated, and a *Not*I fragment containing the *HIS4* gene was inserted.

Yeast transformations were performed by the lithium acetate method (27). For β -galactosidase assays, cells were grown in selective media with glucose to an OD₆₀₀

of approximately 1.0 and activity was measured in glass bead extracts as described by Rose and Botstein (26). For p14x2His activity, cells were grown in minimal media supplemented with adenine. For all other assays, cells were grown in synthetic complete media lacking only the amino acids and nucleotide used to select for plasmids. β -galactosidase activity was measured as nmoles/min/mg protein. Other general yeast techniques were performed as described by Guthrie and Fink (16).

***In vitro* transcription/translation.** *In vitro* transcription was performed with 2.5 μ g of linearized template in a 25 μ l volume of 1x T7 buffer (Pharmacia), 0.5 mM rATP, rCTP, and rGTP, 0.1 mM rGTP, 100 mg/ml BSA, 2 mM $MgCl_2$, 10 mM DTT, 40 U RNAsin (Promega), 500 mM $m^7G(5')ppp5'G$ (Boehringer Mannheim) and 20 U T7 polymerase (Pharmacia). The reaction was incubated at 37°C for 30 min, 1 μ l of 10 mM rGTP was added, and the reaction was incubated at 37°C for an additional 30 min. RNA was purified by two phenol chloroform extractions followed by two ethanol precipitations.

In vitro translations were performed using a rabbit reticulocyte lysate system (Promega). 17.5 μ l of rabbit reticulocyte lysate, 20 U RNAsin, 0.5 μ l of 1 mM amino acid mix minus methionine, 20 mCi of [^{35}S]methionine (1200 Ci/mmol) and 0.3 to 1 μ g of each RNA were mixed and incubated for 90 min at 30°C. Proteins were either used directly or stored at -80°C until used. The luciferase used as a negative control in Figure 3 was supplied as a control mRNA from Promega.

Far western analysis. Plasmids for the expression of recombinant ADA3(580-702) and ADA3(452-702) were constructed by cloning the appropriate ADA3 fragments generated by PCR (see above) into the T7 expression vector, pET21d (Novagen). Recombinant glutathione S-transferase (GST) was expressed from pGEX-KG(14). ADA2, and dihydrofolate-reductase (DHFR) were expressed from pUH24.2 Δ CAT (22).

The ADA3 expression constructs were transformed into BL21-DE3 (30) cells, and the pGEX-KG and pUH24.2ΔCAT constructs were transformed into AG115 cells (20). Expression was induced as recommended (Novagen). The 6-histidine tagged ADA3 fragments and DHFR were purified using a nickel column as recommended (Qiagen) while the other proteins were used as unpurified extracts.

Proteins and extracts were subjected to SDS polyacrylamide gel electrophoresis (PAGE) and subsequently stained with Coomassie Brilliant Blue or transferred to nitrocellulose. Nitrocellulose blots were denatured in 8 M urea and stepwise renatured in far western buffer (20 mM Hepes pH 7.3, 60 mM KCl, 7.5 mM MgCl₂, 5% glycerol, and 25 mM DTT). During each renaturation step, the urea concentration was diluted two-fold in the buffer until the urea concentration was below 10 mM. The blots were blocked for 1 h in far western buffer containing 5% BSA followed by an overnight incubation with [³⁵S]methionine-labelled *in vitro* translated probe in buffer. The blots were subsequently washed three times in far western buffer, dried, and exposed to film.

Coimmunoprecipitations. Reactions contained 20 μl immunoprecipitation (IP) buffer (50 mM Hepes pH 7.3, 100 mM K-glutamate, 6 mM MgOAc, 1 mM EGTA, 0.1% NP40, 0.5 mM DTT, 0.5 mg/ml BSA, and 10% glycerol), 1 μl antibody, and 5 μl reticulocyte lysate translation product. The antibodies used were either anti-HA monoclonal (BAbCo), or anti-ADA2 polyclonal (29). Reactions were pipetted onto 10 μl of protein A Sepharose beads which had previously been equilibrated in IP buffer. Following gentle mixing on ice, the reactions were rotated for 3 h at 4°C. The reactions were centrifuged for 15 sec and the supernatant was discarded. The beads were washed three times with 1 ml IP buffer. If the antibody used in the immunoprecipitation was anti-ADA2 polyclonal, the immunoprecipitated complexes were separated from the beads by boiling in protein gel loading buffer. If the antibody used was the anti-HA monoclonal, the immune complexes were eluted

from the beads by incubating for 1 h at 4°C in 1 mg/ml of a 12CA5 epitope peptide (BAbCo) in IP buffer without BSA. The immunoprecipitated complexes were analyzed by SDS-PAGE.

Results

ADA3 has two functional domains. A strain deleted of *ADA3* has a severe slow growth phenotype on minimal media (24). A wild type copy of *ADA3*, or a truncated allele of *ADA3*, lacking the first 214 codons of the 702 codon gene, fully complements the deletion (Figure 1A, C). Expression of either the amino-terminal half of *ADA3* (*ADA3*(1-346)) or the carboxyl-terminal half of *ADA3* (*ADA3*(364-702)) does not allow complementation. However, when both non-overlapping clones, *ADA3*(1-346) and *ADA3*(364-702), are expressed in a $\Delta ada3$ strain simultaneously, growth is fully complemented. These results suggest an unusual interaction between two non-overlapping domains of *ADA3*. We propose two possible models to explain this observation. First, the amino-terminal domain and the carboxyl-terminal domain of *ADA3* may independently fold into functional units that do not need to interact with each other or, second, the amino-terminal domain and carboxyl-terminal domain may interact with each other without having to be covalently linked.

A $\Delta ada3$ strain transformed with two plasmids, one expressing a truncated amino-terminal domain, *ADA3*(214-346), and the other expressing the full carboxyl-terminal domain, *ADA3*(364-702), grows as well as a wild type strain (Figure 1B, C). Growth is restored to an intermediate extent when the $\Delta ada3$ strain is transformed with plasmids expressing a truncated carboxyl-terminal domain, *ADA3*(452-702), and the full length amino-terminal domain, *ADA3*(1-346). However, when both the truncated amino-terminal domain and the truncated carboxyl-terminal domain are expressed, no complementation is evident. This type of interaction can be thought of

as a synthetic phenotype, and suggests that both domains of ADA3 interact in the same pathway, i.e. do not have completely separate functions.

Both the amino-terminal and carboxyl-terminal domains of ADA3 are required to complement the defect in transcription of a $\Delta ada3$ strain. We assayed two *lacZ* reporters in wild type and $\Delta ada3$ strains expressing various *ADA3* constructs. p14x2His contains *lacZ* under the control of two synthetic GCN4 binding sites upstream of a minimal *CYC1* promoter. pRBHis contains a single *lexA* binding site upstream of a *CYC1* minimal promoter and was transformed in combination with a pLexA-GCN4, which expresses a fusion consisting of residues 1-202 of *lexA* fused to residues 9-172 of GCN4, or pLexA202, which expresses residues 1-202 of *lexA* alone. As shown in Table 1, the activity measured from the p14x2His reporter is reduced six fold in a $\Delta ada3$ strain compared to wild type and the activity of *lexA*-GCN4 is reduced 18 fold. Expressing only the amino-terminal domain or only the carboxyl-terminal domain has no effect on this transcription defect, whereas expressing both domains together restores levels to that observed in the presence of full length *ADA3*. Thus, GCN4 and *lexA*-GCN4 seem to require both domains of *ADA3* for full activity.

***lexA*-*ADA3* activity is *ADA2* dependent.** When *ADA2* is fused to a *lexA* moiety, it can activate transcription from reporters containing *lexA* binding sites (29, also see Table 3 controls). This activity is reduced 3.5-fold in a $\Delta ada3$ strain. Furthermore, *lexA*-*ADA2* activity can be hyper-stimulated when *ADA3* is overexpressed. One explanation for this hyper-stimulation is that *ADA2* and *ADA3* interact in a heteromeric complex. *lexA*-*ADA2* is expressed from the strong *ADH1* promoter on a high copy plasmid. Thus, there is a large excess of *lexA*-*ADA2* compared to *ADA3*. If a complex of *lexA*-*ADA2* and *ADA3* is required for activity from a *lexA* operator, overexpressing *ADA3* should lead to more complexes and a hyper-stimulation of activity.

The dependence of lexA-ADA2 upon ADA3 for activity from lexA sites prompted us to determine whether lexA-ADA3 can also activate transcription and if so, whether this activation requires ADA2. As shown in Table 2, lexA-ADA3 activates transcription to an similar level as lexA-ADA2. This activation is dependent upon ADA2 to the same extent as lexA-ADA2 activation is dependent upon ADA3. Finally, lexA-ADA3 activity can be hyper-stimulated by overexpressing ADA2 in a manner identical to lexA-ADA2 activity being hyper-stimulated by excess ADA3. This mutual dependence that each ADA has for the other in terms of lexA activity is consistent with a model in which ADA2 and ADA3 form a heteromeric complex. Combined with the results described later that ADA3 interacts with ADA2 *in vitro*, this provides supporting evidence that an ADA3/ADA2 heteromeric complex exists *in vivo*.

The carboxyl-terminal domain of ADA3 alone can activate LexA-ADA2. We wanted to determine whether the amino-terminal domain, the carboxyl-terminal domain or both together were required for the stimulation of lexA-ADA2 activity by ADA3. As shown in Table 3, overexpressing the amino-terminal domain of ADA3 alone has no effect on lexA-ADA2 activation in both wild type and $\Delta ada3$ strains. However, overexpressing the carboxyl-terminal domain of ADA3 alone stimulates lexA-ADA2 activity in wild type and $\Delta ada3$ strains 7 fold and 4 fold respectively. In wild type cells, this stimulation is even greater than that by full length ADA3. In $\Delta ada3$ cells, stimulation by the carboxyl-terminal domain is observed, but is not as great as with full length ADA3. These data support the model that the carboxyl-terminal domain is the region of ADA3 responsible for complexing with ADA2 *in vivo*.

The carboxyl-terminal domain of ADA3 interacts with ADA2 via far western analysis. In order to demonstrate an interaction between the ADA3 carboxyl-terminal domain and ADA2 biochemically, far western experiments were performed.

In Figure 2A, purified recombinant DHFR, ADA3(452-702), and ADA3(580-702), as well as an extract from *Escherichia coli* expressing GST were run on denaturing gels and either stained with Coomassie Blue or transferred to nitrocellulose. The nitrocellulose blot was subsequently washed under renaturing conditions and probed with *in vitro* translated [³⁵S]methionine-labelled ADA2. As seen in Figure 2A, only the lane containing ADA3(452-702) shows any radiolabelling. Thus ADA2 does not interact with GST, DHFR, or the carboxyl-terminal 122 amino acids of ADA3 by this method. The 37kd band in the lane marked ADA3(452-702) is full length ADA3(452-702). The lower molecular weight bands observed are most likely degradation products from the amino terminal end (see Figure 2, legend). These results indicate that a region larger than the carboxyl-terminal 122 amino acids yet smaller than the carboxyl-terminal 252 amino acids of ADA3 is sufficient to bind to ADA2.

The converse experiment is shown in Figure 2B. In this case, purified DHFR, and *E. coli* extracts expressing GST, vector, or ADA2 were run on gels and either stained with Coomassie Blue or transferred to nitrocellulose, renatured and probed with *in vitro* translated [³⁵S]methionine-labelled ADA3(452-702). It is clear that only the lane in which recombinant ADA2 is present shows any radiolabeling. The major radiolabelled band runs at a position identical to that of recombinant ADA2. The faster migrating and fainter band corresponds to a degraded form of ADA2 (data not shown).

The carboxyl-terminal domain of ADA3 can coimmunoprecipitate ADA2 *in vitro*. In a further attempt to demonstrate the interaction between the ADA3 carboxyl-terminal domain and ADA2, coimmunoprecipitation experiments with *in vitro* translated products were performed (Figure 3). Two forms of ADA3(452-702), one tagged with an hemagglutinin epitope and one lacking the epitope, were cotranslated with either ADA2 or a negative control protein, luciferase. The

translation products were immunoprecipitated using monoclonal antibody to the hemagglutinin epitope. Lanes 1-4 show the cotranslation products and lanes 5-8 are the immunoprecipitation products. As seen in lane 7, ADA2 clearly coprecipitates with ADA3 whereas luciferase does not (lanes 3 and 6). Neither ADA2 nor luciferase are recognized nonspecifically by the hemagglutinin antibody (lanes 4 and 5) and neither ADA2 nor ADA3(452-702) are precipitated when ADA3 is untagged (lanes 1 and 8). This result in combination with the far western results argues strongly for a specific interaction between ADA2 and the carboxyl-terminal domain of ADA3.

GCN5 binds to the ADA3/ADA2 complex. As mentioned earlier, a third gene, *GCN5*, was also isolated from the adaptor screen. We wanted to determine whether GCN5 might also bind to the ADA2/ADA3 complex. Thus, the three proteins, ADA3(214-702), ADA2 and GCN5 were cotranslated *in vitro*. As described above, two forms of ADA3 were used, one tagged with the hemagglutinin epitope and one untagged (Figure 4, lanes 3 and 4). When immunoprecipitated with monoclonal antibodies directed against the HA epitope, ADA2 and GCN5 were coprecipitated with tagged ADA3, while none of the proteins were precipitated when ADA3 was untagged (lanes 1 and 2). Furthermore, antibody directed against ADA2 could immunoprecipitate both GCN5 and both forms of ADA3 (lanes 5 and 7). Preimmune serum failed to precipitate any of the three proteins (lane 6). Thus, GCN5 binds to either ADA2, ADA3 or both.

Both the ADA3 carboxyl-terminal domain and GCN5 bind to ADA2 to form a trimeric complex. Figure 4 demonstrates an interaction between the three proteins ADA2, ADA3(214-702) and GCN5. However, it does not address the question of which proteins make direct contact or whether all three comprise a single complex. To address this issue, all three proteins, ADA3(452-702)HA, ADA2 and GCN5 were cotranslated as well as each combination of two proteins. ADA3(452-702)HA was also translated alone as a negative control. As shown in Figure 5, lane 7, when all

three proteins are cotranslated, all three are precipitated with anti-HA-epitope monoclonal antibody. When ADA2 and ADA3(452-702)HA are cotranslated, ADA2 is coimmunoprecipitated with ADA3. However, when GCN5 is cotranslated with ADA3(452-702)HA in the absence of ADA2, only ADA3 is precipitated. Further, GCN5 is not precipitated with the amino-terminal domain, ADA3(1-346) (data not shown). This indicates that there is no direct interaction between ADA3 and GCN5. These findings suggest that ADA2, ADA3, and GCN5 form a trimeric complex with ADA2 serving as a linchpin. Consistent with this view, it has been shown that ADA2 and GCN5 can form a complex in the absence of ADA3 (22).

Discussion

In this paper, we demonstrate the formation of an ADA2/ADA3/GCN5 complex *in vitro* and begin a structural dissection of this complex. It is now emerging that several factors that are generally important in transcription are heteromeric complexes, such as TFIID (9), the SRBs (21), and the SWI/SNF complex (23). Also, the similarity in phenotypes of mutations in several *SPT* genes suggests that their products might exist in a complex (31).

Two domains in ADA3. Two non-overlapping segments of the *ADA3* gene, one amino-terminal and the other carboxyl-terminal, work together to complement defects in a $\Delta ada3$ strain. Expression of both domains of the protein restores wild type growth to the mutant strain, while expression of one or the other has no effect. Do these two domains have unrelated functions, or do they work together in the same pathway of transcriptional activation? Two findings suggest that they function in the same pathway. First, full activity of the GCN4 activation domain, as assayed from a GCN4 responsive reporter as well as from a *lexA* reporter in the presence of *lexA*-GCN4, requires both domains of ADA3. Expression of one domain or the other

is without effect in this assay. Second, synthetic effects are observed when shortened versions of the amino and carboxyl-terminal domains are expressed. Each of these shortened domains is functional in a strain expressing the unshortened version of the other. However, when the two shortened domains are expressed in the same strain, they do not provide function. This synthetic interaction implies that the two domains of ADA3 function in the same pathway, possibly at the same step of the pathway (15). For example, the combination of two weakened interactions might destabilize a multi-component complex, whereas a weakening of either single interaction might not.

Function of the carboxyl-terminal domain--assembly of a hetero-trimeric complex. Our *in vitro* experiments show that ADA2, ADA3, and GCN5 form a trimeric complex. This complex was first demonstrated by immuno-precipitation of co-translated ADA2, GCN5, and an HA epitope-tagged version of ADA3 containing both the amino- and carboxyl-terminal domains. Precipitation of this translation mix with either monoclonal antibody to HA or antiserum to ADA2 brings down all three proteins.

What region of ADA3 is required for formation of this complex? The carboxyl-terminal domain is clearly sufficient for assembly of the trimeric complex as demonstrated by the following assays. First, this domain binds to ADA2 when the latter has been transferred to nitrocellulose in a far-western experiment. Second, ADA2 binds to the carboxyl-terminal domain of ADA3 in the converse far-western experiment. Third, HA antibody precipitates the three proteins in a co-translation of ADA2, GCN5, and the HA-tagged carboxyl-terminal domain of ADA3. The region of ADA3 that is functional in these assays, residues 452-702, is partially active in the complementation experiments *in vivo* (Figure 1B).

Architecture of the ADA complex. What are the binary protein-protein contacts that hold ADA2, ADA3, and GCN5 together? In an important experiment,

when ADA2 is omitted from the translation mix, the HA-tagged ADA3 carboxyl-terminal domain is unable to precipitate GCN5. This finding indicates that there is no direct contact between ADA3 and GCN5, and that GCN5 is recruited to the complex by ADA2. This model proposes that ADA2 is the linchpin in the complex, binding to both ADA3 and GCN5 (Figure 6). An alternative explanation for our findings is that the conformation of ADA3 is altered when it binds to ADA2, allowing it to make direct contact with GCN5. In a separate analysis of GCN5, however, we found that it can bind directly to ADA2 (22). Therefore, we conclude that the simplest model from our data is that ADA2 is the linchpin in the trimeric complex.

Function of the amino-terminal domain of ADA3. The amino terminal domain of ADA3 is not required for formation of the ternary complex, but clearly is required for function *in vivo*. Neither slow growth nor the ability of the GCN4 activation domain to function are rescued by the ADA3 amino-terminal domain in a $\Delta ada3$ strain. What is the role of this domain in transcriptional activation? One clue is provided by the activation properties of a *lexA-ADA2* fusion. This fusion is highly dependent on ADA3 for activation at the *lexA* site. In a $\Delta ada3$ strain, activation is reduced 3.5-fold, and in a strain with ADA3 on a 2 μ plasmid, activation is increased 4-fold. Interestingly, the activity of *lexA-ADA2* is increased 7-fold in a strain with the carboxyl-terminal domain of ADA3 on a 2 μ plasmid. Further, the carboxyl-terminal domain partially restores activation by *LexA-ADA2* in a $\Delta ada3$ strain. We surmise from these findings that the requirement of the *lexA-ADA2* fusion for ADA3 can be met by the carboxyl-terminal domain of ADA3. If activation by *lexA-ADA2* reflects the normal activity of the ADA complex when tethered to an activation domain, we can surmise that the amino-terminal domain of ADA3 is not required for this activity.

Thus, there are several possible explanations for the requirement of the amino-terminal domain of ADA3 for activation by *lexA-GCN4*. In the first model, this domain is required for the interaction between the acidic activation domain of GCN4

(and other activators that use this adaptor) and the ADA complex, as depicted in Figure 6. Alternatively, the amino-terminal domain may be required to recruit one or more additional subunits to the ADA complex, which are themselves necessary for interaction with activation domains. Our genetic selection for GAL4-VP16 mutants turned up at least one additional gene which we are studying to determine if it is related to *ADA2*, *ADA3*, and *GCN5*.

Model of the ADA complex. Figure 6 shows two parallel pathways, one direct and the other *ADA*-dependent, that connect activators to basal factors. Because *lexA-GCN4* still has a low level of activity in the absence of the *ADA* genes, we envision that an *ADA*-independent pathway may be acting in concert with an *ADA*-dependent pathway for activation. However, at this point, it is unclear whether the final target of the ADA complex is the basal factors or some other target such as nucleosomes. We favor the idea that the ADAs interact with basal factors since the transcriptional defect of a strain mutated for *ADA2* has been demonstrated in *in vitro* transcription experiments where the template is added as naked DNA (3).

The finding that *ADA2*, *ADA3*, and *GCN5* comprise a single complex will allow us to relate structural domains that reside in different subunits to a common function. *ADA2* contains a Cys-rich domain that is conserved in the mammalian factor CBP (3). The latter protein has been proposed to be a coactivator because it binds to the transcription factor CREB and potentiates its activity (6). *GCN5* contains a bromo-domain, which is found in several factors in other important transcription complexes, such as TAF250, SWI2, SPT7, and others (17, 32). The bromo-domain has been shown to be important in *GCN5* function (22), and may exemplify some common function that all of these complexes share.

The *ADA* genes were isolated in yeast and are required for activator dependent transcription for a subset of activators including *GCN4* and *GAL4-VP16*. We show in this report that at least three of the ADA proteins form a heteromeric

complex. Combined with the observation that the ADA complex binds to activators (29), we believe we have identified a complex recruited by activators to help transcriptional activation.

Acknowledgements

We thank N. Austriaco, R. Knaus, D. McNabb, R. Pollock, and S. Treadway for helpful discussions and critical reading of the manuscript. This work was supported by NIH grant GM50207 and ACS NP-755. J. H. was a Howard Hughes Predoctoral Fellow.

References

1. **Alani, E., L. Cao and N. Kleckner.** 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics*. **116**:541-545.
2. **Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg and L. Guarente.** 1990. Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell*. **61**:1199-1208.
3. **Berger, S. L., B. Pina, N. Silverman, G. A. Marcus, J. Agapite, J. L. Regier, S. J. Triezenberg and L. Guarente.** 1992. Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell*. **70**:251-265.
4. **Cairns, B. R., Y.-J. Kim, M. H. Sayre, B. C. Laurent and R. D. Kornberg.** 1994. A multisubunit complex containing the *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* gene products isolated from yeast. *Proc. Natl. Acad. Sci. USA*. **91**:1950-1954.
5. **Carlson, M. and B. C. Laurent.** 1994. The SNF/SWI family of global transcriptional activators. *Current Opinion in Cell Biology*. **6**:396-402.
6. **Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy and R. H. Goodman.** 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature*. **365**:855-859.
7. **Cote, J., J. Quinn, J. L. Workman and C. L. Peterson.** 1994. Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science*. **265**:53-60.
8. **Dalton, S. and R. Treisman.** 1992. Characterization of SAP-1, a protein recruited by serum response factor to the *c-fos* serum response element. *Cell*. **68**:597-612.

9. **Dynlacht, B. D., T. Hoey and R. Tjian.** 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell*. **66**:563-576.
10. **Eisenmann, D. M., K. M. Arndt, S. L. Ricupero, J. W. Rooney and F. Winston.** 1992. SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae*. *Genes Dev.* **6**:1319-1331.
11. **Georgakopoulos, T. and G. Thireos.** 1992. Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. *EMBO J.* **11**:4145-4152.
12. **Gill, G., E. Pascal, Z. H. Tseng and R. Tjian.** 1994. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. *Proc. Natl. Acad. Sci. USA.* **91**:192-196.
13. **Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon and R. Tjian.** 1993. *Drosophila* TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell.* **75**:519-530.
14. **Guan, K. and J. E. Dixon.** 1991. Eukaryotic Proteins Expressed in *Escherichia coli*: An Improved Thrombin Cleavage and Purification Procedure of Fusion Proteins with Glutathione S-Transferase. *Analytical Biochemistry.* **192**:262-267.
15. **Guarente, L.** 1993. Synthetic enhancement in gene interaction: a genetic tool come of age. *Trends Genet.* **9**:362-366.
16. **Guthrie, C. and G. R. Fink.** 1991. Guide to yeast genetics and molecular biology. *Methods Enzymol.* **194**:835.
17. **Haynes, S. R., C. Dollard, F. Winston, S. Beck, J. Trowsdale and I. B. Dawid.** 1992. The bromodomain: A conserved sequence found in human, *Drosophila* and yeast proteins. *Nucleic Acids Res.* **20**:2603.

18. **Hinnebusch, A. G., G. Lucchini and G. R. Fink.** 1985. A synthetic *HIS4* regulatory element confers general amino acid control on the cytochrome c gene (*CYC1*) of yeast. *Proc. Natl. Acad. Sci. USA.* **82**:495-502.
19. **Hirschhorn, J. N., S. A. Brown, C. D. Clark and F. Winston.** 1992. Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev.* **6**:2288-2298.
20. **Ireton, K., D. Z. Rudner, K. J. Siranosian and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. *Genes Dev.* **7**:283-294.
21. **Koleske, A. J. and R. A. Young.** 1994. An RNA polymerase II holoenzyme responsive to activators. *Nature.* **368**:466-469.
22. **Marcus, G. A., N. Silverman, S. L. Berger, J. Horiuchi and L. Guarente.** 1994. Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. *EMBO J.* **13**:4807-4815.
23. **Peterson, C. L., A. Dingwall and M. P. Scott.** 1994. Five SWI/SNF gene products are components of a large multisubunit complex required for transcriptional enhancement. *Proc. Natl. Acad. Sci. USA.* **91**:2905-2908.
24. **Pina, B., S. Berger, G. A. Marcus, N. Silverman, J. Agapite and L. Guarente.** 1993. ADA3: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. *Mol. Cell. Biol.* **13**:5981-5989.
25. **Poon, D. and P. A. Weil.** 1993. Immunopurification of yeast TATA-binding protein and associated factors. Presence of transcription factor III B transcriptional activity. *J. Biol. Chem.* **268**:15325-15328.
26. **Rose, M. and D. Botstein.** 1983. Structure and function of the yeast URA3 gene. Differentially regulated expression of hybrid beta-galactosidase from overlapping coding sequences in yeast. *J. Mol. Biol.* **170**:883-904.

27. **Sherman, F., G. R. Fink and J. B. Hicks.** 1986. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Sikorski, R. S. and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. **122**:19-27.
29. **Silverman, N., J. Agapite and L. Guarente.** 1994. Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. *Proc. Natl. Acad. Sci. USA*. **91**:11665-11668.
30. **Studier, F. W., A. H. Rosenberg, J. J. Dunn and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol*. **185**:60-89.
31. **Swanson, M. S. and F. Winston.** 1992. SPT4, SPT5 and SPT6 interactions: effects on transcription and viability in *Saccharomyces cerevisiae*. *Genetics*. **132**:325-336.
32. **Tamkun, J. W., R. Deuring, M. P. Scott, M. Kissinger, A. M. Pattatucci, T. C. Kaufman and J. A. Kennison.** 1992. Brahma: a regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell*. **68**:561-572.
33. **Thompson, C. M., A. J. Koleske, D. M. Chao and R. A. Young.** 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell*. **73**:1361-1375.
34. **Verrijzer, C. P., K. Yokomori, J. L. Chen and R. Tjian.** 1994. *Drosophila* TAFII150: similarity to yeast gene TSM-1 and specific binding to core promoter DNA. *Science*. **264**:933-941.

TABLE 1. Effects of expressing ADA3 amino- and carboxyl-terminal domains on GCN4 and LexA-GCN4 activation

Plasmid#1 ^d	Plasmid#2 ^e	p14x2His ^a reporter		pRbHis ^b reporter with pLexA-GCN4 ^c	
		wild type	Δ ada3	wild type	Δ ada3
vector	vector	74 ^f	12	5300	290
ADA3 (1-346)	vector	86	10	5000	330
vector	ADA3 (364-702)	84	16	3800	270
ADA3 (1-346)	ADA3 (364-702)	107	80	3300	3100
vector	ADA3	nd	82	4200	2100

^a p14x2His - lacZ expression is regulated by two 14mer GCN4 binding sites upstream of a minimal CYC1 promoter.

^b pRbHis - lacZ expression is regulated by a single lexA operator site upstream of a minimal CYC1 promoter.

^c pLexA-GCN4 expresses a fusion consisting of residues 1-202 of lexA fused to residues 9-172 of GCN4. A control plasmid expressing only residues 1-202 of lexA gives 5-15 units of activity in wild type and Δ ada3 strains.

^d For p14x2His assays vector was pRS316. ADA3(1-346) refers to an ADH1 promoter terminator cassette expressing ADA3(1-346) cloned into pRS316. For pRbHis assays, vector was pDB20L. ADA3(1-346) refers to ADA3(1-346) cloned behind the ADH1 promoter of pDB20L.

^e For p14x2His assays, vector was pDB20L. ADA3 and ADA3(364-702) were cloned behind the ADH1 promoter of pDB20L. For pRbHis assays, vector was pRS314. ADH1 promoter terminator cassettes expressing ADA3 and ADA3(364-702) were cloned into pRS314.

^f Measurements presented in this table were carried out in quadruplicate with standard errors, in most cases, less than 20% of the mean. The value labelled nd was not determined.

TABLE 2. Effects of deleting or overexpressing ADA2 on LexA-ADA3 activity.

Strain	lexA-ADA3 ^a activity ^b	
	vector ^c	ADA2
wt	140	440
Δ ada2	32	nd ^d

^a A control plasmid, plex202 (21) expressing only residues 1-202 of lexA gives 5-15 units of activity in wild type, and Δ ada2 cells.

^b β galactosidase measurements represent the average of assays performed on four independent transformants. Standard errors were less than 20% of the mean.

^c Vector refers to pDB20L. ADA2 refers to a construct where ADA2 was cloned behind the ADH1 promoter of DB20L.

^d Not determined.

TABLE 3. Effects of overexpressing ADA3 amino-terminal or carboxyl-terminal fragments on LexA-ADA2 activity.

strain	lexA-ADA2 ^a activity ^b			
	vector ^c	ADA3	ADA3 (1-346)	ADA3 (364-702)
wt	110	370	100	700
Δ ada3	31	380	24	130

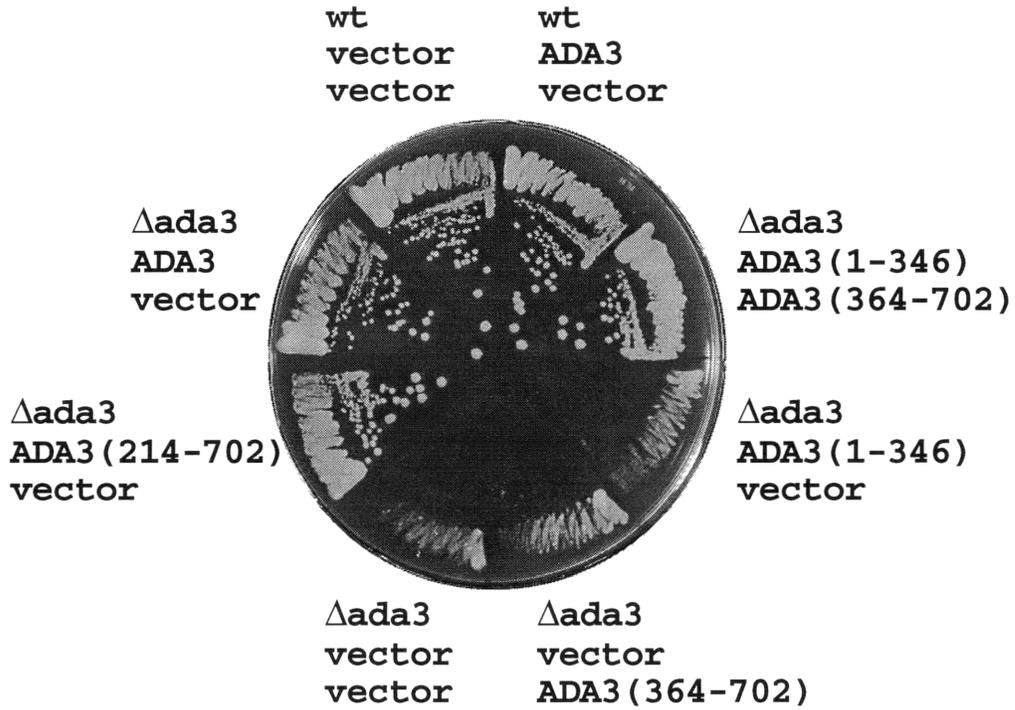
^a A control plasmid, plex202 (21) expressing only residues 1-202 of lexA gives 5-15 units of activity in wild type, and Δ ada3 cells.

^b β galactosidase measurements were performed as described in Table 2. Standard errors were less than 20% of the mean.

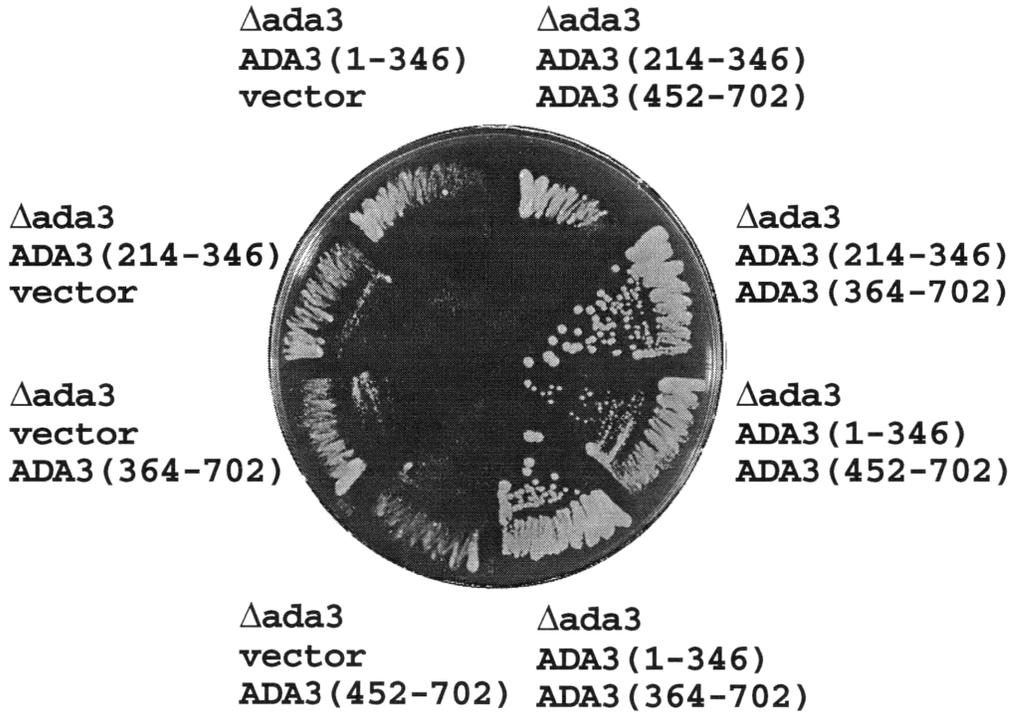
^c The vector used was pDB20L. ADA3, ADA3(1-346) and ADA3(364-702) refer to constructs where the appropriate gene or gene fragment was cloned behind the ADH1 promoter of pDB20L.

Figure 1. Complementation of the slow growth phenotype on minimal media of a $\Delta ada3$ strain by various ADA3 fragments. BWG1-7a (wild type) or 1-7a $\Delta ada3$ were transformed with combinations of plasmids and restreaked onto plates containing no amino acid or nucleoside supplements except histidine and uridine. Each restreak is labelled first with the strain, followed by the fragment of ADA3 expressed from pJH1 followed by the fragment of ADA3 expressed from DB20L. Vector refers to either pJH1 or DB20L with no insert. ADA3 with no parentheses refers to the full length protein. (A) shows complementation tests of a $\Delta ada3$ strain expressing the 1-346 domain of ADA3, the 364-702 domain, and both domains together. Wild type cells and $\Delta ada3$ cells transformed with vectors, ADA3, and ADA3(214-702) are shown in comparison. (B) shows complementation tests of a $\Delta ada3$ strain expressing the following domains of ADA3: the amino-terminal domains, 1-346 and 214-346, the carboxyl-terminal domains, 364-702 and 452-702, and each combination of an amino- and a carboxyl-terminal domain. (C) shows data from (A) and (B) in schematic form. "++++" corresponds to wild type growth while "+" corresponds to growth of a $\Delta ada3$ strain with vector alone. Full length ADA3 extends from residues 1 to 702.

A.



B.



C.

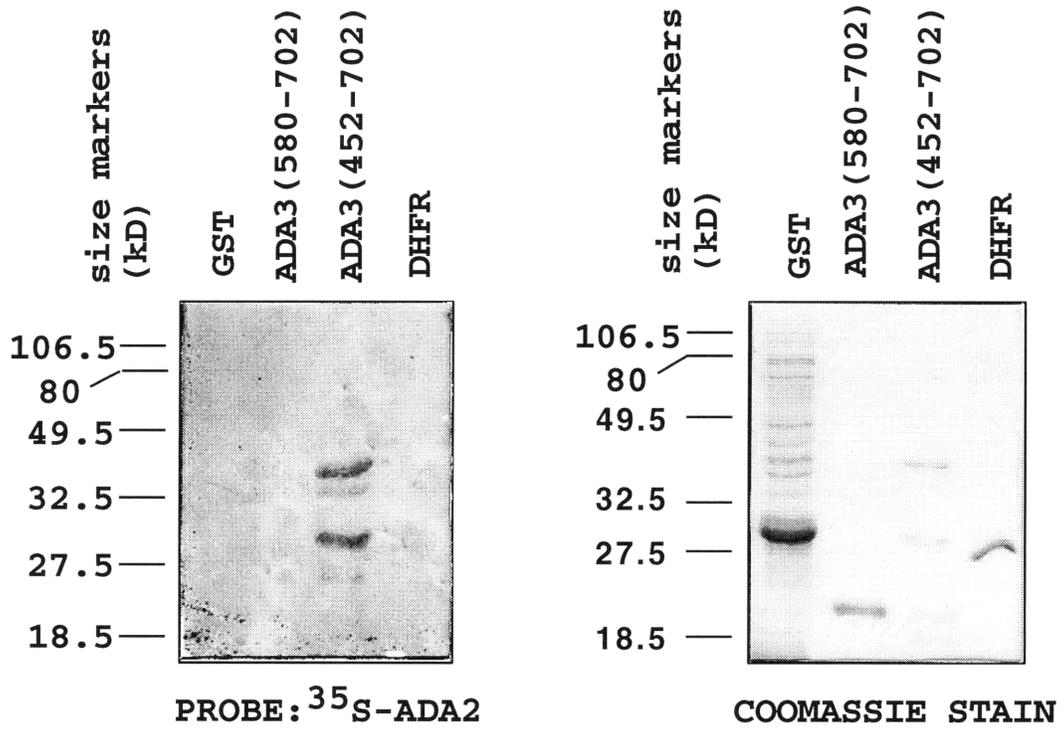
Fragment of ADA3
expressed in a $\Delta ada3$ strain

Growth on
minimal media

vector alone	Growth on minimal media
1 702 [1-702]	++++
214 702 [214-702]	++++
364 702 [364-702]	+
1 346 [1-346]	+
214 346 [214-346]	+
452 702 [452-702]	+
1 346 364 702 [1-346] [364-702]	++++
214 346 364 702 [214-346] [364-702]	++++
1 346 452 702 [1-346] [452-702]	+++
214 346 452 702 [214-346] [452-702]	+

Figure 2. Far western experiments of ADA2 and ADA3. (A) An extract from *E. coli* expressing GST, as well as purified recombinant ADA3(580-702), ADA3(452-702) and DHFR were subjected to SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose, denatured, stepwise renatured and probed with [³⁵S]-methionine labelled ADA2. The 37kDa band in the lane labelled ADA3(452-702) represents full length ADA3(452-702). Smaller bands result from amino-terminal degradation, since the fragment is expressed with a carboxyl-terminal 6-histidine fusion and purified from a nickel column. For the gel transferred to nitrocellulose and probed with radiolabelled ADA2, extract from 5x10⁷ cells expressing GST, and 5μg each of ADA3(580-702), ADA3(452-702) and DHFR were loaded. For the Coomassie Blue stained gel, extract from 10⁷ cells expressing GST, 1μg of ADA3(580-702), 400ng of ADA3(452-702) and 0.5μg of DHFR were loaded. (B) *E. coli* extracts expressing ADA2, vector (pUH24.2ΔCAT), GST, and purified recombinant DHFR were subjected to SDS-PAGE. As above, the gels were either stained or transferred to nitrocellulose. The nitrocellulose blot was probed with ³⁵S labelled ADA3(452-702). The 50 kDa band represents ADA2 and the smaller band is a degradation product of ADA2 (data not shown). Extracts from 2x10⁸ cells expressing ADA2, vector, or GST and 20 μg of purified DHFR were loaded on both gels.

A.



B.

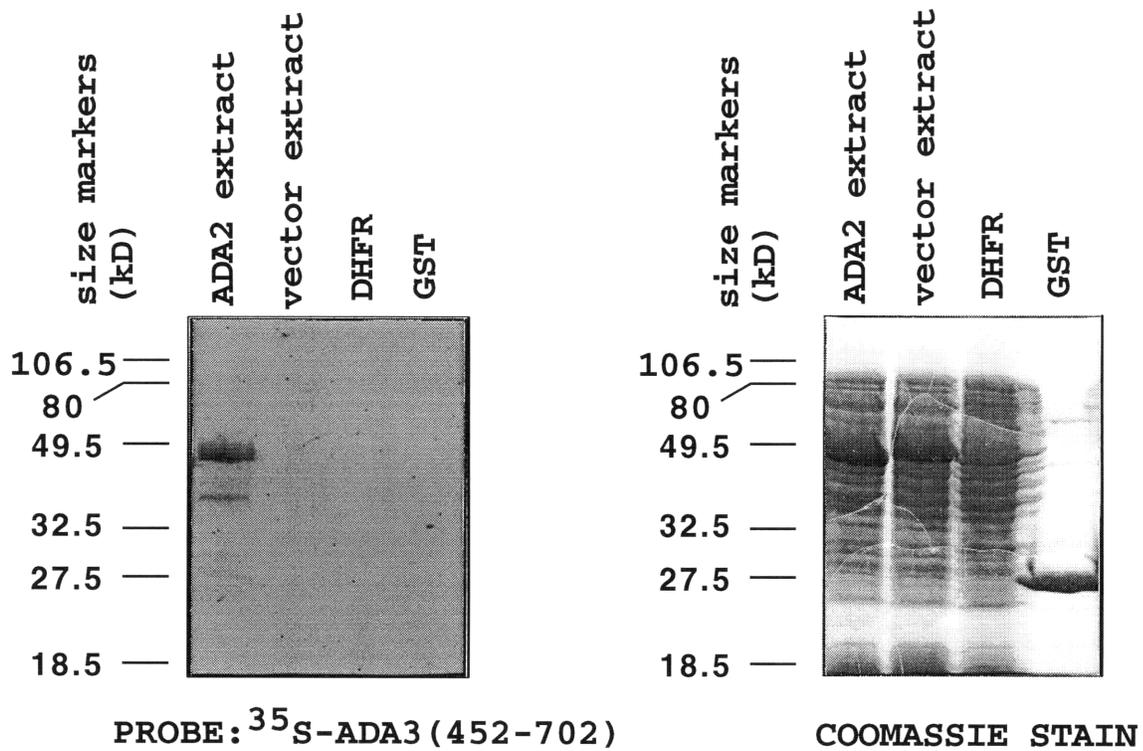


Figure 3. Coimmunoprecipitation experiments of ADA2 with the C-terminal domain of ADA3. Combinations of HA epitope-tagged or untagged ADA3(452-702), ADA2 and luciferase were cotranslated in an *in vitro* reticulocyte lysate system. Lanes 1-4 show SDS-PAGE analysis of the cotranslated products. The cotranslated products were immunoprecipitated using antibody directed against the HA epitope and analyzed by SDS-PAGE (lanes 5-8). The lower molecular weight bands in lanes 6 and 7 seem to be a degradation product of ADA3(452-702)HA.

	Starting				IP (HA ab)			
Luciferase			+	+	+	+		
ADA2	+	+		+	+		+	+
ADA3 (452-702) HA		+	+			+	+	
ADA3 (452-702)	+							+
Lane	1	2	3	4	5	6	7	8

Luciferase →
 ADA2 →
 ADA3-451CHA →
 ADA3-451 →

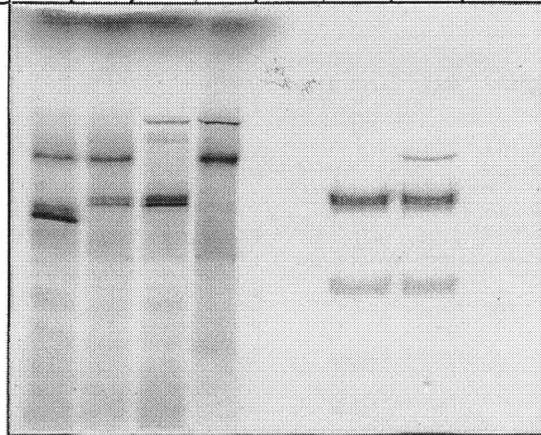


Figure 4. Coimmunoprecipitation experiments of ADA2, ADA3 and GCN5. ADA2, GCN5, and either HA epitope-tagged or untagged ADA3(214-702) were cotranslated (lanes 3 and 4). Lanes 1 and 2 show anti-HA antibody precipitated products, lanes 5 and 7 show complexes immunoprecipitated with anti-ADA2 antibody, and lane 6 shows products immunoprecipitated by preimmune serum.

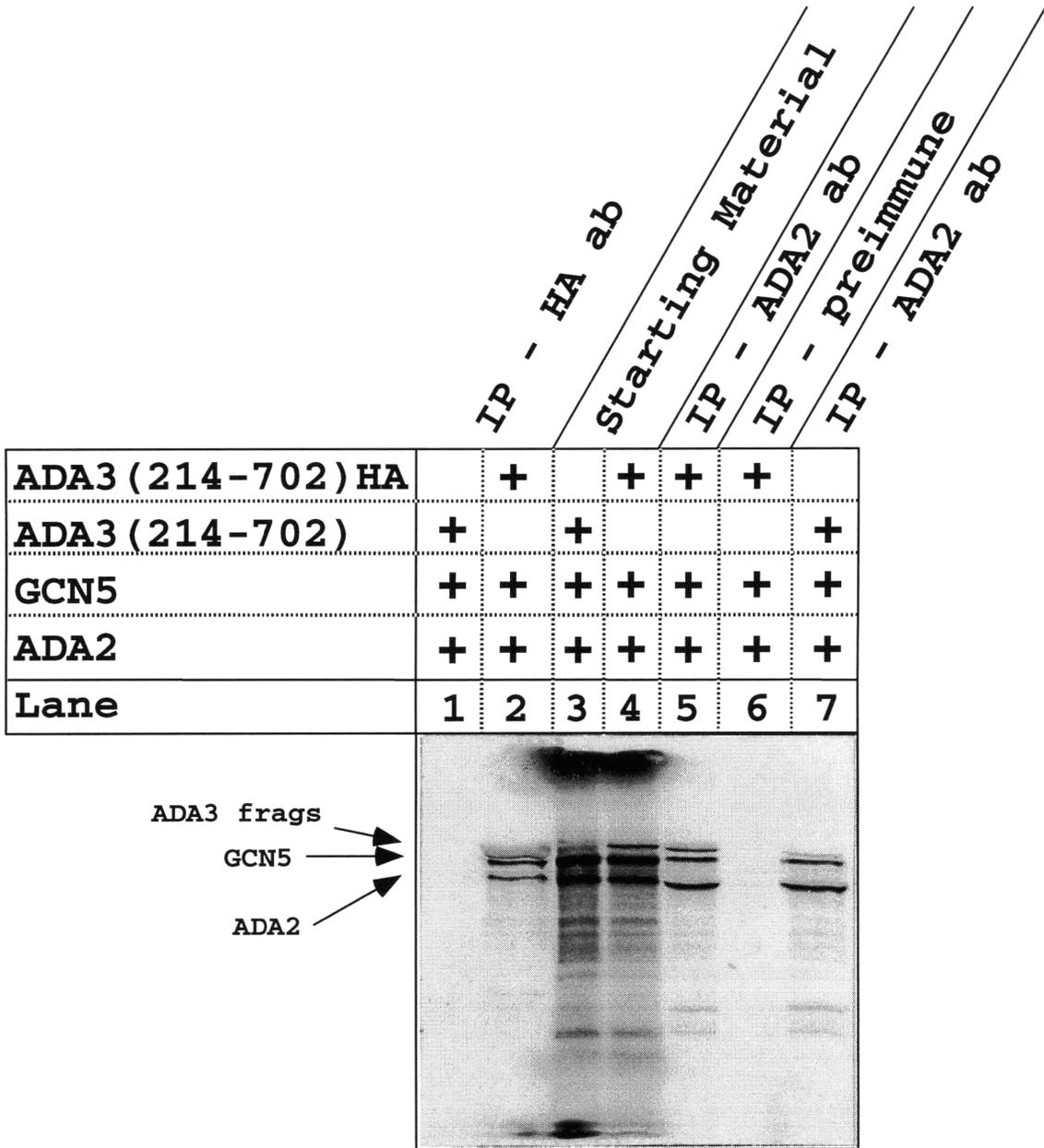


Figure 5. ADA3, GCN5 coimmunoprecipitation experiments. Combinations of two of the three proteins in the ADA complex were cotranslated along with controls. Lanes 1-5 show cotranslation products. Lanes 6-10 show products immunoprecipitated with anti-HA antibody.

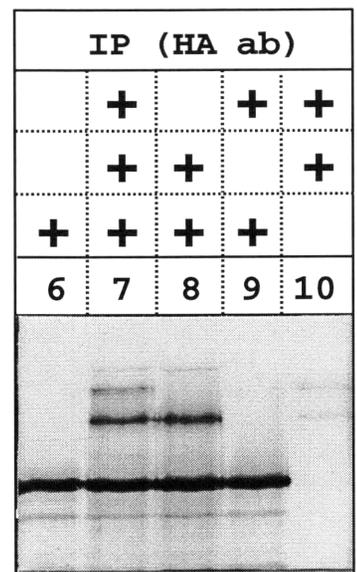
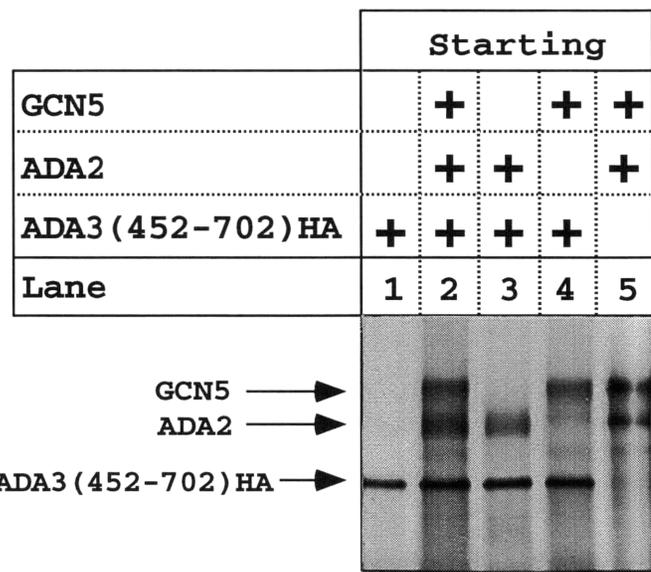
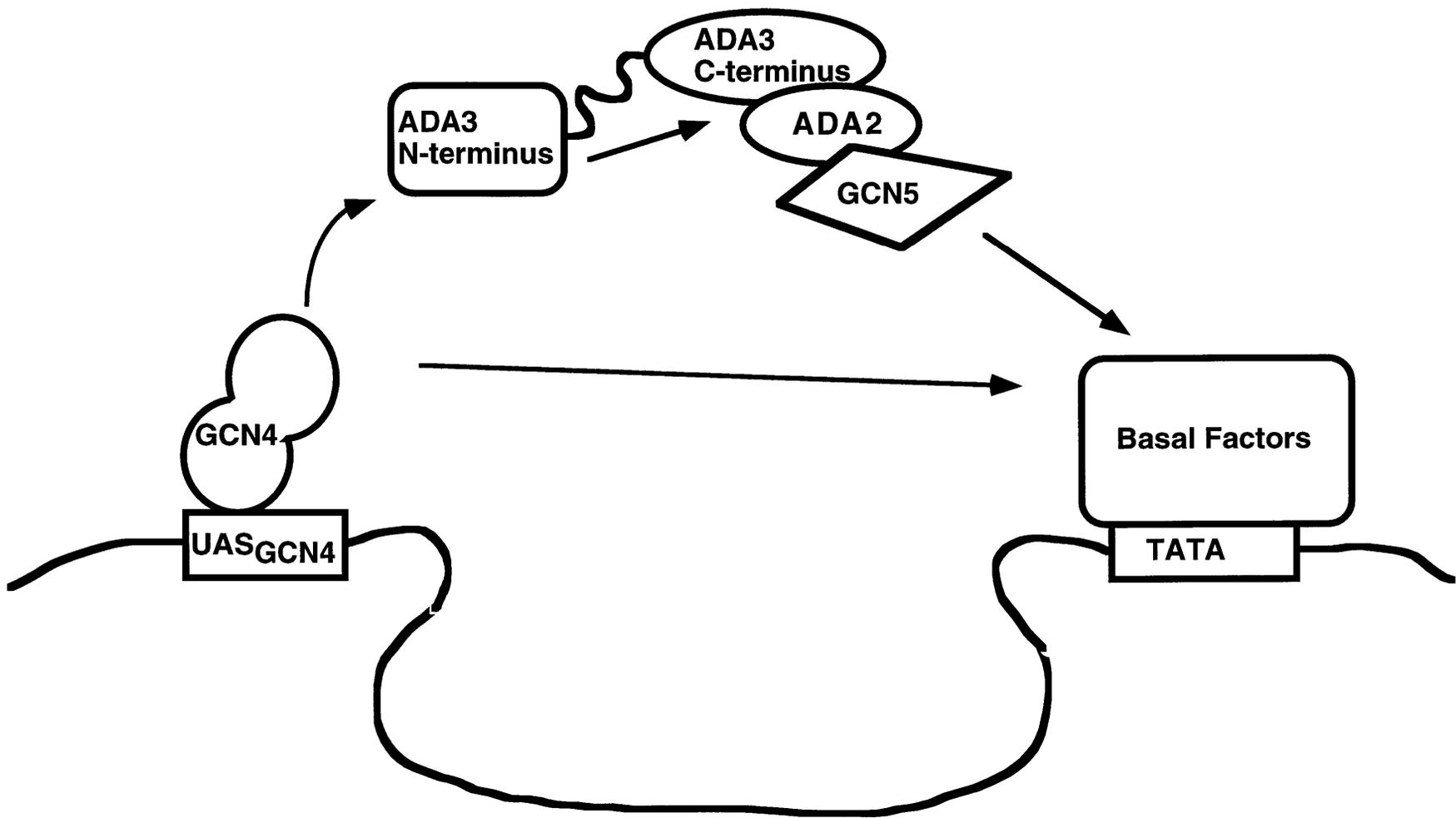


Figure 6. Model for the role of the ADA genes in transcriptional activation. The dark black line represents DNA. GCN4 is depicted binding to its UAS and basal transcription factors are shown binding to the TATA region of the promoter. We envision two parallel activation pathways between activators and basal factors that may act in synergy to activate transcription, an ADA-dependent pathway and an ADA-independent, perhaps direct, pathway. The ADA3 carboxyl-terminal domain/ADA2/ GCN5 complex is required for the ADA-dependent pathway. ADA2 serves as a linchpin type molecule binding to both ADA3 and GCN5 at the same time. The amino-terminal domain is shown between GCN4 and the carboxyl-terminal complex since it is required for GCN4 dependent transcription but is not for LexA-ADA2 dependent transcription.



Chapter III

ADA1, a Novel Component of the ADA/GCN5 Complex has Broader Effects than GCN5, ADA2, or ADA3

This chapter will be published in *Molecular and Cellular Biology* in June 1997.
The authors are Junjiro Horiuchi, Neal Silverman, Benjamin Piña,
Gregory A. Marcus and Leonard Guarente.

Summary

The *ADA* genes encode factors which are proposed to function as transcriptional coactivators. Here we describe the cloning, sequencing, and initial characterization of a novel *ADA* gene, *ADA1*. Similar to the previously isolated *ada* mutants, *ada1* mutants display decreases in transcription from various reporters. Furthermore, *ADA1* interacts with the other ADAs in the *ADA/GCN5* complex as demonstrated by partial purification of the complex and immunoprecipitation experiments. We estimate that the complex has a molecular weight of approximately 2 MDa. Previously it had been demonstrated that *ada5* mutants displayed more severe phenotypic defects than the other *ada* mutants (G. A. Marcus, J. Horiuchi, N. Silverman, and L. Guarente, *Mol. Cell. Biol.* 16:3197-3205, 1996; S. M. Roberts and F. Winston, *Mol. Cell. Biol.* 16:3206-3213, 1996). *ada1* mutants display defects similar to *ada5* mutants and different from the other mutants with respect to promoters affected, inositol auxotrophy, and *Spt⁻* phenotypes. Thus the ADAs can be separated into two classes, suggesting that the *ADA/GCN5* complex may have two separate functions. We present a speculative model on possible roles of the *ADA/GCN5* complex.

Introduction

RNA polymerase II (Pol II) dependent transcription in eukaryotes requires general transcription factors and gene specific transcriptional activators. The general factors include Pol II itself, the TATA binding protein (TBP), TFIIB, and other factors required for preinitiation complex formation at promoters.

Activators bind to upstream activating sequences (UAS) or enhancers, which can be hundreds of base pairs upstream from the site of initiation of transcription, and stimulate transcription through their activation domains. In addition to these two types of factors, a third type has been isolated which are referred to as mediators, coactivators or adaptors (3, 32, 44). These have been proposed to function by several mechanisms, including bridging interactions between activator proteins and basal factors, and by counteracting the repressive effects on transcription by nucleosomes. Many factors believed to belong to this coactivator group form large heteromeric complexes. For example, the SWI/SNF complex is approximately 2 MDa in size (11, 40, 60) and functions by countering chromatin mediated repression (14, 27). The SRB complex is required for robust activated and basal transcription and interacts with basal factors to form a RNA Pol II holoenzyme complex (25, 35, 58). The TFIID complex consists of the basal transcription factor TBP and associated TAF proteins which are thought to be required for activated transcription (15, 51, 59).

Two other groups of factors which seem to function as adaptors or coactivators, the SPTs and the ADAs, were identified genetically in yeast. The *SPT* genes were isolated as suppressors of the auxotrophies resulting from transposon insertions at the promoters of various biosynthetic genes. The *SPT* genes fall into two classes based upon the insertions that they suppress, and upon various other genetic and molecular characteristics (61). One class is the histone

class which includes *SPT4*, *SPT5*, *SPT6*, *SPT11* and *SPT12*. *SPT11* and *SPT12* encode histones H2A and H2B respectively. *SPT4*, *SPT5*, and *SPT6* also encode negative regulators of transcription that form a complex and are thought to be functionally related to H2A and H2B (57). The second class, the TBP class, includes *SPT3*, *SPT7*, *SPT8*, *SPT15*, and *SPT20*. *SPT15* encodes TBP, while the others encode products which are thought to interact with TBP and play a role in transcriptional initiation site selection (16, 17, 61).

The *ADA* genes were isolated as suppressors of GAL4-VP16 toxicity (4). When GAL4-VP16 is overexpressed, it is thought to kill yeast cells by sequestering the general transcription factors away from productive transcription complexes. Therefore, mutations that suppress toxicity may be in genes encoding factors that mediate the interaction between the general factors and activation domains. The *ada* mutants fell into five complementation groups, four of which have been characterized, *ADA2*, *ADA3*, *GCN5*, and *ADA5* (4, 37, 38, 43). *GCN5* had been previously isolated as a gene whose product was a factor required for full transcriptional activation by *GCN4* (21). *ADA3* has been subsequently isolated as *NGG1*, a gene whose product is required for glucose repression (5, 6).

ADA2, *ADA3*, and *GCN5* are all required for full transcriptional activation by a subset of activators, including GAL4-VP16 and *GCN4* (4, 38, 43). This is consistent with a model in which these ADAs function as adaptors for a subset of activators. Also, mutations in the three genes yield similar phenotypes, such as temperature sensitivity and slow growth. Mutants carrying null alleles of any two of the three genes display phenotypes identical to single mutants (20, 38). It has been demonstrated that *ADA2*, *ADA3*, and *GCN5* interact to form a complex (12, 29, 38).

ADA5 is similar to the other ADAs except that mutations result in some broader transcriptional defects (37, 45). For example, the *ADH1* promoter is not

affected by mutations in *ADA2*, *ADA3* or *GCN5* but is reduced in *ada5* mutants. Furthermore, *ADA5* has been shown to be identical to *SPT20* linking the *ADA* genes and the *SPT* genes (45). The other *ADAs* have not been isolated as *spt* mutants and do not display *Spt*⁻ phenotypes. Despite these differences, *Δada5Δada3* mutants do not demonstrate any phenotypes worse than a *Δada5* mutant. Furthermore, there is evidence that *ADA5* interacts with *ADA3* (37), suggesting that it too is part of an *ADA/GCN5* complex.

Recently, a functional role for the *ADA/GCN5* complex has been suggested. A histone acetyltransferase activity has been purified from *Tetrahymena* macronuclei (7). The gene responsible for this activity was cloned and determined to be a homologue of *GCN5* (10). Yeast *GCN5* has also been shown to have histone acetyltransferase activity. Combined with data that *ADA2* and *ADA5* interact with activators (2, 37, 39, 55), this suggests that the role of the *ADA/GCN5* complex may be to acetylate nucleosomes at transcriptionally active sites (8).

In this study, we describe the cloning and characterization of a novel *ADA* gene, *ADA1*. We present evidence that *ADA1* is part of a large *ADA/GCN5* complex and that *ada1* mutants display phenotypes similar to *ada5* and broader than *ada2*, *ada3*, and *gcn5* mutants. Thus, although the *ADA* proteins bind to form a complex, they can be separated into two classes suggesting that the *ADA/GCN5* complex may have a second function in addition to histone acetylation.

Materials and Methods

Strains and Media. Strains used in this study are shown in Table 1. BWG1-7a, 1-7a Δ *ada1* and 1-7a Δ *ada2* were used for purification and immunoprecipitation

experiments. BWG1-7a, 1-7a Δ ada1, PSY316 and 316 Δ ada1 were used for β -galactosidase assays. Inositol studies were performed in PSY316 and PSY316 disrupted for the appropriate *ADA* gene. Strains FY56, FY710, and FY630, used to determine Spt phenotypes, were gifts from S. Roberts and F. Winston. Yeast disruptions of *ADA1* were generated by transforming the appropriate yeast strains with pADA1KO (described below) that had been linearized with *Xho*I. *ADA3* was deleted using pADA3KO (38). Disruptions were verified by Southern analysis.

Rich, synthetic complete, and synthetic minimal media were prepared as previously described (53).

Cloning and Sequencing of ADA1. Three original clones complementing the slow growth, morphological defect, and GAL4-VP16 resistance phenotypes of the *ada1-1* mutant (4) were originally isolated. One clone, YCP50ADA1, had an 11 kb insert. Two others had identical 16 kb inserts. Physical mapping using restriction enzymes revealed that these clones overlapped over a region of 5.7 kb. This region had a convenient *Hind*III site. Using a second *Hind*III site in the vector (YCP50) and a third in a nonoverlapping region in YCP50ADA1, the 5.7 kb region was split into two fragments which were separately subcloned into YCP50. Neither subclone complemented the slow growth phenotype of the *ada1-1* mutant suggesting that the relevant gene had been cut in half. The *Hind*III fragments were subcloned into pBluescript (Stratagene) and sequenced from the internal *Hind*III site. Sequencing was performed by the dideoxy chain termination method (49). Further sequence was obtained by either subcloning convenient restriction fragments into pBluescript, or by using custom synthetic oligonucleotide primers. This revealed an open reading frame of 488 codons (later verified as *ADA1*) with an internal *Hind*III site.

Plasmid Constructions. PCR primers were designed such that the coding region of *ADA1* could be amplified with *Bam*HI and *Afl*III sites on the 5' end and

a 6-histidine tag followed by a stop codon and a *Bam*HI site on the 3' end of the gene. The PCR product was digested with *Bam*HI and ligated into the *Bam*HI site of pBluescript to generate BluescriptADA1. The BluescriptADA1 *Bam*HI fragment was cloned into the *Bgl*II site of DB20LeuBgl (4) to generate DB20LADA1.

To verify that the cloned *ADA1* gene actually corresponded to the gene that was mutated in *ada1-1*, it was necessary to mark the genomic locus of the cloned gene. The *Bam*HI fragment from DB20LADA1 which contains the cloned gene under control of the *ADH1* promoter was inserted into the *Bam*HI site in pRS306 (54), a *URA3* marked integrating plasmid. This construct was digested with *Bcl*I to direct integration to the cloned locus and used as described in Results.

The disruption construct of *ADA1*, pADA1KO, was generated in several steps. The vector, pUC19Xho-Hind, was constructed by inserting a *Xho*I linker into the *Sma*I site of pUC19 and destroying the *Hind*III site by cutting, filling-in with Klenow, and ligating. A 5.4 kb *Xho*I fragment containing the coding and flanking sequences of *ADA1* was digested from YCP50ADA1 and ligated into the *Xho*I site of pUC19Xho-Hind. This was subsequently digested with *Hind*III and *Eco*RV, filled-in with Klenow and ligated to a *Bgl*II linker. The *hisG-URA3* cassette from pNKY51 (1) was ligated into the *Bgl*II site. This construct disrupts *ADA1* between amino acids 113 and 408.

For recombinant expression of *ADA1*, the bacterial expression vector pUHE24.2ΔCAT (38) was used. The *ADA1* coding region fused to a 6-histidine tag was digested from BluescriptADA1 using *Afl*III and *Bam*HI. This fragment was cloned into the *Nco*I and *Bam*HI sites of pUHE24.2ΔCAT to generate pUHE24.2ADA1.

For recombinant expression of *GCN5*, the *GCN5* coding sequence was amplified by PCR using primers GCN5N and GCN5C (38). The product was

digested with *EcoRI* and *NotI* and cloned into the *EcoRI* and *NotI* sites of pET28a (Novagen). The resulting plasmid, pET28a-EcoGCN5, could be used to express a carboxyl terminal fragment of GCN5 with a 6-histidine tag at its amino terminal end.

To make the plasmid pPADA26HIS, pDB200CADA26HIS, an ARS/*cen* derivative of pADA26HIS (55), was digested with *SpeI*, filled-in with T4 DNA polymerase, and then digested with *AflIII*. The fragment containing the C-terminal one-third of *ADA2* fused to a 6-histidine tag followed by the *ADH1* terminator was purified and cloned into pNS3.8 (4) that had been digested with *AflIII* and *MscI*. This created a plasmid which contains the *ADA2* promoter expressing the *ADA2*-6-histidine fusion gene followed by the *ADH1* terminator.

Yeast manipulations. Transformations were performed by the lithium acetate method (53). For β -galactosidase assays, cells were grown to an optical density at 600 nm of approximately 1.0 and activity was measured in glass bead extracts as previously described (46). Cells were grown in synthetic complete medium lacking uracil. For HIS(2)^{14x2} and HIS(1)⁶⁶ activity, cells were induced in minimal media complemented only for the auxotrophies of the yeast for eight hours. β -galactosidase activity was measured as nanomoles per minute per milligram of protein. Reporters used were pLG Δ AluXho (22) for CYC1 UAS1 which binds the activator HAP1, pLG265UP1 (18) for CYC1 UAS2 which binds the HAP2/3/4/5 heterotetrameric activator, HIS(1)⁶⁶ (26) and HIS(2)^{14x2} (26) which bind GCN4, pCP0 (50) which contains the *ADH1* promoter, and pdAT2 (36) which contains the thymidine rich UAS of the *DED1* gene. Other general yeast techniques were performed according to standard protocols (23).

Generation of Antisera. pUHE24.2ADA1 was transformed into AG115 (30) cells. Cells were grown to an optical density of 0.5, induced with 2 mM isopropyl- β -D-thiogalactopyranoside and grown for another two hours. Cells were lysed by

sonication and the insoluble material, which contained recombinant ADA1 fused to a 6-histidine tag, was resuspended in 8 M urea. Recombinant ADA1 was purified on a Ni-NTA agarose column (Qiagen) as recommended by the manufacturer.

To obtain polyclonal antisera to ADA1, 1 mg of recombinant ADA1 was dialyzed into 2 ml of phosphate buffered saline solution, mixed with RIBI adjuvant (RIBI ImmunoChem Research, Inc.) and injected into two rabbits according to standard protocols (24). After several boosts, antibodies to ADA1 were purified from crude serum by binding to immunoblots of recombinant ADA1 and eluting at pH 2.5 or by binding and eluting from recombinant ADA1 crosslinked to cyanogen bromide activated sepharose beads.

In order to generate antibodies to GCN5, a carboxyl terminal fragment of GCN5, tagged with 6-histidines, was purified similar to ADA1 above, except that the plasmid pET28a-EcoGCN5 and the strain BL21:DE3 (Novagen) was used. The eluate from the Ni-NTA agarose column was subjected to SDS-PAGE and acrylamide slices containing the GCN5 protein fragment were lyophilized and resuspended into phosphate buffered saline. Antibodies to GCN5 was generated as described for ADA1 except that 400 μ g of protein were injected into each rabbit. Antibodies to ADA2 (38), ADA3 (37), ADA5 (37), TBP and TFIIB (33) have been described previously.

Extracts and fractionation. The purification of the ADA/GCN5 complex was performed in 1-7a Δ ada2 (4) carrying pPADA26HIS. Cells were grown in minimal media to yield an approximate wet weight of 500 g. Preparation and fractionation of whole cell extract on a Bio-Rex 70 column (5x10cm) was performed as described by others (52) except elutions were performed with 250, 600 and 1200 mM potassium acetate. Elutions were analyzed for the presence of ADA2 and ADA3 proteins by Western blot. The relevant fractions from the

600mM potassium acetate elutions were pooled and dialyzed against a buffer containing 20 mM Hepes pH 7.6, 10% glycerol, 300 mM potassium acetate, 10 mM β -mercaptoethanol, and protease inhibitors. This material was then bound to an Ni-NTA agarose column (1.5x10cm) and eluted with an imidazole gradient. Fractions enriched in ADA2 and ADA3 were pooled and diluted into Buffer H (20 mM Hepes pH 7.6, 10% glycerol, 1 mM DTT, 1 mM EDTA, and protease inhibitors) to a final salt concentration of 100mM potassium acetate. This material was then fractionated over a Poros HQ-20 column (4.6x100mm) (PerSeptive Biosystems) with an elution gradient from 100mM to 1500mM potassium acetate of 10 column volumes using the BioCAD Sprint apparatus (PerSeptive Biosystems). Fractions that contained the peak of ADA proteins were pooled and loaded onto a Superose 6 HR 10/30 gel filtration column (Pharmacia Biotech). The column was then washed with buffer H containing 300 mM potassium acetate and fractions were collected. As a control for determining the approximate molecular weight of the ADA/GCN5 complex, protein size standards (Biorad) were fractionated on the Superose 6 column under identical conditions.

For quantifying purification, a densitometer (LKB) was used to measure the relative band intensities on immunoblots of ADA2, ADA3, and ADA1.

Immunoprecipitations. Preparation and fractionation of extracts of BWG1-7a and 1-7a Δ *ada1* were performed as described (37). Crosslinking of antisera to protein A-Sepharose beads and immunoprecipitation experiments were also performed according to published methods (11) except that 1 ml of the 600 mM eluate from the Bio-Rex 70 column was used for the precipitations and bound material was eluted off of the antisera coated beads using 20 μ l of 50 mM glycine at pH 2.5, neutralized with 3 μ l of 1 M Tris pH 7.5 and mixed with 4x SDS/PAGE loading buffer.

Nucleotide sequence accession number. The sequence of *ADA1* has been assigned GenBank accession number U76735. The sequence of *HF11*, a gene identical to *ADA1*, is U41324 (19).

RESULTS

Cloning and sequencing of *ADA1*. *ada* mutants were isolated from a screen for GAL4-VP16 resistant mutants as previously described (4), with the exception that the first *ada1* allele, *ada1-1*, was isolated from non-mutagenized cells. The *ada1* mutants isolated have severe slow growth defects and morphological changes (28). These phenotypes are recessive. We therefore used the slow growth phenotype of *ada1-1* to isolate complementing clones from a yeast genomic library on an ARS-CEN containing vector (4, 42). Three overlapping clones were isolated which not only restored wild type growth but also restored wild type morphology and toxicity to overexpressed GAL4-VP16. By analyzing the complementation of subclones, and sequencing, a putative open reading frame of 488 codons was identified (see Materials and Methods). The putative open reading frame was amplified by PCR and cloned into an episome under control of the *ADH1* promoter (see Materials and Methods). This construct also complemented the slow growth phenotype, restored wild type morphology and restored sensitivity to GAL4-VP16 overexpression. To verify that the cloned gene corresponded to *ADA1*, the *URA3* marker was integrated at the cloned locus in a wild type strain (see Materials and Methods) and mated to *ada1-1*. The resultant diploid was sporulated and in 6 out of 6 four spore tetrads tested, resistance to GAL4-VP16 toxicity segregated 2:2 in opposition to the Ura⁺ phenotype. It was determined through data base searches that a gene identical to *ADA1*, termed

HFI1, had been cloned (19). There are no other genes with any significant homologies to *ADA1*.

ADA1 was deleted in yeast cells as described in the Materials and Methods. The deletion behaved similarly to the mutants with respect to slow growth and resistance to GAL4-VP16 toxicity. Both phenotypes were fully complemented by the cloned gene. The morphological changes mentioned above for *ada1* mutants were determined to be strain specific. Disrupting *ADA1* in BWG1-7a resulted in extremely elongated cells similar to the morphology of the original *ada1-1* mutant, which was in a BWG1-7a background. Disrupting *ADA1* in PSY316 resulted in a slight change in cell morphology which was much less apparent than that of BWG1-7a.

***ada1* mutants display transcriptional defects.**

Consistent with a model in which the ADA proteins function as transcriptional coactivators or adaptors, the ADAs isolated previously have specific defects in transcription (4, 37, 38, 43). In order to determine whether $\Delta ada1$ strains were also defective for transcription from certain activators, β -galactosidase assays were performed on wild type and $\Delta ada1$ strains transformed with various reporters. As demonstrated in Table 2, $\Delta ada1$ strains show decreased activity from several reporters. Reporters, which bind the activators HAP1 (*CYC1 UAS1*) and HAP2/3/4/5 (*CYC1 UAS2UP1*) are reduced 21 fold and 9 fold, respectively, and reporters which bind the activator GCN4 (*HIS66* and *HIS(2)14x2*), are reduced 15 and 34 fold, respectively. The *ADH1* reporter is also reduced 5 fold in a $\Delta ada1$ strain. This is of particular interest because $\Delta ada2$, $\Delta ada3$ and $\Delta gcn5$ strains do not do not show this decrease (4, 38) while $\Delta ada5$ strains do (37). These results suggest that the *ADA* genes can be separated into two classes based upon transcriptional differences. A reporter containing the thymidine rich sequences of the *DED1* gene as a UAS, dAdT, is not reduced in a $\Delta ada1$ strain. In fact, activity may be slightly

elevated. This argues that it is not a general defect in transcription that occurs in *Δada1* strains but specific defects that affect only a subset of promoters.

***ada1* mutants are auxotrophic for inositol and display Spt⁻ phenotypes.**

Mutations in many factors that are thought to be coactivators result in inositol auxotrophy. These include the *SRB* genes (34), the *SWI/SNF* genes (41), and some *SPT* genes, including *ADA5/SPT20* (45). In order to determine whether deleting *ADA1* resulted in inositol auxotrophy, various strains were grown on plates lacking inositol. As shown in Figure 1, a *Δada1* strain does not grow on media lacking inositol whereas *Δada2*, *Δada3*, and *Δgcn5* strains do. This further links *ADA1* to *ADA5* phenotypically and separates them from *ADA2*, *ADA3*, and *GCN5*.

To determine whether *ada1* mutants displayed other phenotypes similar to *ada5/spt20* mutants, we tested whether *Δada1* strains displayed an Spt⁻ phenotype. *ADA1* was disrupted in strain FY56, which contains δ element insertions at the *HIS4* and *LYS2* loci. As demonstrated in Figure 2A, a *ΔADA1* mutant is indeed able to suppress the *his4-912 δ* and *lys2-128 δ* alleles present in the yeast strain FY56. In contrast, a *ΔADA3* mutant is unable to suppress these alleles, further delineating the two classes of *ADA* genes. As a positive control, strain FY710 (*hta1-htb1 Δ*) is also able to suppress both the *his4-912 δ* and *lys2-128 δ* alleles.

As mentioned previously, the *SPT* genes can be separated into two classes: the histone class and the TBP class. Paradoxically, *ADA5* had been isolated as an *SPT* of the TBP class (45) while *GCN5* has been shown to acetylate histones (10). Thus we were interested in knowing whether *ADA1* is of the TBP or histone class of *SPTs*. Since the *his4-912 δ* and *lys2-128 δ* alleles do not distinguish between the two classes, *ADA1* and *ADA3* were disrupted in strain FY630 which contains *his4-917 δ* , an allele specifically suppressed by the TBP class but not the histone class. As demonstrated in Figure 2B, a deletion of *ADA1* is able to suppress the *his4-*

917 δ allele while a deletion of *ADA3* is not. This demonstrates that *ADA1* is of the TBP class of *SPT* genes.

Partial copurification of an ADA complex. 1-7a Δ *ada2* was transformed with pPADA26His, a plasmid which expresses a 6-histidine tagged version of ADA2 from its own promoter and fully complements a deletion of the gene (56). The resulting strain was grown in minimal media and used to make a whole cell extract which was fractionated over a Bio-Rex 70 column (see Materials and Methods). After step elution with potassium acetate, the ADA proteins were detected by Western blot analysis in two fractions: the 600 mM fraction and the 1200 mM fraction. The 600 mM fraction contained about 60% of the ADA proteins and the 1200 mM fraction accounted for the remainder (56). We estimate that both the 600 mM and 1200 mM eluent fractions represent a 10-fold purification of the ADA/GCN5 complex (Table 3).

Next, the 600 mM eluent was bound to a Ni-NTA agarose column. The Ni-NTA column was eluted with a gradient of imidazole, and the ADA proteins were quantitatively recovered in the peak fractions of the eluent. This step represented only a 1.6 fold purification of the ADA/GCN5 complex (56) because a relatively large fraction of proteins in the yeast extract bound to the column under the conditions we used.

The peak fractions eluted from the Ni-NTA column were fractionated on a HQ-20 anion exchange column, and eluted with a gradient of potassium acetate. As shown in Figure 3, cofractionation of ADA1, ADA2, ADA3, GCN5 and ADA5 was observed. The peak of the ADA/GCN5 complex eluted from the column at approximately 1.1 M potassium acetate. The ADA proteins are purified approximately 160 fold in the peak fractions from this column (Table 3). The fractions eluted from the HQ-20 column were also probed for the basal transcription factor, TBP. While TBP was present in the material loaded onto the

column, it did not cofractionate with the ADA complex (Fig. 3), and, in fact, flowed through the column. Fractions from the HQ-20 column were also probed with antibodies to other transcription factors as well. TFIIB and TFIIE behaved similarly to TBP and flowed through the column while SRB4, SRB5, SWI3 and TFIIF fractionated together, eluting prior to the ADA/GCN5 complex (56).

Finally, the fractions from the HQ-20 column containing the peak amounts of ADA proteins were pooled and subjected to fractionation on a Superose 6 gel filtration column. Again, all ADA proteins cofractionated over this column (Fig. 4). Comparison of the elution profiles of the ADA proteins with that of known protein standards indicated that the ADA proteins eluted at an approximate molecular weight of 2 MDa. The sizing column resulted in an 18 fold purification of the ADA proteins bringing up the total purification to approximately 2,880 fold (Table 3). These data suggest that the ADA gene products, including GCN5, form a complex in yeast.

Fractionation of the 1200 mM potassium acetate elution from the Bio-Rex 70 column on either an HQ-20 column or a Superose 6 column also resulted in co-fractionation of all five ADA proteins (56).

ADA1 interacts with the ADA complex by immunoprecipitation. In order to verify that ADA1 interacts with the other ADAs, whole cell extracts were made from wild type and $\Delta ada1$ strains grown in rich media. The extracts were then passed over a Bio-Rex 70 column and the flowthrough and 250 mM, 600 mM and 1200 mM potassium acetate elutions were examined by Western blot. As demonstrated in Figure 5A, ADA1 elutes from this column in a profile identical to ADA3. The band that is seen in the 600 mM fraction of the $\Delta ada1$ strain is a background band that migrates at a slightly higher molecular weight than ADA1 (compare wt and $\Delta ada1$ START lanes in the second panel of Fig. 5B). In contrast to the partial purification of the ADA/GCN5 complex described above some

ADA1 and ADA3 was observed in the flowthrough fraction. The flowthrough fraction was run over a Superose 6 column and all five ADA proteins co-fractionated as determined by Western blot (28) suggesting that the column residue may have been limiting in this second preparation.

ADA1 was immunoprecipitated from the 600 mM potassium acetate elutions from the Bio-Rex 70 column using ADA1 antibodies crosslinked to protein A sepharose beads. As expected, antibody to ADA1 is able to precipitate ADA1 from wild type but not $\Delta ada1$ extracts and preimmune antisera is unable to precipitate ADA1 from either extract (Fig. 5B second panel). As shown in the top panel of Figure 5B, ADA3 co-immunoprecipitates with ADA1, implying that ADA1 and ADA3 interact. Importantly, ADA3 is not precipitated in a $\Delta ada1$ extract nor by preimmune serum demonstrating that this interaction is specific and dependent on ADA1. Two other proteins probed in this experiment, TBP and TFIIB, do not co-precipitate with ADA1 indicating that they either do not interact with ADA1 or that an interaction is too weak to be detected.

To demonstrate that the other ADA gene products also interact either directly or indirectly with ADA1, antibody to ADA2, GCN5, ADA5 and, as controls, antibody to ADA1 and pre-immune serum were crosslinked to protein A sepharose beads and used in immunoprecipitation experiments. As shown in Figure 5C, antibodies to ADA2, GCN5, ADA5 and ADA1, but not preimmune serum, are able to precipitate ADA1 from wild type extracts, demonstrating that there is an interaction between all of the ADA gene products. To verify that the protein co-precipitated by these antibodies is ADA1, the lower panel in Figure 5C demonstrates that the protein is not precipitated from a $\Delta ada1$ extract. These results suggest that ADA1, ADA2, ADA3, GCN5, and ADA5 are all found in a single complex.

Discussion

We report here the cloning, sequencing, and characterization of *ADA1*, which encodes a novel member of the ADA/GCN5 complex. *ADA1* encodes a 488 amino acid protein. *ada1* mutants display defects in transcription similar to those of *ada5* mutants and broader than those found in *ada2*, *ada3* and *gcn5* mutants. Notably, mutations in *ADA2*, *ADA3*, and *GCN5* do not affect transcription from the *ADH1* promoter while mutations in *ADA1* and *ADA5* decrease activity of this reporter approximately five fold. Indeed, since the activator, GAL4-VP16, that was used in the ADA screen was overexpressed using the *ADH1* promoter, it was initially thought that *ADA1* was totally unrelated to *ADA2*, *ADA3* and *GCN5* because resistance could arise from a reduction in the amount of GAL4-VP16 produced. On the other hand, all *ada* mutants display similar decreases in transcription from GCN4 dependent promoters. Importantly, activity from the dAdT reporter was not reduced in a $\Delta ada1$ strain demonstrating that not all transcription is dependent on *ADA1*. It has been proposed that the dAdT UAS activates transcription because this DNA sequence by itself disrupts nucleosomes (31). It is possible that disruption of chromatin by dAdT bypasses the need for the histone acetyl transferase activity of the ADA/GCN5 complex in transcription.

Two classes of ADA genes. The similarity in transcription defects between $\Delta ada1$ and $\Delta ada5$ strains led us to test whether the strains had other phenotypes in common. We have determined that $\Delta ada1$ strains are auxotrophic for inositol and are Spt⁻, two phenotypes described for *ada5* mutants (45). Furthermore, both *ADA1* and *ADA5* are in the TBP class of *SPTs*, not the histone class. This is in agreement to previous genetic and biochemical data suggest a link between the functions of the ADA/GCN5 complex and TBP (2, 37). As controls, we have

determined that representative mutants of the *ADA2*, *ADA3*, and *GCN5* class of *ADAs* are neither auxotrophic for inositol, nor *Spt*⁻. Thus, the *ADA* genes can be separated into two distinct classes based on three criteria: transcriptional reporters that are affected, inositol auxotrophy, and *Spt* phenotype.

The five ADA gene products form a complex. With two different classes of *ADA* genes, it seemed possible that two different complexes may be formed. Previously, it has been shown that *ADA2*, *GCN5* and *ADA3* form a complex *in vitro* (2, 29). Also, various individual interactions between these three *ADA* proteins have been demonstrated *in vivo* (29,38). Therefore, it is clear that components of one of the classes form a complex. Interestingly, an interaction between *ADA3* and *ADA5* has also been demonstrated (37), indicating a possible interaction between members of different classes of *ADAs*. Here we present evidence that all five *ADA* proteins bind together in a complex by two methods. First, we demonstrate that the five proteins remain together throughout an approximately 2,880 fold purification over four successive columns. In each of these columns, the *ADA* proteins eluted with identical profiles (Fig. 3 and Fig. 4) (56). Second, we demonstrate, using immunoprecipitation experiments, specific interactions between *ADA1* and each of the other *ADA* proteins (Fig. 5). These two lines of evidence, taken together, strongly support a model in which all five *ADA* proteins form a complex *in vivo*.

The elution profile of the *ADA* proteins through the Superose 6 column indicates that the complex migrates with a molecular weight of approximately 2 MDa. This is much larger than the sum of the molecular weights of the five known individual subunits suggesting that there may be other components that have yet to be identified, or that the known components are present as multimers. Many other transcription factors have been shown to be associated in large molecular weight complexes such as the TFIID complex, the SWI/SNF

complex, and the holoenzyme complex. One possibility is that the ADA proteins could be part of one of these complexes. However, probing the partially purified ADA fractions with antibodies to TBP, Pol II, SWI3, and various SRBs demonstrated that these proteins do not peak with the ADA proteins (56). Thus the ADA/GCN5 complex may be distinct and novel.

In all the purification steps performed, all five ADA proteins co-fractionate. This co-fractionation is not limited to the ADAs in the 600 mM potassium acetate elution of the initial Bio-Rex 70 column but also to the ADAs in the flowthrough and 1200 mM potassium acetate elutions (28, 56). Thus, although we cannot rule out the formal possibility that ADA1 and ADA5 may form an alternate complex without the other ADAs, we have not found any evidence of this and it seems more likely that all five ADA proteins are always associated.

It has recently been shown that a *Tetrahymena* GCN5 complex contains a protein that crossreacts with antibody to ADA1 (9). Also, human homologues of ADA2 and GCN5 have been isolated and these homologues have been demonstrated to interact with each other (13). These data suggest that the ADA/GCN5 complex is evolutionarily conserved.

One complex with two separate functions? As mentioned before, it has been determined that GCN5 functions as a histone acetyltransferase (10) and can acetylate free histone H3 *in vitro*. However, it is unable to acetylate whole nucleosomes. Thus, one function proposed for the other ADAs in the complex is to alter the conformation of either GCN5 or of nucleosomes, allowing the acetylation of whole nucleosomes (47). We have demonstrated here that there are at least five ADA proteins in the ADA/GCN5 complex and that they can be separated into two distinct classes. We propose that the ADA proteins of one class, ADA2, ADA3, and GCN5, function together to acetylate nucleosomes, resulting in the clearing away of repressive nucleosomes from some promoters.

The ADAs of the other class, ADA1 and ADA5, have an additional function. This is supported by the observation that *ada1* and *ada5* mutants have more pronounced phenotypic defects than *ada2*, *ada3* and *gcn5* mutants. If GCN5 dependent acetylation of histones was the sole function of the ADA/GCN5 complex, one would not expect to observe any *ada* mutants with phenotypes more severe than $\Delta gcn5$ mutants.

The proposed second function of the ADA/GCN5 complex has not been defined yet. One possibility is that GCN5 is not the only histone acetyltransferase in the ADA complex and there is a second one that interacts with ADA1 and ADA5. On the other hand, it is fascinating that both *ADA1* and *ADA5*, but not the other *ADAs*, are in the TBP class of *SPTs*. Recently, it has been demonstrated that some mutants in *SPT15*, which encodes TBP, are resistant to GAL4-VP16 toxicity (37). It is extremely interesting to speculate, therefore, that the function of this second class of ADAs may be to interact with basal factors, possibly TBP. The ADA/GCN5 complex may be brought to promoters through interactions between activators and ADA2 (2, 39, 55) and ADA5 (37). Once there, the ADA2, ADA3, GCN5 class of ADAs may function to acetylate nucleosomes, opening up the promoter region, while the ADA1 and ADA5 class of ADAs recruit TBP and possibly other basal factors to bind to the TATA box. The observation that $\Delta ada1$ or $\Delta ada5$ strains display broader defects than $\Delta ada2$, $\Delta ada3$, or $\Delta gcn5$ strains, combined with the observation that $\Delta ada3\Delta ada5$ strains do not display phenotypes more severe than $\Delta ada5$ strains (37) suggests the following. Disrupting a member of the ADA2, ADA3, GCN5 class of ADA will not disrupt the activity of the ADA1, ADA5 class whereas disrupting ADA1 or ADA5 may disrupt the activity of the entire ADA/GCN5 complex.

It should be mentioned that TBP is not present in our partially purified ADA fractions and is not immunoprecipitated by ADA antibodies (Fig. 3 and 4).

However, other groups have demonstrated such interactions (2, 48) and it is possible that TBP is not a stably bound component of the ADA/GCN5 complex but is only transiently associated, ie only when bound to DNA or when activators are bound to the ADA complex. Alternatively, it is possible that the ADAs in the 1200 mM potassium acetate fraction from the Bio-Rex 70 column have different properties from the ADAs in the 600 mM fraction which we have been studying. Indeed, it has recently been reported that several ADA/GCN5 complexes may exist in yeast (48). It will be interesting to determine whether the ADAs in the 1200 mM potassium acetate fraction may be bound to TBP or other basal factors.

Acknowledgments

We would like to thank the Young lab, including A. Koleske, D. Chao, C. Wilson, and C. Hengartner for sharing antibodies and for technical assistance. We also thank A. Greenleaf and C. Peterson for sharing antisera and K. Kaplan for assistance with the Sprint BIOCAD apparatus. We are grateful to S. Roberts and F. Winston for strains, T. D. Fox and D. Allis for sharing unpublished results, and D. McNabb for critical comments on the manuscript. G. M. thanks C. Armstrong and S. Treadway for help with the GCN5 antibody. J. H. was a HHMI predoctoral fellow. This work was supported by a grant from NIH, #GM50207 to L. G.

References

1. **Alani, E., L. Cao and N. Kleckner.** 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics*. **116**:541-545.
2. **Barlev, N. A., R. Candau, L. Wang, P. Darpino, N. Silverman and S. L. Berger.** 1995. Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein. *J. Biol. Chem.* **270**:19337-19344.
3. **Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg and L. Guarente.** 1990. Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell*. **61**:1199-1208.
4. **Berger, S. L., B. Piña, N. Silverman, G. A. Marcus, J. Agapite, J. L. Regier, S. J. Triezenberg and L. Guarente.** 1992. Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell*. **70**:251-265.
5. **Brandl, C. J., A. M. Furlanetto, J. A. Martens and K. S. Hamilton.** 1993. Characterization of *NGG1*, a novel yeast gene required for glucose repression of GAL4p-regulated transcription. *EMBO J.* **12**:5255-5265.
6. **Brandl, C. J., J. A. Martens, A. Margaliot, D. Stenning, A. M. Furlanetto, A. Saleh, K. S. Hamilton and J. Genereaux.** 1996. Structure/functional properties of the yeast dual regulator protein NGG1 that are required for glucose repression. *J. Biol. Chem.* **271**:9298-9306.
7. **Brownell, J. E. and C. D. Allis.** 1995. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in *Tetrahymena* macronuclei. *Proc. Natl. Acad. Sci. USA.* **92**:6364-6368.

8. **Brownell, J. E. and C. D. Allis.** 1996. Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr. Opin. Genet. Dev.* **6**:176-184.
9. **Brownell, J. E., T. A. Ranalli and C. D. Allis.** Personal communication.
10. **Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth and C. D. Allis.** 1996. *Tetrahymena* histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell.* **84**:843-851.
11. **Cairns, B. R., Y.-J. Kim, M. H. Sayre, B. C. Laurent and R. D. Kornberg.** 1994. A multisubunit complex containing the *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* gene products isolated from yeast. *Proc. Natl. Acad. Sci. USA.* **91**:1950-1954.
12. **Candau, R. and S. L. Berger.** 1996. Structural and functional analysis of yeast putative adaptors. Evidence for an adaptor complex *in vivo*. *J. Biol. Chem.* **271**:5237-5245.
13. **Candau, R., P. A. Moore, L. Wang, N. Barlev, C. Y. Ying, C. A. Rosen and S. L. Berger.** 1996. Identification of human proteins functionally conserved with the yeast putative adaptors ADA2 and GCN5. *Mol. Cell. Biol.* **16**:593-602.
14. **Cote, J., J. Quinn, J. L. Workman and C. L. Peterson.** 1994. Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science.* **265**:53-60.
15. **Dynlacht, B. D., T. Hoey and R. Tjian.** 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell.* **66**:563-576.

16. **Eisenmann, D. M., K. M. Arndt, S. L. Ricupero, J. W. Rooney and F. Winston.** 1992. SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae*. *Genes Dev.* **6**:1319-1331.
17. **Eisenmann, D. M., C. Chapon, S. M. Roberts, C. Dollard and F. Winston.** 1994. The *Saccharomyces cerevisiae* *SPT8* gene encodes a very acidic protein that is functionally related to SPT3 and TATA-binding protein. *Genetics.* **137**:647-657.
18. **Forsberg, S. L. and L. Guarente.** 1989. Identification and characterization of HAP4: a third component in the CCAAT-bound HAP2/HAP3 heteromer. *Genes Dev.* **3**:1166-1178.
19. **Fox, T. D.** Personal communication.
20. **Georgakopoulos, T., N. Gounalaki and G. Thireos.** 1995. Genetics evidence for the interaction of the yeast transcriptional co-activator proteins GCN5 and ADA2. *Mol. Gen. Genet.* **246**:723-728.
21. **Georgakopoulos, T. and G. Thireos.** 1992. Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. *EMBO J.* **11**:4145-4152.
22. **Guarente, L., B. Lalonde, P. Gifford and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene. *Cell.* **36**:503-511.
23. **Guthrie, C. and G. R. Fink.** 1991. Guide to yeast genetics and molecular biology. *Methods Enzymol.* **194**:835.
24. **Harlow, E. and D. Lane.** 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory.
25. **Hengartner, C. J., C. M. Thompson, J. Zhang, D. M. Chao, S. M. Liao, A. J. Koleske, S. Okamura and R. A. Young.** 1995. Association of an activator with an RNA polymerase II holoenzyme. *Genes Dev.* **9**:897-910.

26. **Hinnebusch, A. G., G. Lucchini and G. R. Fink.** 1985. A synthetic *HIS4* regulatory element confers general amino acid control on the cytochrome c gene (*CYC1*) of yeast. *Proc. Natl. Acad. Sci. USA.* **82**:495-502.
27. **Hirschhorn, J. N., S. A. Brown, C. D. Clark and F. Winston.** 1992. Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev.* **6**:2288-2298.
28. **Horiuchi, J. and L. Guarente.** unpublished results.
29. **Horiuchi, J., N. Silverman, G. A. Marcus and L. Guarente.** 1995. ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. *Mol. Cell. Biol.* **15**:1203-1209.
30. **Ireton, K., D. Z. Rudner, K. J. Siranosian and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. *Genes Dev.* **7**:283-294.
31. **Iyer, V. and K. Struhl.** 1995. Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. *EMBO J.* **14**:2570-2579.
32. **Kelleher, R. J. I., P. M. Flanagan and R. D. Kornberg.** 1990. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell.* **61**:1209-1215.
33. **Knaus, R., R. Pollock and L. Guarente.** 1996. Yeast SUB1 is a suppressor of TFIIB mutations and has homology to the human co-activator PC4. *EMBO J.* **15**:101-108.
34. **Koleske, A. J., S. Buratowski, M. Nonet and R. A. Young.** 1992. A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell.* **69**:883-894.

35. **Koleske, A. J. and R. A. Young.** 1994. An RNA polymerase II holoenzyme responsive to activators. *Nature*. **368**:466-469.
36. **Lue, N. F., A. R. Buchman and R. D. Kornberg.** 1989. Activation of yeast RNA polymerase II promoters by a thymidine-rich upstream element in vitro. *Proc. Natl. Acad. Sci. USA*. **86**:486-490.
37. **Marcus, G. A., J. Horiuchi, N. Silverman and L. Guarente.** 1996. *ADA5/SPT20* links the *ADA* and *SPT* genes, which are involved in yeast transcription. *Mol. Cell. Biol.* **16**:3197-3205.
38. **Marcus, G. A., N. Silverman, S. L. Berger, J. Horiuchi and L. Guarente.** 1994. Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. *EMBO J.* **13**:4807-4815.
39. **Melcher, K. and S. A. Johnston.** 1995. GAL4 interacts with TATA-binding protein and coactivators. *Mol. Cell. Biol.* **15**:2839-2848.
40. **Peterson, C. L., A. Dingwall and M. P. Scott.** 1994. Five SWI/SNF gene products are components of a large multisubunit complex required for transcriptional enhancement. *Proc. Natl. Acad. Sci. USA*. **91**:2905-2908.
41. **Peterson, C. L., W. Kruger and I. Herskowitz.** 1991. A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. *Cell*. **64**:1135-1143.
42. **Pfeifer, K., B. Arcangioli and G. Guarente.** 1987. Yeast HAP1 activator competes with the factor RC2 for binding to the upstream activation site UAS1 of the *CYC1* gene. *Cell*. **49**:9-18.
43. **Piña, B., S. Berger, G. A. Marcus, N. Silverman, J. Agapite and L. Guarente.** 1993. *ADA3*: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of *ADA2*. *Mol. Cell. Biol.* **13**:5981-5989.
44. **Pugh, B. F. and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell*. **61**:1187-1197.

45. **Roberts, S. M. and F. Winston.** 1996. *SPT20/ADA5* encodes a novel protein functionally related to the TATA-binding protein and important for transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**:3206-3213.
46. **Rose, M. and D. Botstein.** 1983. Structure and function of the yeast *URA3* gene. Differentially regulated expression of hybrid beta-galactosidase from overlapping coding sequences in yeast. *J Mol Biol.* **170**:883-904.
47. **Roth, S. Y. and C. D. Allis.** 1996. The subunit exchange model of histone acetylation. *Trends in Cell Biology.* **6**:371-375.
48. **Saleh, A., V. Lang, R. Cook and C. Brandl.** 1997. Identification of native complexes containing the yeast coactivator/repressor proteins NGG1/ADA3 and ADA2. *J. Biol. Chem.* **272**:5571-5578.
49. **Sanger, F., S. Nicklen and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74**:5463-5467.
50. **Santangelo, G. M., J. Tornow, C. S. McLaughlin and K. Moldave.** 1988. Properties of promoters cloned randomly from the *Saccharomyces cerevisiae* genome. *Mol. Cell. Biol.* **8**:4217-4224.
51. **Sauer, F., S. K. Hansen and R. Tjian.** 1995. Multiple TAF_{II}s Directing Synergistic Activation of Transcription. *Science.* **270**:1783-1788.
52. **Sayre, M. H., H. Tschochner and R. D. Kornberg.** 1992. Reconstitution of transcription with five purified initiation factors and RNA polymerase II from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **267**:23376-23382.
53. **Sherman, F., G. R. Fink and J. B. Hicks.** 1986. *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory.
54. **Sikorski, R. S. and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* **122**:19-27.

55. **Silverman, N., J. Agapite and L. Guarente.** 1994. Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. *Proc. Natl. Acad. Sci. USA.* **91**:11665-11668.
56. **Silverman, N. and L. Guarente.** Unpublished results.
57. **Swanson, M. S. and F. Winston.** 1992. SPT4, SPT5 and SPT6 interactions: effects on transcription and viability in *Saccharomyces cerevisiae*. *Genetics.* **132**:325-336.
58. **Thompson, C. M., A. J. Koleske, D. M. Chao and R. A. Young.** 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell.* **73**:1361-1375.
59. **Thut, C. J., J. L. Chen, R. Klemm and R. Tjian.** 1995. p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science.* **267**:100-104.
60. **Wilson, C. J., D. M. Chao, A. N. Imbalzano, G. R. Schnitzler, R. E. Kingston and R. A. Young.** 1996. RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell.* **84**:235-244.
61. **Winston, F.** 1992. Analysis of *SPT* genes: a genetic approach toward analysis of TFIID, histones, and other transcription factors of yeast. *Transcriptional Regulation.* McKnight and Yamamoto ed. Cold Spring Harbor Laboratory Press. 1271-1293.

Table 1. Strains used in this study.

<u>Strain</u>	<u>Genotype</u>	<u>Source</u>
ada1-1	<i>MATa ura3-52 leu2-3,112 his4-519 ade1-100 gal4::HIS4 ada1-1</i>	(4)
BWG1-7a	<i>MATa ura3-52 leu2-3,112 his4-519 ade1-100</i>	Lab strain
1-7a Δ ada1	BWG1-7a Δ ada1::hisG	This study
1-7a Δ ada2	BWG1-7a Δ ada2::hisG	(4)
PSY316	<i>MATα ura3-52 leu2-3,112 his3-Δ200 ade2-101 lys2-801</i>	Lab strain
316 Δ ada1	PSY316 Δ ada1::hisG	This study
316 Δ ada2	PSY316 Δ ada2::hisG	(4)
316 Δ ada3	PSY316 Δ ada3::hisG	(34)
GMy27	PSY316 Δ gcn5::hisG	(34)
FY56	<i>MATα ura3-52 LEU2 his4-912∂ lys2-128∂</i>	Fred Winston
FY56 Δ ada1	FY56 Δ ada1::hisG	This study
FY56 Δ ada3	FY56 Δ ada3::hisG	This study
FY710	<i>MATa ura3-52 leu2Δ1 his4-912∂ lys2-128∂ Δ(hta1-htb1)::LEU2</i>	Fred Winston
FY630	<i>MATa ura3-52 leu2Δ1 his4-917∂ lys2-173R2 trp1Δ63</i>	Fred Winston
FY630 Δ ada1	FY630 Δ ada1::hisG	This study
FY630 Δ ada3	FY630 Δ ada3::hisG	This study

Table 2. β -galactosidase assays^a of wild type and *Δada1* strains.

Reporter	Strain	
	wt	<i>Δada1</i>
CYC1 UAS1	144	6.7
CYC1 UAS2up1	265	28
HIS(1)66	8160	547
HIS(2)14x2	2990	89
ADH1	12800	2400
dAdT	319	627

^a Assays were performed as described in the Materials and Methods.

Measurements are the averages of assays performed on four individual transformants. Standard errors were less than 20% of the mean.

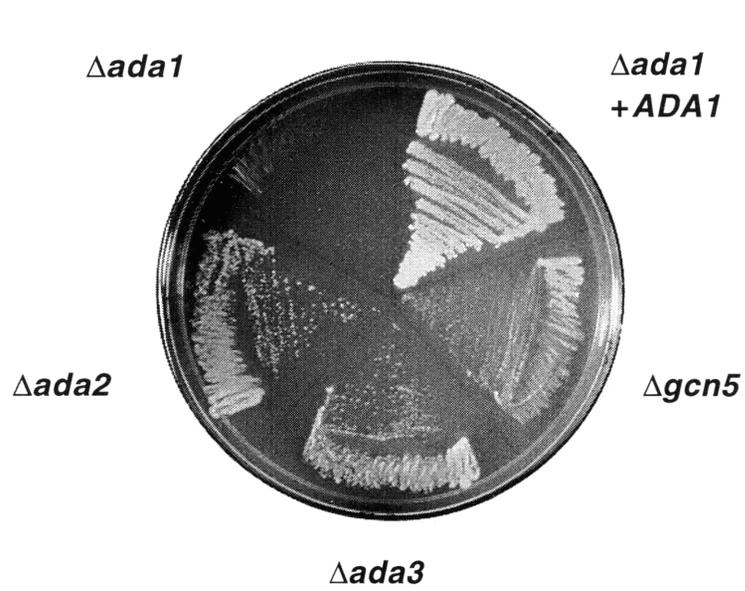
Table 3. Partial purification of the ADA/GCN5 complex.

Fraction	Protein (mg)		Yield (%)	Fold Purification ^a	
	Onput	Pooled fractions		Column	Total
Crude extract	-	2975	(100)	-	-
Bio-Rex 70	2975	179.5	60	10	10
Ni-NTA agarose	149.5	72	38	1.6	16
Poros HQ-20	72	3.6	19	10	160
Superose 6 HR ^b	0.23	0.0004	0.03	18	2880

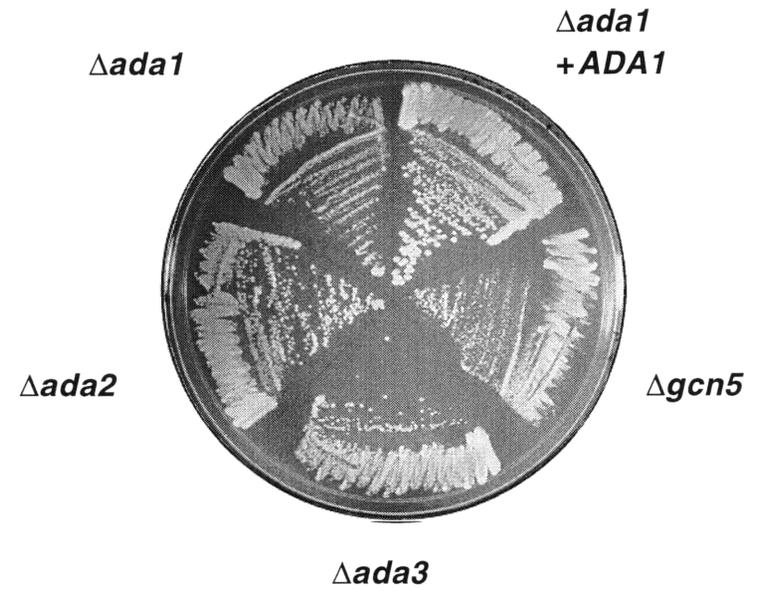
^a The fold purification by column is the ratio of the yield divided by the protein in the pooled fractions of the current column to the previous column. The total fold purification is the product of the previous fold purifications.

^b The Superose 6 HR column was an analytical column with a low capacity and yield.

Figure 1. Inositol phenotype of a $\Delta ada1$ strain. $316\Delta ada1$, $316\Delta ada2$, $316\Delta ada3$, GMy27 ($316\Delta gcn5$), and as a positive control, $316\Delta ada1$ transformed with YCP50ADA1 ($\Delta ada1+ADA1$) were grown on synthetic complete media lacking inositol (-Inositol) and an identical plate on which 200 μ l of 200 mM inositol had previously been spread (+Inositol).



-Inositol

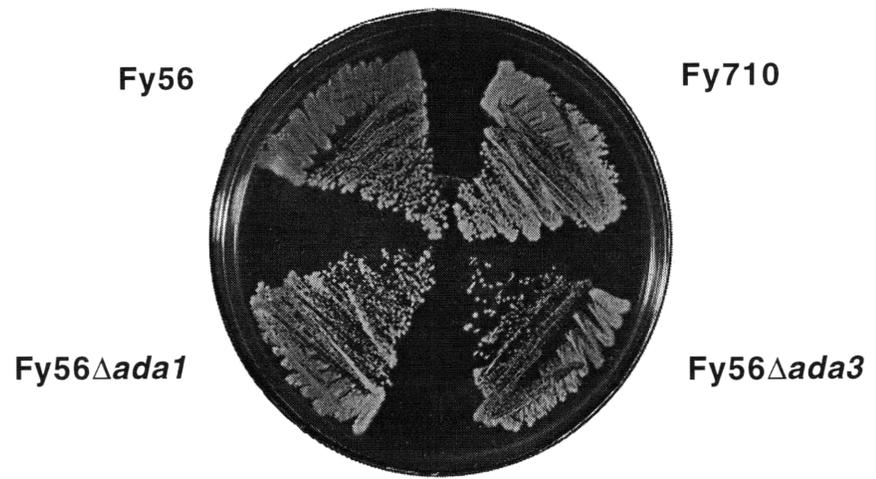


+Inositol

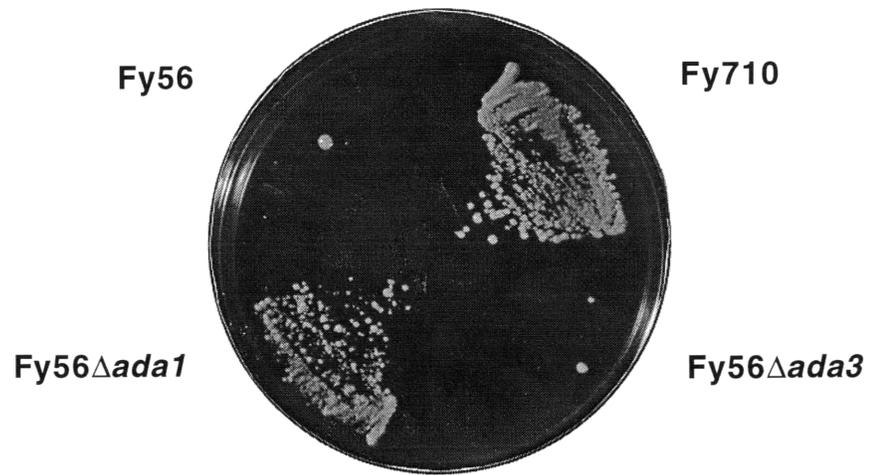
Figure 2. Spt^- phenotype of a $\Delta ada1$ strain. (A) The indicated strains were streaked on synthetic complete media (Complete) or media lacking lysine (-lysine) or histidine (-histidine) to determine whether they suppressed the $his4-912\delta$ and $lys2-128\delta$ alleles present. FY710, which is disrupted for the histone $hta1-htb1$ locus, was used as a positive control. (B) The indicated strains were patched on synthetic complete media (Complete) or media lacking histidine (-histidine) to determine whether they suppressed the $his4-917\delta$ allele, which is specifically suppressed by mutants in the TBP class of $SPTs$.

A.

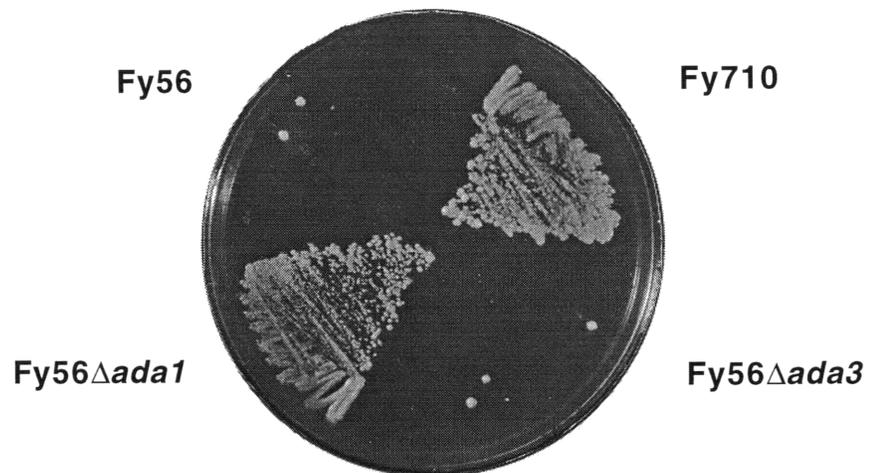
Complete



-lysine



-histidine



B.

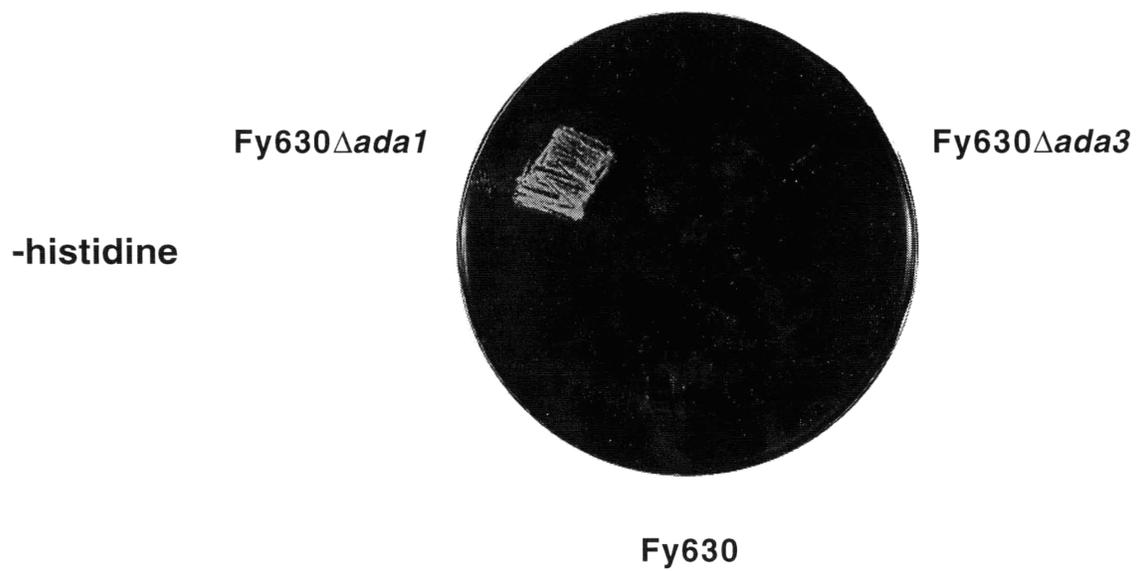
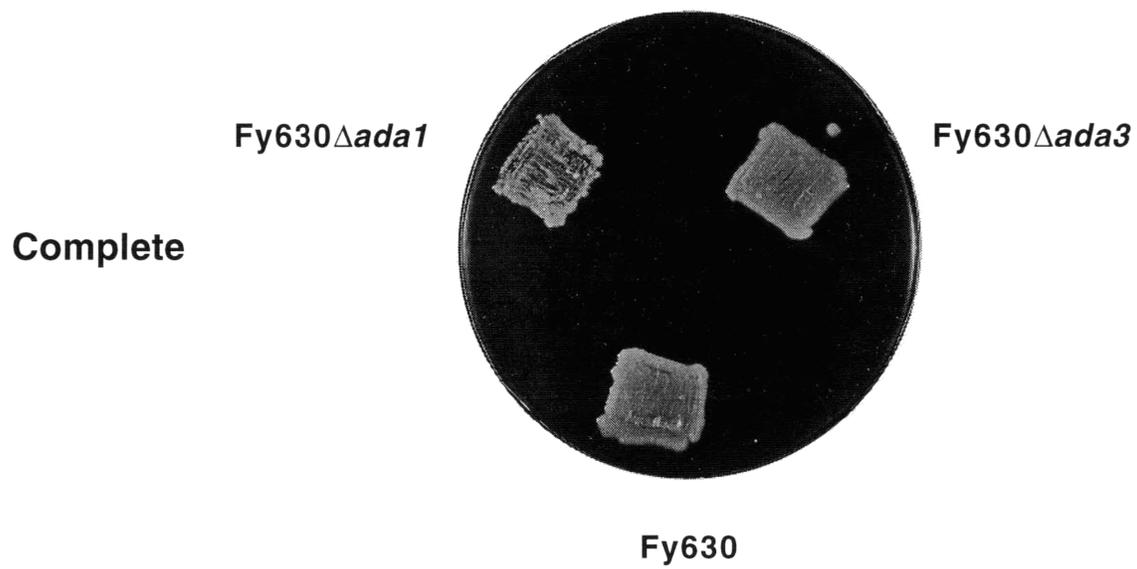


Figure 3. Cofractionation of ADA1, ADA2, ADA3, GCN5, and ADA5 over an HQ-20 anion exchange column. All panels show immunoblots of fractions from the HQ-20 column eluted with a potassium acetate gradient. The output (OP), flow through (FT) and every other fraction between fractions 13 and 23 were probed with antibody to the indicated proteins. The band recognized by the GCN5 antibody runs slightly faster than expected. It is possible that GCN5 is slightly degraded in our extracts.

OP FT



ADA1



ADA2



ADA3



GCN5



ADA5



TBP

Figure 4. Cofractionation of the ADA proteins over a Superose 6 gel filtration column. Aliquots of the onput (OP) to the column and the indicated fractions were TCA precipitated and analyzed by Western blot. Antibodies to the indicated proteins were used to probe the blots. Aliquots corresponded to 3% of the onput and 25% of the fractions.

ST 15 17 19 21 23 25 27 29



ADA1



ADA3



ADA2



GCN5

ST 16 18 20 22 24 26 28 30



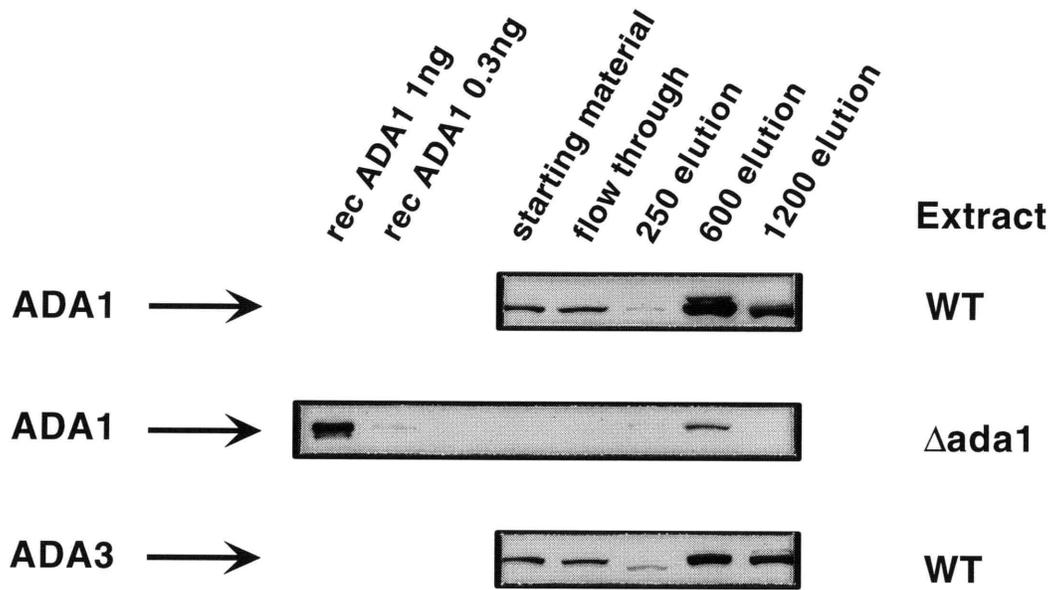
ADA3



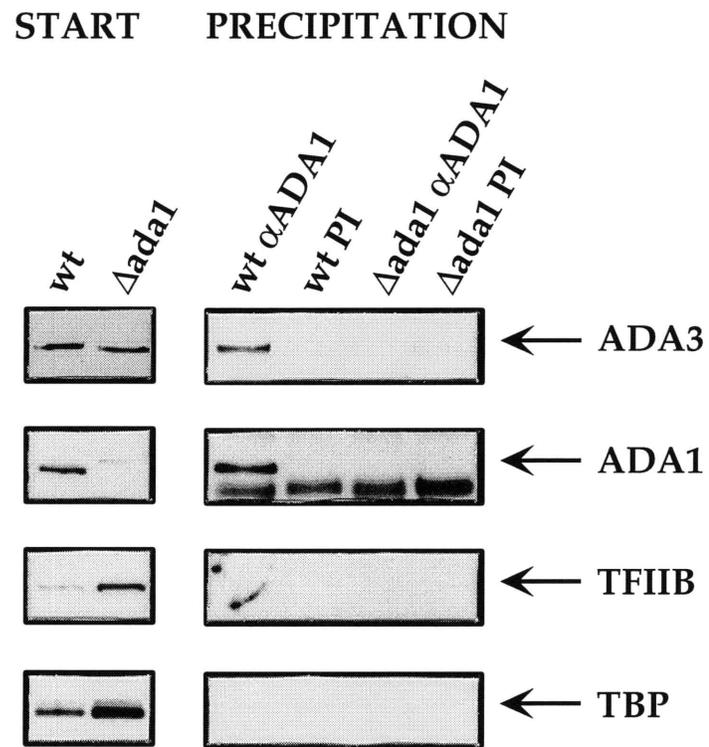
ADA5

Figure 5. Co-precipitation experiments between ADA1 and other ADA proteins. (A) Whole cell extracts from BWG1-7a (WT) or 1-7a Δ ada1 (Δ ada1) strains were fractionated on a Bio-Rex 70 column as described in the Materials and Methods. Proteins were eluted stepwise in 250, 600 and 1200 mM potassium acetate. 20 μ g each of whole cell extract (starting material), flow through, and each of the elutions were assayed for ADA1 and ADA3 by Western blot analysis. Recombinant ADA1 (rec ADA1) was used as a control in the Δ ada1 blot to determine the migration of ADA1 on SDS-PAGE. The band in the 600 mM elution in the Δ ada1 extract is a background band that migrates slightly above ADA1. (B) Immunoprecipitations were performed on 1 ml of the 600 mM elution from wt and Δ ada1 extracts from (A) using either protein A sepharose beads crosslinked to ADA1 antibody (α ADA1) or pre-immune serum (PI). 100 μ l of the 600 mM elution (START) from wt and Δ ada1 extracts as well as the precipitations were subjected to SDS-PAGE and immunoblotted with the antibody to the indicated proteins (ADA3, ADA1, TFIIIB, and TBP). The band below ADA1 corresponds to uncrosslinked IgG heavy chain which was eluted off of the beads and crossreacts with the secondary antibody. (C) Immunoprecipitations were performed on 1 ml of the 600 mM elution from the wt and the Δ ada1 extracts shown in (A). Protein A sepharose beads crosslinked to pre-immune serum (PI) or antibody to ADA2 (α ADA2), GCN5 (α GCN5), ADA5 (α ADA5) and ADA1 (α ADA1) were used for immunoprecipitations. 250 μ l of the 600 mM elution from the extracts (START) as well as each of the precipitations was subjected to SDS-PAGE and analyzed by Western blot for the presence of ADA1. As in (B), the band below ADA1 in the precipitation lanes corresponds to IgG heavy chain. Rec ADA1 refers to 5 ng of recombinant ADA1.

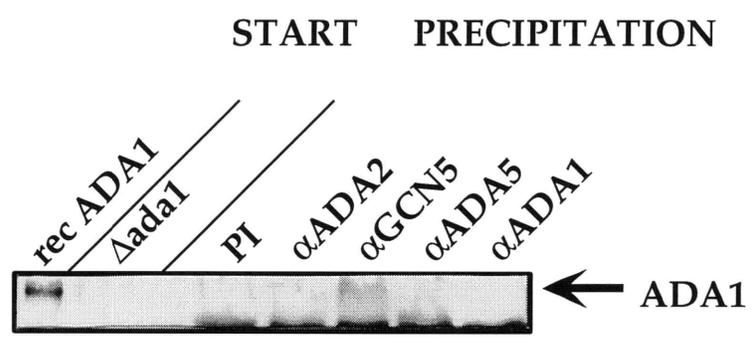
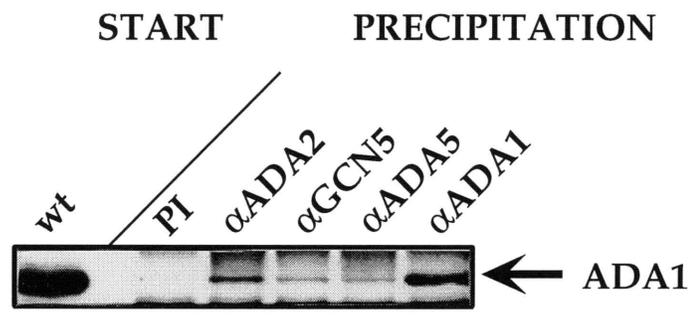
A.



B.



c.



Appendix I

***ADA5/SPT20* links the *ADA* and *SPT* genes involved in yeast transcription**

This appendix was previously published in *Molecular and Cellular Biology*, Vol. 16, pages 3197-3205, June 1996. The authors were Gregory A. Marcus, Junjiro Horiuchi, Neal Silverman and Leonard Guarente. My contribution to this work was the biochemical experiments demonstrating that ADA5 is part of the ADA/GCN5 complex.

Summary

In this report we describe the cloning and characterization of *ADA5*, a gene identified by resistance to GAL4-VP16-mediated toxicity. *ADA5* binds directly to the VP16 activation domain, but not to a transcriptionally defective VP16 double point mutant. Double mutants of *ada5* and other genes isolated by resistance to GAL4-VP16 (*ada2* or *ada3*) grow like *ada5* single mutants, suggesting that *ADA5* is in the same pathway as the other *ADA* genes. Further, *ADA5* cofractionates and coprecipitates with *ADA3*. However, an *ada5* deletion mutant exhibits a broader spectrum of phenotypes than null mutants in the other *ADA* genes. Most interestingly, *ADA5* is identical to *SPT20* (S. M. Roberts and F. Winston, Mol. Cell. Biol. 16: 3206-3213, 1996), showing that it shares phenotypes with the *ADA* and *SPT* family of genes. Of the other *SPT* genes tested, mutants in *SPT7* and strikingly, *SPT15* (encoding the TATA-binding protein) show resistance to GAL4-VP16. We present a speculative pathway of transcriptional activation involving the *ADA2/3/GCN5/ADA5* complex and the TATA-binding protein.

Introduction

In eukaryotes, transcriptional activation is a central means of gene regulation. RNA polymerase II, which is responsible for transcribing mRNA, is recruited to transcriptional start sites via its interaction with the general transcription factors. These factors recognize the TATA or initiator elements found near the start site. Moreover, transcriptional activators, which bind enhancer/UAS elements found hundreds of base pairs away from the TATA box, can greatly stimulate the initiation of transcription. These transcriptional activators are often modular in structure; one domain is responsible for recognizing the DNA and another domain is responsible for transcriptional activation (18). The mechanism by which these activation domains function remains unknown although it is the focus of much research.

It is clear that proteins other than the general transcription factors and activators are necessary for activated transcription (41). These factors, termed coactivators or adaptors, have been identified using various genetic and biochemical methods and are thought to function by mediating the interaction between basal factors and activation domains (3, 31, 41). TFIID, a complex of the TATA binding protein (TBP) and associated TAFs (TBP associated factors) is capable of supporting activated transcription whereas TBP alone cannot (9). Further, different TAF subunits bind to and mediate activation by different classes of activation domains (17, 28, 48, 56). Additionally, other non-TAF coactivators identified in metazoans or their viruses include PC4/p15 (15, 35), CBP (36), and the X protein from hepatitis B virus (24). These proteins have been shown to have the properties expected of coactivators, including the ability to mediate transcriptional activation.

Yeast genetics has proven to be very useful in identifying potential coactivators genes. For example, the *SRB* genes were isolated as suppressors of truncations in the conserved carboxy terminal domain (CTD) of RNA polymerase II (33, 55). These gene products copurify in a complex called the mediator that can bind activators (25), and has coactivator activity (25, 32). This mediator complex together with the core RNA polymerase II subunits form the RNA polymerase II holoenzyme, which can support activated transcription (32, 34). The products of the *SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5* and *SNF6* genes, identified as positive regulators of *SUC2* and *HO* transcription (39, 54), are part of a subcomplex of the mediator/holoenzyme (57) that antagonizes histone repression *in vivo* and *in vitro* (7, 27).

Other yeast coactivator candidates include *ADA2*, *ADA3* and *GCN5*, isolated in a selection for mutants resistant to GAL4-VP16 mediated toxicity (4, 5, 38). Mutants in these genes relieve toxicity by reducing the ability of the VP16 activation domain to activate transcription without altering the levels of GAL4-VP16 in cells. Moreover, *ada2*, *ada3*, and *gcn5* mutants all have similar phenotypes including slow growth on minimal medium, temperature sensitivity, and a reduced ability to support activation by certain activation domains *in vivo* and *in vitro* (4, 38, 40). *ADA2*, *ADA3* and *GCN5* form a complex when translated *in vitro* (30), and they cofractionate from yeast extracts (53). The fact that double mutants of these genes have phenotypes indistinguishable from single mutants also supports the model that *ADA2*, *ADA3* and *GCN5* function together in a complex (38). *ADA2* can bind VP16 and GCN4 activation domains (2, 52) and may be necessary for a TBP-VP16 interaction in yeast extracts (2).

Another set of yeast genes important for proper transcriptional regulation and promoter selection, the *SPT* genes, were identified as suppressors of Ty insertions in yeast promoters (59). These genes may be grouped into two classes,

those that affect transcription via chromatin and those that affect transcription in a chromatin independent manner. (58). The latter class includes *SPT15*, which encodes the TATA binding protein, TBP (12, 22), as well as *SPT3*, *SPT7* and *SPT8* (12). Genetic evidence suggests that *SPT3*, *SPT7*, *SPT8* may act as a complex with TBP, and *SPT3* can be coimmunoprecipitated with TBP (10, 11, 14). Strains harboring *spt3*, *spt7*, *spt8* or *spt15* mutations show reduced expression of the Ty element, reduced expression of other yeast genes, and an alteration in promoter selection at Ty loci (58).

Here we report the cloning and initial characterization of *ADA5*. The phenotypes of *ada5* mutants and of double mutants suggest that *ADA5* operates in the same pathway as the other *ADA* genes (*ADA2/ADA3/GCN5*). Moreover, *ADA5* cofractionates with *ADA3* in yeast extracts. *ADA5* is identical to *SPT20* (45), and is the only *ada* gene to date to display *Spt*⁻ phenotypes. We present a speculative pathway of interaction of *ADA5* and the other *ADAs* in connecting activators to basal factors at promoters.

Materials and Methods

Yeast strains and manipulations. All yeast strains are derivatives of BWG1-7a (*MATa*, *ade1-100*, *ura3-52*, *leu2-3,2-112*, *his4-519*) (20), BP1 (*MATa*, *ade1-100*, *ura3-52*, *leu2-3,2-112*, *his4-519*, *gal4::HIS4*) or PSy316 (*MAT α* *ura3-52*, *leu2-3,2-112*, *his3- Δ 200*, *lys2*) (4). GMy37p (*MATa*, *ura3-52*, *leu2*, *his4*, *gal4::HIS4*, *ada5-1*) was isolated as a strain resistant to GAL4-VP16 toxicity (38).

Yeast transformations were performed using the LiOAc method (16). Tetrad analysis and other yeast manipulations were done according to standard techniques (21). Slow growth phenotypes of *ada* mutants were assayed on synthetic dextrose (SD) minimal medium supplemented with only the necessary

amino acids and adenine (Difco). Otherwise, strains were grown on synthetic complete (SC) medium containing all amino acids except those needed for plasmid selection.

ADA5 cloning and plasmids. To clone the wildtype *ADA5* gene, GMy37p, the *ada5-1* mutant, was transformed with a yeast genomic library (55) and colonies that grew as well as wild type were selected. From these, a clone p3,1 was isolated with an 8.5 kb insert that restored wild type growth and sensitivity to GAL4-VP16 toxicity. p3,1 was subject to partial digestion with *Sau3AI* and 1-3 kb fragments were isolated and ligated into *Bam*HI site of pRS316 (51) to create a subgenomic library. GMy37p was transformed with this subgenomic library, and colonies that grew as well as wild type were again selected. Two truncated subclones were isolated for further study: pL1B1 (with a 2.2 kb insert) which partially complements the slow growth phenotype of GMy37p and pL1G1 (with a 1.8 kb insert) which fully complements the slow growth phenotype. Both subclones complement the toxicity resistance phenotype of *ada5-1*. Sequence analysis (see below) revealed that pL1B1 is lacking the upstream sequences of *ADA5* as well as the region encoding the first 10 amino acids. Presumably, the remaining region of *ADA5* is expressed from a cryptic promoter in the vector and an internal methionine in the *ADA5* coding sequence. pL1G1 is a C-terminal *ADA5* truncation, containing approximately 500 bp of upstream sequences and 1368 bases of the coding region, which encodes residues 1-456.

pRS316-*ADA5*, a subclone with the entire *ADA5* coding region, was created in several stages. The *Bst*XI site in pRS316 (51) was destroyed by digesting, blunting with T4 polymerase and ligating to create pRS316-*Bst*XI. A 1.9 kb *Eco*RI fragment containing the sequences encoding the first 437 amino acids of *ADA5* as well as the upstream sequences was cloned into the *Eco*RI site to create pRS316-*ADA5*_{437a} and pRS316-*ADA5*_{437b}. pRS316-*ADA5*_{437a} is oriented such that the

*Bst*XI site at the 5' end of *ADA5* is proximal to the *Sac*I site in the polylinker. pRS316-*ADA5*_{437b} is in the other orientation. A 1.9 kb *Bst*XI -*Hind*III fragment from pL1B1, was cloned into the *Bst*XI *Hind*III sites of pRS316-*ADA5*_{437a} to create pRS316-*ADA5*. A 2.6 kb *Xho*I *Not*I fragment from pRS316-*ADA5* was cloned into pRS315 (51) cut with *Xho*I and *Not*I to create pRS315-*ADA5*.

The *ADA5* coding sequence was amplified using PCR (Perkin Elmer) with primers *ADA5N* (CCCGGGAGATCTGCGCCGCAATGAGTGCCAATAGCCCGACAGG) and *ADA5C* (CCCGGGGGATCCGCGGCCGCCTAAGATCTTGACATTGTAGTAGAA GAGGGCG). The resulting fragment was digested with *Not*I, and cloned into the *Not*I site of pDB20L (4) to form pDB20L-*ADA5*. pBluescript-*ADA5* was generated by cloning a 2.2 kb *Bam*H1 *Hind*III fragment from pL1B1 into Bluescript KS+ (Stratagene) cut with *Bam*H1 and *Hind*III.

To show that the cloned gene corresponds to the *ada5-1* mutation, pRS306-*ADA5* was generated by cloning a 1.8 kb *Xba*I fragment from p3,1 into the *Xba*I site of a version of the *URA3* integrating vector pRS306 (51) with the *Eco*RI site destroyed. pRS306-*ADA5* was cut with *Eco*R1 (a unique site in the 1.8 kb insert) and transformed into PSy316. A colony with an integration at the cloned locus was mated to GMy37p, and the resulting diploid was sporulated. In each of five tetrads dissected, two spores gave rise to slow growing Ura- colonies, and two spores gave rise to Ura+ colonies with wild type growth, showing that the cloned gene maps to the *ada5-1* mutant locus.

***ADA5* sequencing.** All sequencing was done using the Sequenase kit (USB). A deletion series from the *NOTI* site of pL1B1 was generated with the *Exo*III and *Exo*VII enzymes and sequenced with the -20 primer. The sequence of the other strand was obtained by using the T3 primer to sequence a second deletion series from the *Kpn*I site of pL1B1, or from sequence specific primers.

Since pL1B1 does not fully complement the *ada5-1* mutant, and the largest open reading frame on pL1B1 was found to be open at the 5' end, the other subclone, pL1G1, was partially sequenced. Sequence obtained using the -20 primer in pL1G1 showed that it is lacking sequences after residue +1368 in the putative *ADA5* open reading frame, and thus encodes a C-terminal truncation of *ADA5*. Using a sequence specific primer, it was determined that the incomplete open reading frame identified in pL1B1 continues for an additional thirty base pairs in pL1G1. To confirm that the *ADA5* open reading frame is continuous across the truncation junctions present in pL1B1 and pL1G1, the full length *ADA5* genomic clone (p3,1) was used to sequence across these junctions using sequence specific primers.

***ADA5* deletion plasmid and strains.** The *ADA5* deletion plasmid was created in several steps. A 550 bp *XhoI* blunted *BstXI* fragment from pL1G1 containing sequence encoding the first 12 amino acids of *ADA5* and 5' flanking sequence was cloned into the *XhoI* and *EcoRV* sites of pBluescript KS+ (Stratagene) to form pBluescript-A5BstX. Next, a 2.4 kb *BamHI BglII* fragment containing the *hisG URA3* cassette from pNKY51 (1) was cloned into the *BamHI* site of pBluescript-A5BstX. A clone, pADA5nko, was chosen such that the reformed *BamHI* site was distal to the *ADA5* 5' sequence. Finally, pBluescript-*ADA5* was cut with *DraIII*, ligated to a *NotI* linker (New England Biolabs), and cut with *NotI* and *BstYI*. The 400 bp *BstYI NotI* fragment containing sequences encoding the C-terminal 136 amino acids and 3' flanking sequence was cloned into the *BamHI NotI* site of pADA5nko to form pADA5KO. This plasmid will delete the coding sequence for 437 amino acids from the N-terminus of *ADA5*, which should produce a null phenotype.

ADA5 deletion strains ($\Delta ada5$) were generated by transforming yeast with pADA5KO cut with *XhoI NotI*. Slow growing Ura⁺ transformants were tested for

resistance to GAL4-VP16, and mated to previously characterized *ada5* mutant strains of the opposite mating type when available. Strains that were resistant to GAL4-VP16 were grown on 5-fluoroorotic acid (5-FOA) to select for strains that had looped out the *URA3* sequence. Ura⁻ derivatives were transformed with pRS316-ADA5 to confirm that wild type growth and sensitivity to GAL4-VP16 were restored by the *ADA5* clone. In this manner, the Ura⁺ and Ura⁻ deletion strains GMy29 and GMy30 were generated in the parent strain BWG1-7a; GMy31 and GMy32 in BP1; and GMy33 and GMy34 in PSy316.

The $\Delta ada2\Delta ada5$ and the $\Delta ada3\Delta ada5$ double deletion strains were generated in the following manner. GMy30 containing the plasmid pDB20L-ADA5, was transformed with pADA2KO (4) cut with *Bam*HI and *Xho*I, or pADA3KO cut with *Pvu*II and *Bam*HI (38). *ada2* or *ada3* deletion strains were identified by mating slow growing transformants to $\Delta ada2$ or $\Delta ada3$ strains. Strains that failed to complement the cognate Δada strain were grown on 5-FOA to select strains that had looped out the *URA3* sequence.

The resulting Ura⁻ derivatives were grown on YPD plates and replica plated to identify strains that had lost the *LEU2* plasmid containing the *ADA5* clone. GMy36 (BWG1-7a $\Delta ada2\Delta ada5$) can only be restored to wildtype growth by transformation with both the *ADA5* (pRS315-ADA5) and *ADA2* clones (pNS3.8, (4)), confirming that it is a double mutant. Similarly, GMy38 (BWG1-7a $\Delta ada3\Delta ada5$) was confirmed by transforming with the *ADA5* and the *ADA3* (pADA3HHV, (40)) clones.

ADA5 expression, antisera and Western Blot analysis. pET15b-ADA5, an *ADA5* expression vector, was created by exploiting the single *Bam*H1 site in pL1B1, created when the 2.2 kb *Sau*3AI fragment from p3,1 was cloned into the *Bam*H1 site of pRS316. This *Bam*H1 site, derived from the *Sau*3AI site at position +28 in the *ADA5* coding sequence, is in frame with the *Bam*H1 site of pET15b

(Novagen), a vector that fuses six histidine residues at the N-terminus of expressed proteins. pL1B1 was digested with *DraIII*, ligated with a *BamHI* linker (New England Biolabs), and then digested with *BamHI*. The resulting 2.2 kb fragment was cloned into the *BamHI* site of pET15b (Novagen) to form pET15b-ADA5. In the bacterial strain BL21 (Novagen), this plasmid produces insoluble ADA5, which was purified in urea on a Nickel column (Qiagen).

Polyclonal rabbit ADA3 or ADA5 antiserum was generated using standard procedures (23). For ADA5, two rabbits were immunized with lyophilized acrylamide slices containing 400 µg ADA5 resuspended in saline followed by six boosts, three containing 200 µg ADA5 and three boosts containing 100 µg. IgGs were purified from serum on a protein A column (23). Antisera to ADA3 was generated by injecting rabbits 6 times with 0.5 mg of a fragment of ADA3, ADA3(580-702), in RIBI adjuvant (RIBI ImmunoChem Research, Inc.). Expression and purification of ADA3(580-702) have been previously described (30). Antisera was purified by binding and eluting from immunoblots of ADA3(580-702) (23).

Westerns Blots were performed as follows: SDS-PAGE was performed on yeast extracts or recombinant proteins were run in the absence of a reducing agent to eliminate a contaminating keratin band that co-migrates with ADA5. Proteins were transferred to PVDF (DuPont) using a milliblot graphite electroblotter (Millipore), and filters were probed with using standard techniques (23) with affinity purified anti-ADA3 sera or purified anti-ADA5 IgG. Proteins were visualized using a horse radish peroxidase conjugated secondary antibody with the ECL system(Amersham).

Fractionation and Immunoprecipitation of yeast extracts. BWG1-7a was grown in 5 liters of YPD to an optical density at 600 nm of approximately 4. GMy30 was grown in 10 liters of YPD to an optical density of approximately 2.

This yielded a wet weight of 35 grams of cells each. Preparation of yeast whole cell extract (WCE) and fractionation using a Bio-Rex 70 (Bio-Rad laboratories) column was performed as described elsewhere(49, 55) with the following modifications. 1.5 cm x 7 cm Bio-Rex 70 columns were used and protein was eluted from the columns successively with buffer A (49) containing 250 mM, 600 mM, and 1.2 M potassium acetate.

Immunoprecipitation experiments were performed on the 600 mM elution fractions from the Bio-Rex 70 columns. Antibody from crude preimmune sera and from crude anti-ADA5 sera were crosslinked to protein A-Sepharose beads (Sigma) as previously described (6, 23). Comparing antibody eluted off of the protein A beads before and after crosslinking by boiling, PAGE, and staining with Coomassie brilliant blue demonstrated that equal amounts of antibody were crosslinked from both sera. Beads were washed several times with 100 mM glycine (pH 2.5) to remove uncrosslinked antibody, then washed several times and equilibrated in IP buffer (6) with 0.1 M potassium acetate.

Immunoprecipitations were performed as described (6). Briefly, 100 µg of the 600 mM fraction from BWG1-7a or 150 µg of the 600 mM fraction from GMy30 were precleared with protein A-Sepharose beads and then incubated with antibody crosslinked beads by rotating at 4° C for three hours. Beads were sedimented and washed four times with 1 ml IP buffer with 0.1 M potassium acetate. Bound proteins were then eluted from the beads by two successive elutions with 12 µl of 100 mM glycine (pH 2.5). The elutions were pooled and analyzed by Western blot.

GST-VP16 precipitations. The *ADA5 in vitro* transcription/translation plasmid pCITE2b-ADA5 was generated by cloning the 2.2 kb *Bam*HI fragment containing the *ADA5* coding sequence and 3' sequence from pET15b-ADA5 into the *Bam*HI site of pCITE2b (Novagen). *In vitro* translations were performed as previously described (30).

Precipitations experiments were performed by loading 10 μg of GST-VP16(413-490), GST-VP16FA442,FA475, or GST-VP16 Δ (413-456) on 10 μl glutathione-Sepharose beads (Pharmacia) that were preblocked in *E.coli* extract. 10 μl *in vitro* translated ADA5 and 200 μl S3001%T buffer (20 mM HEPES pH 7.6, 300 mM Potassium acetate, 25 mM Magnesium acetate, 1% Triton X-100, 20% glycerol, plus 1mg/ml *E. coli* extract) were added to the GST-VP16 beads and incubated for one hour at 4°C. Samples were washed four times with 1 ml S300 1%T buffer, eluted from the beads with 20 mM reduced glutathione (Sigma) in S300 buffer, and electrophoresed on an SDS-polyacrylamide gel. The gel was dried and exposed on XAR film (Kodak). GST-VP16 and GST-VP16 Δ expression constructs and purification techniques were described previously(37). GST-VP16FA442FA475 was made by cloning a *Bam*HI *Sph*I fragment from pMSVP16FA442/FA475 (42) into the *Bam*HI and *Sph*I sites of pGVP (the GST-VP16 expression plasmid (37)).

Gel shifts and β -galactosidase assays. β -galactosidase assays were carried out on glass bead yeast extracts (46). The activity of β -galactosidase was normalized to total protein. Gel shift analysis was performed as previously described (4).

Nucleotide sequence accession number. The GenBank accession number for the *ADA5* sequence is U43153.

Results

Properties of the *ada5-1* mutant. A screen for relief of GAL4-VP16 induced toxicity (4) identified multiple alleles of *ADA2*, *ADA3* and *GCN5* and a single allele of *ADA5*, *ada5-1*(38). The *ada5-1* mutant grows slowly on rich as well as minimal media. This is in contrast to the other *ada* mutants, which have a pronounced slow growth phenotype only on minimal media (38). This slow

growth phenotype segregated 2 slow:2 wild type in six complete tetrads, and co-segregated with resistance to GAL4-VP16 (data not shown).

ADA2, *ADA3* and *GCN5* mutants survive GAL4-VP16 toxicity by reducing the ability of GAL4-VP16 to activate transcription, rather than reducing the level of the toxic protein (4, 38, 40). Since GAL4-VP16 is toxic to wildtype cells, we assayed levels of a less toxic derivative, GAL4-VP16FA, expressed on a low copy plasmid also under control of the *ADH1* promoter, by gel shift (4). Compared to wildtype, less of the GAL4-VP16 specific complex was found in the *ada5* mutant (data not shown). Thus, unlike the other ADA genes studied to date, it is possible that ADA5 escapes toxicity by reducing levels of GAL4-VP16.

Cloning, mapping and sequencing of ADA5. *ADA5* was cloned by complementation of the slow growth phenotype of the *ada5-1* mutant (see Materials and Methods). The *ADA5* clone has the ability to restore wild type growth and sensitivity to GAL4-VP16 to the *ada5* mutant strain (data not shown). To confirm that the clone indeed corresponds to the *ADA5* gene, we directed integration of *URA3* in plasmid pRS306-*ADA5* to the cloned locus in a wild type strain (Materials and Methods). This strain was mated to the *ada5-1* strain, the diploid was sporulated, and tetrads were dissected. In all five tetrads, two spores grew slowly and were Ura⁻, and two spores grew normally and were Ura⁺ showing linkage between the clone and the *ada5-1* mutation.

In order to map *ADA5* to the yeast physical map, a fragment from the gene was radiolabeled and hybridized to a phage grid representing over 90% of the yeast genome (43). Two overlapping clones were identified, showing that *ADA5* maps to the right arm of Chromosome XV.

ADA5 was further subcloned and sequenced (Materials and Methods). The gene encodes a novel protein with 604 amino acids and a predicted molecular weight of 68kD. The *ADA5* protein contains two glutamine rich regions, several

Ser/Thr rich regions, a proline rich region, an acidic region, and a basic region (Figure 1). As a final confirmation that the open reading frame we identified corresponds to *ADA5*, the open reading frame was amplified using the Polymerase Chain Reaction (PCR) and placed under the control of the *ADH1* promoter (see Materials and Methods). This plasmid complements the *ada5-1* mutation as well as the genomic clone.

Characterization of *ADA5* deletion mutants. *ada5* deletion mutants ($\Delta ada5$) were constructed by homologous recombination as described in the Materials and Methods. The deletion mutant is viable but grows more slowly than the *ada5-1* mutant (Figure 2) and more slowly than *ada2*, *ada3*, or *gcn5* deletion mutants (not shown). Like other *ada* mutants, the *ada5* mutant is temperature sensitive for growth and completely resistant to GAL4-VP16. However, unlike the other *ada* mutants, the *ada5* mutant is auxotrophic for inositol (data not shown).

Analysis of an amino-terminal fragment of *ADA5* for complementation of cell growth and toxicity by GAL4-VP16. In the process of subcloning *ADA5*, we discovered that a portion of the *ADA5* coding sequence could be deleted without loss, or with only partial loss of the ability to complement an *ada5* mutant strain (Materials and Methods). As shown in Figure 3a and 3b, *ADA5*₄₃₇, the first 437 amino acids of *ADA5*, can complement a $\Delta ada5$ strain for growth on rich medium and partially complement for growth on minimal medium. However, *ADA5*₄₃₇ strains remain resistant to GAL4-VP16 (Figure 3c). These findings show that the *ADA* fragment 1-437 complements the growth phenotype but does not restore the toxicity of GAL4-VP16. It is thus possible that the deletion in *ADA5* separates two functions of the gene product. Alternatively, the observed properties of the deletion may result from a quantitative reduction in *ADA5* activity.

***In vivo* activation defects of *ada5* strains.** The lower levels of GAL4-VP16FA in *ada5* mutants may indicate that *ADA5* is required for activation at the *ADH1* promoter. To determine whether the *ADH1* promoter or other yeast promoters require *ADA5* for activation, we introduced β -galactosidase reporter plasmids under the control of eight different yeast UAS sequences into the wild type, the *ada5-1* mutant, and the *ada5* deletion strains.

The activity of these reporters, as measured by β -galactosidase assays, is shown in Table 1. The activities of the *HO* and *INO1* promoters are the most drastically reduced. The activity of the GCN4-responsive *HIS66* and *14x2* promoters, known to require *ADA2/ADA3/GCN5* (4, 38, 40), are also severely reduced in the *ada5* mutant and deletion strains. The *CYC1* UAS1 promoter, which is known to be independent of the other ADAs, is reduced three fold in the *ada5-1* mutant, and 10 fold in the *ada5* deletion strain. Interestingly, the activities of the *CYC1* UAS2, *ADH1*, and the *GAL1-10* promoters, which are not affected by *ada2* mutations, are reduced only 3-5 fold in the *ada5* deletion.

In summary, the *ada5* deletion strain has a broad defect in transcription affecting all of the promoters tested. However, there may be a relationship between the spectrum of effects in *ada5* versus *ada2* mutants. In particular, the *SD5*, *ADH1*, and UAS2 promoters, which are the least affected in the *ada5* mutants, are promoters that are not affected at all in *ada2* mutants (4, 40). The reduction in activation of the *ADH1* and *INO1* promoters explains the novel phenotypes of *ada5* mutants; a small reduction in GAL4-VP16 levels and inositol auxotrophy.

Double mutants between *ADA5* and other *ADA* genes. The above findings raise the question of whether *ADA5* functions in the same pathway as the other *ADA* genes. To begin to address this issue, we constructed double mutants between *ada5* and *ada2* or *ada3* mutants (Materials and Methods). We found that both $\Delta ada2\Delta ada5$ and $\Delta ada3\Delta ada5$ double deletion mutants are viable and grow

no more slowly than $\Delta ada5$ single mutants (Figure 4 and data not shown). This shows that *ADA5* is not solely responsible for activation in the absence of *ADA2* or *ADA3*, and suggests that *ADA5* may operate in the same pathway as the other *ADA* genes. In the Discussion we speculate how *ADA5* may operate in the same pathway as the other *ADA* genes, yet have a more general function in transcription.

Specific binding of ADA5 to functional VP16 activation domains. We began a biochemical analysis of *ADA5* by studying whether the protein bound to the activation domain of VP16. *ADA5* was translated *in vitro*, radiolabeled, and added to glutathione-Sepharose beads coupled to GST-VP16. After extensive washing, the GST fusion was eluted from the beads with glutathione, and the eluent electrophoresed on an SDS polyacrylamide gel. Autoradiography of the gel indicated a band the size of the *ADA5* output protein, showing that the protein bound to the beads (Figure 5). Two mutant VP16 proteins were also fused to GST and used as controls. The first contained a deletion of residues 457-490 of VP16, leaving intact residues 413-456. This VP16 derivative shows greatly reduced activity in yeast cells (52). The second mutant changes only the Phe residues at positions 442 and 475 to Ala. This mutant shows a large reduction in activity in mammalian cells (42). In both cases, the mutations abolished the binding of *ADA5*. Following autoradiography, Coomassie staining of the gel showed that the same amount of all three GST fusion proteins were bound to and eluted from the beads (data not shown). This experiment shows that *ADA5* binds to the activation domain of VP16 but not to non-functional mutant domains. Models for *ADA5* function must take into account this activity.

Cofractionation of ADA5 and ADA3. Genetic and biochemical findings summarized in the Introduction indicate that *ADA2*, *ADA3*, and *GCN5* function together in a complex (30, 38). Because the growth phenotypes of $\Delta ada5\Delta ada2$ and

Δada5Δada3 double mutants suggest that *ADA5* may be acting in the same pathway as *ADA2*, *ADA3* and *GCN5*, we examined whether *ADA5* co-purifies with a member of the ADA complex, *ADA3*.

The first step in purification of the ADA complex (which will be described in detail elsewhere) was to chromatograph yeast whole cell extract on a Bio-Rex 70 cation exchange column. We assayed the flow-through and three salt elution fractions from this column by Western blot analysis for *ADA5* and *ADA3*. As shown in Figure 6A, *ADA3* and *ADA5* eluted primarily in the 600 and 1200 mM KAc fractions in very similar proportions. *ADA2* and *GCN5* also elute in the 600 mM and 1200 mM fractions with a profile identical to *ADA3* and *ADA5* (53). As a control, an extract from a *Δada5* strain was also fractionated, clearly showing that the indicated band is *ADA5*.

To obtain more direct evidence for the association between *ADA3* and *ADA5*, the 600 mM fraction from the Bio-Rex column was immunoprecipitated with anti-*ADA5* or preimmune serum. As a control, this immunoprecipitation was also performed using the 600 mM fraction of the *ada5* deletion extract. Western analysis of the precipitates with anti-*ADA3* (Figure 6B) shows that *ADA3* was precipitated using anti-*ADA5* but not preimmune serum. Moreover, *ADA3* was not precipitated from the extract missing *ADA5*. Note, the lanes labeled starting material were loaded with the same amount of total protein. The observation that less *ADA3* is detected in the *Δada5* extract may indicate that less *ADA3* is present in the absence of *ADA5*. We conclude from these findings that *ADA5* and *ADA3* are physically bound to one another in a complex in yeast extracts.

Identification of *ADA5* as *SPT20*. By comparing sequences, it was determined that *ADA5* is identical to *SPT20* (45) a newly isolated *SPT* gene in the TBP class (12, 22). *spt20* mutants, like the other *spt* mutants of this class, have

pleiotropic mating, growth and sporulation defects. Furthermore, transcription of Ty1 and other yeast genes is compromised. Thus, unlike the other *ada* mutants (29, 45), *ada5* mutants also have an Spt⁻ phenotype.

Ada⁻ phenotype in two SPT15 (TBP) mutants. We examined *spt3, 7, 8, 15* and *20* mutants (all in the TBP class), as well as *spt5, 6, and 4* for resistance to GAL4-VP16 mediated toxicity (the Ada⁻ phenotype). As shown in Table 2, the *spt20* mutant (as expected) and the *spt7* mutant were completely resistant to GAL4-VP16. As in *ada5* mutants, *spt7* mutants show reduced activation of the ADH1 promoter (44) and we can not rule out that this is the basis of the resistance. Interestingly, other *spt* mutants, including *spt 3* and *8* in the TBP class, were sensitive to GAL4-VP16. Roberts and Winston find that *spt7* and *spt20* have very similar phenotypes, including inositol auxotrophy and slow growth on rich media, that are not shared by *spt8* or *spt3* (45).

Two *spt15* alleles (TBP), *spt15-21* and *spt15-122*, showed partial resistance to GAL4-VP16 (Table 2). Interestingly, *spt15-21*, the most resistant *spt15* allele, also causes inositol auxotrophy (45). Because of the possible pleiotropic nature of *spt15* mutations, resistance to GAL4-VP16 could be due to reduced levels of the toxic chimera in the mutant strains. To address this issue, levels of GAL4-VP16 were determined in both the *spt15-21* and *spt15-122* strains by gel shift analysis (Figure 7). Levels in the *spt15-122* strain were indistinguishable from the wild type, while levels in the *spt15-21* were somewhat higher than wild type. In addition, for reasons that are not clear, the form of the chimera which corresponds to full length (i.e. the unproteolytically cleaved form) is favored in the *spt15-21* extract. Thus, the Ada⁻ phenotype of these mutants is not due to a reduction in levels of GAL4-VP16. These findings provide the first genetic clue that the ADA functional pathway includes a basal factor, TBP, and is consistent with *in vitro* findings (2) (see Discussion).

Discussion

Here, we report the cloning and initial characterization of *ADA5*, a novel gene selected by resistance to GAL4-VP16-mediated toxicity. Mutants in *ADA5* display some phenotypes that are distinct from mutants in the previously described genes *ADA2*, *ADA3*, and *GCN5*, which arose from the same selection. First, $\Delta ada5$ strains show reduced levels of GAL4-VP16, unlike *ada2*, *ada3* or *gcn5* deletion strains. Second, *ada5* mutants grow more slowly on rich and minimal medium than mutants of other *ADA* genes. Third, *ada5* strains are inositol auxotrophs and have Spt⁻ phenotypes. Fourth, promoters that function independently of *ADA2*, such as *UAS1* and *ADH1* are dependent on *ADA5*. The effects on the *ADH1* promoter account for the reduction in GAL4-VP16 levels.

Although *ADA5* appears to be distinct from the other *ADA* genes, some phenotypes suggest they may all function in the same pathway. The activators that require *ADA2* for activation are also dependent on *ADA5*. GCN4 mediated activation which is highly *ADA2* dependent (40), is also *ADA5* dependent. HAP4 and GAL4 mediated activation, which are only slightly *ADA2* dependent (40), are less *ADA5* dependent than most of the other activators tested (Table 1, activities of *UAS2* and *SD5*). Moreover, double mutants of *ada5* and either *ada2* or *ada3* have growth phenotypes no more severe than *ada5* single mutants. If the *ADA2* complex and *ADA5* work through different activation pathways, then we would expect these double mutants to have a more severe growth defect than either of the single mutants. These genetic data suggest that *ADA5* and *ADA2/ADA3/GCN5* function in the same pathway of transcriptional activation in cells.

Biochemical studies provide direct evidence that ADA5 is a part of the ADA complex. Fractionation of a yeast extract on a Bio-Rex 70 column shows cofractionation of ADA5 and ADA3. Further, immunoprecipitation of ADA5 coprecipitates ADA3. If an extract from a $\Delta ada5$ strain is used as a control in this experiment, no ADA3 is precipitated.

ADA5 as an SPT gene. The more general effects of *ada5* mutations on transcription is underscored by its identity with *SPT20* (45). It is intriguing that this gene influences promoter selection when two promoters are in competition, as well as transcriptional activation (as do other *ADA* genes). The identity of *ADA5* and *SPT20* prompted us to examine a panel of *SPT* mutants for the *Ada*⁻ phenotype, i.e. resistance to GAL4-VP16. *spt7* mutants were also resistant and, like *spt20* mutants, their resistance was complete. Other *spt* mutants, including *spt 3* and *8*, in the TBP phenotypic class, were sensitive. These findings suggest the possibility that SPT7 and SPT20 function together in the transcription process, a surmise also indicated by the findings of Roberts and Winston (45).

SPT15 (TBP) mutants show resistance to GAL4-VP16. We also observed that two *spt15* mutants were partially resistant to GAL4-VP16. This result provided the first possible genetic link between the ADA pathway of transcriptional activation and a basal factor, namely TBP. To further characterize this finding, we showed that the resistance of these *spt15* mutants did not arise from a reduction in levels of the toxic chimera. The similarity in phenotypes between *spt7*, *ada5*, and *spt15* alleles, including inositol auxotrophy and toxicity-resistance, suggests that the target of the ADA activation pathway could be TBP. Our genetic findings are consistent with the earlier biochemical findings of Barlev et al., (2) who also proposed that ADA2/3/GCN5 may contact the basal machinery by binding to TBP. They found that TBP in yeast extracts from a wild type strain bound to a VP16 column, while TBP from *ada2* mutant extracts did not.

We suggest a pathway which leads from the activation domain of transcriptional activators to a complex, ADA2/ADA3/GCN5/ADA5, and to TBP. Several findings are consistent with this model. First, ADA2 has been shown to bind to the activation domain of VP16 (2, 52), GCN4 (Barlev et al., 1995), and ADR1 (8). We show here that ADA5 binds to VP16 and that amino acid substitutions in VP16 can abolish this binding. Second, *ada5* (and *spt7*) mutations can affect promoter selection (i.e. have Spt⁻ phenotypes) suggesting that TBP may be a target of the ADA complex. Third, mutations in *SPT15* (TBP) can give rise to partial resistance to GAL4-VP16. This finding, along with the earlier biochemical data (2), also indicates that TBP might be the ultimate target of ADA mediated activation.

How can *ada5* mutants share some phenotypes with *ada2*, *ada3*, and *gcn5* mutants, yet exert a broader range of effects? We suggest the possibility that ADA5 (and possibly SPT7) retain a partial activity in the absence of ADA2, ADA3, and GCN5. This activity may suffice in *ada2* mutants for transcription of *ADH1*, *INO1*, and perhaps other genes. Deletion of *ADA5*, by this model, would destroy all activity of the complex, and give rise to a broader spectrum of phenotypes (i.e. Spt⁻, Ino⁻, and slower growth).

In summary, we present the analysis of a novel gene, *ADA5*, that shares phenotypes of two broad classes of yeast genes affecting transcription, the *ADAs* and the *SPTs*. It will be interesting to see how this genetic connection between these different classes of factors reflects the physical arrangement of the coactivators at promoters.

Acknowledgements

We would like to thank S. Roberts and F. Winston for the *spt* strains and the sharing of unpublished results; W. Gao for pCP0, C. Peterson for pCP8, the Young lab for the INO1 reporter, S. Triezenberg for VP16FAFA plasmids, S. Treadway and R. Kindt for comments on the manuscript, and Y. Clark for the endnote library. This work was supported by NIH grant #GM 50207 to LG, NIH predoctoral grants to GM, JH and NS, and a HHMI predoctoral training grant to JH.

References

1. **Alani, E., L. Cao and N. Kleckner.** 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics*. **116**:541-545.
2. **Barlev, N. A., R. Candau, L. Wang, P. Darpino, N. Silverman and S. L. Berger.** 1995. Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein. *J. Biol. Chem.* **270**:19337-19334.
3. **Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg and L. Guarente.** 1990. Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell*. **61**:1199-1208.
4. **Berger, S. L., B. Piña, N. Silverman, G. A. Marcus, J. Agapite, J. L. Regier, S. J. Triezenberg and L. Guarente.** 1992. Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell*. **70**:251-265.
5. **Brandl, C. J., A. M. Furlanetto, J. A. Martens and K. S. Hamilton.** 1993. Characterization of NGG1, a novel yeast gene required for glucose repression of GAL4p-regulated transcription. *EMBO J.* **12**:5255-5265.
6. **Cairns, B. R., Y.-J. Kim, M. H. Sayre, B. C. Laurent and R. D. Kornberg.** 1994. A multisubunit complex containing the *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* gene products isolated from yeast. *Proc. Natl. Acad. Sci. USA*. **91**:1950-1954.
7. **Cote, J., J. Quinn, J. L. Workman and C. L. Peterson.** 1994. Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science*. **265**:53-60.

8. **Denis, C.** Personal communication.
9. **Dynlacht, B. D., T. Hoey and R. Tjian.** 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell*. **66**:563-576.
10. **Eisenmann, D., C. Chapon, S. Roberts, C. Dollard and F. Winston.** 1994. The *Saccharomyces cerevisiae* *SPT8* gene encodes a very acidic protein that is functionally related to SPT3 and TATA-binding protein. *Genetics*. **137**:647-657.
11. **Eisenmann, D. M., K. M. Arndt, S. L. Ricupero, J. W. Rooney and F. Winston.** 1992. SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae*. *Genes Dev.* **6**:1319-31.
12. **Eisenmann, D. M., C. Dollard and F. Winston.** 1989. *SPT15*, the gene encoding the yeast TATA binding factor TFIID, is required for normal transcription initiation *in vivo*. *Cell*. **58**:1183-1191.
13. **Forsberg, S. L. and L. Guarente.** 1989. Identification and characterization of HAP4: a third component in the CCAAT-bound HAP2/HAP3 heteromer. *Genes Dev.* **3**:1166-1178.
14. **Gansheroff, L., C. Dollars, P. Tan and F. Winston.** 1995. The *Saccharomyces cerevisiae* *SPT7* gene encodes a very acidic protein important for transcription *in vivo*. *Genetics*. **139**:523-536.
15. **Ge, H. and R. Roeder.** 1994. Purification, cloning, and characterization of a human coactivator, PC-4, that mediates transcriptional activation of class II genes. *Cell*. **78**:513-523.
16. **Gietz, D., A. St. Jean, R. A. Woods and R. H. Schiesti.** 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20**:1425.

17. **Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon and R. Tjian.** 1993. Drosophila TaffII 40 Interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell.* **75:**519-530.
18. **Guarente, L.** 1992. Mechanism and regulation of transcriptional activation in Eukaryotes: conserved features from yeast to humans. *Transcriptional Regulation.* McKnight and Yamamoto ed. Cold Spring Harbor Laboratory Press.
19. **Guarente, L., B. Lalonde, P. Gifford and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene. *Cell.* **36:**503-511.
20. **Guarente, L. and T. Mason.** 1983. Heme regulates transcription of the *CYC1* gene of *S. cerevisiae* via an upstream activation site. *Cell.* **32:**1279-1286.
21. **Guthrie, C. and G. R. Fink.** 1991. Guide to Yeast Genetics and Molecular Biology in *Methods in Enzymology*, eds. Abelson and Simon. Academic Press, Inc. Harcourt Brace Jovanovich: San Diego.
22. **Hahn, S., S. Buratowski, P. A. Sharp and L. Guarente.** 1989. Isolation of the gene encoding the yeast TATA binding protein TFIID: a gene identical to the *SPT15* suppressor of Ty element insertions. *Cell.* **58:**1173-1181.
23. **Harlow, E., and Lane, D.** 1988. *Antibodies: A laboratory manual.* Cold Spring Harbor Laboratory: Cold Spring Harbor, New York.
24. **Haviv, I., D. Vaizel and Y. Shaul.** 1995. The X protein of hepatitis B virus coactivates potent activation domains. *Mol Cell Biol.* 1079-1085.
25. **Hengartner, C. J., C. M. Thompson, J. Zhang, D. M. Chao, S. M. Liao, A. J. Koleske, S. Okamura and R. A. Young.** 1995. Association of an activator with an RNA polymerase II holoenzyme. *Genes Dev.* **9:**897-910.

26. **Hinnebusch, A. G., G. Lucchini and G. Fink.** 1985. A synthetic *HIS4* regulatory element confers general amino acid control on the cytochrome c gene (*CYC1*) of yeast. *Proc. Natl. Acad. Sci. USA.* **82**:498-502.
27. **Hirschhorn, J. N., S. A. Brown, C. D. Clark and F. Winston.** 1992. Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev.* **6**:2288-98.
28. **Hoey, T., B. D. Dynlacht, M. G. Peterson, B. F. Pugh and R. Tjian.** 1993. Molecular cloning and functional analysis of *Drosophila* TAF110 reveal properties expected of coactivators. *Cell.* **72**:247-260.
29. **Horiuchi, J. and L. Guarente.** unpublished results.
30. **Horiuchi, J., N. Silverman, G. Marcus and L. Guarente.** 1995. ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. *Mol. Cell. Biol.* **12**:1203-1209.
31. **Kelleher, R. J. I., P. M. Flanagan and R. D. Kornberg.** 1990. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell.* **61**:1209-1215.
32. **Kim, Y. J., S. Björklund, Y. Li, M. H. Sayre and R. D. Kornberg.** 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell.* **77**:599-608.
33. **Koleske, A. J., S. Buratowski, M. Nonet and R. A. Young.** 1992. A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. *Cell.* **69**:883-894.
34. **Koleske, A. J. and R. A. Young.** 1994. An RNA polymerase II holoenzyme responsive to activators. *Nature.* **368**:466-469.

35. **Kretzschmar, M., K. Kaiser, F. Lottspeich and M. Meisterernst.** 1994. A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. *Cell*. **78**:525-534.
36. **Kwok, R. P. S., J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. E. Roberts, M. R. Green and R. H. Goodman.** 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature*. **370**:223-226.
37. **Lin, Y. S., and Green, M. R.** 1991. Mechanism of action of an acidic transcriptional activator *in vitro*. *Cell*. **64**:971-981.
38. **Marcus, G. A., N. Silverman, S. L. Berger, J. Horiuchi and L. Guarente.** 1994. Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. *EMBO J*. **13**:4807-4815.
39. **Neigeborn, L. and M. Carlson.** 1984. Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. *Genetics*. **108**:845-858.
40. **Piña, B., S. Berger, G. A. Marcus, N. Silverman, J. Agapite and L. Guarente.** 1993. *ADA3*: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of *ADA2*. *Mol. Cell. Biol*. **13**:5981-5989.
41. **Pugh, B. F. and R. Tjian.** 1990. Mechanism of transcriptional activation by Spt1: evidence for coactivators. *Cell*. **61**:1187-1197.
42. **Regier, J. L., F. Shen and S. J. Triezenberg.** 1993. Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. *Proc. Natl. Acad. Sci. USA*. **90**:883-887.
43. **Riles, L., J. E. Dutchik, A. Baktha, B. K. McCauley, E. C. Thayer, M. P. Leckie, V. V. Braden, J. E. Depke and M. V. Olson.** 1993. Physical maps of the six smallest chromosomes of *Saccharomyces cerevisiae* at a resolution of 2.6 kilobase pairs. *Genetics*. **134**:81-150.

44. **Roberts, S. and F. Winston.** Personal communication.
45. **Roberts, S. M. and F. Winston.** 1996. *SPT20/ADA5* encodes a novel protein functionally related to the TATA-binding protein and important for transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**:3206-3213.
46. **Rose, M. and D. Botstein.** 1983. Structure and function of the yeast *URA3* gene: Differentially regulated expression of hybrid β -galactosidase from overlapping coding sequences in yeast. *J. Mol. Biol.* **170**:883-904.
47. **Santangelo, G. M., J. Tornow, C. S. McLaughlin and K. Moldave.** 1988. Properties of promoters cloned randomly from the *Saccharomyces cerevisiae* genome. *Mol. Cell. Biol.* **8**:4217-4224.
48. **Sauer, F., S. K. Hansen and R. Tjian.** 1995. Multiple TAF_{II}s directing synergistic activation of transcription. *Science.* **270**:1783-1788.
49. **Sayre, M. H., H. Tschochner and R. D. Kornberg.** 1992. Reconstitution of transcription with five purified initiation factors and RNA polymerase II from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **267**:23376-23382.
50. **Scafe, C., D. Chao, J. Lopes, J. P. Hirsch, S. Henry and R. Young.** 1990. RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. *Nature.* **347**:491-494.
51. **Sikorski, R. S. and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* **122**:19-27.
52. **Silverman, N., J. Agapite and L. Guarente.** 1994. Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. *Proc. Natl. Acad. Sci. USA.* **91**:11665-11668.
53. **Silverman, N. and L. Guarente.** unpublished results.
54. **Stern, M. J., R. E. Jensen and I. Herskowitz.** 1984. Five *SWI* genes are required for expression of the *HO* gene in yeast. *J. Mol. Biol.* **178**:853-868.

55. **Thompson, C. M., A. J. Koleske, D. M. Chao and R. A. Young.** 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell*. **73**:1361-1375.
56. **Thut, C. J., J. L. Chen, R. Klemm and R. Tjian.** 1995. p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science*. **267**:100-104.
57. **Wilson, C. J., D. M. Chao, A. N. Imbalzano, G. R. Schnitzler, R. E. Kingston and R. A. Young.** 1996. RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell*. **84**:235-244.
58. **Winston, F.** 1992. Analysis of SPT genes: a genetic approach toward analysis of TFIID, histones, and other transcription factors of yeast. *Transcriptional Regulation*. McKnight and Yamamoto ed. Cold Spring Harbor Laboratory Press.
59. **Winston, F., D. T. Chaleff, B. Valent and G. R. Fink.** 1984. Mutations Affecting Ty-Mediated Expression of the *HIS4* Gene of *Saccharomyces cerevisiae*. *Genetics*. **107**:179-197.

Table 1. Requirement of *ADA5* for promoter activation^a

Promoter	β-Galactosidase activity in:		
	wt	<i>ada5-1</i> mutant	<i>Δada5</i> mutant
CYC1 UAS1	989	282	92
CYC1 UAS2UP1	347	251	71
HIS(1)66	217	19	23
HIS(2)14x2	20	3	4
HO	206	23	10
INO1	101	4	<1
SD5	9588	ND ^b	3027
ADH1	8943	3245	1600

^a BWG1-7a (wild-type strain [WT]) and *ada5-1* and *Δada5* mutants were transformed with the following β-galactosidase reporter plasmids: pLG312ΔAluXho, which contains the *CYC1* UAS1 (19); p265UP1, which contains the *CYC1* UAS2 (13); pHIS66 which contains the *HIS4* UAS (26); p14x2 which contains two synthetic GCN4 binding sites (26); pCP8 (gift of C. Peterson), which contains the *HO* URS1 (position -1516 to -901); p*INO1* (50); pLGSD5, which contains the *GALI-10* promoter, and pCP0, which contains the *ADH1* promoter (47). β-Galactosidase gene activities are means of results of three different assays differing by less than 20% and are normalized to the level of total protein. The activity of pLGSD5 was measured after induction with galactose, and the activity of the *INO1* reporter was assayed after 5 h of induction in inositol-free medium (50).

^b ND, not determined

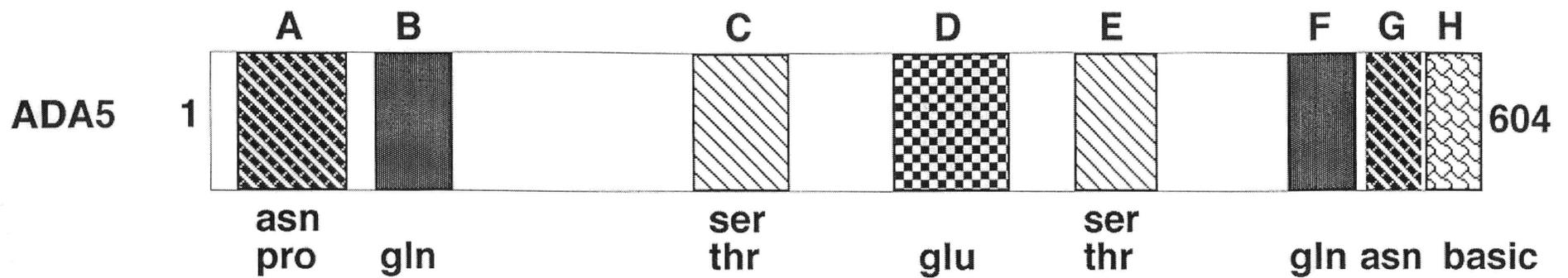
Table 2. Resistance of *spt* mutants to GAL4-VP16 mediated toxicity^a

Strain	<i>SPT</i> genotype	Resistance to GAL4-VP16 ^b
FY3	Wild type	-
L881	<i>spt3-401</i>	-
FY51	<i>spt3Δ203::TRP1</i>	-
YSMR247	<i>spt4Δ::URA3</i>	-
FY363	<i>spt5-194</i>	-
FY137	<i>spt6-140</i>	-
FY963	<i>spt7Δ::LEU2</i>	++++
FY463	<i>spt8-302::LEU2</i>	-
FY383	<i>spt15-21</i>	++
FY508	<i>spt15-122</i>	+
YSMR191	<i>spt20-61</i>	++++
YSMR210	<i>spt20Δ::URA3</i>	++++

^a Strains were transformed with pGAL4-VP16Ura (38) and matching pRS426 vector, plated on SC medium, and scored for growth after 3 days. L881, FY191, FY210, and FY247 were transformed with the *LEU2*-marked plasmids pSB202 (4) and pRS425 because these strains are Ura⁺Leu⁻. GAL4-VP16 plasmids with either marker are equally toxic to yeast cells (unpublished data).

^b -, only pinpoint colonies form, +, ++, +++, the size of the colonies expressing GAL4-VP16 is the same as that of the vector controls.

Figure 1. Sequences of *ADA5* domains. The sequence of *ADA5* was determined as described in the Materials and Methods and contains an open reading frame encoding 604 amino acids with a predicted molecular mass of 68 kDa. Eight domains with unusual amino acid compositions are listed.



A(4-59): SPTGNDPHVFGIPVNATPSNMGSPGVNVPPPMNPAVANVNHPVMRTNSNSNAN

B(69-104): QIQQLQQRQRLLLLQQRLLEQQQRKQQALQNYEAQFYQ

C(235-269): SSSSPSNNNSTQDNSKIQQPSEPNSGVANTGANT

D(335-382): EHRDMLEETAFSEPHWDSEKKSFIHEHRAESTREGTKGVVGHIEERDE

E(401-446): TTTITNSTFAVSLTKNAMEIASSSSNGVIRGASSSTSNSASNTRNNS

F(518-533): QQQLLQRQQQALEQQQ

G(540-559): NANKRSGNNATSNNNNNNNN

H(562-591): KPKVKRPRKNAKKSESGTPAPKKKRMTKKK

Figure 2. The *ada5* deletion strain grows more slowly than the *ada5-1* strain. GMy30 ($\Delta ada5$), GMy37p (*ada5-1*), and BWG1-7a (isogenic wildtype [WT] strain) were streaked on rich (YPD) medium. Growth was scored after 2 days (A) and 3 days (B).

A

ada5-1

$\Delta ada5$

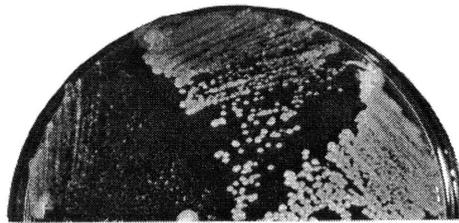


WT

B

ada5-1

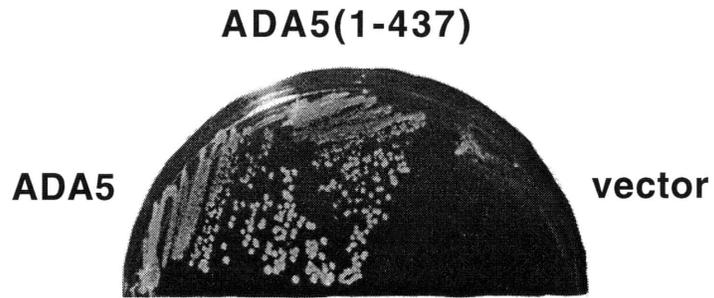
$\Delta ada5$



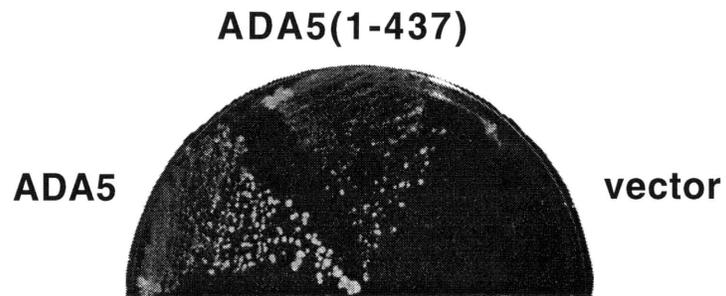
WT

Figure 3. The sequence encoding the first 437 amino acids of *ADA5* is sufficient to complement the slow growth phenotype of $\Delta ada5$ on rich medium, but only partially complement on minimal medium. (A) GMy30, a $\Delta ada5$ mutant, was transformed with the full length *ADA5* clone, the *ADA5*₄₃₇ fragment (containing the first 437 amino acids of *ADA5*), or a vector control. Transformants were restreaked on either SC medium and scored after two days (A), or restreaked on synthetic dextrose medium and scored after three days (B). (C) *ADA5*₄₃₇ does not restore sensitivity to GAL4-VP16 to an *ada5* deletion strain. GMy30 complemented by full length *ADA5* or *ADA5*₄₃₇ was transformed with pSB201, a 2 μ m plasmid expressing GAL4-VP16 from the *ADH1* promoter, or a vector control and plated on SC medium. Transformation plates were scored for growth after three days.

A



B



C

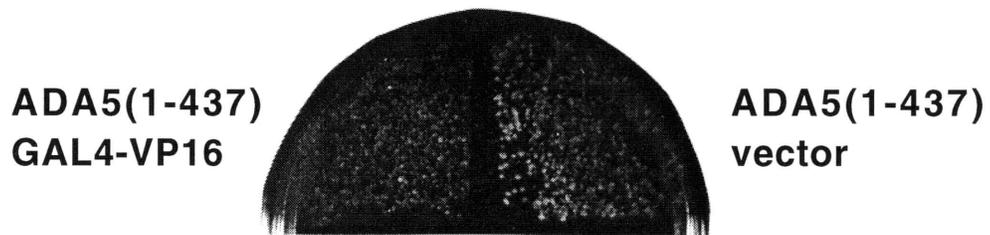
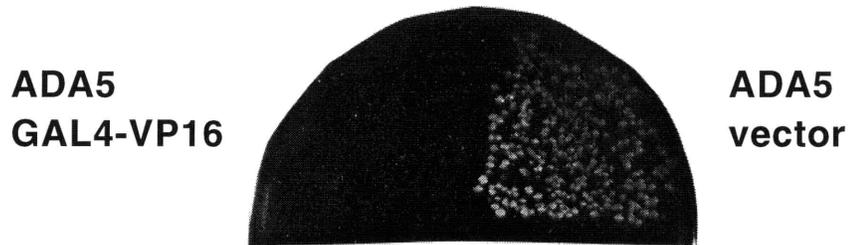


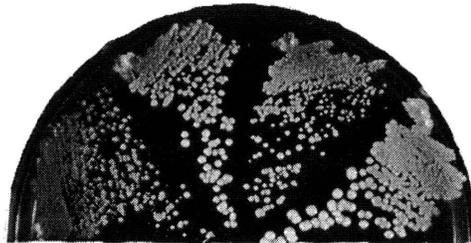
Figure 4. $\Delta ada3\Delta ada5$ double deletion mutants grow as well as *ada5* deletion mutants. GMy38, a $\Delta ada5\Delta ada3$ double deletion mutant was transformed with all pairwise combinations of pRS315 $ADA5$ or pRS315 (a *LEU2* vector) and pADA3HHV or pRS316, (a *URA3* vector). Transformants indicated by plasmid-borne genes were restreaked on drop out medium, and scored after three days. Note that the $\Delta ada3\Delta ada5$ transformant (pRS316pRS315) grows comparably to the $\Delta ada5$ transformant (pADA3 pRS315). Growth of the $\Delta ada3$ transformant (pRS316pADA5) is intermediate between the double deletion strain and wild type (pADA3pADA5).

$\Delta ada3\Delta ada5$

**pRS316
pADA5**

**pADA3
pRS315**

**pRS316
pRS315**



**pADA3
pADA5**

Figure 5. ADA5 binds to VP16. ADA5 was translated in a reticulocyte lysate and labeled with [³⁵S]Met. GST-VP16 (WT) and, as controls, GST fused to two mutant VP16 derivatives were coupled to glutathione-Sepharose beads. The experiment in the lane marked FF utilized an activation domain which changes Phe442 and Phe475 to Ala, while the experiment in the lane marked Δ utilized an activation domain that has residues 457-490 removed. GST-VP16 beads were incubated with [³⁵S]ADA5, washed extensively, and eluted with glutathione. The eluent was electrophoresed on an SDS-polyacrylamide gel and autoradiographed. OP shows 1/10 the output of translated ADA5 used in these experiments.

GST-VP16

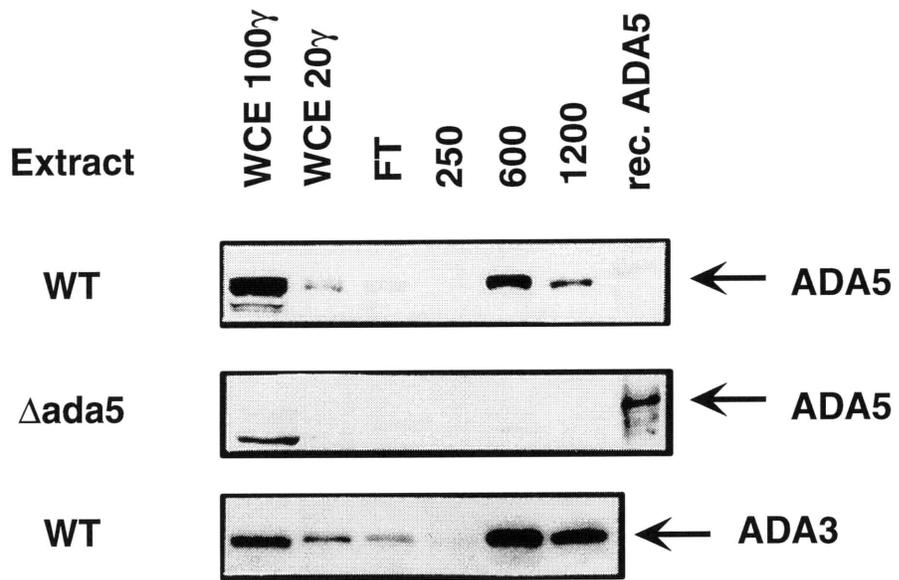
OP WT FF Δ

ADA5 →



Figure 6. ADA5 and ADA3 are physically bound to each other in a yeast extract. (A) Whole cell yeast extracts from a wild type (WT) or a *Δada5* strain were fractionated on a Bio-Rex 70 column. Proteins were eluted stepwise in 250 mM, 600 mM and 1200 mM potassium acetate. 100 and 20 μg (γ) of the whole cell extract (WCE), 20 μg of the flowthrough (FT), and 20μg of each elution were assayed for ADA5 and ADA3 by Western blot analysis. ADA5 and ADA3 cofractionate over this column. Samples from a *Δada5* extract fractionated similarly are shown, demonstrating that the indicated band is ADA5. (B) Protein from the 600 mM fraction of the Bio-Rex 70 column (100 μg from the wild type extract and 150 μg from the *Δada5* extract) was immunoprecipitated with anti-ADA5 (αADA5) or with preimmune (PI) serum. The precipitate was assayed by Western blotting for ADA3. Note that ADA3 is precipitated using the anti-ADA5 serum but not the preimmune serum. Moreover, ADA3 is not precipitated from a *Δada5* extract. Also shown are Western blots of 20 μg of starting material for each extract.

A.



B.

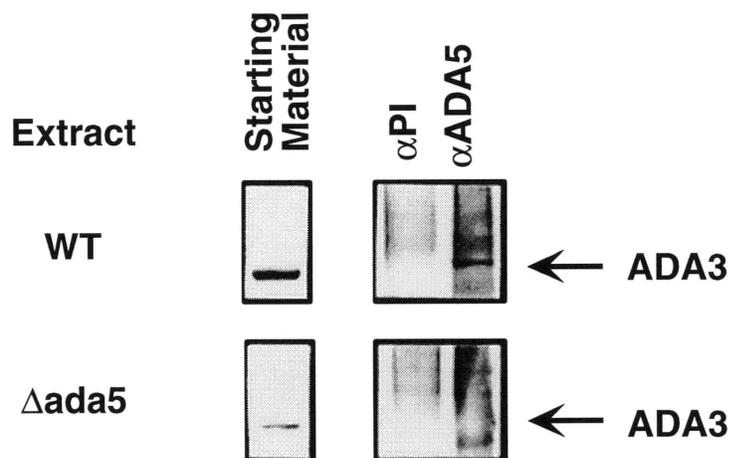


Figure 7. Levels of GAL4-VP16 are not reduced in *spt15-21* or *spt15-122* strains. The indicated *SPT*⁺ or *spt15* strains were transformed with pGAL4-VP16FA a/c, an ARS-CEN plasmid that expresses GAL4-VP16FA from the *ADH1* promoter. 50 µg of whole cell protein extracts were mixed with a radiolabeled GAL4 oligonucleotide probe. Lane 1, which utilized an extract from a strain without GAL4-VP16, shows complexes due to host proteins (host). GAL4-VP16 complexes are indicated (4).

SPT+

SPT15-21

SPT15-122

GAL4-VP16

-

+

+

+

+

+

+

+

+

Lane

1

2

3

4

5

6

7

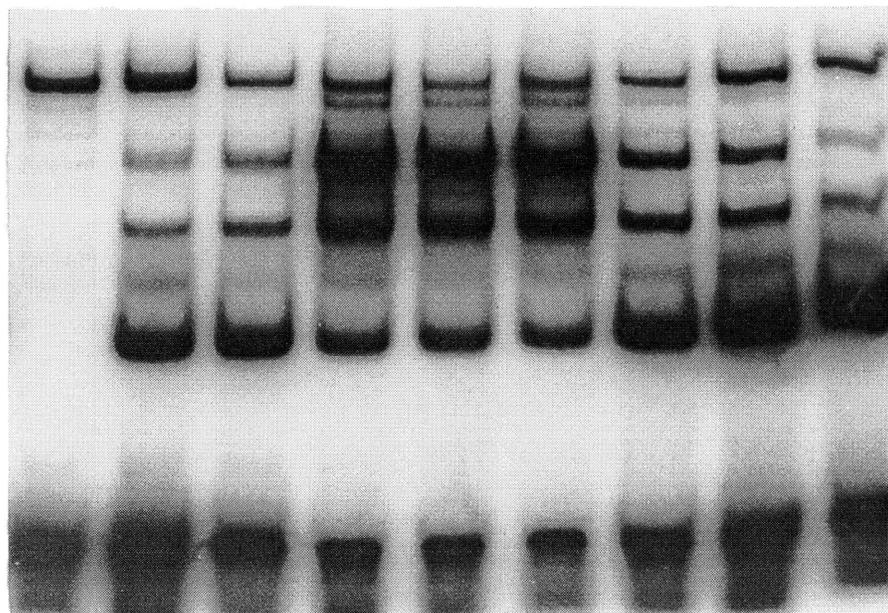
8

9

Host →

GAL4-VP16 →

Probe →



Appendix II

Further interaction studies of the ADAs

Comparison of ADA1 and ADA3 in various fractions of whole cell extract separated on a Bio-Rex 70 column. An explanation, proposed in Chapter 3, for the broader phenotypic defects of $\Delta ada1$ mutants compared to $\Delta ada3$ mutants is that the ADA/GCN5 complex may have two separate functions. Only one function is dependent on ADA3 while both may be dependent on ADA1. An alternative proposal is that ADA1 may have a separate role outside of the ADA/GCN5 complex. ADA1 may act alone or in a separate complex not including the GCN5 class of ADAs. Determining whether ADA1 always cofractionated with members of the GCN5 class of ADAs or whether ADA1 was present in fractions where the other members were absent, would add support for one or the other of the proposed models for ADA1 function.

Yeast whole cell extract was prepared and fractionated on a Bio-Rex 70 column as described in the immunoprecipitation section of Chapter 3. ADA1 cofractionates with ADA3 during this Bio-Rex 70 fractionation and is present in the flowthrough fraction (FT) and the 600 and 1200 mM potassium acetate elutions as shown in Chapter 3 figure 5A. The FT, 600 and 1200 mM fractions were then all subjected to further fractionation on a superose 6 gel filtration column and the elution profiles of various ADAs were determined and compared by Western blotting.

As expected, in the 600 mM Bio-Rex 70 fraction subjected to superose 6 fractionation, ADA1 and ADA3 cofractionated in an identical elution profile (Fig. 1). Also as expected, the peak fractions of ADA1 and ADA3 corresponded to fractions where proteins or complexes of an estimated molecular mass of approximately 2 mDa would elute.

When the FT Bio-Rex 70 fraction was subjected to superose 6 fractionation, ADA1, ADA2, and ADA3 cofractionated identically (Fig. 2). Interestingly, they eluted in the void volume of the column suggesting that they were in a complex

of 5 mDa or larger. Similarly, when the 1200 mM fraction was run through the sizing column, ADA1 and ADA3 again cofractionated in the void volume (Fig. 3). It should be noted, unfortunately, that in the lane marked 14+15 in this figure size markers were also loaded and the presence of the markers obscures the ADA3 band.

In the conditions that we have tested, ADA1 and ADA3 cofractionate. Thus, we think it is less likely that there are separate types of complexes: some containing all ADAs and other containing only one class of ADAs. Rather, we believe that it is more likely that all five subunits are always associated. The observations that ADA1 and ADA3 are eluted from a sizing column in two peaks, one at 5 mDa or larger and one at 2 mDa, suggests that more than one ADA/GCN5 complex (containing both classes of ADAs) may exist. Although the exact relationship between the two molecular mass species remains to be determined, it is interesting to conjecture that the larger mass complex may consist of the smaller one bound to DNA or chromatin.

Interactions between ADA2, ADA3, and GCN5 in extracts from *Δada1* cells.

The data in Chapter 2 demonstrate that ADA3, ADA2, and GCN5 can form a complex *in vitro*. This *in vitro* complex can form in the absence of added ADA1 and ADA5. The data in Chapter 3 demonstrate that all five ADA gene products interact in a complex. Chapter 3 also ascribes two separate functions to this complex. The observation that *Δada1* strains have broader phenotypic defects than *Δada3* strains and the observation that *Δada3Δada5* strains have no defects worse than *Δada5* strains are consistent with a model where disrupting a member of the ADA1/ADA5 class of ADAs might disrupt both functions of the ADA/GCN5 complex while disrupting a member of the GCN5 class may only disrupt the GCN5 dependent histone acetyl transferase function of the complex.

If disrupting ADA1 prevents the GCN5 class of ADAs from functioning, it is possible that in $\Delta ada1$ strains ADA2, ADA3, and GCN5 proteins may not be stable or may not interact with each other. Thus, immunoprecipitation experiments were performed on wildtype and $\Delta ada1$ extracts to compare interactions between ADAs in the presence and absence of ADA1.

Protein A sepharose beads were crosslinked to antibody to ADA1, ADA2, GCN5, ADA5, and preimmune serum. Immunoprecipitations were performed on wildtype (wt) and $\Delta ada1$ extracts as described in Chapter 3. Precipitated proteins were subjected to polyacrylamide gel electrophoresis and Western blotting with antibody to ADA1 and ADA3. As shown in Fig. 4, in the wildtype extract, all ADA antibodies immunoprecipitated ADA1 and ADA3 with the exception of GCN5 antibody which worked extremely inefficiently in this experiment (see Chapter 3 for an example of a more efficient immunoprecipitation by GCN5 antibody). Preimmune serum did not precipitate either ADA1 or ADA3. In the $\Delta ada1$ extract, as expected, in no case was ADA1 precipitated. Also as expected, antibody to ADA1 could not precipitate ADA3 in the absence of ADA1. Interestingly, ADA5 antibody also could not precipitate ADA3 in the absence of ADA1 while antibody to ADA2 and GCN5 (which again worked inefficiently in this experiment) could.

These results demonstrate that in $\Delta ada1$ strains, ADA2, ADA3, and GCN5 could still form a complex. ADA5 is either unable to interact with this complex or is degraded in the absence of ADA1. Whether this subcomplex that is formed when ADA1 is not present has *in vivo* histone acetyl transferase activity is as of yet unclear. The genetic data mentioned above suggest that ADA1 and ADA5 association may be required for activity.

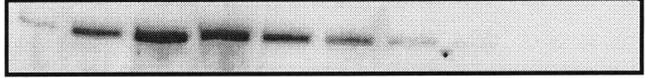
Figure 1. ADA3 and ADA1 in the 600 mM potassium acetate elution of the Bio-Rex 70 column cofractionate over a Superose 6 column. 0.5 ml of the 600 mM potassium acetate elution (see Chapter 3, Fig. 5A, WT extract) was fractionated over a Superose 6 HR column. 0.5 ml fractions were collected of which 0.25 ml of each indicated fraction was precipitated with TCA and subjected to PAGE. Gels were blotted and analyzed by Western with antibody to the indicated proteins. Methods are described in Chapter 3.

600 mM
fraction

12+13
14+15
16+17
18+19
20+21
22+23
24+25
26+27
28+29
30+31



ADA3

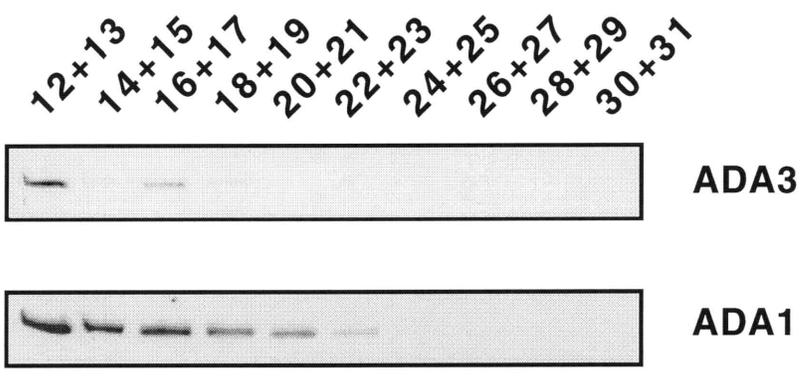


ADA1

Figure 2. ADA3, ADA1, and ADA2 in the FT fraction of the Bio-Rex 70 column cofractionate over a Superose 6 column. 0.5 ml of the flowthrough fraction (see Chapter 3, Fig. 5A, WT extract) was treated as described in Fig. 1.

Figure 3. ADA3 and ADA1 in the 1200 mM potassium acetate elution of the Bio-Rex 70 column cofractionate over a Superose 6 column. 0.5 ml of the 1200 mM potassium acetate elution (see Chapter 3, Fig. 5A, WT extract) was treated as described in Fig. 1. In the lane marked 14+15, protein size marker was run as well obscuring the ADA3 band.

1200 mM
fraction



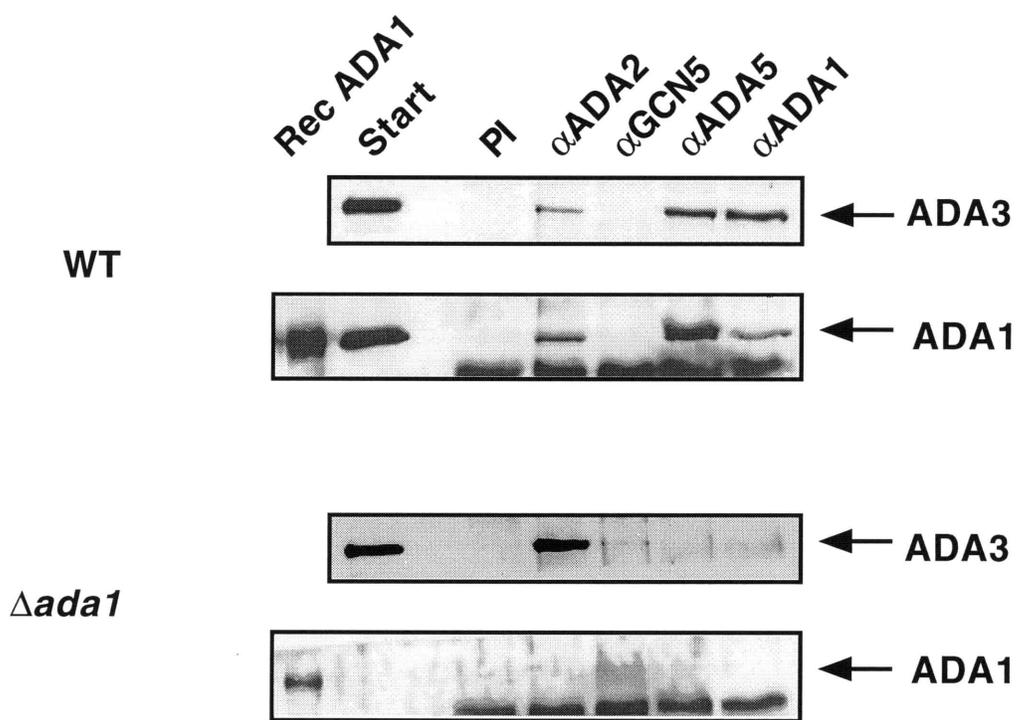


Figure 4. Immunoprecipitation experiments indicate that ADA2, ADA3, and GCN5, but not ADA5, bind together in the absence of ADA1. Starting material refers to the 600 mM potassium acetate elution of the Bio-Rex 70 column of the indicated extracts (see Chapter 3, Fig 5A). Immunoprecipitations and Western blots were performed as described in Chapter 3. In this particular experiment, the antibody to GCN5 did not work well. Rec ADA1 refers to recombinant ADA1 used to mark the expected position of ADA1 in experiments using *Δada1* extract.