Isolation Of *GCN5* And *ADA5* In A Selection For Transcriptional Adaptors

by

Gregory A. Marcus

B.A., Biology

Cornell University, 1989

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

November 1995

Signature of Author _____

Gregory A. Marcus
Department of Biology
November 6, 1995

Certified by _____

Leonard Guarente, Ph.D.
Thesis Supervisor

Accepted by _____

Phillip Sharp, Ph.D.
Thesis Committee Chairman

To my father, my mother and my brother,

for teaching me to persevere

ACKNOWLEDGEMENTS

There are many people I would like to thank for their friendship and support over the years. Some of them helped with the science too:

Lenny Guarente, for being an excellent advisor, running a great lab, providing many ideas, support, and prodding, as well as demonstrating that there is basketball after age 40 (The Chief notwithstanding). I would also like to thank the other members of my thesis committee, Phil Sharp, Fred Winston, Chris Kaiser and Rick Young for their time and efforts.

Neal Silverman, Junjiro Horiuchi, Thomas Oehler, Tod Smeal and Sheri Treadway for their helpful suggestions on parts of this thesis. I would also like to thank Neal for the ADA enriched fractions used in Chapters 3 and 4, and for doing the VP16 pull down experiments in Chapter 4. Also, thanks to Yvette Clark and Myrna Helfenstein for assistance with the Endnote library as well as their administrative support over the years.

Neal Silverman and Jun Horiuchi for being such great friends and partners in the study of the ADA genes. It has been great to work with you guys.

I would also like to thank Shelley Berger, Rainer Knaus, Benjamin Piña, Julie Agapite, Roy Pollock, Paul Spellman, Thomas Oehler, and Sheri Treadway for their many past and future contributions to the ADA project and for the great atmosphere in the lab. To Dave McNabb and Jennifer Zamanian for the above as well as making me feel good that I chose not to do an ADA2 synthetic lethal screen.

Nick Austriaco and Brian Kennedy, for longtime friendship in the lab, and giving Lenny something else to think about. Also to Francesca Cole, James Claus, Alex "Prion Woman" Van Geel, Kevin Mills, Dave Lombard and Tod Smeal for continuing the aging project and contributing to the fun and intellectually stimulating atmosphere in the lab.

I would like to thank other past members of the lab, Paul "Hong Kong Alley" Sugiono, Yan Yan Xing, Maryann Haldi and especially John Fikes and Bernard Turcotte, for their time and patience with a young grad student.

Ruth and Josie for good work, good food, and for being good friends.

Mario, for your encouragement and friendship on many long, lonely evenings.

I would like to thank my parents David and Elaine Marcus, my fathers wife Wendy Bousfield, my brother Eugene Marcus and my grandmother Anne Marcus for their love and encouragement during the long dark years of grad school. I would also like to thank my grandparents Phil and Dotty Menchel for the above as well as for providing a safe haven outside of Boston.

Dave Goldberg, Chris Moore and Rick Rosenthal for making the good times great and the bad times better.

And finally, to my beloved Rachel (Kindt), for making my life so happy and full.

Isolation Of *GCN5* And *ADA5* In A Selection For Transcriptional Adaptors

by

Gregory A. Marcus

Submitted to the Department of Biology on November 6, 1995 in partial fulfillment of the requirement for the Degree of Doctor of Philosophy in Biology

ABSTRACT

Overexpression of a fusion between the GAL4 DNA binding domain and the powerful VP16 activation domain is toxic to yeast. *ADA2* and *ADA3* were isolated in a selection for mutants resistant to GAL4-VP16 toxicity. *ada2* and *ada3* mutants grow slowly on minimal medium, are temperature sensitive, and cannot support activation by certain activation domains *in vivo* and *in vitro*. This last property suggests that ADA2 and ADA3 function as transcriptional adaptors to mediate the interaction of activation domains and basal factors.

In the initial selection, only 2 alleles of *ada2*, and one allele of *ada3* were isolated, suggesting that the selection was not saturated. Here, I report the isolation and characterization of two additional genes, *GCN5* and *ADA5* in the toxicity selection. *gcn5* mutants are phenotypically similar to *ada2* and *ada3* mutants. *ada2gcn5* or *ada3gcn5* double deletion mutants grow no more slowly than single deletion mutants, arguing that these genes are in the same pathway or complex. In fact, GCN5 can bind ADA2 *in vivo* and *in vitro*, forms a complex with ADA2 and ADA3 *in vitro*, and copurifies with ADA2 and ADA3 from yeast extracts.

ADA5, on the other hand, is in a phenotypically different class from the *ADA2* complex genes. *ada5* mutants grow more slowly than *ada2* mutants, and have more general activation defects. Genetic evidence suggests *ADA5* works in the same pathway as *ADA2*, but ADA5 does not copurify with the ADA2 complex. Finally, ADA5 can bind directly to the VP16 activation domain, and is identical to *SPT20*, a gene that may regulate the binding of TBP to promoters *in vivo*. This suggests that ADA5 may function as an adaptor by contacting activation domains, and regulating the binding of TBP to promoters.

Table of Contents

Overview: Transcription requires activators and basal factors

An RNA Polymerase II promoter has at least two different kinds of cis acting elements, a proximal element, often a TATA box, and a distal enhancer or UAS element. Activation requires three different classes of transcription factors: basal factors, activators, and coactivators. The basal factors, such as RNA polymerase, assemble at the TATA box. Activators are sequence specific DNA binding proteins or complexes that recognize UAS/enhancer elements and interact directly or indirectly with the basal transcription factors at the TATA box to promote transcriptional initiation. Coactivators are necessary for activated but not basal transcription. Transcriptional adaptors are one class of coactivators that bind activators and basal factors to mediate activation. In order to understand the mechanism of activation, we must first understand basal factors, activators and coactivators.

Basal Factors

It is possible to reconstitute transcription that accurately initiates at a TATA box *in vitro*. Purification of cell extracts has identified eight fractions designated TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, TFIIJ and RNA polymerase II can be used to reconstitute not only basal, but also activated transcription (Conway and Conway, 1993; Zawel and Reinberg, 1993). Many of these factors are now available as recombinant proteins or as highly purified fractions (Buratowski, 1994).

TFIID is the only basal factor that can bind the TATA box in a sequence specific manner, and thus promoter recognition and binding by TFIID is the first step in transcriptional initiation in most models (Buratowski, 1994). It is important to note that TFIID is a complex that can be separated into two distinct

activities. TBP, the TATA binding protein.is capable of binding to the TATA box alone and is sufficient for basal but not activated transcription (Pugh and Tjian, 1990). The TAFs (TBP-associated factors) are tightly associated with TBP and are necessary for activated but not basal transcription (Dynlacht, et al., 1991).

A preinitiation complex can be assembled by ordered addition of basal factors to template DNA (Buratowski, et al., 1989). In the first step, TBP binds the TATA box. TBP binding is stabilized by the binding of TFIIA. TFIIB can bind to TBP in the presence or absence of TFIIA, although the TBP/TFIIA/TFIIB (DAB) complex is more stable than the TBP/TFIIB (DB) complex. The DB or DAB complex recruits RNA polymerase, which pre-assembles with TFIIF. TFIIF is thought to stabilize the TFIIB-polymerase interaction. Finally, TFIIE and TFIIH are recruited to the complex sequentially (reviewed in Buratowski, 1994; Conway and Conway, 1993; Zawel and Reinberg, 1993).

It is also possible to assemble mini-complexes that have only a fraction of the activity of the complete preinitiation complex. For example, on certain negatively supercoiled templates, TBP, TFIIB, TFIIF and RNA polymerase II are sufficient for transcriptional initiation (Parvin, et al., 1994). In fact, for one template, TFIIF is dispensable. For initiation complexes using this mini-complex, the level of transcription is correlated with the number of negative supercoils, suggesting that the supercoils may be providing a source of energy to drive the transcription process (Parvin, et al., 1994).

The TBP/TFIIB/TFIIF/polymerase mini-complex cannot transcribe linear templates, although it can produce abortive (three nucleotide) RNAs (Goodrich and Tjian, 1994; Parvin, et al., 1994). This suggests that on linear templates, transcription by this minicomplex is blocked after RNA synthesis begins, but very early in its synthesis. This theoretical stage, called promoter clearance which proceeds on negatively supercoiled DNA templates can occur on linear

templates only if TFIIE, TFIIH and ATP are included in the initiation complex (Goodrich and Tjian, 1994). This suggests that TFIIE and TFIIH play a role in promoter clearance (Goodrich and Tjian, 1994). However, others suggest that the abortive initiation assay may be an artifact, and that TFIIH plays a role in open complex formation (R. Young and P. Sharp, personal communication).

During the transition from an initiation to an elongation complex, the carboxy terminal domain (CTD) of RNA polymerase is phosphorylated (Laybourn and Dahmus, 1990). The CTD is a highly conserved structure consisting of a heptapeptide sequence that is repeated 26 times in yeast RPB1 and 52 times in human RPB1 and is essential for viability (Young, 1991). The phosphorilation of the CTD may help distinguish elongating polymerase from initiating polymerase because polymerase with an unphosphorylated CTD preferentially binds to initiation complexes (Lu, et al., 1991), and polymerase with a phosphorylated CTD is isolated from elongating transcription complexes (Payne, et al., 1989). Several kinases, including the MO15 subunit of TFIIH are capable of phosphorilating the CTD (Serizawa, et al., 1995). However, the significance of CTD phosphorylation is not entirely understood. Polymerase with an unphosphorylated CTD shows normal transcriptional initiation and elongation *in vitro* (Mälakä, et al., 1995). Finally, analysis of CTD truncation mutants *in vitro* suggests that the CTD functions in the response to transcriptional activators, and does not influence elongation (Liao, et al., 1991).

Genetic analysis of basal factors in yeast suggests additional properties of basal factors not necessarily identified by biochemical experiments *in vitro*. For example, mutants in *SUA7* which encodes yeast TFIIB show altered start site selection (Pinto, et al., 1992), an activity not apparent in the biochemical analysis of TFIIB. Further, it is important whenever possible to confirm that a basal factor has a given activity *in vivo*. To this end, CTD truncation mutants

have demonstrated that the CTD is necessary for viability, and may mediate the response to some activators. Progressive CTD truncations result in first temperature/cold sensitivity and then inviability (Nonet, et al., 1989). The conditional mutants are also inositol auxotrophs which results from decreased expression of the *INO1* gene (Scafe, et al., 1990).

TBP mutants have also been extensively studied *in vivo*. Several of these confirm the importance of TBP properties described *in vitro*. Mutants in*spt15*, which encodes the yeast TBP were isolated as suppressors of Ty insertions in yeast promoters (Eisenmann, et al., 1989; Hahn, et al., 1989). Some of these have altered DNA binding specificity *in vitro*, and show altered promoter selection for transcriptional initiation *in vivo*, confirming that promoter selection can indeed be mediated by TBP (Arndt, et al., 1992). A TBP mutant unable to bind TFIIA is able to support constitutive but not activated transcription *in vivo* (Stargell and Struhl, 1995). This suggests that the TFIIA-TBP interaction is critical for activation *in vivo* (Stargell and Struhl, 1995). Other TBP mutants will be cited later to illustrate how TBP responds to activators.

Transcriptional activators

Transcriptional activators have been biochemically or genetically shown to be sequence specific DNA binding proteins that bind to UAS or enhancer elements and promote activation. Initial characterization of transcriptional activators showed that they are modular in nature, with separable activation and DNA binding domains (Hope and Struhl, 1986). Since then, the complex nature of the transcriptional activator has been further unraveled, such that domains for DNA binding, activation, dimerization, and cofactor binding (e.g. hormone receptors) have been identified. Each of these can make important contributions to the activation process. For example, the DNA binding

specificity of an activator, or its ability to activate, can vary with its dimerization partner.

In addition, there is growing evidence that DNA binding domains play a role in activation beyond tethering an activation domain to a specific DNA sequence. The existence of positive control mutants in DNA binding domains that do not alter DNA binding but reduce activation suggests that the DNA binding domain makes specific contacts with cofactors necessary for activated transcription (Turcotte and Guarente, 1992). In fact, one putative coactivator, TAF55, has been shown to bind to the SP1 DNA binding domain (Chiang and Roeder, 1995).

Activation domains were initially characterized by their amino acid content. Acidic, Glutamine rich, Serine/Threonine rich, Proline rich and Isoleucine rich activation domains have been described (Triezenberg, 1995). Only acidic activation domains activate in yeast, suggesting that the coactivators for these other types of activation domains are present only in Metazoans.

Recent analysis of activation domains suggests that characterization of activation domains by amino acid content is an oversimplification in two ways. First, although acidic and glutamine residues characterize the VP16 and SP1 activation domains respectively, individual acidic or glutamine residues to not make essential contributions to trans-activation (Cress and Triezenberg, 1991; Gill, et al., 1994). In fact, it is large bulky hydrophobic amino acids that are the critical residues within an activation domain (Cress and Triezenberg, 1991; Drysdale, et al., 1995; Gill, et al., 1994). Second, two activation domains that appear similar by amino acid composition often behave differently in activation assays. For example in yeast, the acidic activation domains GCN4 requires the transcriptional adaptor *ADA2* in order to activate, whereas the HAP4 acidic activation domain activates in an *ADA2* independent manner (Piña, et al.,

1993). Similarly, different glutamine rich activation domains show variable abilities to interact with the coactivator TAF110 (Hoey, et al., 1993).

Additionally, activation domains themselves are modular and can be subdivided into smaller units that themselves have the ability to activate transcription (Drysdale, et al., 1995; Seipel, 1992; Silverman, et al., 1994). In some cases, these units appear to have different specificities, and may trans-activate by different mechanisms (Silverman, et al., 1994). These issues might be resolved if the structure of activation domains were known. However, several studies have failed to find any discernible structure in an activation domain, which suggests that activation domains may adopt a structure by induced fit when they bind their targets (Triezenberg, 1995).

Activators activate by several pathways. Activators can counteract nucleosomal repression, and influence the activity of basal factors *in vivo.* Some models to explain the mechanism of activation have suggested that direct interactions between activation domains and basal factors as one component. In addition, proteins called coactivators or transcriptional adaptors have been discovered that are necessary for activated but not basal transcription. In the following sections, I will discuss experiments that relate to chromatin anti-repression, the response of TBP to activators *in vivo*, direct interactions between activators and basal factors, and the discovery of coactivators.

Activators counter nucleosomal repression

Complete nucleosomes or Histone H1 alone can repress transcription *in vitro* (Grunstein, et al., 1992). The repressive effects of histone H1 or nucleosomes can be overcome by transcriptional activators (Croston and Kadonaga, 1993; Workman, et al., 1991). Thus, one function of activators is to alleviate repression by chromatin. Furthermore, genetic evidence in yeast

suggests that histones regulate transcriptional activation *in vivo* (Grunstein, et al., 1992). For example, repressing the synthesis of certain histones results in elevated expression of some yeast genes (Han, 1989). In addition, the non-conserved N-terminal arms of histones H3 and histone H4 are required for transcriptional repression and transcriptional activation respectively of the GAL1-10 genes *in vivo* (Durrin, et al., 1991; Mann and Grunstein, 1992). Similarly, different mutations the gene encoding histone H2A can cause positive or negative changes in the transcription of the *SUC2* gene (Hirschhorn, et al., 1995; Hirschhorn, et al., 1992). However, anti-repression cannot account for the total effect of trans-activation (Wolffe, 1994). Moreover, activated transcription can occur on "naked DNA" *in vitro* in the absence of histones, and thus must involve a more direct regulation of basal factors.

TBP responds to activators *in vivo*

Mutant analysis suggests that one way activators function *in vivo* is to regulate TBP binding to the TATA box. For example, some mutants in TBP that display lower affinity for the TATA box *in vitro*, show activation defects *in vivo* (Arndt, et al., 1995). Importantly, the activation defects are not governed by the TATA box but by the activator, suggesting that binding of TBP to some promoters may be regulated by activators (Arndt, et al., 1995). If TBP is tethered to DNA by a DNA binding domain, it can interact with the TATA element and activate transcription without an upstream activator (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao, et al., 1995). Thus, decreasing TBP binding can result in activator dependent transcription decreases, and increasing TBP DNA binding by tethering it to DNA allows activation without activators. Together, these results suggest TBP binding to the TATA box can be regulated by activators. Moreover, there are human TBP mutants that show

activator specific transcription defects *in vivo*. This activator specificity suggests that *in vivo*, TBP responds to different activators in different ways (Arndt, et al., 1995; Tansey, et al., 1994).

Direct interactions between activation domains and basal factors *in vitro*

Clearly, activators operate by countering nucleosomal repression, and influencing basal factors. Mechanistically, activation domains presumably operate through protein-protein interactions with other members of the transcriptional apparatus. By identifying the target or targets of activation domains, we will begin to unravel the mechanism of its action. The basal transcription factors represent one obvious target for activation domains, and in fact several basal factors have been shown to interact directly with activation domains.

TBP was the first basal factor that was shown to bind directly to an activation domain (Stringer, et al., 1990). Since the binding of TBP or TFIID to the TATA box is the first step in activation, and may be limited or regulated *in vivo*, there is a good rational for a direct interaction between TBP and an activation domain (Triezenberg, 1995). The interaction between TBP and VP16, a well studied, powerful activation domain, is sensitive to mutations in VP16 that reduce its ability to trans-activate (Ingles, et al., 1991). In addition, TBP mutants have been isolated that can support basal but not activated transcription (Kim, et al., 1994a; Tansey, et al., 1994). One of these is no longer able to bind directly to VP16, suggesting that VP16 TBP interactions may indeed play a role in activated transcription (Kim, et al., 1994a). However, there is no evidence whether direct interactions between VP16 and TBP facilitate activated transcription *in vivo*.

VP16 has also been shown to interact directly with TFIIB (Lin, 1991; Lin, et al., 1991). As was the case with TBP, this interaction is also sensitive to mutations in the VP16 activation domain (Lin, et al., 1991). Moreover, double point mutants within a domain of TFIIB argue that direct interactions are indeed important for activation. These double mutants only weakly bind VP16, and support basal but not activated transcription (Roberts, 1993). In addition, VP16 induces a conformational change in TFIIB that may expose a site within TFIIB that binds basal factor(s), facilitating the assembly of the initiation complex (Roberts and Green, 1994).

Additionally, the VP16 and p53 activation domains can bind to the basal factor TFIIH through its p62 subunit (Xiao, et al., 1994). The strength of the VP16 TFIIH interaction correlates with the ability of VP16 to activate transcription. Mutants that reduce the ability of VP16 to activate also reduce its binding to TFIIH (Xiao, et al., 1994). TFIIH has several properties that make it an interesting target for activation domains. First, TFIIH contains helicase and kinase activities that could potentially be regulated by activators. However, activation domains have not been shown to alter either of these activities, and these activities have not yet been shown to be important for transcription. Second, TFIIH acts in promoter clearance (Goodrich and Tjian, 1994), which is after the stages in transcription where TBP and TFIIB first operate. This suggests that activation domains could function at several stages in activation by contacting basal factors that operate in different stages of transcription.

Finally, the artificial activation domain AH or VP16 can recruit TFIIB into preinitiation complexes (Choy and Green, 1993; Lin, 1991). In fact, in one system, the activation domain appears to be acting twice in transcription, early in a TAF independent manner to recruit TFIIB, and then again in a TAF dependent manner, to recruit the other general transcription factors (Choy and

Green, 1993). However, recruitment of TFIIB does not result in activation in the absence of TAFs. Nevertheless, one important step may be activator mediated binding of TFIIB to TBP. An activation specific TBP mutant is defective in TFIIB binding and does not allow VP16 mediated TFIIB recruitment into promoter complexes (Kim, et al., 1994a). Thus, because activation has not been observed in absence of TAFs, direct interactions between activation domains and basal factors are not sufficient for activation. However, one important step in activation may be the binding of TFIIB to TBP, which can be mediated by direct interactions (Lin, 1991)(Lin, 1991; Kim, et al., 1994a).

Discovery of coactivators and their isolation by biochemical means

Several lines of evidence suggest the existence of factors necessary for activated but not basal transcription called transcriptional adaptors or coactivators. First, recombinant TBP can only support basal but not activated transcription whereas the TFIID fraction can support activated transcription as well. It was therefore reasoned that TFIID must contain other proteins necessary for activated but not basal transcription termed coactivators (Pugh and Tjian, 1990).

Another line of evidence derives from studies of squelching *in vitro* by GAL4-VP16. (Berger, et al., 1990). GAL4-VP16 can inhibit transcription from a heterologous promoter in two different ways. Both basal and activated transcription are inhibited by GAL4-VP16 when it is free to bind non-specific sites on the DNA template. This "cis inhibition" results from trapping of basal factors by GAL4-VP16, preventing transcription. Interestingly, only activated transcription is inhibited when GAL4-VP16 is prevented from binding to the template by a GAL4 oligonucleotide. This "trans-inhibition" suggests that GAL4-VP16 is titrating a factor necessary for activated but not basal transcription,

which must be distinct from the basal factors. If a long oligo is used with a GAL4 site and a TATA box, both activated and basal transcription are inhibited, arguing that cis inhibition is indeed due to basal factor sequestration (Wang, et al., 1995). They called the factor necessary for activated but not basal transcription an adaptor because mechanistically, it may be needed to bridge the interaction between activation domains and basal factors.

Finally, a putative coactivator called the mediator was purified from yeast that had the ability to overcome the squelching of basal and activated transcription by GAL4-VP16 (Kelleher, et al., 1990). However, the composition of the mediator was uncharacterized, and it was unclear whether the mediator contained basal factors or other factors specific for activated transcription (Kelleher, et al., 1990).

Since the existence of coactivators was discovered, many different proteins have been proposed to be coactivators. In the following sections, some of these coactivators will be discussed. Particular attention will be paid to whether these molecules have demonstrated a stimulatory activity *in vivo* or *in vitro*, what activators regulate this stimulatory activity, and what basal factors respond to this activity. Some coactivators are previously isolated yeast mutants with transcription defects. I will begin with coactivators for the chromatin pathway, and then discuss coactivators that target basal factors. Many of these coactivators operate by different mechanisms, which demonstrates the complexity of transcription in eukaryotes.

Chromatin associated HMG proteins can function as coactivators *in vitro*

HMG2 and HMG17 are both components of chromatin, and both can act as coactivators (Paranjape, et al., 1995; Shykind, et al., 1995). HMG2 was

isolated by conventional chromatography as a coactivator activity that stimulated GAL4-VP16 activation in the presence of TAFs (Shykind, et al., 1995). Unlike the TAFs and other coactivators that will be discussed in the following sections, a direct interaction between HMG2 and activation domains or basal factors cannot be detected (Shykind, et al., 1995). Its strong stimulatory activity is mediated through the TFIIA-TFIID complex, which may adopt a more active conformation in the presence of HMG2 (Shykind, et al., 1995). The ability of HMG2a to act as a coactivator on chromatin templates, a more physiological context, has not been reported.

HMG17, on the other hand, acts as a coactivator to stimulate activated but not basal transcription on chromatin templates (Paranjape, et al., 1995). On naked DNA in the presence of basal factors and TBP, HMG17 mildly inhibits transcription thus showing chromatin specifity. The mechanism of how HMG17 acts as a coactivator is unknown, but may function by allowing the basal machinery to transcribe more effectively on a chromatin template (Paranjape, et al., 1995). However, it was not reported whether HMG17 can stimulate transcription in the presence of TAFs. In fact, the stimulatory activity of HMG2 and HMG17 have never been compared in the same assay. Thus, they may operate by the same mechanism. Whether or not they do, chromatin factors can clearly act as coactivators to stimulate transcription *in vitro*.

The SWI1, SWI2/SNF2, SWI3, SNF5 and SNF6 proteins are part of a multi-subunit anti-histone complex

The SWI1, SWI2 and SWI3 genes and the SNF2, SNF5 and SNF6 genes were originally isolated in separate genetic selections for regulators of HO endonuclease transcription and regulators of SUC2 expression respectively (Neigeborn and Carlson, 1984; Stern, et al., 1984). SWI2 and SNF2 are the

same gene, and mutants in any of these SWI/SNF genes have similar pleiotropic phenotypes (Peterson and Herskowitz, 1992). Importantly, all of these genes are necessary for expression of several genes including HO, SUC2, INO1, and GAL1-10 (Peterson and Herskowitz, 1992). In addition, the snf and swi mutants can all be suppressed by mutations in *SPT6* or *SPT11*, strong genetic evidence that the SWI and SNF proteins have related functions (Winston and Carlson, 1992).

The SWI1,2,3 and SNF5,6 proteins have been purified as members of a large multi-subunit complex (Cairns, et al., 1994; Peterson, et al., 1994). This complex contains at least 4 additional polypeptides by silver staining (Cairns, et al., 1994). Recently a novel gene, *SNF11*, was identified by a two-hybrid interaction with SNF2/SWI2, and shown to be an additional member of this complex that can only be visualized by Coomassie blue staining (Treich, et al., 1995).

Genetic and biochemical evidence suggests that the SNF/SWI complex acts to antagonize histone repression. Mutations in genes encoding histone or non-histone chromatin proteins such as H2A, H2B and SPT6, can suppress swi or snf mutations suggesting that the SWI/SNF genes affect chromatin structure (Winston and Carlson, 1992). Moreover, *snf2* and *snf5* mutants change the micrococcal nuclease digestion patterns at the SUC2 promoter, perhaps indicating a change from an open to a closed nucleosome structure. In *(h2a1-h2b1)Δ snf5* double mutants, *SUC2* expression, as well as an open nucleosomal cleavage pattern are restored (Hirschhorn, et al., 1992). *In vitro*, the SWI/SNF complex has the ability to allow GAL4-DNA binding domain derivatives to bind to nucleosomal DNA (Cote, et al., 1994). The human SWI/SNF complex also increases the binding of GAL4 as well as basal factors to nucleosomal DNA (Imbalzano, et al., 1994; Kwon, et al., 1994).

In addition, the SWI/SNF complex may be recruited to promoters by specific activators. A reporter regulated by GAL4 sites alone is SWI dependent, suggesting the SWI genes are regulating the activity of GAL4 and not some other element of the GAL1-10 promoter (Peterson and Herskowitz, 1992). Moreover, one SWI dependent activator, the glucocorticoid receptor immunoprecipitates with SWI3 (Yoshinaga, et al., 1992). This interaction depends on SWI1 and SWI2 (Yoshinaga, et al., 1992), which argues that GR may be physically associated with the entire SWI/SNF complex.

The need for an activator can be bypassed if a member of the SWI/SNF complex is tethered to DNA by the lexA DNA binding domain. For example, lexA-SNF2 activates transcription. Mutants in *SNF5, SNF6* or *SWI1* reduce the activity of lexA-SNF2 fusions, suggesting that an intact complex is necessary for activation by lexA SNF fusions. This may indicate that the SWI/SNF complex activates when recruited to DNA by SWI/SNF dependent activators. It is currently not known why SWI/SNF dependent activators like GAL4 require the SWI/SNF complex in order to alleviate chromatin repression, and how other SWI/SNF independent activators deal with chromatin.

Two classes of *SPT* genes regulate transcription in chromatin and non-chromatin pathways

Insertion of a Ty or ∂ element (the Ty LTR) into a yeast promoter inhibits or alters transcription of adjacent genes. Mutations in the *SPT* genes were isolated as suppressors of certain Ty or ∂ insertions. For example, in the his4-912∂ insertion, the ∂ element TATA box is used preferentially over the HIS4 TATA box, in wildtype (SPT+) strains to produce a longer non-functional RNA transcript. The longer transcript contains upstream ATG initiation codons which

initiate translation out of frame with the *HIS4* coding sequence (revieved in Winston, 1992).

spt mutants were classified according to which Ty or ∂ insertions are suppressed, as well as other common phenotypes. One group will be referred to as the chromatin class because it includes SPT11 and SPT12 that encode one of the two copies of the histone H2A and H2B genes (Clark-Adams and Winston, 1988). The chromatin class also includes the SPT4, SPT5, SPT6 genes There is physical evidence that SPT5 and SPT6 physically interact, and strong genetic evidence that SPT4, SPT5 and SPT6 are part of the same complex (Swanson and Winston, 1992).

The chromatin class of SPT genes regulates Ty transcription, and act to negatively regulate the transcription of several genes. Chromatin class spt mutants can suppress *snf2* mutants to restore expression of the *SUC2* gene (Hirschhorn, et al., 1992), and can suppress *adr1* mutants to allow ADH2 expression (Denis and Malvar, 1990). The putative SPT4, 5, 6 complex may be acting to establish or maintain chromatin repression (Swanson and Winston, 1992). In fact, based on the ability of spt mutants to suppress snf/snf mutants, the SNF/SWI complex may be negatively regulating the SPT4, 5, 6 complex that in turn negatively regulates transcription via chromatin.

The other major class of SPT genes includes SPT15, which encodes the yeast TBP gene (Eisenmann, et al., 1989; Hahn, et al., 1989). The SPT15/TBP class also includes SPT3, SPT7 and SPT8 which share a number of pleiotropic phenotypes, including slow growth, mating defects, and sporulation defects (Eisenmann, et al., 1989). In addition, transcription of Ty elements, and other yeast genes such as MFA1 is reduced (Hirschman and Winston, 1988). For Ty or ∂ insertions at the HIS4 locus, the decrease in the Ty transcript correlates with a shift from the ∂ TATA box to the normal HIS4 TATA box, allowing

expression of the normal HIS4 transcript. However, a decrease in Ty transcription is not sufficient to give an spt phenotype, because Ty transcription is also lower in *snf2* mutants, which do not have an SPT phenotype (Happel, et al., 1991).

Because TBP binds to the TATA box, mutations in *spt15* could change its binding specificity and thus promoter selection *in vivo*. Indeed, seven of the eight original *spt15* alleles have the same amino acid substitution in the TBP coding sequence, which changes its DNA binding specificity *in vitro* (Arndt, et al., 1992). This change appears to favor the natural HIS4 TATA box over the TATA within the ∂ element *in vivo*. Interestingly, the other allele *spt15-21* has the same DNA binding properties as wt TBP *in vitro*, and yet still shows a change in promoter preference *in vivo* (Eisenmann, et al., 1992). The mutation in *spt15-21* may be destroying an interaction with auxiliary factors that influence promoter selection (Eisenmann, et al., 1994).

Mutations in *spt3, spt7* and *spt8* show the same promoter preference alteration as the *spt15* mutants (Winston, 1992). Allele specific suppression and co-immunoprecipitation provides evidence for an interaction between SPT3 and SPT15 (Eisenmann, et al., 1992). Further, some *spt3* alleles that suppress *spt15* also suppress an *spt8* deletion, suggesting that SPT8 functions to modulate the SPT3-TBP interaction (Eisenmann, et al., 1994). Although SPT7 has not been directly linked to the other members of this group by genetic or physical means, the phenotypes of *spt7* mutants are the same as the other members of this group, suggesting it may be in the same complex (Gansheroff, et al., 1995). Thus, SPT3, SPT7 and SPT8 may be acting as coactivators in a complex with TBP to regulate promoter selection (Winston, 1992).

The TBP-associated factors (TAFs) are coactivators tightly associated with TBP

The TAFs are one of the earliest identified and best characterized examples of coactivators. They co-purify with TBP in Drosophila and Hela extracts, and are necessary for activated but not basal transcription (Dynlacht, et al., 1991). Several of the TAFs show striking conservation from Drosophila to humans in terms of sequence similarity and biochemical activity (Thut, et al., 1995). TAFs have also been identified in yeast (Poon and Weil, 1993; Reese, et al., 1994).

Studies of individual TAFs and specific TAF subcomplexes reconstituted from recombinant TAFs suggest several general principles for TAF mediated activation. First, there is specificity in TAF-activation domain interactions. For example, TAF110 interacts specifically with the Glutamine rich activation domain of SP1, whereas TAF 40 and TAF60 interact with VP16 and p53, acidic activation domains (Goodrich, et al., 1993; Hoey, et al., 1993; Thut, et al., 1995). Second, for a TAF subcomplex to respond to an activator, it must have a TAF subunit that can bind to its activation domain. For example, a TAF60, TAF250 and TBP subcomplex can mediate activation by VP16 or p53 but not SP1. Alternatively, a subcomplex with TAF110, TAF250 and TBP can mediate activation by SP1 but not VP16. As expected, subcomplexes with both TAF110 and TAF60 can mediate activation by either activator (Chen, et al., 1994). Third, a TAF that binds an activator must itself interact with a TAF or TAFs binding TBP in order to mediate activation. An activation domain-TAF interaction alone is not sufficient for activated transcription. For example, TAF110 is sufficient to promote activated transcription by SP1 when it is tethered to TBP by either TAF250 or TAF30α (Yokomori, et al., 1993). Hence, the TAF complex acts as a

transcriptional adaptor, that mediates activation by specific protein-protein interactions with activation domains and basal factors.

In addition, different TAF complexes with distinct activities have been isolated from human cells (Brou, et al., 1993). TAF30 was cloned as a member of one particular complex (Jacq, et al., 1994). This TAF, and its specific TAF complex, is necessary for activation by an Estrogen Receptor (ER) activation domain, but not by the VP16 activation domain, which itself activates through other TAFs and a different TAF complex (Jacq, et al., 1994). This work is particularly important because it supports the aforementioned model that a TAF complex will support activation only if a component of the complex can bind to the activator. Furthermore, it suggests that the composition of TAF complexes may vary from cell to cell or from promoter to promoter, and determine which activation domains a promoter can respond to. In fact, TAF150 and TFIIA may help govern the developmental switch from the proximal to the distal ADH promoter in *Drosophila* (Hansen and Tjian, 1995).

Multiple TAF complexes have also been isolated in yeast (Poon and Weil, 1993; Reese, et al., 1994). Immunoprecipitation of TBP from yeast extracts simultaneously isolates at least three TAF complexes, including a specific Pol III complex, a putative repression complex containing MOT1,[1] and a Pol II complex (Poon, et al., 1994). Thus far, none of the SPT15-associated SPT genes have been identified as a TAFs. One member of the Pol II complex is *TSM-1*, the yeast homolog of the Drosophila TAF150 gene(Poon, et al., 1994) (Verrijzer, et al., 1994). The Pol II TAFs were also isolated by purification of an activity retained on a GST-TBP column necessary for activated but not basal

[1]MOT1 is an ATP dependent negative regulator of TBP binding in vitro, and acts as a negative regulator of basal transcription in vivo (Auble, et al., 1994; Davis, et al., 1992).

transcription *in vitro* (Reese, et al., 1994). Two of these genes were cloned and show sequence homology to Drosophila TAFs (Reese, et al., 1994).

Several experiments have also addressed the role of TAFs *in vivo*. TAF250 is identical to CCG1, originally cloned by complementation of a recessive temperature sensitive mutation in a cell line (Hisatake, et al., 1993; Ruppert, et al., 1993; Sekiguchi, et al., 1988). This *taf250* allele results in activator specific activation defects and cell cycle arrest at the non-permissive temperature showing that TAF250 mediates activation *in vivo* (Wang and Tjian, 1994) In another study, mutants in TBP with reduced affinity for TAF250 *in vitro* show reduced ability to support activation *in vivo*, suggesting that TAF250 helps mediate activation *in vivo* by interacting with TBP (Tansey, et al., 1994). Finally, the yeast TAFs *TSM-1, yTAF145* and *yTAF90* are essential for viability (Reese, et al., 1994; Verrijzer, et al., 1994).

CBP and p300 are members of a family of coactivators targeted by E1A during tumorogenesis.

Unlike TAFs, which were identified as coactivators by association with the basal factor TBP, CBP was identified by its ability to bind specifically to the transcriptionally active (i. e. phosphorylated) form of CREB, a transcriptional activator (Chrivia, 1993). Transfection experiments show that CBP can potentiate CREB activation *in vivo*, and that CBP itself can trans-activate when tethered to DNA (Chrivia, 1993; Kwok, et al., 1994). Furthermore, CBP binds the basal factor TFIIB (Kwok, et al., 1994). This suggests that like the TAFs, CBP functions as an adaptor molecule, in this case mediating the interaction between CREB and TFIIB.

CBP is a member of a family of related proteins, including the E1A-associated protein p300 (Lundblad, et al., 1995). p300 had previously been

proposed to be a coactivator for the SV40 enhancer locus that is inactivated *in vivo* by E1A binding (Eckner, et al., 1994). In fact, p300 is indistinguishable from CBP for CREB binding and CREB mediated activation. Moreover, E1A also binds to CBP (Lundblad, et al., 1995). Interestingly, part of the tumorogenic activity of E1A derives from its ability to bind and inactivate the p300/CBP adaptors, thereby repressing transcription (Eckner, et al., 1994; Lundblad, et al., 1995).

Promiscuous adaptors respond to a variety of activation domains

The TAFs and p300/CBP are transcriptional adaptors that mediate activation via interactions between one type of activation domain and one particular adaptor. There are other types of coactivators that function as promiscuous adaptors that can bind and respond to different types of activators. For example, TAF55 binds to the diverse transcriptional activators Sp1, YY1, USF and CTF (Chiang and Roeder, 1995). Furthermore, TAF55 interacts with the DNA binding domain of SP1 (Chiang and Roeder, 1995), unlike TAF110 which responds to SP1's glutamine rich activation domain (Hoey, et al., 1993). This suggests that TAF55 may act by a different and perhaps more general mechanism (Chiang and Roeder, 1995). Unlike many other TAFs, however, the role of TAF55 in transcription is only inferred by its association with a human TAF complex and its ability to bind activators. Further characterization, including *in vitro* transcription and mutant analysis is needed to confirm and elucidate its role in transcription.

PC4/p15 is a more thoroughly characterized promiscuous adaptor. PC4 binds directly to acidic activation domains (Ge and Roeder, 1994) as well as to a TBP TFIIA complex (Ge and Roeder, 1994; Kretzschmar, et al., 1994) . The importance of these interactions are confirmed in two ways. First, recombinant

PC4/p15 can stimulate activation from a variety of activation domains in a manner dependent on TAFs (Ge and Roeder, 1994; Kretzschmar, et al., 1994). Further, depletion of p15 from crude transcription systems lowers activated but not basal transcription (Kretzschmar, et al., 1994). The TAF dependent PC4/p15 activation may be analagous to the TAF dependent activation associated with VP16 mediated TFIIB recruitment into initiation complexes (Choy and Green, 1993). PC4/p15 can which can interact with basal factors and activation domains is not sufficient for activation, just as direct interactions between TFIIB and activation domains are not sufficient for activation. CBP, which binds the activator CREB and TFIIB, may also require the TAFs for activation in vivo.

Genetic isolation of the SRB genes led to the identification of the RNA Polymerase II Holoenzyme

Suppression analysis uses the awesome power of yeast genetics to isolate novel factors (G. Fink, personal philosophy). Nine SRB genes were isolated as dominant and recessive allele specific suppressors of a conditional truncation mutant of the CTD of RPB1 (Hengartner, et al., 1995, and references therein). The SRB proteins play an important if not essential role in transcription of mRNA *in vivo* and *in vitro*. All nine SRBs co-fractionate in a large multi-subunit complex called the RNA Polymerase II holoenzyme (Hengartner, et al., 1995; Koleske and Young, 1994). In addition to the SRBs, this complex contains RNA Polymerase II, TFIIB, TFIIF, TFIIH as well as a number of unidentified polypeptides(Koleske and Young, 1994). A closely related holoenzyme was purified that did not contain TFIIB and TFIIH (Kim, et al., 1994b). With the addition of the missing basal factors, the holoenzyme is capable of both basal and activated transcription (Kim, et al., 1994b; Koleske

and Young, 1994). Importantly, core polymerase (i.e. the 11 subunit complex isolated by affinity purification) in reconstituted transcription systems using TBP and basal factors is not able to respond to transcriptional activators.

The SRBs can also be isolated as part of a complex independent of Pol II called the mediator. The mediator stimulates basal transcription and allows activated transcription when added to a reconstituted *in vitro* transcription system (Hengartner, et al., 1995; Kim, et al., 1994b). Because addition of the mediator is sufficient to allow activation, it can be considered a transcriptional coactivator. In addition, both the mediator and holoenzyme are capable of binding to the VP16 activation domain (Hengartner, et al., 1995). Because the mediator is sufficient to allow activated transcription, and can bind activation domains and basal factors, it has the properties of a transcriptional adaptor complex.

Characterization of individual SRBs demonstrates the diverse functions of the holoenzyme. For example, dominant mutations in SRB2 and SRB5 were isolated as suppressors of conditional CTD truncations (Nonet and Young, 1989; Thompson, et al., 1993). Deletion mutants of either of these genes, however, display a phenotype similar to CTD truncation mutants, and are inviable when the CTD contains less than 20 repeats (Koleske, et al., 1992; Thompson, et al., 1993). *In vitro*, SRB2 and SRB5 associate with pre-initiation complexes, and are necessary for basal and perhaps activated transcription (Koleske, et al., 1992; Thompson, et al., 1993). In addition, SRB2 interacts with TBP (Koleske, et al., 1992).

The SRB10 and SRB11 genes, isolated as recessive suppressors of conditional CTD truncations, encode kinase and cyclin like proteins and function together as a kinase/cyclin pair (Liao, et al., 1995). *srb10* deletion mutant strains are defective in GAL induction, thus displaying a transcription

defect *in vivo* (Liao, et al., 1995). *In vitro,* holoenzyme lacking SRB10 show normal activated and basal transcription (Liao, et al., 1995). However, the CTD is underphosphorilated, suggesting that SRB10 has CTD kinase activity (Liao, et al., 1995). This is in agreement by work by others who have shown that CTD phosphorilation is unnecessary for transcription *in vitro* (Mälakä, et al., 1995). Presumably, the factors that respond to SRB10 regulation *in vivo* are not present or active in this *in vitro* system (Liao, et al., 1995).

Finally, the SRB4, SRB6 and SRB7 genes are essential for viability (Thompson, et al., 1993). Interestingly, inactivation of SRB4 using a temperature sensitive allele results in a shutdown of all mRNA synthesis in the cell (Thompson and Young, 1995). This argues that SRB4 and perhaps the holoenzyme is essential for all mRNA transcription *in vivo* (Thompson and Young, 1995).

GAL11 and SUG1, putative coactivators in the holoenzyme

GAL11 was first identified as a factor needed for full expression of Galactose inducible genes (Nogi and Fukasawa, 1980). In fact, GAL4 levels are unaffected while GAL4 activity is reduced five fold in *gal11* mutants (Suzuki, et al., 1988). Additionally, *gal11* mutants were isolated in selections for Ty suppressors and SUC2 regulators, suggesting that GAL11 also regulates GAL4 independent activity. Indeed, *gal11* mutants have pleiotrophic phenotypes, some of which correlate with reduced gene expression (Fassler and Winston, 1989; Nishizawa, et al., 1990). For example, *gal11* mutants mate poorly, and show reduced expression of the MATα locus (Fassler and Winston, 1989). In certain contexts, including Ty suppression, GAL11 acts as a negative regulator. Thus, genetic analysis reveals that GAL11 is both a positive and negative regulator of gene expression *in vivo* (Fassler and Winston, 1989).

A particular allele of GAL11, called *GAL11P* (for Potentiator) was isolated as a dominant mutation that has the ability to make certain weak activators with GAL4 DNA binding domains behave as strong activators (Himmelfarb, et al., 1990). *GAL11P* and *gal11* phenotypes argue that GAL11 binds GAL4, and acts as a cofactor in trans-activation (Himmelfarb, et al., 1990). The detection of GAL11 in the holoenzyme is consistent with this model (Kim, et al., 1994b).

In fact, the GAL11P mutation creates a novel contact between an inert portion of the GAL11 protein and the dimerization region of GAL4 (Barberis, et al., 1995). This suggests that contact between a DNA binding protein and a component of the holoenzyme is sufficient for activation, a mechanism that may be used by bonafide activators *in vivo*. This does not, however, explain the normal function of *GAL11* to promote transcription as a member of the holoenzyme. It may itself be a target of activation domains, acting as an adaptor associated with basal factors. On the other hand, it may act to stabilize the holoenzyme without interactions outside of the complex.

SUG1 is a putative coactivator that superficially shares some characteristics with GAL11. Like the GAL11P allele, mutations in SUG1 were isolated in a selection for suppressors of a weak activator, in this case a GAL4 derivative missing its activation domain (Swaffield, et al., 1992). This recessive mutant allele increases the activity of the GAL4 variant by at least ten fold, but does not alter the activity of wildtype GAL4 (Swaffield, et al., 1992). Thus, it was argued that SUG1 acts as a transcriptional coactivator . Moreover, like GAL11, SUG1 was found to be a part of the holoenzyme (Kim, et al., 1994b).

However, SUG1 was also identified as a member of the 26S protease, which cast some doubt on its role as a coactivator, but would not necessarily rule out a function in transcription (Ghislain, et al., 1993). This contention was refuted by a low resolution experiment showing that epitope tagged SUG1 did

not co-sediment with the proteosome but rather with the holoenzyme (Swaffield, et al., 1995). However, in recent analysis of the Young lab holoenzyme, SUG1 could not be detected. Given the sensitivity of the α-SUG1 antibodies, if SUG1 is present in the holoenzyme, there are only trace amounts (1% or less) compared to the level of the SRB proteins, (Rick Young, personal communication). Thus, at present, the role of SUG1 in transcription is unclear.

Isolation of ADA genes in a selection for transcriptional adaptors

Mutations in five genes, ADA1, ADA2, ADA3, GCN5 and ADA5 were isolated in a selection for mutations resistant to toxicity mediated by overexpression of a powerful transcriptional activator, GAL4-VP16 (Berger, et al., 1992; Marcus, et al., 1994). It was hypothesized that toxicity is caused by the trapping of basal factors at nonspecific sites on genomic DNA. Mutations in adaptor molecules that mediate the interaction between the activation domain and basal factors would free the basal factors to allow transcription (Berger, et al., 1992). The ADA genes are necessary for activation by certain activation domains *in vivo* and *in vitro* (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). Characterization of the ADA genes will be the subject of this thesis.

Isolation and initial characterization of *ada2* and *ada3* mutants has been described elsewhere. *ada2* and *ada3* deletion mutants have similar phenotypes including slow growth on minimal medium, and temperature sensitivity (Berger, et al., 1992; Piña, et al., 1993). Furthermore, they are defective in activation mediated by the VP16 and GCN4 but not the HAP4 and GAL4 activation domains *in vivo* (Berger, et al., 1992; Piña, et al., 1993). In addition, nuclear extracts from *ada2* mutants have normal basal transcription and can support activated transcription from the HAP4 activation domain. GAL4-VP16 or GCN4, on the other hand, poorly activate in *ada2* extracts

(Berger, et al., 1992). Thus, activation domains have the same ADA2 specifity *in vivo* and *in vitro*.

ada2ada3 double deletion mutants have the same slow growth phenotype as the single deletion mutants, suggesting that ADA2 and ADA3 act in the same pathway or as a complex *in vivo* (Piña, et al., 1993). In fact, in vitro translated ADA2 and ADA3 coimmunoprecipitate (Horiuchi, et al., 1995)., and ADA2 and ADA3 copurify through four chromotography steps from yeast extracts (N. Silverman and LG, unpublished data).

In Chapter 2 and Chapter 3, I will discuss the isolation of GCN5 in the toxicity screen, and its characterization. Biochemical and genetic evidence argues that GCN5 functions in a complex with ADA2 and ADA3 *in vivo*. In addition, I show that the bromodomain, conserved in many different coactivators, is necessary for full activity by GCN5-dependent activators. In Chapter 4, I report the cloning and characterization of ADA5. ADA5 mutants have different characteristics than *ada2*, *ada3* and *gcn5* mutants. Moreover, ADA5 is not part of the ADA2 complex. Therefore, ADA5 is a novel class of ADA gene. Chapter 5 will discuss the ADA genes in the context of the coactivator field.

REFERENCES

Arndt, K., Ricupero-Hovasse, S. and Winston, F. (1995). TBP mutants defective in activated transcription *in vivo*. EMBO J. **14**, 1490-1497.

Arndt, K.M., Ricupero, S.L., Eisenmann, D.M. and Winston, F. (1992). Biochemical and genetic characterization of a yeast TFIID mutant that alters transcription in vivo and DNA binding *in vitro*. Mol Cell Biol **12**, 2372-82.

Auble, D.T., Hansen, K.E., Mueller, C., Lane, W.S., Thorner, J. and Hahn, S. (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.

Barberis, A., Pearlberg, J., Simkovich, N., Farrell, S., Reinagel, P., Bamdad, C., Sigal, G. and Ptashne, M. (1995). Contact with a component of the polymerase holoenzyme suffices for gene activation. Cell **81**, 359-368.

Berger, S.L., Cress, W.D., Cress, A., Triezenberg, S.J. and Guarente, L. (1990). Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61**, 1199-1208.

Berger, S.L., Piña, B., Silverman, N., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J. and Guarente, L. (1992). Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. Cell **70**, 251-265.

Brou, C., Chaudhary, S., Davidson, I., Lutz, Y., Wu, J., Egly, J.M., Tora, L. and Chambon, P. (1993). Distince TFIID complexes mediate the effect of different transcriptional activators. EMBO J. **12**, 489-499.

Buratowski, S. (1994). The basics of basal transcription by RNA polymerase II. Cell **77**, 1-3.

Buratowski, S., Hahn, S., Guarente, L. and Sharp, P.A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56**, 549-561.

Cairns, B.R., Kim, Y.-J., Sayre, M.H., Laurent, B.C. and Kornberg, R.D. (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.

Chatterjee, S. and Struhl, K. (1995). Connecting a promoter-bound protein to TBP bypassed teh need for a transcriptional activation domain. Nature **374**, 820-822.

Chen, J.L., Attardi, L.D., Verrijzer, C.P., Yokomori, K. and Tjian, R. (1994). Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. Cell **79**, 93-105.

Chiang, C.M. and Roeder, R.G. (1995). Cloning of an Intrinsic Human TFIID Subunit That Interacts with Multiple Transcriptional Activators. Science **267**, 531-536.

Choy, B. and Green, M.R. (1993). Eukaryotic activators function during multiple steps of preinitiation complex assembly. Nature **366**, 531-536.

Chrivia, J.C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature **365**, 855-859.

Clark-Adams, C.D. and Winston, F. (1988). Changes in histone gene dosage alter transcription in yeast. Genes Dev. **2**, 150-159.

Conway, R.C. and Conway, J.W. (1993). General initiation factors for RNA polymerase II. Annu. Rev. Biochem. **62**, 161-190.

Cote, J., Quinn, J., Workman, J.L. and Peterson, C.L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science **265**, 53-60.

Cress, W.D. and Triezenberg, S.J. (1991). Critical structural elements of the VP16 transcriptional activation domain. Science **251**, 87-90.

Croston, G.E. and Kadonaga, J.T. (1993). Role of chromatin structure in the regulation of transcription by RNA polymerase II. Curr. Opinion in Cell Biol. **5**, 417-423.

Davis, J.L., Kunisawa, R. and Thorner, J. (1992). A presumptive helecase (MOT1 gene product) affects gene expression and is required for viability. Mol. Cell. Biol. **12**, 413-421.

Denis, C.L. and Malvar, T. (1990). The CCR4 gene from Saccharomyces cerevisiae is requried fo rbith nonfermentive and spt-mediated gene expression. Genetics **108**, 845-858.

Drysdale, C.M., Duenas, E., Jackson, B.M., Reusser, U., Braus, G. and Hinnebusch, A.G. (1995). The transcriptional activator GCN4 contains multiple activation domains that are critically dependent on hydrophobic amino acids. Mol. Cell. Biol. **15**, 1220-1233.

Durrin, L.K., Mann, R.K., Kayne, P.S. and Grunstein, M. (1991). Yeast histone H4 n-terminal sequence is required for promoter activation *in vivo*. Cell **65**, 1023-1031.

Dynlacht, B.D., Hoey, T. and Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell **66**, 563-576.

Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B. and Livingston, D.M. (1994). Molecular cloning and functional analysis of teh adenovirus E1A-associated protein 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. **8**, 869-884.

Eisenmann, D., Chapon, C., Roberts, S., Dollard, C. and Winston, F. (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.

Eisenmann, D.M., Arndt, K.M., Ricupero, S.L., Rooney, J.W. and Winston, F. (1992). SPT3 interacts with TFIID to allow normal transcription in Saccharomyces cerevisiae. Genes Dev **6**, 1319-31.

Eisenmann, D.M., Dollard, C. and Winston, F. (1989). *SPT15*, the gene encoding the yeast TATA binding factor TFIID, is required for normal transcription initiation *in vivo*. Cell **58**, 1183-1191.

Fassler, J. and Winston, F. (1989). The Saccharomyces cerevisiae SPT13/GAL11 gene has both positive and negative regulatory roles in transcription. Mol. Cell. Biol. **9**, 5602-5609.

Gansheroff, L., Dollars, C., Tan, P. and Winston, F. (1995). The *Saccharomyces cerevisiae* SPT7 gene encodes a very acidic protein important for transcription *in vivo*. Genetics **139**, 523-536.

Ge, H. and Roeder, R. (1994). Purification, cloning, and characterization of a human coactivator, PC-4, that mediates transcriptional activation of class II genes. Cell **78**, 513-523.

Ghislain, M., Udvardy, A. and Mann, C. (1993). S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature **366**, 358-361.

Gill, G., Pascal, E., Tseng, Z. and Tjian, R. (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.

Goodrich, J.A., Hoey, T., Thut, C.J., Admon, A. and Tjian, R. (1993). Drosophila TafII 40 Interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75**, 519-530.

Goodrich, J.A. and Tjian, R. (1994). Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA ploymerase II. Cell **77**, 145-156.

Grunstein, M., Durrin, L.K., Mann, R.K., Fisher-Adams, G. and Johnson, L.M. (1992). Histones: Regulators of Transcription in Yeast. In Transcriptional Regulation, McKnight, S.L. and Yamamoto, K.R., eds. (Cold Spring Harbor Laboratory Press, Plainview, N.Y.) pp. 1295-1315.

Hahn, S., Buratowski, S., Sharp, P.A. and Guarente, L. (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.

Han, M., and Grunstein, M. (1989). Nucleosome loss activates yeast downstream promoters *in vivo*. Cell **55**, 1137-1145.

Hansen, S.K. and Tjian, R. (1995). TAFs and TFIIA Mediate Differential Utilization of the Tandem ADH Promoters. Cell **82**, 565-575.

Happel, A.M., Swanson, M.S. and Winston, F. (1991). The *SNF2, SNF5*, and *SNF6* genes are required for Ty transcription in *Saccharomyces cerevisiae*. Genetics **128**, 69-77.

Hengartner, C.J., Thompson, C.M., Zhang, J., Chao, D.M., Liao, S.M., Koleske, A.J., Okamura, S. and Young, R.A. (1995). Association of an activator with an RNA polymerase II holoenzyme. Genes & Dev. **9**, 897-910.

Himmelfarb, H.J., Pearlberg, J., Last, D.H. and Ptashne, M. (1990). GAL11P: A yeast mutation that potentiates the effect of weak GAL4-derived activators. Cell **63**, 1299-1309.

Hirschhorn, J., Bortvin, A., Ricupero-Hovasse, S. and Winston, F. (1995). A new class of histone H2A mutations in Saccharomyces cerevisiae causes specific transcriptional defects *in vivo*. Mol Cell Biol 1999-2009.

Hirschhorn, J.N., Brown, S.A., Clark, C.D. and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. Genes Dev **6**, 2288-98.

Hirschman, J.E. and Winston, F. (1988). *SPT3* is required for normal levels of a-factor and a factor expression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**, 822-827.

Hisatake, K., Hasegawa, S., Takada, R., Nakatani, Y., Horikoshi, M. and Roeder, R.G. (1993). The p250 subunit of native TATA-binding factor TFIID is the cell-cycle regulatory protein CCG1. Nature **362**, 179-181.

Hoey, T., Dynlacht, B.D., Peterson, M.G., Pugh, B.F. and Tjian, R. (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72**, 247-260.

Hope, I. and Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell **46**, 885-894.

Horiuchi, J., Silverman, N., Marcus, G. and Guarente, L. (1995). ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. Mol Cell Biol 1203-1209.

Imbalzano, A.N., Kwon, H., Green, M.R. and Kingston, R.E. (1994). Facilitated binding of TATA-binding protein to nucleosomal DNA. Nature **370**, 481-485.

Ingles, C.J., Shales, M., Cress, W.D., Triezenberg, S.J. and Greenblatt, J. (1991). Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature **351**, 588-590.

Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P. and Tora, L. (1994). Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. Cell **79**, 107-117.

Kelleher, R.J.I., Flanagan, P.M. and Kornberg, R.D. (1990). A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell **61**, 1209-1215.

Kim, T.K., Hashimote, S., Kelleher, R.J., Flanagan, P.M., Kornberg, R.D., Horikoshi, M. and Roeder, R.G. (1994a). Effects of activaton-defective TBP mutations on transcriptional initiation in yeast. Nature **369**, 252-255.

Kim, Y.J., Björklund, S., Li, Y., Sayre, M.H. and Kornberg, R.D. (1994b). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77**, 599-608.

Klages, N. and Strubin, M. (1995). Stimulation of RNA polymerase II transcription initiation by recruitment of TBP *in vivo*. Nature **374**, 822-823.

Koleske, A.J., Buratwoski, S., Nonet, M. and Young, R.A. (1992). A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. Cell **69**, 883-894.

Koleske, A.J. and Young, R.A. (1994). An RNA polymerase II holoenzyme responsive to activators. Nature **368**, 466-469.

Kretzschmar, M., Kaiser, K., Lottspeich, F. and Meisterernst, M. (1994). A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. Cell **78**, 525-534.

Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature **370**, 223-226.

Kwon, H., Imbalzano, A.N., Khavari, P.A., Kingston, R.E. and Green, M.R. (1994). Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex. Nature **370**, 477-481.

Laybourn, P.J. and Dahmus, M.E. (1990). Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. J. Biol. Chem **265**, 13165-13173.

Liao, S.M., Taylor, I.C.A., Kingston, R.E. and Young, R.A. (1991). RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators *in vitro*. Genes Dev. **5**, 2431-2440.

Liao, S.M., Zhang, J., Jeffrey, D.A., Koleske, A.J., Thompson, C.M., Chao, D.M., Viljoen, M., van Vuuren, H.J. and Young, R.A. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature **374**, 193-196.

Lin, Y.S., and Green, M. R. (1991). Mechanism of action of an acidic transcriptional activator *in vitro*. Cell **64**, 971-981.

Lin, Y.S., Maldonado, E., Reinberg, D. and Green, M.R. (1991). Binding of general transcription factor TFIIB to an acidic activating region. Nature **353**, 569-571.

Lu, H., Flores, O., Weinmann, R. and Reinberg, D. (1991). The nonphosphorylated form of RNA polymerase preferentially associates with the preinitiation complex. J. Biol. Chem. **88**, 10004-10008.

Lundblad, J.R., Kwok, P.S., Laurance, M.E., Harter, M.L. and Goodman, R.H. (1995). Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. Nature **374**, 85-88.

Mälakä, T.P., Parvin, J.D., Kim, J., Huber, L.J., Sharp, P.A. and Weinberg, R.A. (1995). A kinase-deficient transcription factor TFIIH is functional in basal and activated trasncription. Proc. Natl. Acad. Sci. USA **92**, 5174-5178.

Mann, R.K. and Grunstein, M. (1992). EMBO J. **11**, 3297-3306.

Marcus, G.A., Silverman, N., Berger, S.L., Horiuchi, J. and Guarente, L. (1994). Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. EMBO J **13**, 4807-4815.

Neigeborn, L. and Carlson, M. (1984). Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae.* Genetics **108**, 845-858.

Nishizawa, M., Suzuki, Y., Nogi, Y., K., M. and Fukasawa, T. (1990). Yeast GAL11 protein mediates the transcriptional activation signal of two different transacting factors, GAL4 and general regualtory factor I/repressor/activator site binding protein. Proc. Natl. Acad. Sci. USA **87**, 5373-5377.

Nogi, Y. and Fukasawa, T. (1980). A novel mutation that affects utilization of galactose in *Saccharomyces cerevisiae.* Current Genetics **115**, 115-120.

Nonet, M.L., Sweetser, D. and Young, R.A. (1989). Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. Cell **50**, 909-915.

Nonet, M.L. and Young, R.A. (1989). Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. Genetics **123**, 715-724.

Paranjape, S.M., Krumm, A. and Kadonaga, J.T. (1995). HMG17 is a chromatin-specific transcriptional coactivator that increases teh efficiency of transcriptional initiation. Genes Dev. **9**, 1978-1991.

Parvin, J.D., Shykind, B.M., Meyers, R.E., Kim, J. and Sharp, P.A. (1994). Multiple sets of basal factors initiate transcription by RNA polymerase II. J. Biol Chem **269**, 18414-18421.

Payne, J.M., Laybourn, P.J. and Dahmus, M.E. (1989). The transition of RNA polymerase II from initiation to elongation is associated with phosphorylation of the carboxyl-terminal domain of subunit IIA. J. Biol. Chem. **264**, 19621-19629.

Peterson, C.L., Dingwall, A. and Scott, M.P. (1994). Five *SWI/SNF* gene products are components of a large multisubunit complex required for transcriptional enhancement. Proc. Natl. Acad. Sci. USA **91**, 2905-2708.

Peterson, C.L. and Herskowitz, I. (1992). Characterization of the yeast *SWI1, SWI2*, and *SWI3* genes, which encode a global activator of transcription. Cell **68**, 573-584.

Piña, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J.A. and Guarente, L. (1993). ADA3: a gene, indentified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. Molec. Cell. Biol. **13**, 5981-5989.

Pinto, I., Ware, D.E. and Hampsey, M. (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.

Poon, D., Cambell, A.M., Bai, Y. and Weil, P.A. (1994). Yeast Taf170 is encoded by MOT1 and exists in a TATA box-binding protein (TBP)-TBP-associated factor complex distinct from transcription factor IID. J. Biol. Chem. **269**, 23135-23140.

Poon, D. and Weil, A.P. (1993). Immunopurification of yeast TATA-binding protein and associated factors. J. Biol. Chem. **268**, 15325-15325.

Pugh, B.F. and Tjian, R. (1990). Mechanism of transcriptional activation by Spt1: evidence for coactivators. Cell **61**, 1187-1197.

Reese, J.C., Apone, L., Walker, S.S., Griffin, L.A. and Green, M.R. (1994). Yeast TAFIIS in a multisubunit complex required for activated transcription. Nature **371**, 523-527.

Roberts, S.G.E., Ha, I., Maldonada, E., Reinberg, D., and Green, M. R. (1993). Interaction between an acidic activator and transcription factor IIB is required for transcriptional activation. Nature **363**, 741-744.

Roberts, S.G.E. and Green, M.R. (1994). Activator-induced conformational change in general transcription factor TFIIB. Nature **371**, 717-720.

Ruppert, S., Wang, E.H. and Tjian, R. (1993). Cloning and expression of the human TAF250: a TBP-associated factor implicated in cell cycle regulation. Nature **362**, 175-179.

Scafe, C., Chao, D., Lopes, J., Hirsch, J.P., Henry, S. and Young, R. (1990). RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. Nature **347**, 491-494.

Seipel, K., Georgiev, O., and Schaffner, W. (1992). Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. EMBO Journal **11**, 4961-4968.

Sekiguchi, T., Miyata, T. and Nishimote, T. (1988). Molecular cloning of the cDNA of human X chromosomalgene (CCG1) which complements teh temperature-sensitive G1 mutants, tsBN462 and ts13, of te BHK cell line. EMBO J. **7**, 1683-1687.

Serizawa, H., Makela, T.P., Conaway, J.W., Conaway, R.C., Weinberg, R.A. and Young, R.A. (1995). Association of Cdk-activating kinase subunits with transcription factor TFIIH. Nature **374**, 280-282.

Shykind, B.M., Kim, J. and Sharp, P.A. (1995). Activation of the TFIID-TFIIA complex with HMG-2 as coactivator. Genes Dev. **9**, 1354-1365.

Silverman, N., Agapite, J. and Guarente, L. (1994). Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. Proc. Natl. Sci. USA **91**, 11665-11668.

Stargell, L.A. and Struhl, K. (1995). The TBP-TFIIA interaction in the response to acidic activators *in vivo*. Science **269**, 75-78.

Stern, M.J., Jensen, R.E. and Herskowitz, I. (1984). Five *SWI* genes are required for expression of the *HO* gene in yeast. J. Mol. Biol. **178**, 853-868.

Stringer, K.F., Ingles, C.J. and Greenblatt, J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature **345**, 783-786.

Suzuki, Y., Nogi, Y., Abe, A. and Fukasawa, T. (1988). GAL11 Protein, an auxiliary transcription activator for genes encoding galactose-metabolizing enzymes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**, 4991-4999.

Swaffield, J.C., Bromberg, J.F. and Johnston, S.A. (1992). Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in GAL4. Nature **357**, 698-700.

Swaffield, J.C., Melcher, K. and Johnston, S.A. (1995). A highly conserved ATPase protein as a mediator between acidic activation domains and the TATA-binding protein. Nature **374**, 88-91.

Swanson, M.S. and Winston, F. (1992). SPT4, SPT5 and SPT6 interactions: effects on transcription and viability in Saccharomyces cerevisiae. Genetics **132**, 325-36.

Tansey, W.P., Ruppert, S., Tjian, R. and Herr, W. (1994). Multiple regions of TBP participate in the response to transcriptional activators *in vivo*. Genes Dev **8**, 2756-2769.

Thompson, C.M., Koleske, A.J., Chao, D.M. and Young, R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73**, 1361-1375.

Thompson, C.M. and Young, R.A. (1995). General requirement for RNA polymerase II holoenzymes *in vivo*. Proc Natl Acad Sci USA **92**, 4587-4590.

Thut, C.J., Chen, J.L., Klemm, R. and Tjian, R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. Science **267**, 100-104.

Treich, I., Cairns, B.R., de los Santos, T., Brewster, E. and Carlson, M. (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.

Triezenberg, S.J. (1995). Structure and function of transcriptional activation domains. Curr. Op. Gen. Dev **5**, 190-196.

Turcotte, B. and Guarente, L. (1992). HAP1 positive control mutants specific for one of two binding sites. Genes & Dev. **6**, 2001-2009.

Verrijzer, P., Yokomori, K., Chen, J.L. and Tjian, R. (1994). Drosophila TAFII150: similarity to yeast gene TSM-1 and specific binding to core promoter DNA. Science **264**, 933-941.

Wang, E. and Tjian, R. (1994). Promoter-selective transcriptional defect in cell cycle mutant ts13 rescued by hTAFII250. Science **263**, 811-814.

Wang, L., Turcotte, B., Guarente, L. and Berger, S.L. (1995). The acidic transcriptional activation domains of herpes virus VP16 and yeast HAP4 have different co-factor requirements. Gene In Press.

Winston, F. (1992). Analysis of SPT Genes: A genetic Approach toward Analysis fo TFIID, Histones, and Other Transcription Factors of Yeast. In Transcriptional Regulation, McKnight, S.L. and Yamamoto, K.R., eds. (Cold Spring Harbor Laboratory Press, United States) pp. 1271-1293.

Winston, F. and Carlson, M. (1992). Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. Trends Genet **8**, 387-91.

Wolffe, A.P. (1994). Transcription: in tune with histones. Cell **77**, 13-16.

Workman, J.L., Taylor, I.C.A. and Kingston, R.E. (1991). Activation domains of stably bound GAL4 derivatives alleviate repressions of promoters by nucleosomes. Cell **64**, 533-544.

Xiao, H., Friesen, J.D. and Lis, J.T. (1995). Recruiting TATA-Binding Protein to a promoter: transcriptional activation without an upstream activator. Mol. Cell. Biol. **15**, 5757-5761.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J.L., Triezenberg, S.J., Reinberg, D., Flores, O., Ingles, C.J. and Greenblatt, J. (1994). Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol. Cell. Biol. **14**, 7013-7024.

Yokomori, K., Chen, J.L., Admon, A., Zhou, S. and Tjian, R. (1993). Molecular cloning and characterization of dTAFII30 alpha and dTAFII30 beta: two small subunits of Drosophila TFIID. Genes Dev. **7**, 2587-2597.

Yoshinaga, S.K., Peterson, C.L., Herskowitz, I. and Yamamoto, K.R. (1992). Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. Science **258**, 1598-1604.

Young, R. (1991). RNA polymerase II. Annu. Rev. Biochem. **60**, 689-715.

Zawel, L. and Reinberg, D. (1993). Initiation of transcription by RNA polymerase II: a multi-step process. Progress in Nucleic Acid Research and Molecular Biology **44**, 67-108.

Chapter 2

Functional Similarity and Physical Association Between GCN5 and ADA2: Putative Transcriptional Adaptors.

This chapter is adapted from Marcus, G.A., Silverman, N., Berger, S.L., Horiuchi, J. and Guarente, L. (1994). Functional similarity and physical association between GCN5 and ADA2: Putative transcriptional adaptors. EMBO J **13**, 4807-4815.

INTRODUCTION

Transcriptional activation in eukaryotes involves the functional interaction between transcriptional activators bound at enhancers or UASs and the general transcription factors bound at the TATA box. Activators are modular, containing DNA binding domains and activation domains (Hope and Struhl, 1986). One class of activation domains is enriched in amino acids with acidic side chains and can function in a wide variety of eukaryotes ranging from yeast to mammals (Sadowski, et al., 1988). Acidic activators function when bound at sites very distant from the TATA box. Models for activation include direct protein-protein contact between activation domains and general factors (Lin, 1991; Lin, et al., 1991) (looping out intervening DNA) and disruption of chromatin, which results in an alleviation of repression (Croston and Kadonaga, 1993; Han, 1989; Workman, 1992).

Whatever their mechanism of action, activators require novel protein factors to potentiate their full activity. One class of these factors termed coactivators are tightly associated with the TATA binding protein (TBP) and comprise a TFIID complex (Dynlacht, et al., 1991). These TBP-associated proteins (TAFs) evidently serve as sites in the general machinery to which activators can bind (Goodrich, et al., 1993; Hoey, et al., 1993). Another class are products of yeast genes SWI1-3 and SNF5,.6 which comprise a single complex (Peterson, et al., 1994) (Cairns, et al., 1994). These proteins may function through chromatin because suppressers that bypass the requirement for them lie in histone (Hirschhorn, et al., 1992) and non-histone chromatin proteins (Winston and Carlson, 1992). In addition, the SWI/SNF complex promotes the binding of GAL4 derivatives to nucleosomal DNA in an ATP dependent manner (Verrijzer, et al., 1994).

A third class of cofactors required for activation includes products of the yeast *ADA2* and *ADA3* genes. Mutations in these genes were selected since they confer resistance to the toxic chimeric activator GAL4-VP16, containing the DNA-binding domain of GAL4 and the acidic activation domain of VP16 (Berger, et al., 1992). The toxicity of the chimera correlates with its unusual potency as an activator because mutations in VP16 which reduce activation also reduce toxicity (Berger, et al., 1992). Mutations in *ADA2* and *ADA3* allow cells to tolerate the chimera, and also reduce their ability to respond to certain transcriptional activators, including VP16 and GCN4 (Berger, et al., 1992; Piña, et al., 1993).

We have argued that ADA2 and ADA3 could be adaptors that bridge interactions between activation domains and general factors at promoters. This conclusion comes from two observations. First, the VP16 activation domain can be made to bind and sequester factor(s) needed for transcriptional activation but not for basal transcription *in vitro*, demonstrating that adaptors exist (Berger, et al., 1990). Second, mutations in *ADA2* or *ADA3* reduce activation by some, but not all, acidic activation domains *in vivo* and *in vitro* (Berger, et al., 1992; Piña, et al., 1993). This specificity argues for a functional interaction between the ADAs and specific activation domains.

Another yeast gene product that has been implicated in transcription is *GCN5*. Mutations in *GCN* genes cannot derepress *HIS3* and other genes that respond to the general amino acid control system (Hinnebusch and Fink, 1983; Penn, et al., 1983). This failure to derepress results from a defect in the synthesis, stability, or activity of the activator, GCN4. Whereas mutations in *GCN1-3* exert their effects by lowering translation of *GCN4* mRNA (Hinnebusch, 1985), mutations in *GCN5* do not affect the level of GCN4 protein, but rather reduce its ability to activate transcription (Georgakopoulos and Thireos, 1992).

Thus, it has been proposed that GCN5 could be a coactivator that augments the activity of GCN4 (Georgakopoulos and Thireos, 1992).

The *GCN5* sequence has a domain at the carboxyl terminus, the bromodomain, that is highly conserved in other proteins involved in transcription, including brahma from Drosophila (Tamkun, et al., 1992), yeast *SWI2* (*SNF2*) (Laurent, et al., 1991), yeast *SPT7* (Haynes, et al., 1992), the EIA-associated protein p300 (Eckner, *et al.*, 1994), and mammalian TAF250 (*CCG1*) (Ruppert, et al., 1993). The conservation is very high as illustrated by the 50% identity between *GCN5* and *CCG1* across the 70 amino acid bromodomain. The presence of the bromodomain in this apparently diverse set of transcription factors suggests that it is an important functional domain. However, attempts to show functionality of the bromodomain in these proteins have not yet succeeded (Elfring, et al., 1994; Laurent, et al., 1993).

Previously, we isolated ten alleles of *ADA1*, but only two alleles of *ADA2* and one allele of *ADA3*. Here, we demonstrate use of the same selection on a much larger scale to identify more genes. In addition to isolating more alleles of *ADA1*, *ADA2* and *ADA3*, we identify two new genes with similar properties. We show that one of these genes is *GCN5*, and demonstrate a physical interaction between GCN5 and ADA2 *in vivo* and *in vitro*. This provides the first direct indication that the GAL4-VP16 resistant mutants might define a set of proteins that comprise a single multi-protein complex involved in transcriptional activation. Finally, we show that the bromodomain is important in the function of GCN5.

RESULTS

Selection of GAL4-VP16-resistant mutants

The yeast strain BP1, which was used in the selections that yielded *ada2* and *ada3* mutants, (Berger, et al., 1992) was mutagenized and transformed with a high copy plasmid expressing GAL4-VP16 from the constitutive ADH1 promoter. 300 colonies showing resistance to GAL4-VP16 were analyzed as summarized in Table 1. In order to identify recessive chromosomal mutations, the candidates were mated to a wild type strain. 50 of the resulting diploid strains displayed sensitivity to GAL4-VP16, indicating that the mutation conferring resistance in the haploid was recessive. In the remaining 250 candidates the plasmid was removed and the resulting strains were mated to an *ada2* mutant bearing GAL4-VP16. All 250 diploids were sensitive to GAL4-VP16, indicating that resistance of the haploid mutants was due to a mutation on the original GAL4-VP16 expression plasmid. Thus, in none of the 300 strains was resistance due to a dominant chromosomal mutation.

The recessive mutants were characterized further by mating to *ada1*, *ada2* , or *ada3* tester strains. Candidates that failed to complement an *ada* mutation would give rise to diploids that were resistant to GAL4-VP16. Slow growth of the diploid would provide a further indication of a failure to complement. By these tests, we identified five new alleles of *ADA1*, eight new alleles of *ADA2*, and 12 new alleles of *ADA3*. Among the remaining mutants, complementation tests indicated two new groups termed *ADA4* (three mutants) and *ADA5* (one mutant). Complementation tests in other mutants were incomplete, and further analysis is needed to group them.

TABLE .1Selection for mutants resistant to GAL4-VP16 results in additional alleles of *ADA1*, *ADA2*, and *ADA3*, as well as alleles of two new genes.

PRIMARY TRANSFORMANTS:	**300,000**
PLASMID MUTANTS	**250**
ADA1 ALLELES	**5**
ADA2 ALLELES	**8**
ADA3 ALLELES	**12**
ADA4 ALLELES	**3**
ADA5 ALLELES	**1**

LEGEND. BP1 was mutagenized and transformed with pGAL4-VP16 Ura as described in Methods. Approximately 300 large colonies showing resistance to the toxic plasmid were picked. The majority of these appeared to be linked to the plasmid expressing GAL4-VP16, as described in Methods. Other strains were characterized as *ada1, ada2,* or *ada3* alleles by mating to a mutant tester strain and scoring the growth of the diploid on minimal medium as well as its resistance to GAL4-VP16 overexpression. Representative strains were transformed with the appropriate clone for confirmation. From tetrads, we obtained some of these resistant mutations in strains of the opposite mating type. Crossing among mutants was used to identify the *ADA4* and *ADA5* complementation groups.

Cloning of *ADA4* and its identification as *GCN5*

We chose to focus on *ADA4*, in part, because mutants displayed extremely slow growth on minimal media, a phenotype also seen in *ada2* and *ada3* mutants. Tetrad analysis indicated that slow growth and resistance to GAL4-VP16 co-segregated as a single mutation (not shown). ADA4 was cloned on a 12 Kb fragment from a yeast genomic library by restoration of normal growth to an *ada4* mutant strain. This clone also restored sensitivity to GAL4-VP16. The complementing fragment was subcloned to a 2.2 Kb fragment as described in Methods. The sequence at one end of the subclone corresponded to a portion of the *PUP2* gene which is adjacent to *GCN5* (Georgatsou, et al., 1992). Therefore, we determined whether the gene complementing the *ada4* mutation was indeed *GCN5*. Restriction analysis revealed that the entire *GCN5* coding sequence lay within this 2.2 KB. Furthermore, a 1.8 Kb *Xho*1-*Pst*1 fragment containing the *GCN5* sequence (Georgakopoulos and Thireos, 1992) complemented the *ada4* mutant. Lastly, the specific *GCN5* coding sequence amplified by PCR and placed under control of the *ADH1* promoter also complemented the mutant.

To confirm that the *ada4* mutation was in *GCN5*, the 1.8 Kb *Xho*1-*Pst*1 fragment was cloned into an integrating vector bearing the *URA3* marker and targeted to the *GCN5* locus. The strain containing the integrant was mated to the *ada4-1* mutant and the diploid sporulated. In 6/6 tetrads two segregants grew well and were Ura^+, and two grew slowly and were Ura^-, thus showing linkage between *GCN5* and *ADA4* (hereafter designated *GCN5*).

gcn5 mutants exhibit reduced activation by some activation domains *in vivo*

The *GCN5* gene was deleted as described in Methods. The resulting strain shared several phenotypes with *ada2* and *ada3* deletion mutants, including resistance to GAL4-VP16, slow growth on minimal media, and temperature sensitivity (not shown) on minimal or rich media.

Trans-activation by GAL4-VP16 was tested in the *gcn5* deletion mutant by introducing a low copy plasmid expressing GAL4-VP16 or GAL4-VP16FA, (with a Phe 442-Ala mutation) (Cress and Triezenberg, 1991). As shown in Table 2, the ability of GAL4-VP16 to activate a reporter bearing *lacZ* under control of the GAL1-10 UAS was reduced over 20-fold in the *gcn5* mutant and the activity of GAL4-VP16FA was reduced over 40-fold. The *gcn5-1* mutant strain showed a similar defect in the ability of GAL4-VP16 to activate transcription (not shown). The levels of GAL4-VP16 FA protein in the wild type and mutant strains were determined by gel shift analysis and were similar i. e. protein levels in the mutant were reduced by less than two fold (not shown).

We next tested the acidic activation domains of GCN4, GAL4, and HAP4, which were each fused to the lexA1-202 moiety and assayed using a *lacZ* reporter under control of a single lexA site (Table 2). The activity of the GCN4 domain was reduced about four and a half fold in the *gcn5* deletion, whereas the activities of the GAL4 and HAP4 domains were only affected about two fold. These activation domains had similar activities in the *gcn5-1* mutant (not shown). The levels of the lexA fusion proteins were comparable (less than two fold difference) in the wild type and *gcn5-1*mutant by Western blot analysis using anti-lexA antibody (not shown). This pattern of the activation domain defects in the *gcn5* strain recapitulated effects observed in *ada2* and *ada3* mutant strains (Piña, et al., 1993).

TABLE 2 *(Following page)*

LEGEND. Trans-activation by GAL4-VP16 and lexA activation domain fusions in a *gcn5* mutant and *ada2gcn5* double mutant. An ARS-CEN plasmid expressing GAL4-VP16 or GAL4-VP16FA was transformed into a wild type, and a Δ*gcn5* strain. The strains were also transformed with pLGSD5, a reporter plasmid with *lacZ* under GAL4 control. The lexA activation domain fusions, on an ARS-CEN plasmid, were transformed into those strains, as well as into an isogenic *ada2gcn5* double deletion strain, along with Yep21-Sc3423 (Hope and Struhl, 1986), which contains the *lacZ* gene under the control of a lexA operator site. The specific activity of ß-galactosidase averaged form at least three independent experiments (S.D. <20%) is presented. pLGSD5 gives a background of 4-5 units, and Yep21-Sc3423 plus lexA202 alone gives 10-20 units of activity (not shown). Levels of GAL4-VP16 FA were determined in wild type and *gcn5-1* strains by gel shift of a GAL4 site and were similar (data not shown). Likewise, levels of each lexA fusion protein were compared in extracts from wild type and *gcn5-1* cells by Western analysis using anti-lexA antibody and were comparable (data not shown).

TABLE 2. Trans-activation by GAL4-VP16 and lexA activation domain fusions in a *gcn5* mutant and *ada2gcn5* double mutant.

	WT	*Δgcn5*	*Δgcn5 Δada2*
GAL4-VP16 WT	17872	814	N D
GAL4-VP16 FA	6406	144	N D
LEX-GAL4	4049	1823	1433
LEX-GCN4	1785	404	300
LEX-HAP4	4133	2508	2303

ada2 gcn5 and *ada3 gcn5* double mutants

Since *gcn5* null mutations displayed very similar properties to null mutations in *ADA2* and *ADA3*, we constructed double mutants between *GCN5* and the *ada* mutants. If the genes operated in the same pathway, or as a complex, the double deletion strain should not have a more severe phenotype than either of the single mutants. *gcn5 ada2* and *gcn5 ada3* double deletion mutants were generated in the BWG1-7A background as described in Methods. The slow growth phenotype of these strains could be restored to wild type only if they were transformed with both a plasmid bearing *GCN5* and a plasmid bearing the appropriate *ADA* gene. Importantly, these double mutants behaved similarly to *ada2 ada3* double mutants (Piña, et al., 1993), in that they grew no more slowly than the single mutants did (data not shown). Furthermore, the level of trans-activation by lexA-GCN4, lexA-HAP4, and lexA-GAL4 in an *ada2 gcn5* double mutant is similar to that in a single deletion mutant in *gcn5* (Table 2) or *ada2* (not shown) This is strong genetic evidence that ADA2, ADA3 and GCN5 function in the same pathway or as a complex *in vivo*.

lexA-ADA2 and lexA-ADA3 activate transcription in a GCN5-dependent manner

ADA2 and ADA3 were tested for their ability to activate transcription when fused to the lexA202 moiety. These fusions both complement a mutation of the cognate *ADA* gene. Table 3 indicates that these fusions were transcriptionally active and that their activities were greatly reduced in a *gcn5* mutant strain. Further, the activity of lexA-ADA2 was reduced in a *ada3* mutant, and vice versa (unpublished data). These findings provide further evidence for a functional interdependence between *GCN5* and the *ADA* genes, but they must be interpreted with caution (see Discussion).

TABLE 3. lexA-ADA2 and lexA-ADA3 activate transcription in a GCN5 dependent manner.

	WT	Δgcn5
LEX-ADA2	**179**	**63**
LEX-ADA3	**173**	**42**

LEGEND. The wild type and *gcn5* deletion strains BP1 and GMy25 were transformed with plexA-ADA2 or plexA-ADA3 and the *lacZ* reporter Yep21-Sc3423 (Hope and Struhl, 1986). Levels of ß-galactosidase were measured as in Table 2.

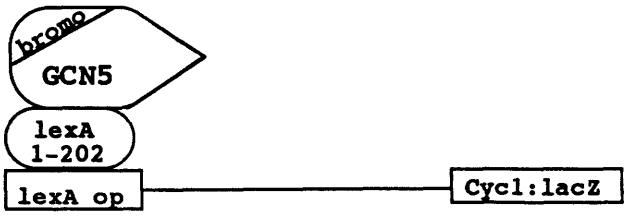
GCN5 binds to ADA2 *in vivo* and *in vitro*

The above observations are consistent with the possibility that GCN5 binds to ADA2. To test whether ADA2 and GCN5 do indeed interact, we carried out two-hybrid studies (Fields and Song, 1989) between lexA-GCN5 and ADA2 fused to a portion of the VP16 activation domain (residues 452-490 see Methods). Both the GCN5 and ADA2 fusion proteins retain the ability to complement the respective mutations *in vivo* and thus retain function. As shown in Figure 1, the activity of lexA-GCN5 is stimulated about 50-fold by ADA2-VP16 as compared to overexpression of ADA2 alone. The lexA DNA binding domain (1-202) alone was not affected at all by ADA2-VP16. This finding suggests that GCN5 and ADA2 interact *in vivo*.

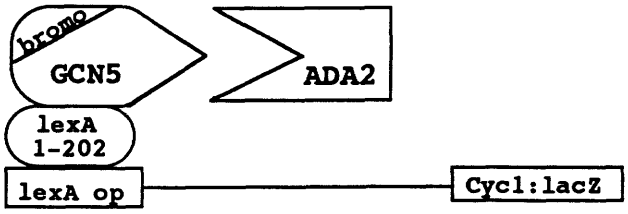
The two-hybrid experiment does not distinguish direct binding of GCN5 to ADA2 from an interaction that may be mediated by other proteins. In order to determine whether GCN5 and ADA2 interact with each other directly, we translated both proteins in a reticulocyte lysate programmed with mRNA from the *ADA2* and *GCN5* genes. As a control we co-translated each gene with luciferase. Precipitation was carried out with antibody to ADA2 (see Methods). Figure 2 shows that GCN5 was clearly co-precipitated with ADA2. In the absence of ADA2, the antibody did not precipitate any GCN5. Further, luciferase was not co-precipitated when translated with ADA2. These results suggest that there is a direct physical interaction between GCN5 and ADA2.

FIGURE 1. GCN5 interacts with ADA2 by Two Hybrid Analysis. BWG1-7a was transformed with a plasmid containing the lexA DNA binding and dimerization domains fused to GCN5 or GCN5Δ bromo. A second plasmid expressed either ADA2, ADA2VP16 or neither protein. The strain also contained the *lacZ* gene under control of a single lexA operator in plasmid pRbHis (gift of John Fikes). Specific activity of ß-galactosidase is shown which represents the mean of at least three independent experiments with an error of less than 20%. In addition the control of lexA 1-202 alone gave 25 units of activity, and varied by less than two units when ADA2 or ADA2VP16 were coexpressed (data not shown).

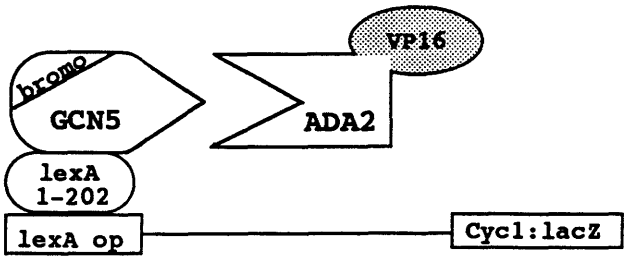
β-Gal Activity



21

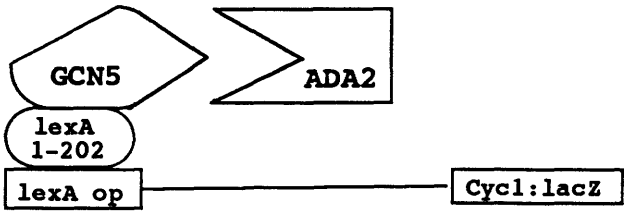


60

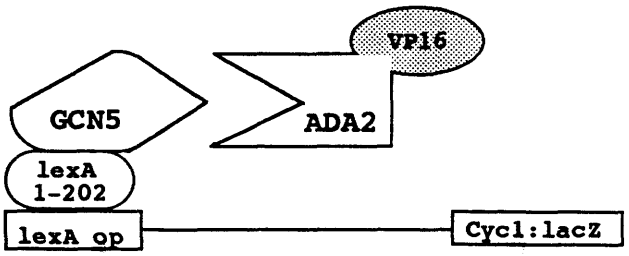


3261

55



101



6214

FIGURE 2 *(Following page)*. The GCN5 protein co-precipitates with ADA2. ADA2 was co-translated with GCN5, GCN5Δbromo, or luciferase in reticulocyte lysate incorporating ^{35}S-Methionine. GCN5 and GCN5Δ were also co-translated with luciferase as a control. Lanes 6-10 show the products of these translations as the "input". '+' indicates which proteins were translated. These lysates were precipitated with anti-ADA2 antibody and the pellets were boiled and loaded on a 10%SDSPAGE gel as described in Methods. Lanes 1-5 show the "precipitate".

	PRECIPITATE					INPUT				
ADA2	+	+	+			+	+	+		
GCN5	+			+		+			+	
GCN5Δ		+			+		+			+
Luciferase			+	+	+			+	+	+
Lane	1	2	3	4	5	6	7	8	9	10

The GCN5 bromodomain is functional

In order to test whether the bromodomain is important in the function of GCN5, we generated a version of GCN5 by PCR that deleted the bromodomain (see Methods). The N-terminal primer was designed to fuse the influenza hemagglutinin (HA) epitope at the amino terminus of the gene. As shown in Figure 3, the HA epitope tag itself had no effect on the ability of GCN5 to complement a mutant. However, GCN5 missing its bromodomain (*GCN5Δ*) only weakly complemented a *gcn5* deleted strain for growth on minimal plates. We suspected the growth defect in a *GCN5Δ* strain was do to a defect in transcription. Therefore, we assayed lexA-GCN4, lexA-HAP4 and lexA-GAL4 for their ability to trans-activate in a *gcn5* deletion mutant complemented with either full length *GCN5*, or *GCN5Δ*. The ADA dependent activation domain of GCN4, showed a partial reducion in its ability to activate transcription in the absence of the bromodomain, whereas the largely ADA independent GAL4 and HAP4 activation domains did not (Figure 4). Finally, to determine if GCN5Δ can restore GAL4-VP16 toxicity, a gcn5 deletion strain was cotransformed with GAL4-VP16 and either GCN5, or GCN5Δ. As shown in Figure 5, cotransformation of GCN5 and GAL4-VP16 results in small, pinpoint colonies, whereas cotransformation of GCN5Δ and GAL4-VP16 results in slightly larger colonies, as well as a greater frequency of large colonies that presumably represent mutant GAL4-VP16 plasmids. (The small colonies do not restreak, and thus the transformation plates must be scored directly. The difference between the ability of GCN5 and GCN5Δ to restore GAL4-VP16 toxicity is subtle, and may be an artifact of the cotransformation assay. The best way to compare the ability of GCN5 and GCN5Δ to restore toxicity would be to express GAL4-VP16 from an inducible promoter, in the presence of GCN5 or GCN5Δ.

However, this has not been done). Thus, in three functional assays, the bromodomain was important for GCN5 function. To demonstrate that deletion of the bromodomain did not result in degradation of GCN5, we carried out Western blot analysis using antibody to the HA epitope (Figure 6). The levels of GCN5 and GCN5Δ proteins were similar in cell extracts.

The bromodomain could be important in aiding the GCN5-ADA2 interaction, or in facilitating the activity of the assembled ADA complex. To determine whether the bromodomain was important for the ADA2-GCN5 interaction, we carried out the *in vivo* and *in vitro* assays for this interaction with GCN5Δ. GCN5Δ was at least as active as full length GCN5 in the two-hybrid

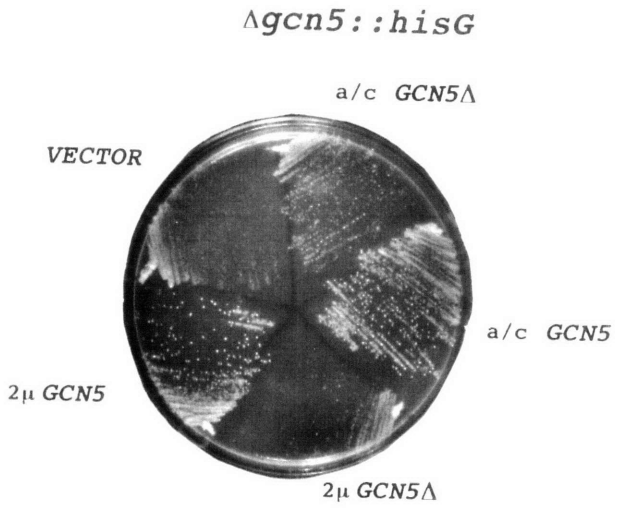


FIGURE 3. ***GCN5*** **deleted of the bromodomain (*GCN5Δ*) has reduced ability to complement a *gcn5* deletion. GMy25, a *gcn5* deletion strain, was transformed with vector, high copy 2 micron(2*μ*) or low copy ARS/cen(a/c) plasmids expressing HA-GCN5 or HA-GCN5Δ from the ADH promoter. Transformants were restreaked on minimal medium containing glucose.**

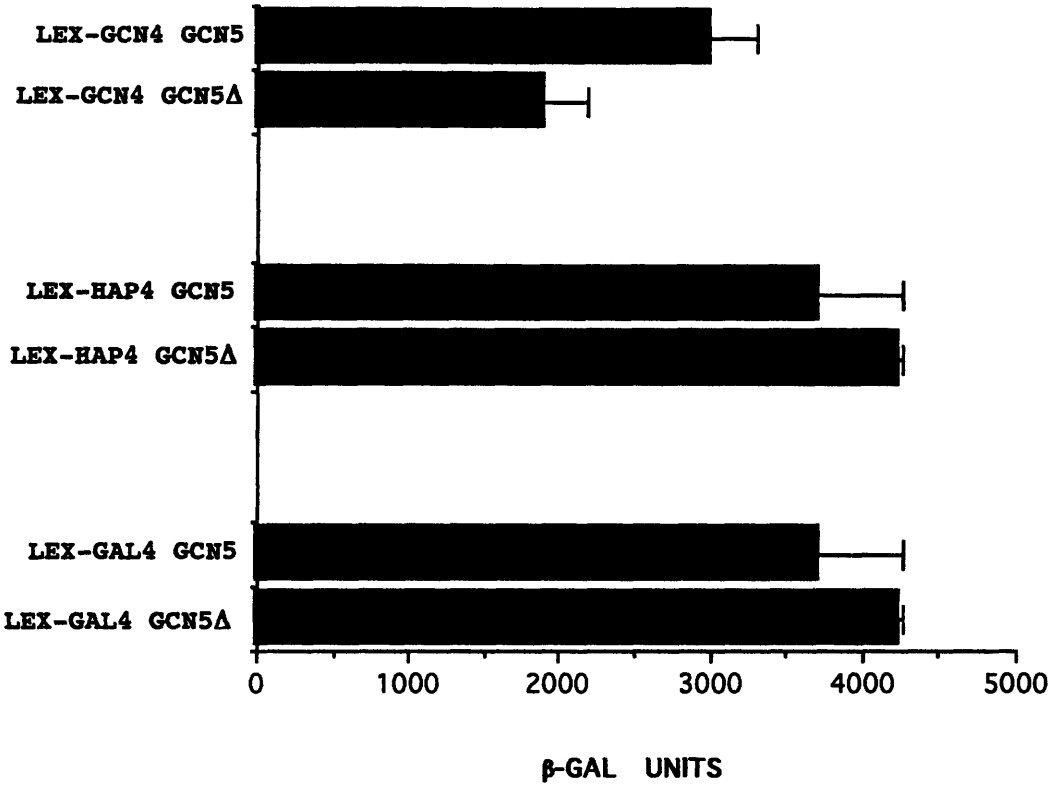
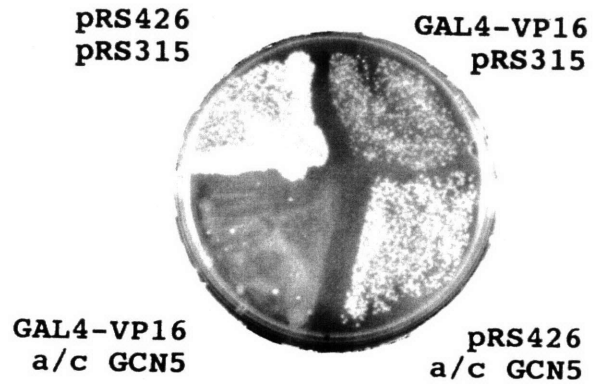


FIGURE 4. GCN4 but not HAP4 or GAL4 mediated activation is reduced in a GCN5 bromodomain deletion mutant (GCN5Δ). GMy23, a *gcn5* deletion strain was transformed with the lexA activation domain fusions, as well as a second plasmid expressing *GCN5* or *GCN5Δ* from the natural *GCN5* promoter. The strain also contained the *lacZ* gene under control of a single lexA operator in plasmid pRbHis. Levels of ß-galactosidase were assayed as in Table 2. Error bars are shown. As an additional control to show that the mutant strain is indeed defective for trans-activation, the lexA fusions were also assayed in the same experiment with a vector that did not express any version of *GCN5*. lexA-GCN4 gave 151 units, lexA-HAP4 gave 1318 units, and lexA-GAL4 gave 1029 units.

FIGURE 5. *(On the following page) GCN5* deleted of the bromodomain only partially restores sensitivity to GAL4VP16 toxicity to a *gcn5* deletion strain. **A.** GMy25 (BP1*Δgcn5*)was doubly transformed with all pairwise combinations of a high copy plasmid expressing GAL4-VP16 (or the matched *URA3* vector control, pRS426) and a low copy ARS-CEN (a/c) plasmid expressing *GCN5* from the ADH1 promoter (or the matched *LEU2* control, pRS315). The transformants were plated on drop out medium on a single plate. The plasmids are listed next to the quadrant in which they were plated. **B.** The transformants here are identical to those in part A, except that a plasmid expressing *GCN5* deleted of the bromodomain (GCN5Δ) was used instead of full length *GCN5*. The severe growth defect of *gcn5* strains observed on minimal medium (FIGURE3a) is not observed on the supplemented drop out medium after three days. The few large colonies observed in the *GCN5*/VP16 quadrant result from mutations, presumably in the GAL4-VP16 expression plasmid. Note that in the *GCN5Δ*/GAL4-VP16 quadrant all transformants grow slightly larger than the transformants in the *GCN5*/VP16 quadrant, and the frequency of large colonies is also greater. This should not be considered more than a mild effect.

A


pRS426
pRS315
GAL4-VP16
pRS315
GAL4-VP16
a/c GCN5
pRS426
a/c GCN5

B

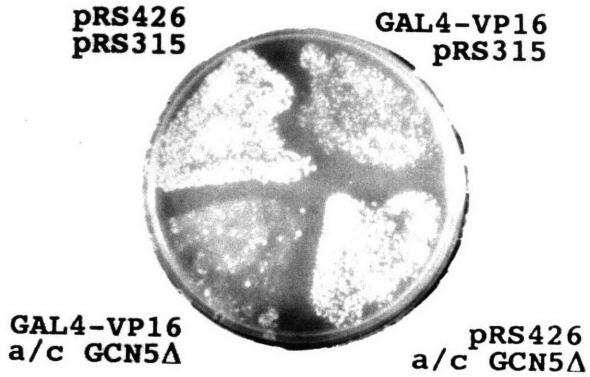
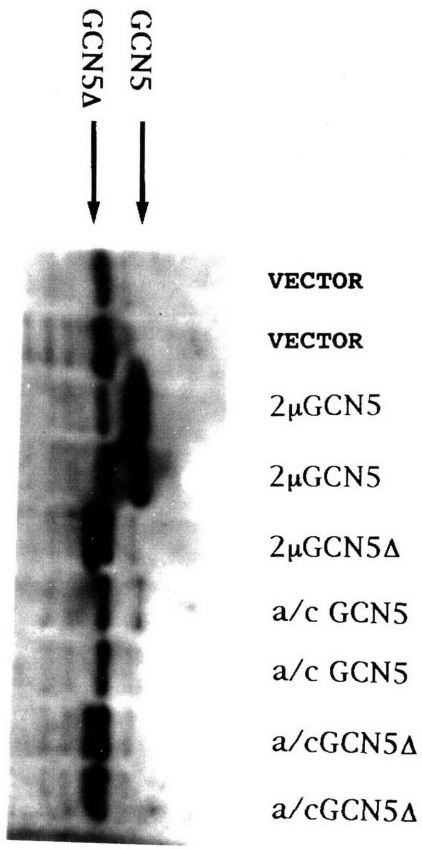

pRS426
pRS315
GAL4-VP16
pRS315
GAL4-VP16
a/c GCN5Δ
pRS426
a/c GCN5Δ

FIGURE 6. *(On the following page)* Western analysis shows similar levels of GCN5 and GCN5Δ protein in a *gcn5* deletion strain. Western analysis using 12CA5 antibody to the HA epitope (Kolodziej, 1991) was performed on whole cell extracts of the transformants of GMy25 described in Figure 3a. The bands corresponding to GCN5 and GCN5Δ proteins are indicated. A background protein, found in all extracts, runs directly above the GCN5Δ band.


GCN5Δ
GCN5
VECTOR
VECTOR
2μGCN5
2μGCN5
2μGCN5Δ
a/c GCN5
a/c GCN5
a/cGCN5Δ
a/cGCN5Δ

assay (Figure 1). Further, GCN5Δ was co-precipitated with ADA2 in a manner similar to GCN5 (Figure2, lanes 1 and 2). Thus, we conclude that the bromodomain is not an important determinant of the GCN5-ADA2 interaction.

DISCUSSION

We describe an exhaustive application of the selection for mutations resulting in resistance to GAL4-VP16. We uncovered more alleles of three genes previously identified, *ADA1*, *ADA2*, and *ADA3*, and also describe mutations in two additional genes that arose from the selection, *ADA5* and *GCN5*. We argued previously that *ADA1* might be mechanistically different from *ADA2* and *ADA3* because *ada1* mutants displayed vastly reduced levels of the toxic chimera, while *ada2* and *ada3* mutants did not (Berger, et al., 1992). Mutations in either *ADA5* (data not shown) or *GCN5* allow accumulation of GAL4-VP16, suggesting that they are similar to *ADA2* and *ADA3*. The properties of the *gcn5* mutant and the interaction between GCN5 and ADA2 are the subject of this report.

On the basis of five criteria, we conclude that GCN5 and ADA2 interact physically and may comprise a part of a multi-protein complex. First, *gcn5* mutants display a very similar phenotype to *ada2* or *ada3* mutants. In particular, strains grow slowly on minimal media, are temperature sensitive on any media, and greatly reduce trans-activation by the GCN4 and VP16 activation domains, with smaller effects on the GAL4 and HAP4 activation domains. Second, doubly null mutants, *ada2 gcn5* or *ada3 gcn5* do not have a more severe phenotype than single mutants. Third, lexA-ADA2 and lexA-ADA3 display trans-activation activities that are dependent upon GCN5. (Other interpretations of this data are possible. For example, lexA-ADA2 and lexA-ADA3 may contain cryptic activation domains that are GCN5 dependent, much as the VP16

activation domain is GCN5 dependent. However, given the other evidence for an ADA2/GCN5 interaction, and the utility of lexA fusions to study interactions among HAP2, HAP3, and HAP4 (Olesen and Guarente, 1990), as well as SNF2,SNF5 and SNF6 (Laurent and Carlson, 1992), it is reasonable to argue that the activity of lexA-ADA2 and lexA-ADA3 represent the activity of an ADA complex). Fourth, ADA2 and GCN5 show a strong interaction *in vivo* by two-hybrid analysis. Fifth, ADA2 and GCN5 co-precipitate. This final experiment suggests that the interaction between the two proteins is direct and requires no other yeast proteins. Furthermore, recent experiments have shown that GCN5 cofractionates with affinity purified ADA2 protein from yeast extracts (N. Silverman, unpublished results).

Thus, we envision a complex containing these two proteins and perhaps ADA3, and ADA5. There may be additional factors in this set among those strains that are resistent to GAL4-VP16 that have not yet been characterized. Several other multi-protein complexes have been shown to play a role in eukaryotic transcription. The *SWI1, SWI2/SNF2, SWI3, SNF5* and *SNF6* genes are important for transcription of many yeast genes. They were first classified together genetically (Winston and Carlson, 1992) and now have been shown to comprise a complex (Cairns, et al., 1994; Peterson, et al., 1994). These factors are evidently important for activity of the glucocorticoid receptor in yeast (Yoshinaga, et al., 1992), and they promote the binding of GAL4 derivatives to nucleosomal DNA *in vitro* (Verrijzer, et al., 1994). Similarly, the *SRB* genes interact genetically with the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Thompson, et al., 1993). The products of these genes form a complex that co-fractionates with RNA polymerase II and comprise an RNA pol II holoenzyme that also includes TFIIB , the 73K subunit of TFIIH , and TFIIF (Koleske and Young, 1994). A third complex may involve products of

some SPT genes, identified as suppressors of TY1 insertions in yeast promoters (Winston, *et al.*, 1984). Based on the similarity of SPT3, 7, 8, and 15 mutants, it is possible that the products of these genes comprise a complex (Winston, 1992). In fact, SPT3 and TBP, the tata binding protein, which is the SPT15 product, have been shown to interact (Eisenmann, *et al.*, 1992). In Drosophilla and mammalian cells, TBP is a part of a multi-protein complex, TFIID, which also contains TBP associated factors (TAFs) (Dynlacht, et al., 1991).

What is the role of the ADA2-GCN5 complex? We have suggested that ADA2 and ADA3 might be transcriptional adaptors which help bridge the interaction between activators and the basal factors. Consistent with this hypothesis, expression of an epitope tagged version of ADA2 in yeast allows co-precipitation of the tagged ADA2 protein and GAL4-VP16 in yeast extracts (N. Silverman, J. Agapite, and L.G., submitted; R. Candau, N. Bordei, D. Darpino, L. Wang, and S.B., unpublished data). We surmise that the ADA/GCN5 complex also contains domains that interact with one or more of the basal factors.

One domain that is a candidate for such interactions is the bromodomain, found at the carboxyl-terminus of GCN5, and also in the mammalian TAF complex, the SNF complex, the E1A associated p300 (Goodrich and Tjian, 1994), and in several factors in Drosophila, such as brahma (Kennison, 1993). In several cases, deletion of the bromodomain was shown to be inconsequential (Laurent, et al., 1993) (Elfring, et al., 1994) (Gansheroff, et al., 1995).

Here, we show that deletion of the bromodomain does not lower the steady-state levels of GCN5, but does reduce the ability of the protein to complement a *gcn5* deletion strain and to support the activity of the GCN4

activation domain. In addition, the truncated protein only partially restores toxicity by GAL4-VP16 compared to the full length GCN5. We have previously proposed that toxicity was due to trapping of basal factors by the potent VP16 activation domain at chromosomal sites (Berger, et al., 1992). The bromodomain may be important in this process by helping the ADA complex bind to activation domains to basal factors, or to DNA.

Although it is also possible that the bromodomain helps interactions within the ADA complex, we do not favor this possibility for two reasons. First, the bromo-deleted GCN5 interacts with ADA2 in the two-hybrid and co-precipitation assays as well as the full length GCN5 does. Second, the fact that the domain is present in proteins found in other transcription complexes suggests that its function is more general. We infer that the function of the bromodomain is partially redundant in the ADA complex, because the truncated protein still has a partial ability to function. The function of the bromodomain may be redundant in other complexes in which it could be deleted without impairing activity.

In summary, we show that our genetic selection has converged on at least two proteins, ADA2 and GCN5, that function together by virtue of comprising a heteromeric complex. The importance of such complexes in transcription is just now coming to light. The precise molecular function of this complex and the activity of the bromodomain in particular, should bring further understanding to the process of eukaryotic transcriptional activation.

MATERIALS and METHODS

Selection of GAL4-VP16 Resistant Mutants

pGAL4-VP16 URA was generated by ligating a 2.8 KB. *Bam*HI fragment from pSB201 (Berger, et al., 1992) containing the ADH promoter/terminator

cassette with GAL4-VP16 into the *Bam*HI site of pRS426 (Sikorski and Hieter, 1989).

The strain BP1 (*MAT a, ade1-100, ura3-52, leu2-3,2-112, his4-519*) was mutagenized with EMS (Guthrie and Fink, 1991), grown for 5 hours in YPD, and then transformed with the 2μ plasmid pGAL4-VP16 URA, and plated on the rich medium sd+ 0.1%case amino acids, 0.006%adenine, 2% glucose. 300,000 primary transformants were screened, the majority of which were tiny, pinpoint colonies. 300 larger colonies were picked and restreaked. Candidate strains with the toxic plasmid were mated to PSY316 (*MAT*α, *ade2-101, ura3-52, leu2-3,2-112, his3-del.200, lys2*), a wild type tester strain, and diploids that retained the plasmid with GAL4-VP16 were selected. Diploid strains that regained sensitivity to the toxic plasmid were obtained when the original haploid strain contained a recessive mutation that gave resistance to GAL4-VP16. The other strains were presumed to have a dominant chromosomal mutation or a mutation in the GAL4-VP16 expression plasmid. These strains were cured of the plasmid by growth on FOA, and mated to strain NSy5B (*MAT*α, *ade2-101, ura3$^-$52, leu2-3,2-112 ada2-2*, his$^-$) containing pGAL4-VP16URA. None of the resulting diploids were clearly resistant to the toxic plasmid, implying that all 250 of these strains had mutations linked to the plasmid. The strains with recessive mutations were mated to *ada1$^-$*, *ada2$^-$*, or *ada3$^-$* tester strains to identify additional alleles of these genes by complementation of the slow growth and toxicity resistance phenotypes. *ADA4* and *ADA5* complementation groups were identified among the remaining resistant strains using a segregant that was obtained during tetrad dissection. Additional strains resistant to GAL4-VP16 were isolated that do not conform to these complementation groups. In most cases this is because they lack secondary phenotypes or appeared to have multiple mutations responsible for the slow growth phenotype. We also isolated

1 sterile strain that conferred resistance to GAL4-VP16. However, no GAL4-VP16 protein was detected (not shown).

Cloning and Sequencing of GCN5

GMy47c (BP1 *gcn5-1*) was transformed with a yeast genomic library (Thompson, et al., 1993) and colonies which grew well on minimal media were selected. From these, we isolated a clone, p15-1,2c with a 12KB insert that restored wild type growth and sensitivity to GAL4-VP16 to GMy47c, as well as to strains with *gcn5-2*, or *gcn5-3* alleles. 15-1,2c was partially digested with *Sau*3a, the DNA was run on a 1.2% agarose gel, and a band was cut out with fragments ranging from 1-3KB. The DNA was gene cleaned (Bio 101) and ligated into pRS316 cut with *Bam*HI to generate a sub-genomic library. GMy47c was transformed with the subgenomic library, and a 2.2KB subclone, p5-1,2D, was isolated from a fast growing colony that restored wild type growth and sensitivity to GAL4-VP16 to GMy47c. Restriction analysis later revealed that 5-1,2D is in its CT3, the vector of 15-1,2c and not in pRS 316. Thus, the subclone is an internal deletion of almost 10KB from the insert of 15-1,2C.

The ends of the insert in 5-1,2D were sequenced using the Sequenase kit (USB) using the T3 and -20 primers. The DNA sequences were analyzed using the Blast program (Altschul, et al., 1990), and the sequence from the -20 primer matched the yeast sequence for the *PUP2* gene (Georgatsou, et al., 1992), which lies adjacent to GCN5

GCN5 Plasmids

pRS316 GCN5 was generated by cutting p5-1,2D with *Pst*I, blunting with T4 polymerase, and cutting again with *Xho*I to get a 1.8 KB. fragment. This was

cloned into pRS 316 cut with *Xho*I and *Sma*I. This same 1.8 KB. fragment was cloned into pRS 306 cut with *Xho*I and *Sma*I to generate pRS306 GCN5.

The PCR generated fragments were cut with *Not*I and cloned into a high copy vector (DB20L) or a low copy vector (RK15) to generate the following ADH expression plasmids: pDB20LGCN5 (using primers GCN5N and GCN5C, Table 4), pDB20LGCN5Δ (using primers GCN5N and GCN5CΔ, Table 4), pDB20LHA-GCN5 (using primers NHAGCN5N and GCN5C, Table 4), pDB20LHA-GCN5Δ (using primers NHAGCN5N and GCN5CΔ, Table 4). PCR primers are listed in Table 4. The same fragments were ligated into the *Not*I site of pRK15 (an ARS/cen ADH expression plasmid based on pRS315 R.Knaus, unpublished data) to generate pRKGCN5, etc.

pRS315GCN5 was generated by cloning a 1.8 KB *Xho*I *Eco*RV fragment containing the *GCN5* gene from pSP72 GCN5 (see below) into the *Xho*I - blunted *Bam*HI site of pRS 315(Sikorski and Hieter, 1989). pRS315GCN5Δ was generated by removing most of the *GCN5* coding sequence from pRS315GCN5 by cleaving at the unique *Hind*III (which cuts 15 base pairs after the stop codon) , filling in the ends with the Klenow fragment of DNA polymerase, and then cleaving with *Bam*HI, which cuts 50 base pairs after the start codon. The remainder of the coding sequence for GCN5Δ was supplied by cutting pRKHAGCN5Δ with *Not*I to release the GCN5Δ insert, treating with the Klenow fragment of DNA polymerase to blunt the ends, and cutting with *Bam*HI.

lexA and VP16 fusion plasmids:

plexA-ADA2 was generated by amplifying the *ADA2* gene using primers ADA2LN, AND ADA2LC (Table 4), cutting with *Not*I, and ligating in frame to the *Not*I site of pADHlexA202 (a 2μplasmid). plexA-ADA3 was generated the same way except primers ADA3N and ADA3CNOT (Table 4) were used to amplify

ADA3. plexA-GCN5 and plexAGCN5Δ were generated the same way except that primers GCN5N and GCN5C or GCN5CΔ (Table 4) were used to amplify *GCN5* and *GCN5Δ* respectively. All three lexA fusions were able to complement the slow growth and toxicity phenotypes in the appropriate *ada* mutant strains (data not shown). lexAGCN5Δ was able to complement GMy25 as well as pRKHA-GCN5Δ.

The ADA2VP16 plasmid was generated in two steps. ADA2 was amplified using primers ADA2PRON and ADA2CNOT (Table 4), cut with *Hind*III and cloned into the *Hind*III site of pRK25 (a 2μ ADH expression plasmid based on pRS425 R.Knaus, unpublished data) to generate pRK25ADA2CNOT. Then, the bases encoding residues 452-490 of VP16 were amplified by PCR using primers V452N and VP16C (Table 4), cut with *Not*I, and cloned into pRK25ADA2CNOT cut with *Not*I, which fuses VP16 452-490 in frame with the C-term of ADA2, to generate pRK25ADA2VP16.

The lexA His reporter pRBHis (gift of John Fikes) was generated by cutting Rb1155 (Brent and Ptashne, 1985)with *Stu*I to excise the *URA3* gene filling in with the DNA polymerase Klenow fragment, and ligating the *HIS4* fragment from pB54 (Donahue, et al., 1982).

TABLE 4 *(Following page)*

LEGEND. PCR Primers. Primers were synthesized at the Biopolymers Laboratory, Howard Hughes Medical Institute, Center For Cancer Research, Department of Biology, MIT. 50 pMol of each primer was used for each PCR reaction.

TABLE4: PCR Primers

NAME	SEQUENCE
GCN5N	CCCGGGAGATCTGCGGCCGCGATGGTCACAAAACATCAG
GCN5C	GAACCCCGGGGCGGCCGCCTAAGATCTTCAATAAGGTGAGAATA TTC
GCN5CΔ	GGCCCGGGGCGGCCGCCTAAGATCTTGCTGCATGATTTTGTAGC
GCN5AADC	CCCGGGAGATCTCTAAGAGGCCGCTCAATAAGGTGAGAATATTC
NHAGCN5	CCCGGGGCGGCCGCATGCTTACCCATACGACGTCCCAGACTACG CCATGGTCACAAAACATCAGATTG
ADA2LN	GGGCCGCGGCCGCATGTCAAACAAGTTTCACTGTGAC
ADA2LC	GGGCCGCGGCCGCTTACATCCAATTCTGGCTCTGGAA
ADA2proN	GGGCCCGGAAGCTTCATGAGCAACAAGTTTCACTGTGACGTTTG
ADA2CNOT	CCCGGGAAGCTTAAGCGGCCGCCATCCAATTCTGGCTCTGG
ADA3N	CCCGGGGCGGCCGCTGGATCCATGCCTAGACATGGAAGAAGAGG
ADA3CNOT	CCCGGGTGCGGCCGCTTAATTTAGTTCCACGTCC
V452N	CCCGGGGCGGCCGCGTCCCCGGGTCCGGGATTTACC
VP16C	CCCGGGATCCGCGGCCGCTACCCACCGTACTCGTCAATTCC

Deletion Plasmids and Strains:

The *GCN5* deletion plasmid was generated in several steps. First, the *Bam*HI site in pSP72 (Promega) was destroyed by cutting, filling in using the DNA polymerase Klenow fragment, and ligation to generate pSP72-Bam. Next, the 1.8KB *Xho*I-*Pst*I fragment from 5-1,2D, containing *GCN5* and flanking sequence was cloned into the *Xho*I-*Pst*I sites of pSP72-Bam to generate pSP72 GCN5. The *GCN5* coding sequence was removed by ligating a *Bam*HI linker to a filled in *Hind*III site, followed by digestion with *Bam*HI. This served as the backbone to which the 2.4 KB. *Bam*HI-*Bgl*II *hisG Ura3* cassette from pNKY51 (Alani, et al., 1987) was ligated, to generate pGCN5KO.

The *ADA3* deletion plasmid was generated in several steps also. A 2.9KB *Xba*I-*Pst*I fragment containing *ADA3* and flanking sequences was cut from the genomic clone pADA3HHV (Piña, et al., 1993) and ligated into the *Xba*I *Pst*I sites of pSP65 (Promega) to generate pSP65 ADA3. A *Nde*I-*Spe*I fragment encoding the first 588 amino acids of the ADA3 protein was removed from this plasmid. The ends were filled in with DNA polymerase Klenow fragment , ligated with BglII linkers, and cut with BglII. The 2.4 KB. *Bam*HI-*Bgl*II *hisG URA3* cassette (Alani, et al., 1987) was ligated into this backbone to generate pADA3KO.

GCN5 deletion strains were generated by transforming yeast with 10 μg of GCN5KO cut with *Xho*I-*Sal*I. Slow growing Ura$^+$ transformants were tested for resistance to GAL4-VP16, and to see if wild type growth was restored by DB20L GCN5. Strains that were resistant to GAL4-VP16 and had wild type growth restored by the clone were streaked on 5-fluoroorotic acid (FOA) to select strains that had looped out the *URA3* sequence. In this manner, Ura$^+$ and ura$^-$ deletion strains GMy22 and GMy23 were generated from BWG1-7a;

GMy24 and GMy25 from BP1; and GMy26 and GMy27 from PSY316. 1-7a*Δada2Δgcn5* was constructed by transforming GMy23 with ADA2KO (Berger, et al., 1992) cut with *Bam*HI and *Xho*I. Transformants were isolated, tested by mating, grown in YPD broth and plated on medium containing FOA to select strains that had excised the *URA3* gene from the hisG cassette. The genotype of the strains were confirmed transformation with the *ADA2* and *GCN5* clones.

GMy28 (BWG1-7a*Δada3Δgcn5*) was constructed in a similar manner except that GMy23 was transformed with pADA3KO plasmid cut with *Pvu*II and *Bam*HI. Double mutants were confirmed by mating and by transforming with the *ADA3* and *GCN5* clones.

PSY316GCN5 was generated by transforming PSY316 with pRS306GCN5 cut with *Hind*III to target the *GCN5* locus. This strain was mated to GMy47c (BP1, *gcn5-1*). The resulting diploid was sporulated and tetrads were discected.

ADA2 anti-sera.

The *ADA2* coding sequence engineered with a *Bsp*HI site at the ATG, 6 Histidines at the C-terminus, as well as flanking *Hind*III sites was generated using PCR and primers ADA2PROC and ADA2PRON (Table 4). This PCR product was cloned into pRK16 (gift of R. Knaus) as a *HindIII* fragment and checked for complementation in yeast. Then, the gene was isolated on a *Bsp*HI/*Hind*III fragment and cloned in a *Nco*I and *Hind*III digested pUH24.2ΔCAT. This vector was contructed by modifying the expression vector pDS56/RBSII, *Nco*I (gift of D. Stüber, identical to pQE-7 from Qiagen) by cutting with *Bsm*I and religating, leaving a unique *Nco*I site. The *ADA2* bacterial expression vector, pA26HE produced large amounts of ADA2 protein which

was insoluble. Denaturing Ni-bead chromotography (Qiagen) was used to purify this protein.

Purified ADA2 protein (0.5-1.0mg/ml in Saline) was mixed with RIBI adjuvant (RIBI ImmunoChem Research, Inc.) and used to immunize two rabbits per standard protocol (Harlow, 1988). After several boosts crude sera was assayed for anti-ADA2 antibodies by Western blot analysys. It was demonstrated that one rabbit produced a good titter of anti-ADA2 sera by virtue of its ability to recognize ADA2 protein in E. coli extracts from strains with pA26HE, but not in control extracts. ADA2 protein could also be detected in yeast extracts from strains overexpressing ADA2 (data not shown).

In vitro transcription/translation

To generate *GCN5* RNA the transcription plasmid pT7GCN5 was generated by amplifying *GCN5* with the primers GCN5N and GCN5AADC (Table 4), cutting with *Bgl*II, and ligating into the *Bam*HI site of T7Plink (Dalton and Treisman, 1992). pT7GCN5Δ was generated in the same way except the PCR fragment was amplified using the GCN5CΔ oligo (Table 4) instead of the GCN5AADC oligo. pT7ADA2 was generated by ligating the *Bsp*HII-*Bgl*II fragment from pA2HA (Silverman, Agapite and Guarente, in preparation) into the *Nco*I-*Bam*HI sites of T7Plink.

Transcription reactions were carried out using 2.5μg of T7GCN5 or T7GCN5Δ linearized with XhoI in 1X T7buffer (GIBCO BRL). Trace amounts of rUTP were included in the reaction to measure percent incorporation. RNA pellets were resuspended in H_2O at .4μg/μl. Translations were carried out in 25μl reactions with .6μg of each RNA following the standare protocol of the Nuclease Treated Lysate (Promega). The -met amino acid mix was used, and ^{35}S methionine (Amersham) was incorporated in the proteins produced.

Immunoprecipitation

Protein A Sepharose beads (CL-4b Sigma) were pre-equilibrated overnight in IP buffer (10% Glycerol, 50mM Hepes KOH pH 7.3, 100mM K glutamate, 0.5mM DTT, 6mM MgOAc, 1mM EGTA, 0.1% NP40 and .5mg/ml BSA). 20μl of bead slurry were spun in a microfuge, and the beads were resupeneded in 20μl fresh IP Buffer. 5μl retic lysate containing translated proteins and 1μl anti-ADA2 sera were added to the beads, mixed, and rotated 3 hrs at 4^{o}. The reactions were then spun 2 min. at 7k and the supernatant was removed. The beads were washed three times with 1ml IP buffer by inverting and vortexing. Following the last wash, the supernatent was removed and the pellets were resuspended in 20μl loading dye (Maniatis). Samples were boiled 3 min, vortexed, and boiled again 3 min prior to loading on 10% SDS PAGE gels. The dried gel was exposed overnight on Hyperfilm-ECL (Amersham).

Yeast Manipulations, Media, Westerns and ß-galactosidase assays.

Transformations were by the LiOAc method (Gietz, et al., 1992). Tetrad analysis and other yeast manipulations were done using standard techniques (Guthrie and Fink, 1991). ß-galactosidase assays were carried out on yeast extracts made from breaking cells with glass beads (Rose and Botstein, 1983). The activity of ß-galactosidase is normalized to total protein. Westerns were performed using standard protocols(Harlow, 1988). Slow growth phenotypes of *ada* mutants were assayed on SD minimal medium supplemented with amino acids and adenine. Otherwise strains were grown in SD rich drop out medium containing all amino acids except those needed for plasmid selection.

ACKNOWLEDGEMENTS

We would like to thank, the Treizman lab for pT7Plink, R. Knaus for pRK325, pRK16 and pRK15, D. Stüber for pDS56/RBSII NcoI, J. Agapite for construction of plexA-ADA3 and J. Fikes for pRBHis. In addition, thanks to Roy Pollack, Rainer Knaus, Nicanor Austriaco for helpful comments on the manuscript, Rachel Kindt for comments on the manuscript and assistance with graphics, members of the Guarente lab for fruitful advice and discussions, as well as Myrna Helfenstein for help in preparation of the manuscript. This work was supported by NIH grant GM50207 and ACS NP-755. SLB gratefully acknowledges the support of Smithkline Beecham Pharmacuticals and Charles King Medical Foundation.

REFERENCES

Alani, E., Cao, L. and Kleckner, N. (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.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. **215**, 403-410.

Berger, S.L., Cress, W.D., Cress, A., Triezenberg, S.J. and Guarente, L. (1990). Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61**, 1199-1208.

Berger, S.L., Piña, B., Silverman, N., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J. and Guarente, L. (1992). Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. Cell **70**, 251-265.

Brent, R. and Ptashne, M. (1985). A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell **43**, 729-736.

Cairns, B.R., Kim, Y.-J., Sayre, M.H., Laurent, B.C. and Kornberg, R.D. (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.

Cress, W.D. and Triezenberg, S.J. (1991). Critical structural elements of the VP16 transcriptional activation domain. Science **251**, 87-90.

Croston, G.E. and Kadonaga, J.T. (1993). Role of chromatin structure in the regulation of transcription by RNA polymerase II. Curr. Opinion in Cell Biol. **5**, 417-423.

Dalton, S. and Treisman, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the *c-fos* serum response element. Cell **68**, 597-612.

Donahue, T.F., Farabaugh, P.J. and Fink, G.R. (1982). Gene **18**, 47-59.

Dynlacht, B.D., Hoey, T. and Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell **66**, 563-576.

Elfring, L.K., Deuring, R., McCallum, C., Peterson, C.L. and Tamkun, J.W. (1994). Identification and Characterization of Drosophila Relatives of the Yeast Transcriptional Activator SNF2/SWI2. MCB **14**, 2225-2234.

Fields, S. and Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature **340**, 246-246.

Gansheroff, LJ., Dollars, C., Tan, P.and Winston, F. (1995). The *Saccharomyces cerevisiae SPT7* gene encodes a very acidic protein important for transcription *in vivo*. Genetics **135**, 523-536.

Georgakopoulos, T. and Thireos, G. (1992). Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. EMBO J. **11**, 4145-4152.

Georgatsou, E., Georgakopoulos, T. and Thireos, G. (1992). Molecular cloning of an essential yeast gene encoding a proteasomal subunit. FEBS **299**, 39-43.

Gietz, D., St. Jean, A., Woods, R.A. and Schiesti, R.H. (1992). Improved method for high efficiency transformation o□f intact yeast cells. Nucleic Acids Res. **20**, 1425.

Goodrich, J.A., Hoey, T., Thut, C.J., Admon, A. and Tjian, R. (1993). Drosophila TafII 40 Interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75**, 519-530.

Goodrich, J.A. and Tjian, R. (1994). TBF-TAF complexes: selectivity factors for eukaryotic transcription. Current Opinion in Cell Biology **6**, 403-409.

Han, M., and Grunstein, M. (1989). Nucleosome loss activates yeast downstream promoters *in vivo*. Cell **55**, 1137-1145.

Haynes, S.R., Dollard, C., Winston, F., Beck, S., Trowsdale, J. and Dawid, I.B. (1992). The bromodomain: a conserved sequence found in human, Drosophila and yeast proteins. Nucleic Acids Res **20**,

Hinnebusch, A.G. (1985). A Hierarchy of trans-acting Factors Modulates Translation of an Activator of Amino Acid Biosynthetic Genes in *Saccharomyces cerivisiae*. MCB **5**, 2349-2360.

Hinnebusch, A.G. and Fink, G.R. (1983). Positive regulation in the general amino acid control of Saccaromyces cerevisiae. PNAS **80**, 5374-5378.

Hirschhorn, J.N., Brown, S.A., Clark, C.D. and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. Genes Dev **6**, 2288-98.

Hoey, T., Dynlacht, B.D., Peterson, M.G., Pugh, B.F. and Tjian, R. (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72**, 247-260.

Hope, I. and Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell **46**, 885-894.

Kennison, J.A. (1993). Transcriptional activation of *Drosophila* homeotic genes from distant regulatory elements. Trends Genet. **9**, 75-79.

Koleske, A.J. and Young, R.A. (1994). An RNA polymerase II holoenzyme responsive to activators. Nature **368**, 466-469.

Kolodziej, P.A., and Young, R. A. (1991). Epitope tagging and protein surveillance. In Guide to Yeast Genetics and Molecular Biology, Guthrie, C. and Fink, G.R., eds. (Academic Press, Inc., San Diego) pp. 508-520.

Laurent, B.C. and Carlson, M. (1992). Yeast SNF2/SWI2, SNF5, and SNF6 proteins function coordinately with the gene-specific transcriptional activators GAL4 and Bicoid. Genes & Dev. **6**, 1707-1715.

Laurent, B.C., Treich, I. and Carlson, M. (1993). The yeast SNF2/SWI2 protein has DNA-stimulated ATPase activity required for transcriptional activation. Genes & Dev. **7**, 583-591.

Laurent, B.C., Treitel, M.A. and Carlson, M. (1991). Functional interdependence of the yeast SNF2, SNF5, and SNF6 proteins in transcriptional acitvation. Proc. Natl. Acad. Sci. **88**, 2687-2691.

Lin, Y.S., and Green, M. R. (1991). Mechanism of action of an acidic transcriptional activator *in vitro*. Cell **64**, 971-981.

Lin, Y.S., Maldonado, E., Reinberg, D. and Green, M.R. (1991). Binding of general transcription factor TFIIB to an acidic activating region. Nature **353**, 569-571.

Olesen, J.T. and Guarente, L. (1990). The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. Genes Dev **4**, 1714-29.

Penn, M.D., Galgoci, B. and Greer, H. (1983). Identification of AAS genes and their regulatory role in general control of amino acid biosynthesis in yeast. Proc Natl Acad Sci U S A **80**, 2704-8.

Peterson, C.L., Dingwall, A. and Scott, M.P. (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.

Piña, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J.A. and Guarente, L. (1993). ADA3: a gene, indentified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. Molec. Cell. Biol. **13**, 5981-5989.

Ruppert, S., Wang, E.H. and Tjian, R. (1993). Cloning and expression of the human TAF250: a TBP-associated factor implicated in cell cycle regulation. Nature **362**, 175-179.

Sadowski, I., Ma, J., Triezenberg, S. and Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. Nature **335**, 563-564.

Sikorski, R.S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**, 19-27.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992). brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell **68**, 561-72.

Thompson, C.M., Koleske, A.J., Chao, D.M. and Young, R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73**, 1361-1375.

Verrijzer, P., Yokomori, K., Chen, J.L. and Tjian, R. (1994). Drosophila TAFII150: similarity to yeast gene TSM-1 and specific binding to core promoter DNA. Science **264**, 933-941.

Winston, F. and Carlson, M. (1992). Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. Trends Genet **8**, 387-91.

Workman, J.L., and Kingston, R. E. (1992). Nucleosome core displacement *in vitro* via a metastable transcription factor-nucleosome complex. Science **258**, 1780-1784.

Yoshinaga, S.K., Peterson, C.L., Herskowitz, I. and Yamamoto, K.R. (1992). Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. Science **258**, 1598-1604.

Chapter 3

GCN5 Cofractionates With ADA2 and ADA3, and Regulates lexA-ADA2 In An *ADA3* Independent Manner

INTRODUCTION

Activation in eukaryotes requires two types of DNA elements, and three classes of transcription factor. Basal factors bind to the TATA box, proximal to the site of transcriptional initiation. Activators bind to the distal UAS/enhanser element and stimultate activation. Coactivators, the third class of transcription factor, are necesary for activated but not basal transcription and mediate the interaction between activators and basal factors (Berger, et al., 1990; Pugh and Tjian, 1990).

Coactivators can bind activators in at least two different ways. Some coactivators only bind to certain activation domains. For example, TAF110 interacts with the glutamine rich activation domain SP1 but not the acidic activation domain VP16 (Hoey, et al., 1993). Conversely, TAF60 binds VP16 but not SP1 (Thut, et al., 1995). Both TAF110 and TAF60 are part of a coactivator complex associated with TBP that can respond to either SP1 or VP16 (Goodrich and Tjian, 1994). Subcomplexes lacking TAF110 are not activated by SP1, and subcomplexes lacking TAF60 do not respond to VP16, indicating that the activator-coactivator interaction determines the specifity for coactivator activity (Chen, et al., 1994). Other coactivators such as PC4/p15 can bind to, and stimulate activation from a number of different types of activator (Ge and Roeder, 1994; Kretzschmar, et al., 1994).

In addition, it is possible to bypass the need for an activator by fusing a coactivator directly to a DNA binding domain. For example, the coactivator CBP fused to the GAL4 DNA binding domain can stimulate a GAL reporter when transfected into cells (Kwok, et al., 1994). The yeast coactivator GAL11 also activated when fused to a DNA binding domain (Himmelfarb, et al., 1990). Similarly, the yeast SNF2/SWI2, SNF5 or SNF6 activate transcription when fused to the lexA DNA binding domain (Laurent, et al., 1991). SNF2/SWI2,

SNF5 and SNF6 are part of a large, multisubunit complex that antagonizes histone repression *in vivo* and *in vitro* (Cote, et al., 1994; Hirschhorn, et al., 1992). The activity of the lexA-SNF2 and lexA-SNF5 fusions decreases in swi/snf mutants strains, which supports the notion that the activity of these fusions depends on the integrity of the SWI/SNF complex *in vivo* (Carlson and Laurent, 1994).

ADA2, ADA3 and *GCN5* were isolated in a selection for mutants resistant to GAL4-VP16 mediated toxicity (Berger, et al., 1992; Marcus, et al., 1994). These genes have been proposed to be coactivators because mutants in any of these strains are unable to support activation from the VP16 or GCN4 activation domains *in vivo* or *in vitro* (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). Other activation domains, such as HAP4 and GAL4 activate independently of *ADA2, ADA3* and *GCN5* (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). Moreover, the ADA2, ADA3 and GCN5 proteins can form a complex *in vitro*, and may act as a complex *in vivo* by genetic criteria (Horiuchi, et al., 1995; Marcus, et al., 1994). The specifity of the ADA2 complex may be governed by the binding of ADA2 to activation domains. ADA2 can bind VP16 and GCN4 (Barlev, et al., 1995; Silverman, et al., 1994), but does not bind HAP4 or GAL4 (Barlev, et al., 1995).

Like CBP, GAL11 and components of the SWI/SNF complex, ADA2 and ADA3 can activate transcription when fused to a DNA binding domain (Marcus, et al., 1994). The activity of lexA-ADA2 can be modulated by increasing or decreasing the level of ADA3 in the cell. lexA-ADA2 does not activate well in *ada3* mutants, but activates extremely well when ADA3 is overexpressed (Silverman, et al., 1994). The activity of lexA-ADA3 is similarly effected by the level of ADA2 in the cell (Horiuchi, et al., 1995). Neither lexA-ADA2 nor lexA-ADA3 can activate in *gcn5* mutants (Marcus, et al., 1994). Together, this argues

that fusing ADA2 or ADA3 to a DNA binding domain is bypassing the need for an activator, allowing the ADA complex to activate directly.

Here, we show that the activity of lexA-ADA2 is superactivated by ADA2 overexpression, and superrepressed by GCN5 overexpression. Further, superrepression occurs in an *ada3* deletion mutant, suggesting that ADA2 and GCN5 can associate *in vivo* in the absence of ADA3. Additionally, we show that GCN5 co-fractionates with ADA2 and ADA3 from yeast extracts. Finally, we report the identification of a human cDNA that encodes a protein with high sequence similarity to GCN5.

RESULTS

The ability of lexA-ADA2 to activate transcription is modulated by overexpression of *ADA2* and *GCN5*

Previously, we have observed that the activity of lexA-ADA2 is superactivated by overexpression of *ADA3*, and the activity of lexA-ADA3 is superactivated by overexpression of *ADA2* (Horiuchi, et al., 1995; Silverman, et al., 1994). Given these results, we took a more systematic approach to investigate the effect of overexpressing ADA genes on the activity of lexA-ADA fusions. lexA-ADA2, lexA-ADA3 and lexA-GCN5 were tested for their ability to activate transcription alone, or when one of the ADA genes is overexpressed. As shown in Table 1, the activity of lexA-ADA2 moiety is superactivated over three fold when *ADA3* is overexpressed, and similarly, the activity of lexA-ADA3 is superactivated three fold by overexpression of *ADA2*. This is in agreement with previous observations, and suggests that either ADA2 or ADA3 is limiting for trans-activation when the other is overexpressed as a lexA fusion (Horiuchi, et al., 1995; Silverman, et al., 1994).

TABLE 1 *(Following page)*

LEGEND. The wildtype strain BWG1-7a containing pRbHis (Marcus, et al., 1994), a plasmid with the ß-galactosidase gene under the control of a lexA operator, was doubly transformed with all pairwise combinations of plexA-ADA2, plexA-ADA3 or plexA-GCN5 as the first plasmid (Marcus, et al., 1994), and pDB20LADA2 (Marcus, et al., 1994), pDB20LADA3 (Silverman, et al., 1994), pDB20LGCN5 (Marcus, et al., 1994) or pDB20L as the second plasmid. pDB20L is a 2μ plasmid used to overexpress genes from the strong constitutive ADH1 promoter (Becker, 1991). The specific activity of ß-galactosidase averaged from at least three independant experiments is presented (SD<20%)

TABLE 1. Overexpression of ADA genes modulates the activity of lexA-ADA fusions

Activator	Vector	ADA2	ADA3	GCN5
lexA-ADA2	351	1624	1240	85
lexA-ADA3	227	754	343	183
lexA-GCN5	21	52	46	23

Surprisingly, the activity of lexA-ADA2 can also be modulated by overexpression of either *ADA2* itself or *GCN5*. lexA-ADA2 is superactivated four fold by *ADA2* overexpression. In contrast, the activity of lexA-ADA2 is superrepressed four fold by overexpression of *GCN5*. The superactivation may be related to possible multimerization by ADA2, and the superrepression may result from saturation of a binding site on a basal factor by GCN5 (see Discussion). In addition, although lexA-GCN5 only activates very weakly, its activity increases two fold when *ADA2* or *ADA3* is overexpressed. Importantly, the superactivation and superepression effects are specific to certain lexA-fusions. The activity of lexA-ADA3 is unaffected by overexpression of either ADA3 or GCN5. Furthermore, we have previously shown that the activity of true activators such as lexA-GCN4 or lexA-HAP4 is not altered by overexpression of ADA2 or ADA3 (Horiuchi, et al., 1995; Silverman, et al., 1994). The activity of lexA-GCN4 is also unchanged when GCN5 is overexpressed (Table 2).

The observation that the activity of lexA-ADA2 can be modulated by overexpression of *ADA2* or *GCN5* is novel. Therefore, we wished to determine whether superactivation and superrepression depend on the copy number of the lexA-ADA2 protein. As shown in Table 2, lexA-ADA2 expressed from a low copy ARS-CEN plasmid is less active than 2μ (high copy) lexA-ADA2, and is still superactivated by *ADA2* overexpression and superrepressed by *GCN5* overexpression.

Previously, we have argued that the activity of lexA-ADA2 represents the activity of the ADA2, ADA3 and GCN5 complex. The activity of lexA-ADA2 decreases in *ada3* or *gcn5* mutants, suggesting that the complex is disrupted (Marcus, et al., 1994; Silverman, et al., 1994). We have also shown that ADA2 and GCN5 can interact *in vitro* in the absence of ADA3 (Marcus, et al., 1994). If superrepression requires the integrity of the ADA2 complex *in vivo*, than GCN5

TABLE 2 *(Following page)*

LEGEND. The wildtype strain BWG1-7a containing pRbHis, a plasmid with the B-galactosidase gene under the control of a lexA operator, was doubly transformed with all pairwise combinations of plexA-ADA2, or plexA-ADA2 a/c as the first plasmid, and pDB20LADA2, , pDB20LGCN5, pDB20LGCN5Δ or pDB20L as the second plasmid. pDB20LGCN5 expresses a varient of GCN5 with the bromodomain deleted (Marcus, et al., 1994). In addition, plexA-ADA3, or plexA-GCN4 were also transformed with pDB20LGCN5 or pDB20L. The specific activity of B-galactosidase averaged from at least three independant experiments is presented (SD<20%). n.d. indicates the experiment was not performed.

TABLE 2 ADA2 superactivation and GCN5 superrepression are specific to lexA-ADA2

Activator	Vector	ADA2	GCN5	GCN5Δ
lexA-ADA2	197	1274	37	28
lexA-ADA2 a/c	62	280	16	16
lexA-ADA3	117	n.d.	93	n.d.
lexA-GCN4	2898	n.d.	2737	n.d.

should not be able to superrepress lexA-ADA2 in an *ada3* deletion strain. However, if superrepression only depends upon the ADA2-GCN5 interaction, then *GCN5* should be able to superrepress in an *ada3* deletion strain. As shown in Table 3, although the activity of lexA-ADA2 is three fold lower in the *ada3* deletion strain, it is still superrepressed by GCN5. This shows that superrepression reflects the ADA2-GCN5 interaction and not a destablization of the ADA complex. Further, it argues that ADA2 and GCN5 can associate *in vivo* in the absence of ADA3.

The bromodomain of *GCN5* contributes to growth and GCN4 activation *in vivo*, and does not mediate the ADA2-GCN5 interaction (Marcus, et al., 1994). Because the bromodomain is conserved in many different coactivators, it may interact with a component of the basal machinery. Conceivably, the bromodomain could be necessary for superrepression. As shown in Table 2, however, a GCN5 bromodomain deletion mutant (GCN5Δ) can still superrepress. Thus, the putative interaction between the bromodomain and another factor is unnecessary for superrepression.

GCN5 shows the same elution profile as ADA2 and ADA3 during purification.

The superrepresion of lexA-ADA2 by *GCN5* adds to a large body of evidence arguing that ADA2, ADA3 and GCN5 act as a complex *in vivo*. ADA2 and GCN5 interact by two hybrid analysis (Marcus, et al., 1994); *in vitro* translated ADA2, ADA3 and GCN5 co-immunoprecipitate as a complex (Horiuchi, et al., 1995); and *ada2ada3*, *ada2gcn5* and *ada3gcn5* double deletion mutants have phenotypes no more severe than single mutant phenotypes, a strong indication that they act as a complex or the the same pathway *in vivo* (Marcus, et al., 1994; Piña, et al., 1993).

TABLE 3 *(Following page)*

LEGEND. BWG1-7a (wildtype) or 1-7aΔada3(*Δada3*) (Piña, et al., 1993), each containing pRbHis were transformed with plexA-ADA2 and pDB20LADA3, pDB20LGCN5 or pDB20L. The specific activity of ß-galactosidase averaged from at least three independant experiments is presented (SD<20%).

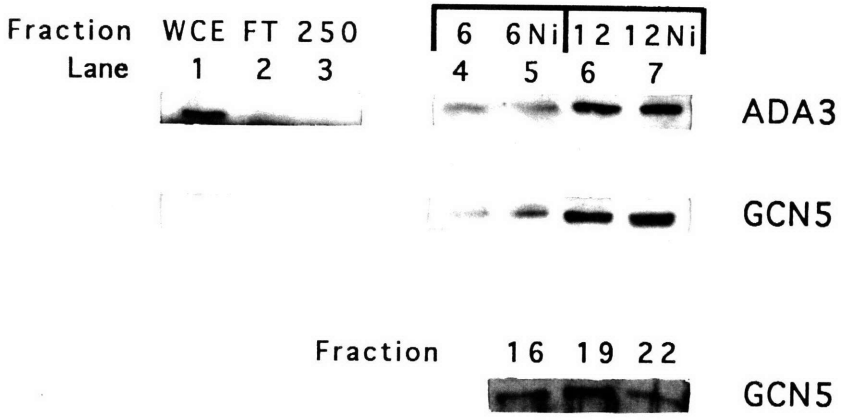
TABLE 3 GCN5 can superrepress lexA-ADA2 in the absence of ADA3.

Genotype	Activator	Vector	ADA3	GCN5
Wildtype	lexA-ADA2	106	370	24
Δada3	lexA-ADA2	31	380	9

In addition, unpublished experiments by Silverman and Guarente have shown that ADA2 and ADA3 copurity from yeast extracts through four columns (see Methods for more details of the purification). A nickel column is used in the second step to affinity purify ADA2, which is tagged with six histidine residues. To determine if GCN5 is copurifying with ADA2 and ADA3, protein fractions from each stage of the purification were assayed for GCN5 by Western analysis using αGCN5 antiserum. GCN5 and ADA3 show an identical elution profile through the first two purification steps (Figure 1 top). Both proteins are retained on the Bio-Rex70 column and elute in 600mM and 1200mM potassium acetate. Moreover, six-his tagged ADA2 is retained on the nickel column (N. Silverman, unpublished data), as well as ADA3 and GCN5 (Figure 1 top, 6N and 12N). Importantly, GCN5 also co-purifies through two additional chromatography steps with ADA2 and ADA3 (data not shown). In summary, ADA2, ADA3 and GCN5 show identical elution profiles through four chromotagraphy steps that give a 300 fold purification (data not shown), arguing that these genes are part of a complex *in vivo*.

ADA2 and ADA3 also copurify over four columns in a different fractionation system (see methods). Three fractions that cross the ADA2/ADA3 peak on Heparin column were assayed for GCN5 by Western blot analysis using GCN5 antiserum. As shown in Figure 1 (bottom), GCN5 peaks in fraction 21, which is the peak of both ADA2 and ADA3 (data not shown). Thus, ADA2, ADA3 and GCN5 show the same elution profile after four purification steps in this second fractionation, which along with the first purification, indicates that they are part of the same complex *in vivo*.

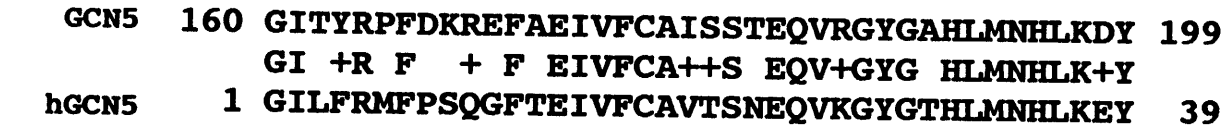
FIGURE 1. *(Following page).* GCN5 copurifies with ADA3. Whole cell yeast extracts were chromatographed on a Bio-Rex 70 column. Proteins were stepwise eluted in 250 mM, 600 mM and 1200 mM potassium acetate. 100 μg of the whole cell extract (WCE) and flow through (FT), and 50 μg of each elution were assayed for GCN5 and ADA3 by Western blot analysis. "250" is the 250mM elution, "6" is the 600 mM elution, and "12" is the 1200 mM elution from the Bio-Rex 70 column. "6Ni" and "12Ni" are the 600 mM and 1200 mM fractions after an additional purification on a nickel column, which should retain the six His tagged ADA2 (see Methods).

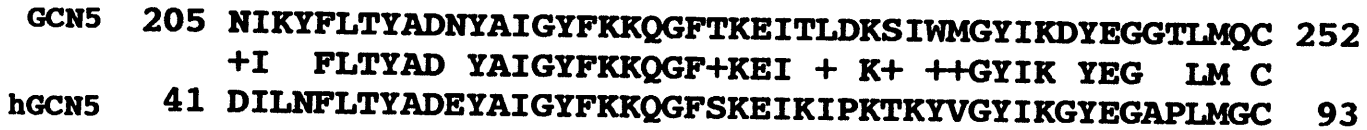

Fraction WCE FT 250 6 6Ni 12 12Ni
Lane 1 2 3 4 5 6 7
ADA3
GCN5
Fraction 16 19 22
GCN5

Identification of a putative human homolog of GCN5

The growing library of expressed sequence tags (EST) is a useful reagent to identify mammalian homologs of yeast genes by amino acid similarity. Using the XREFdb database, we identified three independent ESTs from humans with regions of overlapping DNA sequence (Reeves, et al., 1995). The similarity between the protein sequence of the largest cDNA is compared to GCN5 in Figure 2. Over these two closely spaced regions, there is 66% amino acid identity and 80% amino acid similarity. This homology is located in the N-terminal half of GCN5, and does not contain the highly conserved bromodomain that has been found in many proteins. The high degree of sequence similarity makes this an excellent candidate to be a human homolog of *GCN5*. However, isolation of the entire cDNA, followed by functional analysis will be necessary to confirm this.

FIGURE 2 *(Following page).* GCN5 and a human cDNA share 66% amino acid identity and 80% amino acid similarity. A human cDNA (Accession number H38810) homologous to GCN5 was identified from the XREFdb database (Reeves, et al., 1995).


GCN5 160 GITYRPFDKREFAEIVFCAISSTEQVRGYGAHLMNHLKDY 199
GI +R F + F EIVFCA++S EQV+GYG HLMNHLK+Y
hGCN5 1 GILFRMFPSQGFTEIVFCAVTSNEQVKGYGTHLMNHLKEY 39


GCN5 205 NIKYFLTYADNYAIGYFKKQGFTKEITLDKSIWMGYIKDYEGGTLMQC 252
+I FLTYAD YAIGYFKKQGF+KEI + K+ ++GYIK YEG LM C
hGCN5 41 DILNFLTYADEYAIGYFKKQGFSKEIKIPKTKYVGYIKGYEGAPLMGC 93

DISCUSSION

Previously, we have argued that the ability of lexA-ADA2 and lexA-ADA3 to trans-activate in an ADA dependent manner reflects the activity of the ADA complex (Marcus, et al., 1994). The superactivation of lexA-ADA2 by ADA3, and of lexA-ADA3 by ADA2 suggest that when one is overexpressed as a lexA-fusion, the other is limiting, and thus overexpression of both proteins gives maximal trans-activation (Horiuchi, et al., 1995; Silverman, et al., 1994). Here, we have shown that the activity of lexA-ADA2 can be superactivated by *ADA2* overexpression, and super-repressed by *GCN5* overexpression. (This is in contrast to published reports by others, who show that lexA-ADA2 can be superactivated by *GCN5* overexpression, and super-repressed by *ADA2* overexpression (Georgakopoulos, et al., 1995). This may reflect differences in strains, the lexA-ADA2 fusions, or the use of a lex a reporter with one site in this study, and eight sites in the other study).

Superactivation may result from multimerization of ADA2 with lexA-ADA2. This would create a larger surface to interact with basal factor targets and thus activate at a higher rate. Multimerization of weak activation domains give rise to more powerful activation domains (Seipel, 1992). Furthermore, the human activator SP1 can be superactivated by its own activation domain, or by TAF110, a coactivator for SP1 activation (Courey, et al., 1989; Hoey, et al., 1993). The SP1 superactivation is mediated by multimerization (Pascal and Tjian, 1991). Multimerization may also involve the entire ADA2 complex. Since ADA2 can serve as a linchpin between ADA3 and GCN5 *in vitro* (Horiuchi, et al., 1995), it may be well suited to nucleate the formation of multimeric ADA complexes. Alternatively, superactivation may proceed by titration of a negative regulator of ADA2. There are at present no candidates for this negative factor.

One way to explain the superrepression of lexA-ADA2 by GCN5 is to assume the activity of lexA-ADA2 reflects the activity of a lexA-ADA2, ADA3 and GCN5 complex. If GCN5 mediates the interaction between this complex and basal factors, than excess GCN5 could be binding to the basal factors, blocking access by the lexA-ADA2/ADA3/GCN5 complex. It is unlikely that *GCN5* serves as a negative regulator of *ADA2* because trans-activation by lexA-ADA2 as well as real activation domains is reduced in GCN5 mutants (Georgakopoulos and Thireos, 1992; Marcus, et al., 1994). Finally, *GCN5* does not superrepress lexA-ADA3, lexA-GCN4 and can still superrepress lexA-ADA2 in an *ada3* mutant strain. Thus, whatever its mechanism, superrepression reflects the specific interaction between ADA2 and GCN5.

In addition, there is a strong correlation between superactivation or superrepression *in vivo*, and protein protein interactions *in vitro*. In particular, ADA2 can bind ADA3, and ADA2 and ADA3 can superactivate lexA-ADA3 and lexA-ADA2 respectively. Furthermore, GCN5 binds ADA2, and superrepresses lexA-ADA2. In addition, GCN5 does not bind ADA3, and does not superactivate or superrepress lexA-ADA3. Together, this suggests that the architecture of the ADA complex formed *in vitro*, in which ADA2 acts as a linchpin between ADA3 and GCN5 (Horiuchi, et al., 1995), may also exist *in vivo*. Moreover, GCN5 can superrepress lexA-ADA2 in an *ada3* deletion strain, suggesting that ADA2 and GCN5 can associate *in vivo* in the absence of ADA3.

As a way to address whether the ADA complex exists *in vivo*, whole cell yeast extracts were fractionated using conventional and affinity chromotagraphy in an attempt to purify an ADA complex. In these experiments, ADA2 and ADA3 co-purify using two different methods of purification (N. Silverman, unpublished results). Here, we have shown that GCN5 shows the same elution profile as ADA2 and ADA3 for the final stage of one purification procedure (Figure 1,

bottom). Additionally, the copurification of GCN5 and ADA3 in the first two stages of the other fractionation are shown (Figure 1, top). Moreover, ADA2, ADA3 and GCN5 co-purify through two additional chromatography steps (data not shown and N. Silverman, unpublished data). This is consistent with a model in which ADA2, ADA3 and GCN5 are part of a multi-subunit complex *in vivo*. The purification of the ADA2 complex is still in progress. Thus, it is unknown how many other proteins are in the complex with ADA2, ADA3 and GCN5.

Several large complexes of transcription factors have been purified from yeast. The products of the *SWI1, SWI2/SNF2, SWI3, SNF5* and *SNF6* genes function as a large multi-subunit complex to mediate histone anti-repression (Cote, et al., 1994; Hirschhorn, et al., 1992). The products of the SRB genes, isolated as suppressors of a conditional RNA polymerase II mutant cofractionate as part of the RNA polymerase holoenzyme (Hengartner, et al., 1995; Kim, et al., 1994; Koleske, et al., 1992). In addition, several yeast TAF complexes have been purified (Poon and Weil, 1993; Reese, et al., 1994). One of them supports Pol III transcription (Poon and Weil, 1993), and the other can mediate Pol II activated transcription *in vitro* (Reese, et al., 1994). The purified ADA complex may also be able to regulate transcirption *in vitro*.

Finally, a putative human homolog of *GCN5* from the XREF database has been identified by sequence similarity (Reeves, et al., 1995). The amount of sequence similarity is very high over the entire cDNA fragment. Isolation of the complete cDNA will allow functional studies to be conducted. Additionally, isolation of a human homolog of ADA2 has been reported (Barlev, et al., 1995). This indicates that the important role of the ADA2 complex has been conserved in evolution.

MATERIALS and METHODS

ADA2 complex purification

In the first fractionation, whole cell extracts were chromatographed on a Bio-Rex 70 column, followed by stepwise elution in 100mM, 300mM or 600mM potassium acetate. ADA2 and ADA3 elute in the 600mM fraction. Following dialysis, this fraction was chromatographed on a DE52 column, which was eluted in 100mM, 400mM and 600mM potassium acetate. ADA2 and ADA3 both elute in the 600mM fraction. Following dialysis, this fraction was chromatographed on a Hydroxyapatite column, and was eluted in a potassium acetate gradient. Again, ADA2 and ADA3 have the same elution profile. ADA2 enriched fractions were pooled, and chromatographed on a Heparin column. The gradient elution reveals that, yes, ADA2 and ADA3 copurify once again. The level of ADA2 and ADA3 peaks in fraction 19. To determine if GCN5 is cofractionating with ADA2 and ADA3, fractions 16, 19 and 22, which cross the ADA2/ADA3 peak were assayed for GCN5 by Western blot analysis with αGCN5 protein A purified IgGs.

In the second purification, whole cell yeast extracts from a strain expressing ADA2 that is tagged with six Histadine residues from its own promoter was chromatographed over a Bio-Rex70 column, and stepwise eluted with 250mM, 600mM and 1200mM potassium acetate. ADA2 and ADA3 are retained on theBio-Rex70 column, and elute in the 600mM and 1200mM but not 250mM potassium acetate elutions. The 600 and 1200 elution fractions were dialyzed, and chromatographed separately on a nickel beads column to affinity purify ADA2, followed by an elution with an Imidazole gradient. Both ADA2 and ADA3 are retained on the column by Western analysis (N. Silverman, unpublished results). Each of these fractions was assayed for the presence of GCN5 by Western blot analysis using αGCN5 IgGs. The 600Ni and

standard protocols (Harlow, 1988). Strains were grown in SD rich drop out medium containing all amino acids except those needed for plasmid selection.

Acknowledgments

I would like to thank N. Silverman for the gift of protein fractions from the ADA2 affinity purification; N. Silverman, andT. Koleske for the other purification fractions; and J. Horiuchi for purified aADA3 antiserum. Also thanks to N. Silverman and J. Horiuchi for the ADA2 and ADA3 overexpression plasmids, S. Berger for plexA-ADA2, and J. Agapite for plexA-ADA3.

REFERENCES

Barlev, N.A., Candau, R., Wang, L., Darpino, P., Silverman, N. and Berger, S.L. (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.

Becker, D.M., Fikes, J. D., and Guarente, L. (1991). A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. Proc. Natl. Acac. Sci. USA **88**, 1968-1972.

Berger, S.L., Cress, W.D., Cress, A., Triezenberg, S.J. and Guarente, L. (1990). Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61**, 1199-1208.

Berger, S.L., Piña, B., Silverman, N., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J. and Guarente, L. (1992). Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. Cell **70**, 251-265.

Carlson, M. and Laurent, B.C. (1994). The SNF/SWI family of global transciptional activators. Curr. Op. in Cell Biol. **6**, 396-402.

Chen, J.L., Attardi, L.D., Verrijzer, C.P., Yokomori, K. and Tjian, R. (1994). Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. Cell **79**, 93-105.

Cote, J., Quinn, J., Workman, J.L. and Peterson, C.L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science **265**, 53-60.

Courey, A.J., Holtzman, D.A., Jackson, S.P. and Tjian, R. (1989). Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell **59**, 827-836.

Ge, H. and Roeder, R. (1994). Purification, cloning, and characterization of a human coactivator, PC-4, that mediates transcriptional activation of class II genes. Cell **78**, 513-523.

Georgakopoulos, T., Gounalaki, N. and Thireos, G. (1995). Genetics evidence for the interation of the yeast transcriptional co-activator proteins GCN5 and ADA2. Mol Gen. Genet **246**, 723-728.

Georgakopoulos, T. and Thireos, G. (1992). Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. EMBO J. **11**, 4145-4152.

Gietz, D., St. Jean, A., Woods, R.A. and Schiesti, R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20**, 1425.

Goodrich, J.A. and Tjian, R. (1994). TBF-TAF complexes: selectivity factors for eukaryotic transcription. Current Opinion in Cell Biology **6**, 403-409.

Hengartner, C.J., Thompson, C.M., Zhang, J., Chao, D.M., Liao, S.M., Koleske, A.J., Okamura, S. and Young, R.A. (1995). Association of an activator with an RNA polymerase II holoenzyme. Genes & Dev. **9**, 897-910.

Himmelfarb, H.J., Pearlberg, J., Last, D.H. and Ptashne, M. (1990). GAL11P: A yeast mutation that potentiates the effect of weak GAL4-derived activators. Cell **63**, 1299-1309.

Hirschhorn, J.N., Brown, S.A., Clark, C.D. and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. Genes Dev **6**, 2288-98.

Hoey, T., Dynlacht, B.D., Peterson, M.G., Pugh, B.F. and Tjian, R. (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72**, 247-260.

Horiuchi, J., Silverman, N., Marcus, G. and Guarente, L. (1995). ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. Mol Cell Biol 1203-1209.

Kim, Y.J., Björklund, S., Li, Y., Sayre, M.H. and Kornberg, R.D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77**, 599-608.

Koleske, A.J., Buratwoski, S., Nonet, M. and Young, R.A. (1992). A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. Cell **69**, 883-894.

Kretzschmar, M., Kaiser, K., Lottspeich, F. and Meisterernst, M. (1994). A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. Cell **78**, 525-534.

Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature **370**, 223-226.

Laurent, B.C., Treitel, M.A. and Carlson, M. (1991). Functional interdependence of the yeast SNF2, SNF5, and SNF6 proteins in transcriptional acitvation. Proc. Natl. Acad. Sci. **88**, 2687-2691.

Marcus, G.A., Silverman, N., Berger, S.L., Horiuchi, J. and Guarente, L. (1994). Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. EMBO J **13**, 4807-4815.

Pascal, E. and Tjian, R. (1991). Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev. **5**, 1646-1656.

Piña, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J.A. and Guarente, L. (1993). ADA3: a gene, indentified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. Molec. Cell. Biol. **13**, 5981-5989.

Poon, D. and Weil, A.P. (1993). Immunopurification of yeast TATA-binding protein and associated factors. J. Biol. Chem. **268**, 15325-15325.

Pugh, B.F. and Tjian, R. (1990). Mechanism of transcriptional activation by Spt1: evidence for coactivators. Cell **61**, 1187-1197.

Reese, J.C., Apone, L., Walker, S.S., Griffin, L.A. and Green, M.R. (1994). Yeast TAFIIS in a multisubunit complex required for activated transcription. Nature **371**, 523-527.

Reeves, R., Goebl, M. and Hieter, P. (1995). Comparative genomics, genome cross-referencing and XREFdb. TIG. **11**, 372-373.

Rose, M. and Bostein, D. (1983). Structure and function of the yeast URA3 gene: Differentially regulated expression of hybrid B-galactosidase from overlapping coding sequences in yeast. J. Mol. Biol. **170**, 883-904.

Seipel, K., Georgiev, O., and Schaffner, W. (1992). Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. EMBO Journal **11**, 4961-4968.

Silverman, N., Agapite, J. and Guarente, L. (1994). Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. Proc. Natl. Sci. USA **91**, 11665-11668.

Thut, C.J., Chen, J.L., Klemm, R. and Tjian, R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. Science **267**, 100-104.

Chapter 4:

Cloning And Characterization Of *ADA5*:

A Putative Coactivator That Does Not Copurify With The ADA2 Complex.

INTRODUCTION

Transcription of RNA polymerase II genes in eukaryotes is highly complex, and requires many different polypeptides. These include activators that bind to enhancer/UAS elements, the basal transcription factors that work through TATA or initiator elements, and transcriptional adaptors/coactivators that are necessary for activated but not basal transcription. One model suggests that activation proceeds though direct interactions between activation domains and basal factors (Lin, 1991; Stringer, et al., 1990; Xiao, et al., 1994). Basal factor mutants have been identified that cannot support activated transcription and show decreased binding to activation domains, suggesting that direct interactions indeed play a role in activation. However, activation cannot be reconstituted *in vitro* with basal factors alone and requires the presence of coactivators (Pugh and Tjian, 1990).

Coactivators have been proposed to function by mediating the interaction between basal factors and activation domains (Berger, et al., 1990; Kelleher, et al., 1990; Pugh and Tjian, 1990). Several proteins with coactivator activity have been shown to bind basal factors and activation domains. These include PC4/p15 (Ge and Roeder, 1994; Kretzschmar, et al., 1994), CBP (Kwok, et al., 1994), and the X protein from herpes virus (Haviv, et al., 1995). The TATA binding protein (TBP) associated proteins (TAFs) form a complex with TBP capable of responding to sequence specific activators (Dynlacht, et al., 1991). Different TAF subunits bind to and mediate activation by different classes of activation domain (Goodrich, et al., 1993; Hoey, et al., 1993; Thut, et al., 1995).

Other coactivators have been isolated genetically in yeast. For example, the SRB genes were isolated as suppressors of truncations in the conserved Carboxy terminal Domain (CTD) of RNA polymerase II (Koleske, et al., 1992; Thompson, et al., 1993) These gene products copurify in a complex called the

mediator that can bind activators (Hengartner, et al., 1995), and has coactivator activity (Hengartner, et al., 1995; Kim, et al., 1994). The SRBs are also members of the RNA polymerase holoenzyme, which can respond to activators (Kim, et al., 1994; Koleske and Young, 1994). The products of the *SWI1, SWI2/SNF2, SWI3, SNF5* and *SNF6* genes, identified as positive regulators of *SUC2* and *HO* transcription (Neigeborn and Carlson, 1984; Stern, et al., 1984), are part of another coactivator complex that antagonizes histone repression *in vivo* and *in vitro* (Cote, et al., 1994; Hirschhorn, et al., 1992).

The SPT genes were isolated as suppressors of Ty insertions in yeast promoters (Winston, et al., 1984). The two major classes of these genes act as regulators of transcription in chromatin and non-chromatin pathways (Winston, 1992). The latter class includes *SPT15*, which encodes the TATA binding protein TBP (Eisenmann, et al., 1989; Hahn, et al., 1989), as well as *SPT3, SPT7* and *SPT8* (Eisenmann, et al., 1989). Genetic and biochemical evidence suggests that SPT3, SPT7, SPT8 may act as a complex with SPT15 (Eisenmann, et al., 1994; Eisenmann, et al., 1992; Gansheroff, et al., 1995). Strains harboring mutations in *spt3, spt7, spt8* or *spt15* show reduced expression of the Ty element, reduced expression of other yeast genes, and a start site alteration at the Ty locus (Winston, 1992). Thus, *in vivo*, the putative SPT3 complex may act to regulate promoter selection by TBP (Winston, 1992).

Other genes important in yeast transcription are *ADA2, ADA3* and *GCN5,* isolated in a selection for mutants resistant to GAL4-VP16 mediated toxicity (Berger, et al., 1992; Marcus, et al., 1994). Mutants in these genes relieve toxicity by reducing the ability of the VP16 activation domain to activate transcription without altering GAL4-VP16 expression. 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* (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). *In vitro* translated ADA2, ADA3 and GCN5 form a complex (Horiuchi, et al., 1995), and cofractionate from yeast extracts (N. Silverman, unpublished results, Chapter 3). Genetic evidence also supports the model that ADA2, ADA3 and GCN5 operate *in vivo* as a complex (Marcus, et al., 1994). ADA2 can bind activation domains that are ADA2 dependent, which may determine the specifity for the ADA2 complex (Barlev, et al., 1995; Silverman, et al., 1994) . ADA2 is necessary for a TBP-VP16 interaction in yeast extracts (Barlev, et al., 1995). This supports the model that the ADA2 complex serves as a physical link to strengthen the interaction between activation domains and basal factors.

Here we report the cloning and initial characterization of *ADA5*. *ada5* mutants , unlike the other ada mutants, relieve toxicity at least in part by reduced expression of GAL4-VP16. Furthermore, *ada5* mutants have a more severe slow growth phenotype, and more general transcription defects than the other ada mutants. The phenotypic differences between *ada2* complex mutants and *ada5* mutants suggests that ADA5 is a novel class of genes resistant to GAL4-VP16. Consistent with this view, GCN5 and ADA5 do not co-fractionate in yeast extracts. However, *ada2ada5* and *ada3ada5* double mutants suggest that ADA5 operates in the same pathway as the ADA2 complex. ADA5 may act as a transcriptional adaptor because it can bind the VP16 activation domain, and is identical to *SPT20*, a gene that may regulate the binding of TBP to promoters.

RESULTS

Properties of the *ada5-1* mutant

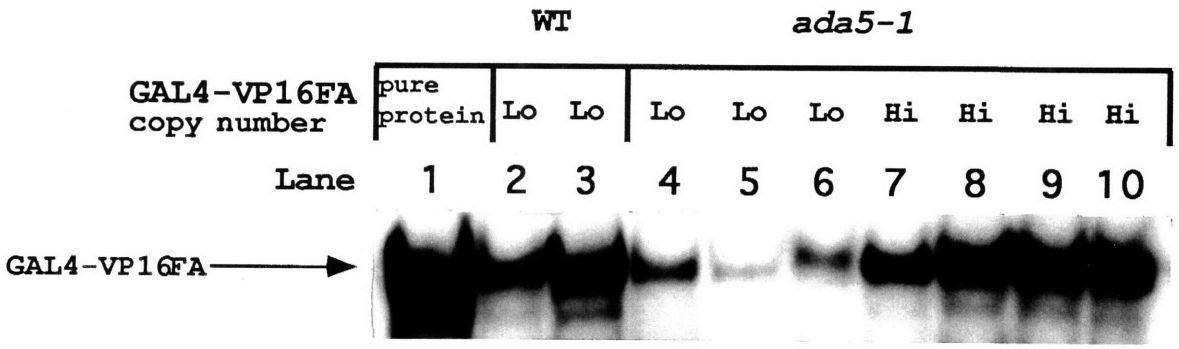
ada5-1 is a recessive mutant isolated in a screen for mutants resistant to GAL4-VP16 mediated toxicity that is described elsewhere (Berger, et al., 1992).

Whereas multiple alleles of *ADA2, ADA3* and *GCN5* were isolated, only one allele of *ada5* was isolated (Marcus, et al., 1994). The *ada5* mutant grows slowly on rich medium as well as minimal, which distinguishes it from the other ada mutants, which only have a pronounced slow growth phenotype on minimal (Marcus, et al., 1994) and references therein). In tetrad analysis, the slow growth phenotype segregated 2:2, and co-segregated with resistance to GAL4-VP16 (data not shown), showing that the slow growth and toxicity resistance phenotypes are the result of a single mutation.

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 (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). To determine whether the *ada5* mutant relieves toxicity by the same mechanism, we made protein extracts from wild type and mutant strains expressing a less toxic derivative of GAL4-VP16, (Phe 442-Ala, designated GAL4-VP16FA (Berger, et al., 1992)) from a low copy plasmid. These extracts were mixed with a radiolabled GAL4 binding site oligonucleotide and electrophoresed. As shown in Figure 1, there is less of the GAL4-VP16 specific complex in the *ada5* mutant strain (lanes 2,3 vs. lanes 4-6). Other, background bands, are identical in the mutant and wildtype extracts, showing these differences are specific to GAL4-VP16 expression. Thus, the expression or stability of GAL4-VP16 FA is somewhat reduced in mutant cells, which may explain why *ada5-1* mutants are resistant to toxicity.

However, a slight reduction in GAL4-VP16 expression from the ADH1 promoter on a 2μ (high copy) may not be sufficient to relieve toxicity because ADH1 driven GAL4-VP16 on an ARS-CEN (low copy) plasmid is toxic to wildtype cells. Moreover, lower expression of GAL4-VP16 does not preclude the possibility that ADA5 functions as a coactivator to mediate activation by the

Figure 1 *(Following page)*. Levels of GAL4-VP16 are reduced in the *ada5-1* mutant strain. BP1, a wild type strain and GMy37p (BP1 *ada5-1*) were transformed with pGAL4-VP16FA a/c, an ARS-CEN plasmid that expresses GAL4-VP16 Phe442-Ala (GAL4-VP16FA, a less toxic VP16 mutant) from the ADH1 promoter. Whole cell protein extracts were made, and used to shift a radiolabled GAL4 oligonucleotide probe (lanes 2-6, labled "Lo"). Extracts were also prepared from GMy37p expressing GAL4-VP16FA from the ADH1 promoter on a 2μ (high copy) plasmid (lanes 7-10, labled "Hi"). Purified recombinant GAL4-VP16 (Gift of S. Treizenberg) was used as a control in Lane 1 to identify the GAL4-VP16 specific band.


WT
ada5-1
GAL4-VP16FA
copy number
pure protein
Lo Lo
Lo Lo Lo Hi Hi Hi Hi
Lane
1 2 3 4 5 6 7 8 9 10
GAL4-VP16FA

VP16 and/or other activation domains. Therefore, we attempted to equalize the levels of GAL4-VP16 in mutant and wildtype cells in order to determine whether VP16 mediated activation depends on ADA5. To do this, we used a low copy (ARS-CEN) plasmid to express GAL4-VP16 FA in wildtype cells, and a high copy (2μ) plasmid to express GAL4-VP16FA in *ada5* mutant cells. Under these conditions, GAL4-VP16FA in the mutant cells is equal to or greater than the level of GAL4-VP16FA expressed in wild type cells (Figure 1, lanes2-3 vs. lanes7-10).

In these conditions, when the level of GAL4-VP16FA is the same in the mutant and wildtype, we measured the ability of GAL-VP16 FA to activate by measuring the activity of the ß-galactosidase gene expressed from the GAL1-10 promoter. As shown in Table 1, there is a two fold difference in activation by low copy GAL4-VP16FA in wildtype cells and high copy GAL4-VP16FA in the mutants. (The activity of ARS-CEN GAL4-VP16FA in mutant cells, and 2μGAL4-VP16FA in mutant cells are included for completeness). We believe that the two fold reduction in activation by GAL4-VP16FA in *ada5* mutants may be underestimating the requirement by VP16 for ADA5 in activation (see Discussion).

Cloning, mapping and sequencing of *ADA5*

ADA5 was cloned by complementation of the slow growth phenotype of *ada5-1* mutant (see Methods). The *ADA5* clone has the ability to restore wild type growth and sensitivity to GAL4-VP16 to the *ada5* mutant strain (Figure 2). To confirm that the clone indeed corresponds to the *ADA5* gene, a 1.8 Kb XbaI fragment from the insert was subcloned into an integrating vector containing the *URA3* gene, and targeted to the *ADA5* locus in a wild type strain. The resulting strain was mated to the *ada5-1* strain, the diploid was sporulated, and tetrads

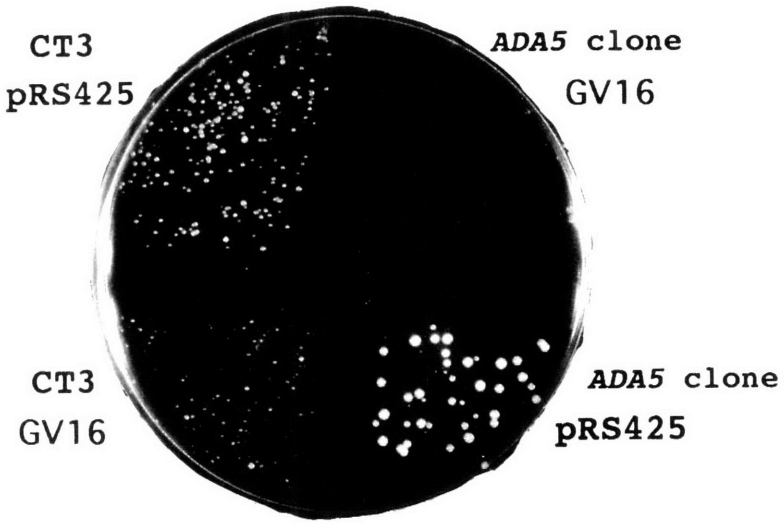


FIGURE 2. The ADA5 clone complements the *ada5-1* mutant for both growth and toxicity. GMy37p, the *ada5-1* mutant strain was transformed with all pairwise combinations of p3,1 the ADA5 clone (or pCT3 its URA3 vector) and either a 2μ high copy plasmid expressing GAL4-VP16 (GV16) from the strong constituative ADH1 promoter (or pRS425, a LEU2 vector). Transformations were plated on the same plate, and scored for growth and sensitivity to GAL4-VP16. Note that wildtype growth and sensitivity to GAL4-VP16 toxicity are restored by the clone.

were dissected. In all tetrads, two spores grew slowly and were Ura-, and two spores grew normally and were Ura+. This shows that the *ADA5* clone is linked to the *ada5-1* mutant locus.

In order to map *ADA5* to the yeast physical map, the 1.8 Kb XbaI fragment was radiolabled and hybridized to a phage grid representing over 90% of the yeast genome (see methods). Two overlapping clones were identified, showing that *ADA5* maps to the right arm of Chromosome XV. There were no genes previously mapped in this region of the genome. Therefore, *ADA5* was further subcloned and sequenced (see Methods).

The sequence of *ADA5* is shown in Figure 3. 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, and an acidic region (not shown). 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 Methods). This plasmid complements the *ada5-1* mutation as well as the genomic clone.

Characterization of ADA5 deletion mutants

ada5 deletion mutants (*ada5Δ*) were constructed by homologous recombination as described in the methods. The *ADA5* gene is not essential, but deletion mutants grow more slowly than the *ada5-1* mutant (Figure 4). It would be unlikely that a null mutant of *ada5* could be isolated in the toxicity screen due to its extremely poor growth, which could explain why only one, presumably hypomorphic, allele of *ada5* was isolated. The *ada5Δ* allele, however, is resistant to toxicity (data not shown). In addition, *ada5Δ* strains are inositol auxotrophs and temperature sensitive (data not shown).

Figure 3 *(Following three pages).* The DNA and protein sequence of the ADA5. The sequence of ADA5 was determined as described in the Methods. The sequence of ADA5 contains an open reading frame of 604 amino acids, with a predicted molecular weight of 68Kd. The Figure was prepared using DNA Strider.

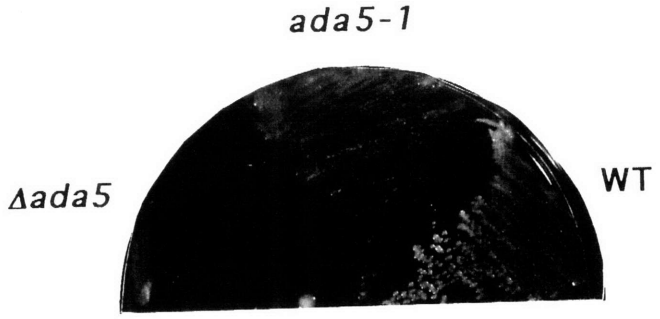
.

```
  1 gtgttaagatgatcgaccagcagagataaaagaatgagaaaagtagccttcaagaggattaggaaggaatagttacggtt 80

 81 aatttgcgcctatatatttcaggg ATG AGT GCC AAT AGC CCG ACA GGA AAC GAT CCC CAT GTA TTT 146
  1                        M   S   A   N   S   P   T   G   N   D   P   H   V   F   14

147 GGT ATT CCT GTG AAC GCA ACA CCA TCC AAT ATG GGT TCG CCA GGC AGT CCA GTT AAT GTA  206
 15 G   I   P   V   N   A   T   P   S   N   M   G   S   P   G   S   P   V   N   V   34

207 CCA CCT CCT ATG AAC CCA GCG GTA GCA AAT GTA AAT CAT CCT GTT ATG AGG ACA AAC AGT  266
 35 P   P   P   M   N   P   A   V   A   N   V   N   H   P   V   M   R   T   N   S   54

267 AAT AGT AAT GCC AAT GAA GGT ACT AGG ACT TTA ACC AGG GAG CAA ATA CAG CAA TTG CAG  326
 55 N   S   N   A   N   E   G   T   R   T   L   T   R   E   Q   I   Q   Q   L   Q   74

327 CAA AGA CAA CGA TTA TTA CTA CAG CAA AGA CTA CTC GAA CAA CAG AGA AAA CAG CAA GCA  386
 75 Q   R   Q   R   L   L   L   Q   Q   R   L   L   E   Q   Q   R   K   Q   Q   A   94

387 CTC CAA AAC TAT GAG GCT CAG TTT TAT CAA ATG CTT ATG ACC TTA AAC AAA AGA CCT AAA  446
 95 L   Q   N   Y   E   A   Q   F   Y   Q   M   L   M   T   L   N   K   R   P   K  114

447 AGA CTT TAC AAT TTT GTG GAA GAT GCA GAC TCA ATT TTG AAA AAA TAT GAG CAA TAT TTA  506
115 R   L   Y   N   F   V   E   D   A   D   S   I   L   K   K   Y   E   Q   Y   L  134

507 CAC AGT TTT GAA TTT CAT ATT TAT GAG AAT AAT TAT AAG ATT TGC GCT CCT GCA AAT AGC  566
135 H   S   F   E   F   H   I   Y   E   N   N   Y   K   I   C   A   P   A   N   S  154

567 AGG TTA CAA CAG CAG CAA AAG CAA CCC GAG CTG ACT AGT GAC GGT TTA ATA TTA ACC AAA  626
155 R   L   Q   Q   Q   Q   K   Q   P   E   L   T   S   D   G   L   I   L   T   K  174

627 AAT AAC GAA ACT TTG AAA GAA TTT TTA GAG TAC GTC GCA AGA GGA AGG ATT CCT GAT GCT  686
175 N   N   E   T   L   K   E   F   L   E   Y   V   A   R   G   R   I   P   D   A  194

687 ATT ATG GAA GTC TTA AGG GAC TGT AAT ATC CAA TTT TAC GAA GGA AAT CTT ATT TTA CAG  746
195 I   M   E   V   L   R   D   C   N   I   Q   F   Y   E   G   N   L   I   L   Q  214

747 GTT TAT GAC CAT ACT AAT ACA GTT GAT GTC ACC CCC AAA GAG AAC AAG CCT AAT TTG AAC  806
215 V   Y   D   H   T   N   T   V   D   V   T   P   K   E   N   K   P   N   L   N  234

807 AGC TCT TCT TCC CCT TCA AAT AAT AAT AGC ACA CAG GAC AAT TCC AAG ATT CAA CAA CCA  866
235 S   S   S   S   P   S   N   N   N   S   T   Q   D   N   S   K   I   Q   Q   P  254
```

```
 867 TCC GAA CCT AAT AGT GGT GTG GCA AAT ACA GGC GCA AAC ACA GCA AAT AAG AAG GCA TCC  926
 255 S   E   P   N   S   G   V   A   N   T   G   A   N   T   A   N   K   K   A   S    274

 927 TTC AAA CGA CCT AGA GTA TAT CGA ACA TTG TTG AAG CCT AAT GAT TTG ACG ACG TAT TAT  986
 275 F   K   R   P   R   V   Y   R   T   L   L   K   P   N   D   L   T   T   Y   Y    294

 987 GAC ATG ATG TCT TAT GCA GAT AAT GCC AGG TTT TCT GAT AGC ATT TAT CAA CAG TTT GAA 1046
 295 D   M   M   S   Y   A   D   N   A   R   F   S   D   S   I   Y   Q   Q   F   E    314

1047 TCT GAA ATT TTA ACA CTT ACC AAG AGG AAC TTG TCA CTA AGT GTG CCA TTA AAT CCA TAT 1106
 315 S   E   I   L   T   L   T   K   R   N   L   S   L   S   V   P   L   N   P   Y    334

1107 GAG CAC CGA GAC ATG TTG GAG GAA ACA GCT TTT TCA GAA CCT CAT TGG GAT AGT GAA AAA 1166
 335 E   H   R   D   M   L   E   E   T   A   F   S   E   P   H   W   D   S   E   K    354

1167 AAA TCC TTC ATT CAC GAA CAT CGT GCT GAG TCT ACG AGA GAA GGC ACG AAG GGC GTT GTA 1226
 355 K   S   F   I   H   E   H   R   A   E   S   T   R   E   G   T   K   G   V   V    374

1227 GGG CAT ATC GAA GAA CGC GAT GAA TTT CCG CAA CAT AGT TCA AAT TAT GAA CAG TTG ATG 1286
 375 G   H   I   E   E   R   D   E   F   P   Q   H   S   S   N   Y   E   Q   L   M    394

1287 TTG ATC ATG AAT GAA CGC ACA ACA ACG ATA ACC AAC TCC ACG TTT GCT GTT TCG TTA ACC 1346
 395 L   I   M   N   E   R   T   T   T   I   T   N   S   T   F   A   V   S   L   T    414

1347 AAG AAT GCG ATG GAG ATT GCT AGC TCC AGT TCC AAC GGG GTA CGT GGA GCT TCG TCC TCA 1406
 415 K   N   A   M   E   I   A   S   S   S   S   N   G   V   R   G   A   S   S   S    434

1407 ACT TCG AAT TCC GCA TCA AAT ACA AGG AAT AAC AGT TTG GCT AAT GGC AAT CAA GTA GCT 1466
 435 T   S   N   S   A   S   N   T   R   N   N   S   L   A   N   G   N   Q   V   A    454

1467 TTG GCA GCG GCA GCG GCT GCG GCT GCA GTG GGA TCT ACC ATG GGT AAC GAT AAT AAT CAA 1526
 455 L   A   A   A   A   A   A   A   A   V   G   S   T   M   G   N   D   N   N   Q    474

1527 TTT AGC AGG CTG AAA TTT ATT GAA CAA TGG AGA ATA AAT AAA GAA AAA AGA AAA CAG CAG 1586
 475 F   S   R   L   K   F   I   E   Q   W   R   I   N   K   E   K   R   K   Q   Q    494

1587 GCT TTA AGT GCA AAC ATC AAT CCA ACC CCC TTC AAC GCT AGA ATA TCG ATG ACA GCA CCA 1646
 495 A   L   S   A   N   I   N   P   T   P   F   N   A   R   I   S   M   T   A   P    514

1647 TTG ACT CCA CAG CAA CAG CTA CTT CAA AGA CAA CAG CAG GCC TTG GAG CAA CAA CAG AAT 1706
 515 L   T   P   Q   Q   Q   L   L   Q   R   Q   Q   Q   A   L   E   Q   Q   Q   N    534
```

```
1707 GGT GGA GCC ATG AAA AAC GCA AAT AAA CGG AGT GGT AAC AAT GCC ACC AGT AAC AAT AAT 1766
 535 G   G   A   M   K   N   A   N   K   R   S   G   N   N   A   T   S   N   N   N   554

1767 AAT AAT AAT AAT AAT TTA GAT AAA CCA AAG GTT AAG CGA CCT AGA AAA AAT GCA AAA AAA 1826
 555 N   N   N   N   N   L   D   K   P   K   V   K   R   P   R   K   N   A   K   K   574

1827 AGC GAG AGC GGT ACA CCA GCT CCT AAA AAG AAA AGA ATG ACT AAG AAG AAA CAG AGC GCA 1886
 575 S   E   S   G   T   P   A   P   K   K   K   R   M   T   K   K   K   Q   S   A   594

1887 AGT AGC ACG CCC TCT TCT ACT ACA ATG TCA TAA tctacttaaatagagttatcattccttatatatata 1955
 595 S   S   T   P   S   S   T   T   M   S   *                                       604

1956 tatatatatatatatatatatatatatattgtattaaagtacatcattatttaaaaaaaattctcacttgggcatctc 2035

2036 acctctgtgctcaa                                                                 2049
```

A.



B.

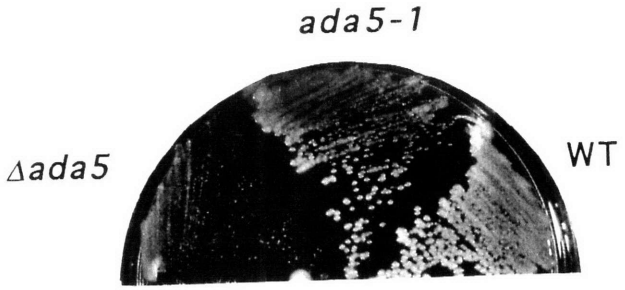


Figure 4. The *ada5* deletion mutant strain grows more slowly than the *ada5-1* mutant strain. GMy30, an *ada5* deletion mutant strain (Δada5), GMy37p, an *ada5-1* strain and BWG1-7a the isogenic wildtype strain were streaked on rich (YPD) medium. Growth was scored after two days (A) and three days (B).

Amino acids 1-437 of ADA5 can complement a *ada5Δ* mutant for growth but not toxicity

In the process of subcloning *ADA5*, we discovered that portions of the *ADA5* coding sequence could be deleted without loss, or with only partial loss of the ability to complement an *ada5* mutant strain. (See Materials and Methods for more details). As shown in Figure 5, $ADA5_{437}$, the first 437 amino acids of ADA5 (with a 22 amino acid tail from vector sequence) can complement a *ada5Δ* strain for growth on rich medium, but can only partially complement for growth on minimal medium. Furthermore, $ADA5_{437}$ strains are resistant to GAL4-VP16 (Figure 6). This is the first time that the growth and toxicity phenotypes have been separated in an ADA gene.

In order to determine whether $ADA5_{437}$ strains are resistant to toxicity by lowering the level of GAL4-VP16 in the cell we compared the levels of ARS-CEN GAL4-VP16FA in a *ada5Δ* strain, an $ada5_{437}$ strain, and a wildtype strain by gel shift analysis. As shown in Figure 7, the level of ARS-CEN GAL4-VP16FA is lower in $ada5_{437}$ strain than the wildtype strain, but higher than the level of protein in the *ada5* deletion strain. As is the case with the *ada5-1* allele, we cannot rule out the possibility that lower expression of the toxic chimera contributes to the resistance of $ada5_{437}$ mutants.

***ADA5* deletion strains show broad activation defects *in vivo*.**

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

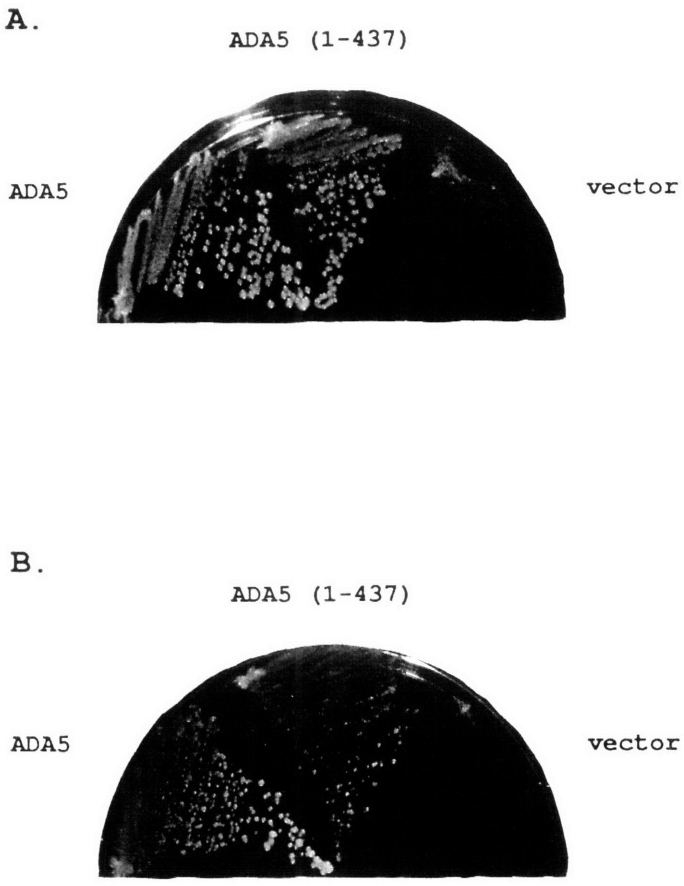


Figure 5. The first 437 amino acids of ADA5 are sufficient to complement the slow growth phenotype of *Δada5* on rich medium, but only partially complement on minimal medium. A+B) GMy30, a *Δ ada5* mutant was transformed with the full length ADA5 clone, the $ADA5_{437}$ fragment (containting the first 437 amino acids of ADA5), or a vector control. Transformants were restreaked on either drop out medium and scored after two days (A), or restreaked on minimal medium and scored after three days (B).

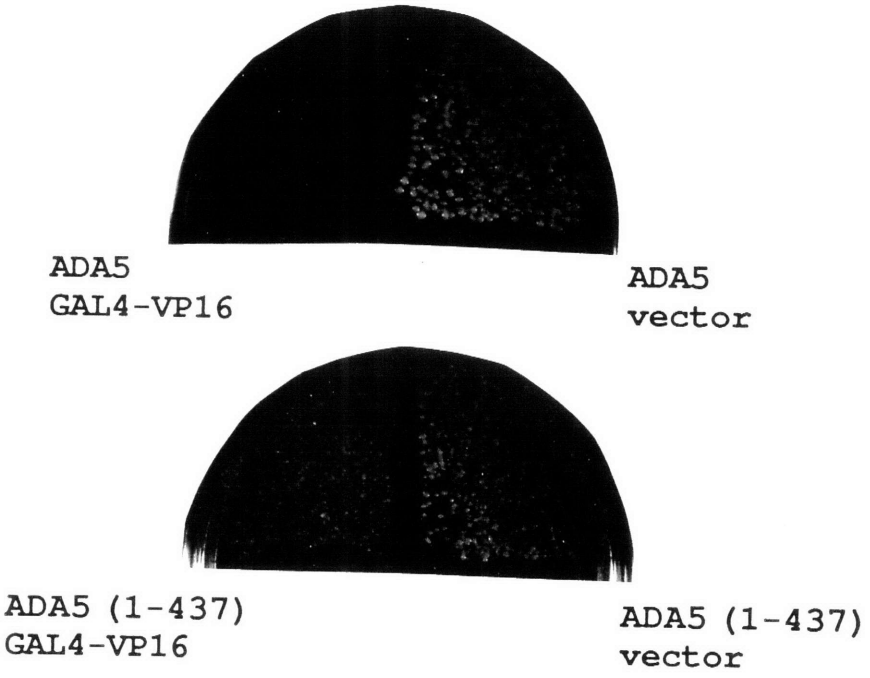


Figure 6. $ADA5_{437}$ does not restore resistance to GAL4-VP16 to an ada5 deletion strain. GMy30 complemented by full length ADA5 or $ADA5_{437}$ were transformed with pSB201, a 2μ plasmid expressing GAL4-VP16 from the ADH1 promoter, or a vector control and plated on drop out medium. Transformation plates were scored for growth after three days.

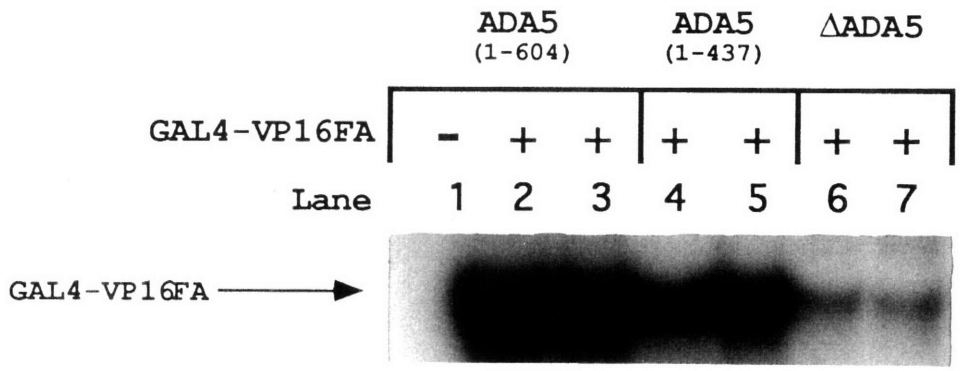


Figure 7. Levels of GAL4-VP16 are reduced in the $ADA5_{437}$ truncation mutant. GMy30 was doubly transformed with pa/cGAL4-VP16FA as well as pRS316 ADA5, pRS316$ADA5_{437}$, or vector. Protein extracts from these strains, as well as from a strain without GAL4-VP16FA (lane 1) were used to shift a radiolabled GAL4 oligonucleotide.

strain. The levels of ß-galactosidase were assayed in each strain as a way to measure activation by these promoters. The results are summarized in Table 1.

The activity of the HIS66 and 14x2 promoters, activated by GCN4, the ADA2/ADA3/GCN5 dependent activation domain, is reduced 7-10 fold in the *ada5* mutant and deletion strains. This suggests that GCN4 requires ADA5 as well as the other ADAs to activate. In addition, UAS1 and ADH1 mediated transcription, which is independent of the other ADAs, is down three fold in the *ada5-1* mutant, and 10 and 5 fold respectively in the *ada5* deletion strain. In addition, transcriptional activation by the *HO* promoter is down 10 fold in the mutant and 20 fold in the deletion, and activation by the *INO1* promoter is down over twenty-five fold in the mutant, and is undetectable in the deletion. Interestingly, the two promoters activated by *HAP4* and *GAL4*, the ADA2 independent activation domains still retain much of their ability to activate in the ADA5 mutant. The activity of UAS2, which uses the HAP4 activation domain is unchanged in the mutant, and is only reduced five fold in the deletion. Similarly, UAS_{GAL} mediated transcription is only down three fold in the *ada5* deletion.

The activation data correlates with some of the growth properties of the *ada5* mutant and deletion strains. In general the *ada5 -1* mutant strain show less severe defects for both growth and activation than the deletion strain. Further, the inability to transcribe the *INO1* gene is a likely reason for the inosital auxotrophy. In addition, *ada5* strains grow slowly on minimal medium, and have defects in GCN4 mediated activation. GCN4 regulates the synthesis of amino acid biosynthetic genes in response to starvation (Hinnebusch, 1985). Finally, activation by the ADH1 promoter is reduced in *ada5-1* and *ada5Δ* strains, which explains the lower levels of GAL4-VP16FA in *ada5-1* and *ada5Δ* mutants.

Table 1 *(Following page)*

LEGEND: (Top) The wildtype strain BP1 (Piña, et al., 1993) was transformed an ARS-CEN plasmid expressing GAL4-VP16FA, and GMy37p, the *ada5-1* strain was transformed with a 2μ plasmid expressing GAL4-VP16FA. In addition, both strains were transformed with pLGSD5 a reporter plasmid with the ß-galactosidase gene under the control of the GAL4 promoter. Activity of the ß-galactosidase gene averaged from multiple experiments is reported. Bottom: BWG1-7a the a wildtype strain, *ada5-1* mutants and *ada5Δ* deletion strains were transformed with the following ß-galactosidase reporter plasmids: pLG312ΔAluXho, which contains theCYC1 UAS1 (Guarente, et al., 1984); p265UP1, which contains the CYC1 UAS2 (Forsberg and Guarente, 1989); pHIS66, which contains the HIS4 UAS (Hinnebusch, et al., 1985); p14x2, which contains two synthetic GCN4 binding sites (Hinnebusch, et al., 1985); pCP8 (Gift of C. Peterson), which contains *HO* URS1 (-1516 to -901; pINO1 (Scafe, et al., 1990); pLGSD5 containing the GAL1-10 promoter and pCP0, containing the ADH1 promoter (Santangelo, et al., 1988). Activity of the ß-galactosidase gene averaged from multiple experiments is reported. Activity of pLGSD5 was measured after induction with Galactose, and the activity of the *INO1* reporter was assayed after five hours induction in -Ino medium (Scafe, et al., 1990).

	WT	*ada5-1*	*Δada5*
a/c GAL4-VP16FA	3429	135	ND
2μ GAL4-VP16FA	6538	1734	ND
UAS1	989	282	92
UAS2	347	251	71
HIS66	217	19	23
14x2	20	3	4
HO	206	23	10
INO1	101	4	<1
SD5	9588	ND	3027
ADH1	8948	3245	1600

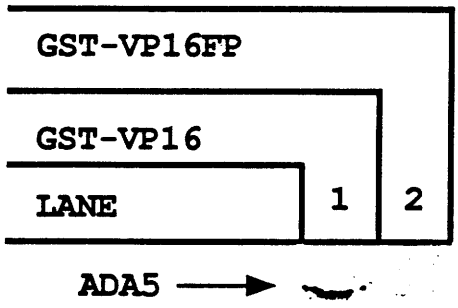
ADA5 binds to VP16

One way that *ADA5* could regulate the activation of these promoters would be as a transcriptional adaptor that mediates the interaction of activation domains and basal factors. One prediction of this model is that ADA5 should be able to bind activation domains. Therefore, we tested whether *in vitro* translated ADA5 can bind directly to the VP16 activation domain in GST- VP16 pull down experiments (see Methods). ADA5 binds the full length VP16 activation domain containing residues 413-490 (Figure 8 Lane 1), but does not bind a VP16 mutant (413-456 Phe442-Proline) that cannot activate (Lane 2). The unmutated N-terminal VP16 activation domain (413-456) also does not bind ADA5 (data not shown). Another ADA gene, GCN5, does not bind VP16 in this assay (data not shown), suggesting that theVP16-ADA5 interaction is specific. The specific ADA5-VP16 interaction suggests that ADA5 may stimulate activation by binding to activation domains.

Double mutants between *ADA5* and other adas

ADA1 is another ada gene that has been shown to regulate the *ADH1* and *UAS1* promoters (J. Horiuchi, unpublished results, Berger, et al., 1992). Furthermore, *ada1* deletion mutants have similar growth properties to *ada5* deletion mutants (data not shown), suggesting ADA1 and ADA5 may act as a complex or in the same pathway *in vivo*. Therefore, we constructed an *ada1ada5* double deletion mutant (see Methods). As shown in Figure 9, *ada1*, *ada5* and *ada1ada5* deletion mutants all have the same slow growth phenotype on rich medium. This is consistent with the model that ADA5 and ADA1 mediate activation in the same pathway or as a complex.

Figure 8 *(Following page)*. ADA5 binds to the VP16 activation domain. Wildtype or mutant VP16 fused to the glutathione transferase protein were tested for their ability to interact with ADA5. "GST-VP16" the wildtype VP16 fusion contains the full length VP16 activation domain (residues 413-490). "GST-VP16FA" contains residues 413-456 phe442-pro. GST-VP16 (Lane 1) or GST-VP16FA (Lane 2) were incubated with *in vitro* translated ADA5, precipitated with Glutathione beads and washed . Samples were eluted with glutathione, electrophoresed on an SDS PAGE gel, which was dried and exposed on a phosphoimager screen for two days.


GST-VP16FP
GST-VP16
LANE
1
2
ADA5

The growth and activation phenotypes are more general and severe in *ada5* deletion mutants than in *ada2, ada3* or *gcn5* deletion mutants. This suggests that ADA5 could be mediating activation by a different mechanism than the ADA2 complex genes. Moreover, ADA5 and ADA2 both can bind to the VP16 activation domain, and may have partially redundant functions. If ADA5 indeed functions in a separate pathway from ADA2 and ADA3, then *ada5ada2* or *ada5ada3* double deletion mutants should have a synthetic phenotype. Surprisingly, *Δada2ada5Δ* and *Δada3ada5Δ* double deletion mutants are viable and grow no more slowly than *ada5Δ* single mutants on rich medium (Figure 10 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. It is not possible to consistently compare the growth of these strains on minimal medium because they are too sick.

GCN5 and ADA5 do not co-fractionate over a Bio-Rex 70 column.

ADA2, ADA3 and GCN5 can form a complex *in vitro* (Horiuchi, et al., 1995), and copurify from yeast extracts (N. Silverman, unpublished results and Chapter 3). Furthermore, double deletion mutants in any two of these genes have slow growth phenotypes no more severe than the single mutant phenotypes, suggesting these genes operate as a complex *in vivo* as well (Marcus, et al., 1994). Given that the growth phenotypes of *ada5ada2* and *ada5ada3* double deletion mutants suggests ADA5 may be in the ADA2 complex, we examined whether ADA5 co-purifies with GCN5, a member of the ADA2 complex.

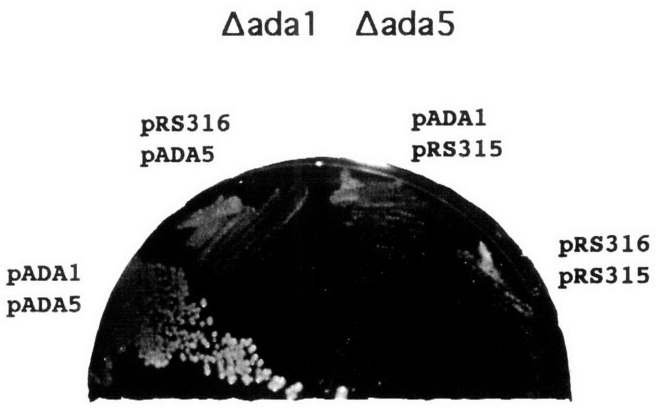


Figure 9. *ada1 ada5* double deletion mutants have similar growth properties. GMy40, an *ada5 ada1* double deletion mutant, was transformed with all pairwise combinations of pRS315ADA5 (or pRS315 a LEU2 vector) and YCp50ADA1, (or pRS316, a URA3 vector). Transformants were restreaked on drop out medium (rich), and scored after two days.

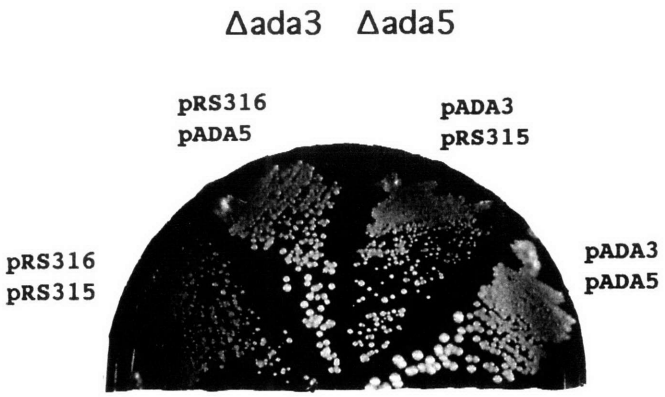


Figure 10. *ada3 ada5* double deletion mutants grow as well as *ada5* deletion mutants. GMy38, an *ada5 ada3* double deletion mutant was transformed with all pairwise combinations of pRS315ADA5 (or pRS315 a LEU2 vector) and pADA3HHV (or pRS316, its URA3 vector). Transformants were restreaked on drop out medium (rich), and scored after three days. Note that the figure names the plasmids in the strain and not the genotype. Thus, the transformant with only the ADA5 clone has the growth properties of an *ada3* mutant, and the transformant with only the ADA3 clone has the properties of the *ada5* mutant.

The three elution fractions as well as the starting material and flowthrough fractions from the Bio-Rex 70 column, the first step in the ADA2 complex purification, were assayed for ADA5 and GCN5 by Western blot analysis (See methods for more detail of fractionation). As shown in Figure 11, ADA5 is not retained on the column and is detected only in the flow-through and starting material. GCN5, on the other hand, although not detectable in the starting material, is clearly in the 600 and 1200 mM elution fractions. ADA2 and ADA3 also elute in the 600mM and 1200mM fractions similar to GCN5 (N. Silverman and LG, unpublished data). Furthermore, ADA5 protein could not be detected in any of the later fractions where ADA2, ADA3 and GCN5 co-purify (data not shown).

Because the ADA5 antiserum is more sensitive than the GCN5 antiserum (data not shown), ADA5 could not be co-fractionating with GCN5 in anything near an equal stochiometry. Therefore, because ADA5 does not copurify with GCN5, and because GCN5 co-purifies with ADA2 and ADA3, ADA5 does not appear to be a member of the ADA complex (hereafter designated the ADA2 complex). In addition, *in vitro* translated ADA5 failed to coimmunoprecipitate with cotranslated ADA2, ADA3 or GCN5 (data not shown).

ADA5 is also *SPT20*

SPT20 is a newly isolated SPT gene that appears to be in the *SPT15* (TBP) (Eisenmann, et al., 1989; Hahn, et al., 1989) class of SPT genes (S. Roberts and F. Winston, personal communication). The other genes in this class are *SPT3, SPT7,* and *SPT8* (Eisenmann, et al., 1989). *spt20* mutants, like the other spt mutants of this class, have pleiotropic mating, growth and sporulation defects. Furthermore, transcription of the Ty LTR and other yeast genes is compromised. The pleiotropic growth and transcription defects in

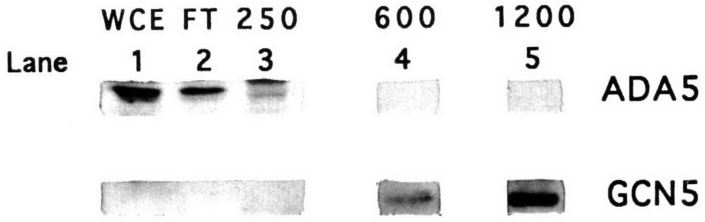


Figure 11. ADA5 and GCN5 do not cofractionate over a Bio-Rex70 column. Whole cell yeast extracts were chromotographed on a Bio-Rex70 column. Proteins were stepwise eluted in 250mM, 600mM and 1200mM potassium acetate. 100μg of the whole cell extract (WCE) and flow through (FT), and 50μg of each elution were assayed for GCN5 and ADA5 by Western blot analysis. Note the presence of an abundant yeast protein smaller than ADA5 in the 250mM fraction that gives a non-specific signal.

ada5 deletion mutants suggests that *ADA5* could be a spt gene. By sequence comparison, we determined that *SPT20* is the same gene as *ADA5* (S. Roberts and F. Winston, personal communication).

Because *ADA5* is also an SPT, it is conceivable that other SPT mutations would also have an ADA phenotype, i.e. resistance to GAL4-VP16 toxicity. Therefore, we challenged 11 different spt strains with GAL4-VP16. The results are summarized in Table 2. As expected, the *spt 20* mutant strains are resistant to GAL4-VP16. In addition, Fy963, an *spt7* deletion strain, is completely resistant to GAL4-VP16, and Fy383 and Fy508, two different *spt15* mutant strains, are somewhat resistant to GAL4-VP16. The remaining spt mutants are not resistant to toxicity. Interestingly, *spt7* and *spt15* mutants are phenotypically similar to *spt20* mutants. Although the levels of GAL4-VP16 in these strains has not been determined, and thus the mechanism of resistance is unknown, it is nonetheless tantalizing that mutants in TBP are resistant to toxicity (see Discussion).

DISCUSSION

Here, we report the cloning and initial characterization of *ADA5*, a novel ADA gene. Unlike *ada2*, *ada3* and *gcn5* mutants, which do not show altered the expression of the toxic chimera GAL4-VP16 but reduce its ability to activate (Marcus, et al., 1994) and references therein, GAL4-VP16 expression is lower in *ada5* mutants. Furthermore, because the levels of GAL4-VP16 are lower in *ada5* mutants, it is difficult to determine whether the VP16 activation domain requires ADA5 in order to activate. However, by expressing GAL4-VP16FA, a less toxic VP16 mutant, from a low copy plasmid in wt cells and a high copy plasmid in mutant cells, the level of GAL4-VP16 is roughly equivalent in the two

Table 2. Most spt mutants are not resistant to GAL4-VP16 toxicity.

STRAIN	SPT GENOTYPE	RESISTANCE TO GAL4-VP16
Fy3	wt	-
Fy363	*spt5-194*	-
Fy137	*spt6-140*	-
L881	*spt3-401*	-
Fy51	*spt3d203::TRP1*	-
FY963	*spt7d::LEU2*	++++
Fy463	*spt8-302::LEU2*	-
Fy383	*spt15-21*	++
Fy508	*spt15-122*	+
Fy191	*spt20-61*	++++
Fy210	*spt20Δ::URA3*	++++
Fy247	*spt4d::URA3*	-

LEGEND. Spt strains were transformed with pGAL4-VP16Ura and matching pRS426 vector, plated on drop out medium, and scored for growth after two or three days. Note that L881, Fy191, Fy210, and Fy247 were transformed with the LEU2 versions of the VP16 toxicity plasmid and vector, pSB202 and pRS425 because they are URA3+. Both GAL4-VP16 derivatives are equally toxic to yeast (G.M., unpublished observation). "-" indicates only pinpoint colonies form. "++++" indicates the size of the colonies expressing GAL4-VP16 is the same as the vector alone colonies.

strains. In these conditions, GAL4-VP16FA mediated activation is two fold lower in the *ada5-1* strain.

However, this may underestimate the dependence of GAL4-VP16WT on ADA5 for activation *in vivo* in two ways. First, GAL4-VP16FA is less active than GAL4-VP16 (Marcus, et al., 1994), which may reflect a loss of the ADA5 interaction. If this is the case, then mutating *ada5* would not further depress the activity of GAL4-VP16FA. This issue could be resolved by comparing the activity of equal levels of unmutated GAL4-VP16 in mutant and wildtype cells. Furthermore, the *ada5-1* mutant is a hypomorph. Evaluation of the ability of VP16 to activate in an *ada5* deletion strain might show a much larger reduction in activity. Unfortunately, GAL4-VP16 expression is lower in the deletion strain than the mutant strain, making it impossible to equalize the levels of GAL4-VP16 in the wildtype and deletion strains.

In addition, two different hypomorphic *ada5* alleles were characterized. The *ada5-1* allele, isolated in the selection does not grow as slowly as the *ada5Δ* mutant, and has less severe activation defects. The $ada5_{437}$ allele can complement a *ada5Δ* strain for growth but is still resistant to GAL4-VP16 toxicity. We cannot rule out the possibility that instability of the $ADA5_{437}$ protein is related to its partial complementation. However, it is unlikely that lower expression of *ADA5* would split the toxicity and growth phenotypes. We favor a model in which the C-terminal 167 amino acids is a domain of ADA5 that interacts with a basal factor or activation domain to mediate toxicity. The remainder of the protein is sufficient to mediate growth on rich medium by interacting with other basal factors, activation domains or coactivators. In support of this view, ADA5 and its *K. lactis* homolog show conservation in four conserved domains (P. Spellman, G.M. and LG, unpublished data). $ada5_{437}$ deletes the last conserved domain (data not shown).

On the basis of five criteria, *ADA5* is classified as a novel type of ADA gene. First, *ada5Δ* strains show reduced levels of GAL4-VP16, unlike *ada2*, *ada3* or *gcn5* deletion strains. Second, *ada5Δ* mutants grow slowly on rich and minimal medium. *ada2* mutants have only a mild slow growth phenotype on rich medium. Third, *ada5Δ* strains are inositol auxotrophs, whereas *Δada2*, *Δada3* and *Δgcn5* mutants are not (J. Horiuchi, unpublished results). Fourth, promoters that are activate independently of ADA2, such as UAS1 and ADH1 are dependent on ADA5. (In *Δada2*, *Δada3* and *Δgcn5* mutants, we have used lexA-GAL4, lexA-HAP4, and lexA -GCN4 fusions to show that the promoter specificity for ADA2 complex dependence resides in the activation domain and not in the DNA binding domain or TATA box (Marcus, et al., 1994; Piña, et al., 1993). Unfortunately, this test is unavailable in the *ada5Δ* strain because the activity of the ADH1 promoter, which drives expression of the lexA fusions is lower in *ada5* mutants.) Fifth, ADA5 does not co-fractionate with GCN5, which itself is in a complex with ADA2 and ADA3 (N. Silverman, unpublished data, Chapter 3).

ADA1 is in the same class as *ADA5* on the basis of three criteria: First, activation by the UAS1 and *ADH1* promoters, which are only ADA5 dependent are also reduced in *ada1* mutants (J. Horiuchi, unpublished data, Berger, et al., 1992)). Second, *ada1*, *ada5* and *ada1ada5* double deletion strains have the same growth properties in rich medium. Third, *ada1* deletion mutants have an spt phenotype (J. Horiuchi, unpublished results). *ada2, ada3* and *gcn5* mutants do not have spt phenotypes (S. Roberts and F. Winston, personal communication; for *ADA3* also J. Horiuchi and LG, unpublished data).

Although *ADA5* and *ADA1* appear to be a novel class of ADA genes, the relationship of this class to the *ADA2* class of ADAs is somewhat unclear. GCN4 mediated activation which is highly ADA2 dependent (Piña, et al., 1993), is also

ADA5 dependent. HAP4 and GAL4 mediated activation, which are only mildly ADA2 dependent (Piña, et al., 1993), are less ADA5 dependent than most of the other activators tested (Table 1, activities of UAS2 and SD5). Furthermore, we have made the genetic argument that ADA2, ADA3 and GCN5 act together *in vivo* because double mutants among any pairwise combination of these genes have a growth phenotype no more severe than the single mutants alone (Marcus, et al., 1994). If the ADA2 complex and ADA5 work through redundant and/or independent activation pathways, then we would expect that *Δada2ada5Δ* double mutants would have a more severe growth defect than either of the single mutants. In contrast, we find that in rich medium, the *ada2 ada5* double mutant and the *ada3ada5* double mutant grows like an *ada5* mutant, which argues that *ADA5* is in the same pathway as the ADA2 complex genes.

Whatever its relationship to the other adas, *ADA5* has several characteristics that suggest it may be a transcriptional adaptor that facilitates or bridges the interaction between activation domains and basal factors. *In vivo*, many promoters require ADA5 for activity. Furthermore, ADA5 binds directly to the VP16 activation domain. This interaction is specific, because ADA5 did not bind to variants of VP16 that cannot activate transcription in yeast.

If ADA5 could be associated with other yeast coactivators, the mechanism it uses to activate might be more apparent. ADA5 does not copurify with, and cannot be detected in the RNA polymerase holoenzyme (data not shown) (Koleske and Young, 1994). Furthermore, it cannot be detected in the SWI/SNF complex (Peterson, et al., 1994), and the Weil lab TAF complex (Poon and Weil, 1993) (data not shown).

However, *ADA5* is identical to *SPT20* which based upon its mutant phenotypes, is in the same class of spt genes as *SPT3, SPT7, SPT8* and

SPT15. These genes regulate start site selection at Ty elements, transcription of Ty, and transcription of *MFA1*. SPT15 encodes the TATA binding protein TBP (Eisenmann, et al., 1989; Hahn, et al., 1989). SPT3, SPT7 and SPT8 may form a complex with TBP that regulates promoter selection (Winston, 1992), (Eisenmann, et al., 1992). SPT20/ADA5 may act in the same pathway or complex as these SPT genes to regulate promoter selection by TBP (S. Roberts and F. Winston, personal communication). Moreover, the specificity determinants for this putative complex may be governed by the interaction of ADA5/SPT20 with activation domains.

Finally, we have previously argued that toxicity results from the trapping of basal factors at non-specific sites on DNA to form an "inhibition complex". According to this model, mutants in basal factors should be able to alter toxicity. In fact, mutants in *TFIIB* have been isolated that are hypersensitive to GAL4-VP16 (R. Knaus and LG, submitted). Furthermore, two different mutants in *spt15* are resistant to GAL4-VP16 toxicity. Although this may be due to lower expression of the toxic chimera, if not, this supports the "inhibition complex" model, and supports the contention that TBP is a target of the VP16 activation domain (Barlev, et al., 1995).

Materials and Methods

Cloning and sequencing of *ADA5*

GMy37p (*mata, ura3-52, leu2, his4, gal4::HIS4, ada5-1*) was transformed with a yeast genomic library on an ARS-CEN plasmid (Thompson, et al., 1993), and colonies that grew well were selected. From these, we isolated a clone p3,1 with an 8.5 KB insert that restored wild type growth and sensitivity to GAL4-VP16 toxicity. After partial digestion with Sau3a, 1-3 Kb fragments from this clone were isolated from a 1.2% agarose gel, and ligated into pRS316 digested

with BamHI to create a subgenomic library. GMy37p was transformed with this subgenomic library, and large colonies were selected. From this, two subclones that restored wild type growth and sensitivity to GAL4-VP16 were isolated, pL1B1 and pL1G1 with 2.2 and 1.8 Kb inserts respectively.

We chose to sequence pL1B1. A unidirectional deletion series from the NotI site in the pRS316 vector was created using ExoIII and ExoVII enzymes. Single and double stranded sequencing, using the Sequenase kit (USB), of deletion subclones from the -20 primer provied sequence on one strand of ADA5 in L1B1. A partial deletion series from the KpnI site, and sequence specific primers were designed to sequence the other strand of pL1B1. However, the largest open reading frame on pL1B1 was open at the 5' end, suggesting the entire ADA5 open reading frame was not complete on the L1B1 subclone. By sequencing the ends of the pL1G1 insert, we learned that pL1G1 is a C-terminal truncation of the same ORF. Using a sequence specific primer, to sequence the L1G1 subclone, I found that the putative ADA5 ORF, incomplete on L1B1, continues in L1G1. The remaining ADA5 sequence derives from L1G1.

ADA5 plasmids

pRS316 ADA5, a subclone with the entire *ADA5* coding region, was created in several stages. The BstXI site in pRS316 (Sikorski and Hieter, 1989)was destroyed by digestion, blunting with T4 polymerase and ligation to create pRS316-BstXI. A 1.9 Kb. EcoRI fragment containing the first 437 aa of ADA5 as well as the upstream sequences was cloned into the EcoRI site to create pRS316 $ADA5_{437a}$ and $ADA5_{437b}$. ($ADA5_{437a}$ is oriented such that the BstXI site at the 5'end of ADA5 is proximal to the SacI site in of the polylinker. $ADA5_{437b}$ is in the other orientation. A 1.9 Kb *BstXI-HindIII* fragment from

pL1B1, was cloned into the *BstXI HindIII* sites of pRS316ADA5$_{437a}$ to create pRS316 ADA5. A 2.6 Kb XhoI NotI fragment from pRS316-ADA5 was cloned into pRS315 cut with XhoI and NotI to create pRS315-ADA5. pRS306-ADA5 was generated by cloning a 1.8Kb *XbaI* fragment from p3,1 into pRS306 cut with *XbaI*. The ADA5 coding sequence was amplified using PCR with primers ADA5N and ADA5C. The resulting fragment was digested with *NotI*, and cloned into the *NotI* site of pDB20L to form pDB20L-ADA5.

ADA5 deletion plasmid and strains

The *ADA5* deletion plasmid was created in several steps. A 550bp *XhoI* blunted *BstXI* fragment from pL1G1 containing the first 12 codons of ADA5 and 5' flanking sequence was cloned into pBluescript KS+ (Stratagene) cut with *XhoI I* and *EcoRV* to form pBluescript A5BstX. Next, a 2.4 Kb. BamHI BglII fragment containing the hisG URA3 cassette from pNKY51 (Alani, et al., 1987) was cloned into the *BamHI* site of pBluescript A5BstX. The resulting plasmid, pADA5nko was chosen because it had the correct orientation of the hisG insert, such that the *BamHI* site not destroyed by ligation with BglII was located farthest from the *BstXI* site. Finally, pBluescript ADA5 was cut with DraIII, ligated to a NotI linker, cut with NotI, and then cut with BstYI. The 400 bp BstYI NotI fragment containing 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 437 amino acids from the N-terminus of ADA5, which should produce a null phenotype.

ADA5 deletion strains were generated by transforming yeast with 10μg of 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 streaked out on FOA to select strains that looped out the URA3 sequence. These ura- derivatives were transformed with pRS316-ADA5 to confirm that wild type growth and sensitivitiy to GAL4-VP16 were restored by the *ADA5* clone. In this manner, the ura+ and ura- deletion strains GMy29 and GMy30 were generated in parent strain BWG1-7a; GMy31 and GMy32 in the parent strain BP1; and GMy33 and GMy34 in PSy316.

The *ada2ada5* , the *ada3ada5* and the *ada1ada5* double deletion strains were generated in the following manner. The strain GMy30 containing the plasmid pDB20L-ADA5, (which contains the ADA5 gene on a plasmid with a Leu2 marker) was transformed with pADA2KO (Berger, et al., 1992) cut with *Bam*HI and *Xho*I, pADA3KO cut with *Pvu*II and *Bam*HI (Marcus, et al., 1994), or pADA1KO (unpublished gift of J. Horiuchi) with PvuII. *ADA1, ADA2* or *ADA3* deletion strains were identified by mating slow growing transformants to *Δada1*, *Δada2* or *Δada3* strains. Strains that failed to complement the cognate ada deletion strain were grown on FOA to select strains that looped out the *URA3* sequence.

The resulting ura- derivatives were grown to saturation in YPD, plated on YPD plates and replica plated to identify strains that had lost the leu2 plasmid containing the ADA5 clone. The double mutant genotype of GMy36 (BWG1-7a *Δada2ada5Δ*), GMy38 (BWG1-7a *Δada3ada5Δ)* and GMy40 (BWG1-7a *Δada1ada5Δ*) was confirmed by transforming these strains with the ADA5 and the ADA1, ADA2 or ADA3 clones.

ADA5 protein expression, antisera and Western analysis of the Bio-Rex 70 ADA fractions

The *Bam*HI site at the N-terminus of pL1B1 is in frame with the *Bam*H1 site of pET15b (Novagen). pL1B1 was digested with DraIII, ligated with a

BamHI linker, and then digested with BamHI. The resulting 2.2 Kb fragment was cloned into the *Bam*HI site of pET15b (Novagen) to form pET15b-ADA5. In the bacterial strain BL21(Novagen), this plasmid produced large amounts of insoluble ADA5 protein. The pET15b vector fused a six histadine tag at the N-terminus of ADA5, which was used for purification on a Ni column (Qiagen).

Lyophylized acrylamide slices containing 400μg ADA5 protein were resuspended in saline, and injected into two different rabbits (Harlow, 1988). After several boosts, the antisera from either rabbit can recognize ADA5 expressed in bacteria or yeast. IgG were purified on a protein A column (Harlow, 1988).

The Bio-Rex70 yeast fractions (gift of Neal Silverman) are described in Chapter 3. Westerns were performed using using HRP conjugated donkey anti-rabbit secondary antibody (ECL) according to standard protocols (Harlow, 1988). Westerns performed with ADA5 antisera were incubated with secondary antibody for two hours, and washed In TBS-Tween no more than three times for five minutes each after incubation with either the primary or secondary antibodies.

Gst-VP16 pull down assay

The ADA5 *in vitro* translation plasmid pCITE2b-ADA5 was generated by cloning the 2.2Kb BamHI fragment containing the ADA5 coding sequence (and some 3' sequence) from pET15b-ADA5 into the BamHI site of pCITE2b (Novagen). *In vitro* translations were performed as previously described (Horiuchi, et al., 1995).

Pull down experiments were performed by incubating 10μg GST-VP16 or 10μg GST-VP16456-490 phe442-pro,10μl Sepharose Glutathione beads (Pharmacia) preblocked in E. coli extract, and 10μl in vitro translated ADA5 in

200μl S300 1%T Buffer (20mM HEPES pH 7.6, 300mM potassium acetate, 1% Triton X100, 20%Glycerol), plus *E. coli* extract to 1mg/ml. Binding proceeds for 1hr, followed by 4 washes with 1ml S300 1%T buffer. Samples were eluted from the beads by 20mM Glutathione in S100 buffer, and electrophoresed on an SDS PAGE gel. The dried gel was exposed 2 days on a phosphoimager screen, and evaluated by phosphoimager (Molecular Dynamics).

Strains, Yeast Manipulations, Media, Gel shifts and ß-galactosidase assays.

All yeast strains are derivatives of BWG1-7a (*mata, ade1, ura3-52, leu2, his4*) unless otherwise noted. Transformations were by the LiOAc method (Gietz, et al., 1992) Tetrad analysis and other yeast manipulations were done using standard techniques(Guthrie and Fink, 1991) ß-galactosidase assays were carried out on yeast extracts made from breaking cells with glass beads (Rose and Bostein, 1983). The activity of ß-galactosidase is normalized to total protein. Gel shift analysis was performed as previously described (Berger, et al., 1992). Slow growth phenotypes of *ada* mutants were assayed on SD minimal medium supplemented with amino acids and adenine. Otherwise strains were grown in SD rich drop out medium containing all amino acids except those needed for plasmid selection.

ACKNOWLEDGEMENTS

GST-VP16 pull down experiments were done by Neal Silverman with *in vitro transation* plasmids generated by GM. I would like to thank Neal Silverman for the gift of protein fractions from the ADA2 affinity purification; Shannon Roberts for the SPT strains and the sharing of unpublished results; Wei Gao for pCP0, Craig Peterson for pCP8, and the Young lab for the INO1 reporter.

REFERENCES

Alani, E., Cao, L. and Kleckner, N. (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.

Barlev, N.A., Candau, R., Wang, L., Darpino, P., Silverman, N. and Berger, S.L. (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.

Berger, S.L., Cress, W.D., Cress, A., Triezenberg, S.J. and Guarente, L. (1990). Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61**, 1199-1208.

Berger, S.L., Piña, B., Silverman, N., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J. and Guarente, L. (1992). Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. Cell **70**, 251-265.

Cote, J., Quinn, J., Workman, J.L. and Peterson, C.L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science **265**, 53-60.

Dynlacht, B.D., Hoey, T. and Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell **66**, 563-576.

Eisenmann, D., Chapon, C., Roberts, S., Dollard, C. and Winston, F. (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.

Eisenmann, D.M., Arndt, K.M., Ricupero, S.L., Rooney, J.W. and Winston, F. (1992). SPT3 interacts with TFIID to allow normal transcription in Saccharomyces cerevisiae. Genes Dev **6**, 1319-31.

Eisenmann, D.M., Dollard, C. and Winston, F. (1989). *SPT15*, the gene encoding the yeast TATA binding factor TFIID, is required for normal transcription initiation *in vivo*. Cell **58**, 1183-1191.

Forsberg, S.L. and Guarente, L. (1989). Identification and characterization of HAP4: a third component in the CCAAT-bound HAP2/HAP3 heteromer. Genes Dev. **3**, 1166-1178.

Gansheroff, L., Dollars, C., Tan, P. and Winston, F. (1995). The *Saccharomyces cerevisiae SPT7* gene encodes a very acidic protein important for transcription *in vivo*. Genetics **139**, 523-536.

Ge, H. and Roeder, R. (1994). Purification, cloning, and characterization of a human coactivator, PC-4, that mediates transcriptional activation of class II genes. Cell **78**, 513-523.

Gietz, D., St. Jean, A., Woods, R.A. and Schiesti, R.H. (1992). Improved method for high efficiency transformation oDf intact yeast cells. Nucleic Acids Res. **20**, 1425.

Goodrich, J.A., Hoey, T., Thut, C.J., Admon, A. and Tjian, R. (1993). Drosophila TafII 40 Interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75**, 519-530.

Guarente, L., Lalonde, B., Gifford, P. and Alani, E. (1984). distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene. Cell **36**, 503-511.

Hahn, S., Buratowski, S., Sharp, P.A. and Guarente, L. (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.

Haviv, I., Vaizel, D. and Shaul, Y. (1995). The X protein of hepatitis B virus coactivates potent activation domains. Mol Cell Biol 1079-1085.

Hengartner, C.J., Thompson, C.M., Zhang, J., Chao, D.M., Liao, S.M., Koleske, A.J., Okamura, S. and Young, R.A. (1995). Association of an activator with an RNA polymerase II holoenzyme. Genes & Dev. **9**, 897-910.

Hinnebusch, A.G., Lucchini, G. and Fink, G. (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.

Hinnebusch, A.G. (1985). A Hierarchy of trans-acting Factors Modulates Translation of an Activator of Amino Acid Biosynthetic Genes in *Saccharomyces cerivisiae*. MCB **5**, 2349-2360.

Hirschhorn, J.N., Brown, S.A., Clark, C.D. and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. Genes Dev **6**, 2288-98.

Hoey, T., Dynlacht, B.D., Peterson, M.G., Pugh, B.F. and Tjian, R. (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72**, 247-260.

Horiuchi, J., Silverman, N., Marcus, G. and Guarente, L. (1995). ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. Mol Cell Biol 1203-1209.

Kelleher, R.J.I., Flanagan, P.M. and Kornberg, R.D. (1990). A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell **61**, 1209-1215.

Kim, Y.J., Björklund, S., Li, Y., Sayre, M.H. and Kornberg, R.D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77**, 599-608.

Koleske, A.J., Buratwoski, S., Nonet, M. and Young, R.A. (1992). A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. Cell **69**, 883-894.

Koleske, A.J. and Young, R.A. (1994). An RNA polymerase II holoenzyme responsive to activators. Nature **368**, 466-469.

Kretzschmar, M., Kaiser, K., Lottspeich, F. and Meisterernst, M. (1994). A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. Cell **78**, 525-534.

Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature **370**, 223-226.

Lin, Y.S., and Green, M. R. (1991). Mechanism of action of an acidic transcriptional activator *in vitro*. Cell **64**, 971-981.

Marcus, G.A., Silverman, N., Berger, S.L., Horiuchi, J. and Guarente, L. (1994). Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. EMBO J **13**, 4807-4815.

Neigeborn, L. and Carlson, M. (1984). Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. Genetics **108**, 845-858.

Peterson, C.L., Dingwall, A. and Scott, M.P. (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.

Piña, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J.A. and Guarente, L. (1993). ADA3: a gene, indentified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. Molec. Cell. Biol. **13**, 5981-5989.

Poon, D. and Weil, A.P. (1993). Immunopurification of yeast TATA-binding protein and associated factors. J. Biol. Chem. **268**, 15325-15325.

Pugh, B.F. and Tjian, R. (1990). Mechanism of transcriptional activation by Spt1: evidence for coactivators. Cell **61**, 1187-1197.

Rose, M. and Bostein, D. (1983). Structure and function of the yeast URA3 gene: Differentially regulated expression of hybrid B-galactosidase from overlapping coding sequences in yeast. J. Mol. Biol. **170**, 883-904.

Santangelo, G.M., Tornow, J., McLaughlin, C.S. and Moldave, K. (1988). Properties of promoters cloned randomly from the *Saccharomyces cerevisae* genome. Mol. Cell. Biol. **8**, 4217-4224.

Scafe, C., Chao, D., Lopes, J., Hirsch, J.P., Henry, S. and Young, R. (1990). RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. Nature **347**, 491-494.

Sikorski, R.S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**, 19-27.

Silverman, N., Agapite, J. and Guarente, L. (1994). Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. Proc. Natl. Sci. USA **91**, 11665-11668.

Stern, M.J., Jensen, R.E. and Herskowitz, I. (1984). Five *SWI* genes are required for expression of the *HO* gene in yeast. J. Mol. Biol. **178**, 853-868.

Stringer, K.F., Ingles, C.J. and Greenblatt, J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature **345**, 783-786.

Thompson, C.M., Koleske, A.J., Chao, D.M. and Young, R.A. (1993). A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73**, 1361-1375.

Thut, C.J., Chen, J.L., Klemm, R. and Tjian, R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. Science **267**, 100-104.

Winston, F. (1992). Analysis of SPT Genes: A genetic Approach toward Analysis fo TFIID, Histones, and Other Transcription Factors of Yeast. In

Transcriptional Regulation, McKnight, S.L. and Yamamoto, K.R., eds. (Cold Spring Harbor Laboratory Press, United States) pp. 1271-1293.

Winston, F., Chaleff, D.T., Valent, B. and Fink, G.R. (1984). Mutations Affecting Ty-Mediated Expression of the *HIS4* Gene of *Saccharomyces cerevisiae*. Genetics **107**, 179-197.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J.L., Triezenberg, S.J., Reinberg, D., Flores, O., Ingles, C.J. and Greenblatt, J. (1994). Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol. Cell. Biol. **14**, 7013-7024.

Chapter 5:

The Role of the ADA genes in transcriptional activation

In Chapter 1, I suggested that coactivators be evaluated by their ability to stimulate activation *in vivo* or *in vitro*, bind activators, and bind basal factors. The interaction with activators determins specifity. The basal factor interaction indicates something about the mechanism used by the coactivator to stimulate activation. The TAF complex is clearly necessary and sufficient for activation *in vitro* (Dynlacht, et al., 1991). Its specificity is governed by interactions between specific TAFs and activation domains (Chen, et al., 1994; Jacq, et al., 1994). Individual TAFs interact with TBP, TFIIB and TFIIA (Tjian and Maniatis, 1994). The yeast SWI/SNF complex is an example of a coactivator complex with a known mechanism (relief of chromatin repression) but with unknown specifity determinants (Peterson and Tamkum, 1995). Similarly, the SPT3, SPT7 and SPT8 genes regulate transcription of certain promoters *in vivo*, perhaps by regulating promoter selection by TBP (SPT15) (Winston, 1992). Again, the mechanism for promoter specificity is unknown. These criteria, regulation of activation, activator specifity and basal factor targets are a useful method with which to evaluate the ADA genes discussed in this thesis.

Mutants in ADA1, ADA2, ADA3, GCN5 (ADA4) and ADA5 were isolated in a selection for mutants resistant to overexpression of GAL4-VP16, which is toxic to yeast. We believe that the mechanism of toxicity is related to the mechanism of transcriptional activation because mutants that reduce the ability of VP16 to activate transcription, or reduce the ability of GAL4 to bind DNA, which also leads to a reduction in activation, lower the toxicity of GAL4-VP16 in a correlated fashion (Berger, et al., 1992).

ADA mutants fall into two phenotypic classes. *ada2, ada3* and *gcn5* deletion mutants releave toxicity by reducing the ability of GAL4-VP16 to activate without changing GAL4-VP16 expression (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). In addition, these mutants are all recessive,

grow slowly on minimal medium, are temperature sensitive, and importantly, are unable to support activation *in vivo* or *in vitro* by certain activation domains (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). Genetic and biochemical evidence suggests that ADA2, ADA3 and GCN5 operate as a complex (Horiuchi, et al., 1995; Marcus, et al., 1994; Piña, et al., 1993), see also Chapter 3 and (Silverman, et al., 1994). In fact, ADA2, ADA3 and GCN5 co-purify from yeast extracts (N. Silverman, unpublished data, Chapter 3). This complex has not been purified to homogeneity, and thus at present the number of proteins in the ADA2 complex is unknown.

ADA1 and ADA5 comprise the other class. *ada1* and *ada5* mutants have more severe and more general slow growth and activation defects than the *ada2* complex mutants (J. Horiuchi, unpublished data, Chapter 4). Moreover, they survive toxicity in part by lower GAL4-VP16 expression, and have an spt- phenotype (J. Horiuchi, unpublished data, Chapter 4). Finally, *ada5* mutants are Ino-, and does not co-purify with the ADA2 complex, which argues that it indeed operates by a different mechanism than the ADA2 complex genes (Chapter 4).

The ADA2 complex genes are required for activation by certain actiators *in vivo* and *in vitro*, and are thus considered coactivators (Berger, et al., 1992; Marcus, et al., 1994; Piña, et al., 1993). The GCN4, HAP4 and GAL4 activation domains when fused to a common DNA binding domain show differential requirements for the ADA2 complex genes *in vivo*, showing that the specifity for the ADA2 complex can be determined by the activation domain per se (Marcus, et al., 1994; Piña, et al., 1993). In fact, the specificity for the ADA2 complex may be determined by the binding of activation domains to ADA2. ADA2 can bind to the GCN4 activation domain and VP16 activation domains, which are ADA2

dependant (Barlev, et al., 1995; Silverman, et al., 1994), but not to the HAP4 activation domain which is ADA2 independent (Barlev, et al., 1995).

Two different basal factor targets of the ADA2 complex have been proposed. GST-VP16 can retain TBP from wild type yeast nuclear extracts but not from *ada2* extracts, suggesting TBP may be a target of ADA2 mediated activation (Barlev, et al., 1995). Alternatively, members of the ADA2 complex have been shown to interact genetically and biochemically with the CTD of RPB1, the largest subunit of RNA polymerase II (N. Silverman, unpublished data). ADA2 and ADA3 are also sub-stochiometric components of the holoenzyme. This argues that the CTD may also be a target of the ADA2 complex.

The activity of the TAF complex is in part mediated by contacts between individual subunits and different activators or components of the basal transcription machinery (Tjian and Maniatis, 1994). The ADA2 complex may operate in an analagous manner. ADA2 directly contacts activators, but only indirectly interacts with TBP (Barlev, et al., 1995). The TBP interaction may be mediated by another member of the ADA2 complex. Moreover, ADA2 does not directly bind the CTD suggesting that another component of the ADA2 complex is binding the CTD. Clearly, it is the complex as a whole that mediates the activation domain basal factor interactions.

Additionally, ADA2 has a Cys rich domain that is found in other coactivators (Arany, et al., 1994). This domain is within a 133 amino acid fragment of CBP that binds TFIIB, and may mediate this interaction (Kwok, et al., 1994). Similarly, GCN5 has a bromodomain, another domain present in many coactivators including TAF250, SWI2, SPT7, GCN5, Brahma, and the CBP/p300 class of proteins (Eckner, et al., 1994; Kennison, 1993). . A GCN5 mutant missing the bromodomain shows reduced growth and less GCN4 mediated

activation, indicating that the bromodomain is important for the activity of the ADA2 complex. Although the function of the Cys domain and the bromodomain are unknown, each may mediate one of many connections between the ADA2 complex and activators or basal factors.

Although not as well characterized as the ADA2 complex genes, ADA5 and ADA1 are putative adapters because of the general activation defects in *ada5* and *ada1* mutants (J. Horiuchi, unpublished data, Chapter 4). Unfortunately, the lexA activation domain fusions cannot be used to evaluate whether activation domains can determine ADA5 specifity because the ADH promoter used to express the lexA fusions is itself regulated by ADA5 (Chapter 4). However, ADA5 binds to the VP16 activation domain, suggesting that specifity for this class of ADAs may be determined by activator-ADA5 interactions (Chapter 4).

Somewhat paradoxically, ADA1 is phenotypically a member of the ADA5 class, but co-purifies with the ADA2 complex (N. Silverman, J. Horiuchi, unpublished data). ADA1 can be also detected in the flowthrough fraction of the Bio-Rex 70 column along with ADA5 (N. Silverman, J. Horiuchi, unpublished data) None of the other ADA proteins can be detected in the Bio-Rex 70 flowthrough. This suggests that ADA1 is not exclusively associated with the ADA2 complex. It may be an abundant protein that happens to be in the flowthrough with ADA5, or based upon common mutant phenotypes, it may be part of a theoretical second complex with ADA5. In the second complex model, ADA1 may be playing a role mediating the interaction between ADA5 and the ADA2 complex. Alternatively, under other conditions, ADA5 may also be a member of the ADA2 complex. Composition of the RNA polymerase holoenzyme and TFIID complexes can varry with preparation conditions (Koleske and Young, 1995; Verrijzer, et al., 1994).

Furthermore, although ADA5 does not cofractionate with the ADA2 complex, it may work in the same pathway. *ada2ada5* and *ada3ada5* double deletion mutants have the growth phenotype of an *ada5* deletion mutant, which argues that ADA5 indeed works in the same pathway as the other ada genes (Chapter 4). Furthermore, GCN4 mediated activation is highly dependent on both ADA2 and ADA5, whereas GAL4 and HAP4 mediated activation are independent of ADA2 and less dependent on ADA5 than any of the other reporters examined (Chapter 4). Whether or not the ADA2 complex works in the same pathway as ADA5 and ADA1, all of the ADA genes are coactivators for several yeast promoters.

The importance of the ADA genes *in vivo* is demonstrated by the isolation of *ada* mutations in several diverse genetic selections. For example, *ADA3* was also isolated as *NGG1*, a negative regulator of GAL4 activity in the absence of GAL80 in glucose (Brandl, et al.,). Negative regulation may be an indirect consequence of ADA3 dependent expression of a repressor, or an indirect effect of promoter competition between ADA dependent and independent activators. Alternatively, ADA3, and perhaps the other ADA genes may in fact be negative regulators of some loci. *GAL11*, a putative coactivator and member of the holoenzyme is both a positive and negative regulator of transcription (Fassler and Winston, 1989).

In addition, the SWI7, SWI8 and SWI9 genes are identical to ADA2, ADA3 and GCN5 (K. Pollard and C. Peterson, personal communication). Mutations in these SWI genes were isolated in a genetic screen for mutants that do not activate an Ho lacZ reporter gene (Breeden and Nasmyth, 1987). The relationship of ADA2, ADA3 and GCN5 to the SWI genes that comprise the SNF/SWI complex and regulate transcription through chromatin is unclear. Activation by three promoters regulated by the other SNF/SWI genes, *INO1*,

SUC2 and *HO* is reduced in *swi7*, *swi8* or *swi9* mutants (K. Pollard and C. Peterson, personal communication).

However, unlike the other swi/snf mutants, strains carrying *swi7*, *swi8* and *swi9* mutants are Inositol prototrophs. Furthermore, the ADA2, ADA3 and GCN5 proteins are not part of the SWI/SNF complex by Western blot analysis, although the complex is less stable in *swi8* (*ada3*) mutants (K. Pollard and C. Peterson, personal communication). Finally, deletion mutants in *ada2* can be suppressed by mutants in TFIIB, suggesting that ADA2 activates transcription in a chromatin independent manner (R. Knaus and LG, unpublished data). At present, it is unclear whether the ADA complex "touches" and coordinates activation with the SWI complex *in vivo*, or whether the ADA genes are simply coactivators for an activator such as *SWI5* that regulates *HO* transcription. Whatever the relationship between the SWI complex and the ADA complex, the isolation of mutants in *ada2*, *ada3* and *gcn5* in a screen for regulators of a yeast promoter underscores the importance of the ADA2 complex genes in the yeast life cycle.

As its name implies, GCN5 was originally isolated in a selection for mutants sensitive to 3-aminotriazole, which mimics Histadine starvation (Hinnebusch and Fink, 1983; Penn, et al., 1983). *gcn5* mutants, unlike the other gcn mutants does not regulate the protein level of the transcriptional activator GCN4, but rather its ability to activate transcription (Georgakopoulos and Thireos, 1992). Thus, it had been proposed to be a coactivator for GCN4 (Georgakopoulos and Thireos, 1992).

ADA5 is identical to SPT20, a newly identified member of the SPT3, SPT7, SPT8 and SPT15 class of Ty suppressors (S. Robberts and F. Winston, personal communication; Chapter 4). Mutations in any of these SPT genes have similar pleiotropic growth and transcription phenotypes (Eisenmann, et al.,

1989). Furthermore, these spt mutants can alter the choice of promoter selection by SPT15(TBP) without changing the DNA binding properties of TBP *in vitro* (Eisenmann, et al., 1992). Based on their similar mutant phenotypes, and genetic and physical interactions among some members of this group, SPT3, SPT7, SPT8, SPT15 may act as a complex (Eisenmann, et al., 1994; Eisenmann, et al., 1992; Winston, 1992). SPT20/ADA5 may act in the same pathway or complex as these SPT genes to regulate promoter selection by TBP (S. Robberts and F. Winston, personal communication). Moreover, the specificity of this putative complex may be determined by the interaction of ADA5/SPT20 with activation domains.

Finally, homologs of ADA genes have been found in other eukaryotes, suggesting that the function of the complex is conserved throughout evolution. Homologs for the ADA2 and ADA5 genes have been isolated from the yeast *K. lactis* (P. Spellman and LG unpublished data); for ADA2 in *S. pombe* (P. Spellman, N. Silverman and LG, unpublished data); and for ADA2 and GCN5 from humans (Barlev, et al., 1995), Chapter 3). Other coactivators have been conserved from yeast to humans as well, including SNF2 and the TAFs (Carlson and Laurent, 1994; Goodrich and Tjian, 1994). Given the important role transcription factors and other coactivators such as Brahma and CCG1(TAF250) play in growth, cell cycle regulation and development in Metazoans (Tamkun, et al., 1992; Wang and Tjian, 1994), it is likely that the ADA homologs play decisive roles as well.

REFERENCES

Arany, Z., Sellers, W., Livingston, D. and Eckner, R. (1994). E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators [letter]. Cell **77**, 799-800.

Barlev, N.A., Candau, R., Wang, L., Darpino, P., Silverman, N. and Berger, S.L. (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.

Berger, S.L., Piña, B., Silverman, N., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J. and Guarente, L. (1992). Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. Cell **70**, 251-265.

Brandl, C.J., Furlanetto, A.M., Martens, J.A. and Hamilton, K. Characterization of NGG1, a novel yeast gene required for glucose repression of GAL4p-regulated transcription. EMBO J. **12**,

Breeden, L. and Nasmyth, K. (1987). Cell Cycle control of the yeast *HO* gene: cis- and trans-acting regulators. Cell **48**, 389-397.

Carlson, M. and Laurent, B.C. (1994). The SNF/SWI family of global transciptional activators. Curr. Op. in Cell Biol. **6**, 396-402.

Chen, J.L., Attardi, L.D., Verrijzer, C.P., Yokomori, K. and Tjian, R. (1994). Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. Cell **79**, 93-105.

Dynlacht, B.D., Hoey, T. and Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell **66**, 563-576.

Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B. and Livingston, D.M. (1994). Molecular cloning and functional analysis of teh adenovirus E1A-associated protein 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. **8**, 869-884.

Eisenmann, D., Chapon, C., Roberts, S., Dollard, C. and Winston, F. (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.

Eisenmann, D.M., Arndt, K.M., Ricupero, S.L., Rooney, J.W. and Winston, F. (1992). SPT3 interacts with TFIID to allow normal transcription in Saccharomyces cerevisiae. Genes Dev **6**, 1319-31.

Eisenmann, D.M., Dollard, C. and Winston, F. (1989). *SPT15*, the gene encoding the yeast TATA binding factor TFIID, is required for normal transcription initiation *in vivo*. Cell **58**, 1183-1191.

Fassler, J. and Winston, F. (1989). The Saccharomyces cerevisiae SPT13/GAL11 gene has both positive and negative regulatory roles in transcription. Mol. Cell. Biol. **9**, 5602-5609.

Georgakopoulos, T. and Thireos, G. (1992). Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. EMBO J. **11**, 4145-4152.

Goodrich, J.A. and Tjian, R. (1994). TBF-TAF complexes: selectivity factors for eukaryotic transcription. Current Opinion in Cell Biology **6**, 403-409.

Hinnebusch, A.G. and Fink, G.R. (1983). Positive regulation in the general amino acid control of Saccaromyces cerevisiae. PNAS **80**, 5374-5378.

Horiuchi, J., Silverman, N., Marcus, G. and Guarente, L. (1995). ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. Mol Cell Biol 1203-1209.

Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P. and Tora, L. (1994). Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. Cell **79**, 107-117.

Kennison, J.A. (1993). Transcriptional activation of *Drosophila* homeotic genes from distant regulatory elements. Trends Genet. **9**, 75-79.

Koleske, A.J. and Young, R.A. (1995). The RNA polymerase II holoenzyme and its implications for gene regulation. Trends Bioch Sci **20**, 113-116.

Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature **370**, 223-226.

Marcus, G.A., Silverman, N., Berger, S.L., Horiuchi, J. and Guarente, L. (1994). Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. EMBO J **13**, 4807-4815.

Penn, M.D., Galgoci, B. and Greer, H. (1983). Identification of AAS genes and their regulatory role in general control of amino acid biosynthesis in yeast. Proc Natl Acad Sci U S A **80**, 2704-8.

Peterson, C.L. and Tamkum, J.W. (1995). The SWI-SNF complex: a chromatin remodeling machine? TIBS **20**, 146.

Piña, B., Berger, S., Marcus, G.A., Silverman, N., Agapite, J.A. and Guarente, L. (1993). ADA3: a gene, indentified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. Molec. Cell. Biol. **13**, 5981-5989.

Silverman, N., Agapite, J. and Guarente, L. (1994). Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. Proc. Natl. Sci. USA **91**, 11665-11668.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992). brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell **68**, 561-72.

Tjian, R. and Maniatis, T. (1994). Transcriptional activation: a complex puzzle with few easy pieces. Cell **77**, 5-8.

Verrijzer, P., Yokomori, K., Chen, J.L. and Tjian, R. (1994). Drosophila TAFII150: similarity to yeast gene TSM-1 and specific binding to core promoter DNA. Science **264**, 933-941.

Wang, E. and Tjian, R. (1994). Promoter-selective transcriptional defect in cell cycle mutant ts13 rescued by hTAFII250. Science **263**, 811-814.

Winston, F. (1992). Analysis of SPT Genes: A genetic Approach toward Analysis fo TFIID, Histones, and Other Transcription Factors of Yeast. In Transcriptional Regulation, McKnight, S.L. and Yamamoto, K.R., eds. (Cold Spring Harbor Laboratory Press, United States) pp. 1271-1293.

APPENDIX I:

Isolation Of Strains Resistant To GAL4-VP16 toxicity

In Chapter 2, I described a selection for mutants resistant to GAL4-VP16 toxicity in the strain BP1. A number of alleles of *ADA1, ADA2* and *ADA3* were isolated in addition to the alleles of the new genes *GCN5* and *ADA5*. In addition, a putative clone for the strain *EMS3* from the original mutagenesis was obtained by complementation of its slow growth phenotype. Its ability to complement toxicity resistance has not been tested. Furthermore , several strains resistant to toxicity have not been named or cloned. Table I contains a listing of all of the ADA mutant strains isolated in this selection.

There are several important characteristics of these strains:

Strain 36n is fully resistant to GAL4-VP16, grows slowly, but does not express GAL4-VP16, as analyzed by Gel shift analysis (data not shown). Because of this, it was not studied further.

Strain 44b is resistant, but does not grow slowly. Therefore it has been very hard to clone. I have not checked the level of GAL4-VP16 by gel shift, and I have not checked whether any of the ADA clones can restore sensitivity to GAL4-VP16.

Strain 42d has a dominant slow growth phenotype, but by mating it is recessive for GAL4-VP16 resistance. I did not check whether the slow growth co-segregated with the toxicity resistance. I attempted to clone this mutation in this strain, and a resistant segregant from tetrad analysis, but failed (data not shown).

Strain 35n is very resistant, but reverts or gets suppressors at a fairly high rate. To work with this strain, get fresh stocks from the perm regularly.

Strain 32s is sterile. It grows slowly, and is not complemented by the ADA1, ADA2 or ADA3 clones. Levels of GAL4-VP16 were not checked.

Strain 48o, *gcn5-3* is rho-.

EMS3 was identified in the original Berger selection. A putative clone has been identified. 20 different clones with related inserts were identified, that complement the slow growth phenotype of *EMS3*. They have not been checked for the ability to restore sensitivity to GAL4-VP16.

The same selection was also carried out in the strain PSY316. $9x10^8$ cells were mutagenized to 50-60% lethality, and transformed with GAL4-VP16. One hundred eighteen colonies were picked and mated to BP1, a strain of the opposite mating type, selecting for the GAL4-VP16 plasmid. 71 diploid strains were able to grow with the plasmid, showing that the mutation conferring resistance was dominant or plasmid linked. These were not studied further. 10 strains did not mate. Of these, only three were resistant upon retransformation. These were not studied further. 37 strains were able to mate, but the resulting

diploid did not grow in the presence of GAL4-VP16, suggesting the mutation giving resistance to GAL4-VP16 is recessive. These strains were mated to *ada1*, *ada2* and *ada3* mutant tester strains. The diploids were tested for slow growth and/or resistance to GAL4-VP16. The results are summarized on TABLE 2.

There were several interesting results of note:

P8f does not grow when mated to the *ADA1* or *ADA3* tester strain. Transformation with the clones shows it to be an *ADA1* allele. Thus, this strain is an unlinked non-complementer with *ada3-1*.

All of the strains that are unknown were originally classified as *ADA3* alleles, because they grow slowly when mated to *ada3-1*. However, their growth is not improved by the ADA3 clone, suggesting that they are not *ADA3* alleles, but rather unlinked non-complementers with *ada3-1*.

P13q was first classified as an *ada3* allele. Later, it was classified as a new gene, and later still it was shown by T. Oheler to be an *ada5* allele. This allele of *ada5* shows unlinked non-complementation with *ada3-1*. This has not been systematically investigated, and should be considered a preliminary finding.

The unknown strains have not been tested with the *GCN5* or *ADA5* clones. These were isolated after these strains were exiled to the freezer. These strains have not been analyzed by tetrad analysis, and may have multiple mutations. In addition, they have not been assayed for the presence of GAL4-VP16 by gel shift analysis. Three strains are sterile, and retransform for resistance to toxicity (Table 2).

In conclusion, there are additional mutant strains resistant to GAL4-VP16 that have not been cloned or classified. These may also have mutations in ADA genes that may be members of the ADA2 complex, or may work in the same pathway as *ADA5*.

Acknowledgments

The putative EMS3 clone was isolated using the hands of Catherine Bae

TABLE 1. Isolation Of Mutants Resistant To GAL4-VP16 Mediated Toxicity In BP1.

STRAIN	ALLELE	CRITERIA
B44a	*ada1-20*	mating
B48l	*ada1-21*	mating
B48n	*ada1-22*	mating
B36u	*ada1-23*	mating
B44h	*ada1-24*	mating
B33h	*ada2-3*	mating
B33l	*ada2-4*	mating
B37b	*ada2-5*	mating
B43b	*ada2-6*	mating
B44d	*ada2-7*	mating
B34q	*ada2-8*	mating
B42c	*ada2-9*	mating
B37o	*ada2-10*	mating
B36i	*ada3-2*	mating
B37g	*ada3-3*	mating
B44i	*ada3-4*	mating
B45h	*ada3-5*	mating
B33u	*ada3-6*	mating
B28g	*ada3-7*	mating
B25a	*ada3-8*	mating
B41g	*ada3-9*	mating
B36u	*ada3-10*	mating
B43i	*ada3-11*	mating
B35s	*ada3-12*	clone
B36r	*ada3-13*	clone
B47c	*gcn5-1*	clone
B36x	*gcn5-2*	clone
B48o	*gcn5-3*	clone
B37p	*ada5-1*	clone
B35n	NEW GENE	ADA clones
B36o	NEW GENE	ADA clones
B42d	NEW GENE	ADA clones
B36n	NEW GENE	ADA clones
B44s	NEW GENE	ADA clones
EMS3	NEW GENE	ADA clones
B32s	NEW GENE	ADA clones

TABLE 2. Isolation Of Mutants Resistant To GAL4-VP16 Mediated Toxicity In PSy316.

STRAIN	GENOTYPE	CRITERIA
P12l	*ada1--25*	mating
P8f	*ada1-26*	mating
P4b	*ada2-11*	mating
P13s	*ada2-12*	mating
P6k	*ada2-13*	mating
P7a	*ada3-14*	mating
P11h	*ada3-15*	clone
P11i	*ada3-16*	clone
P11l	*ada3-17*	clone
P12c	*ada3-18*	clone
P12i	*ada3-19*	clone
P13e	*ada3-20*	clone
P13v	*ada3-21*	clone
P18a	*ada3-22*	clone
P19a	*ada3-23*	clone
P19d	*ada3-24*	clone
P6e	*ada3-25*	clone
P6o	*ada3-26*	clone
P6P	*ada3-27*	clone
P7r	*ada3-28*	clone
P10e	*ada3-29*	clone
P13o	*ada3-30*	clone
P13u	*ada3-31*	clone
P13q	*ada5-2*	clone
P7g	unknown	clone
P7n	unknown	clone
P7P	unknown	clone
P8x	unknown	mating and clone
P8y	unknown	mating and clone
P11b	unknown	mating and clone
P12b	unknown	mating and clone
P12j	unknown	mating and clone
P13m	unknown	mating and clone
P19e	unknown	mating and clone
P6m	unknown	mating and clone
P11f	unknown	mating and clone
P11k	unknown	mating and clone
P4P	unknown	mating and clone
P20b	not tested	sterile
P11g	not tested	sterile
P12f	not tested	sterile