Genetic and Biochemical Characterization of the ADAs, a Transcriptional Adaptor Complex

by

Neal Scott Silverman

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Department of Biology
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Certified by

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Leonard Guarente
Professor, Department of Biology
Thesis Supervisor

Accepted by

_____

Frank Solomon
Chairman, Biology Graduate Committee

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ABSTRACT

Transcriptional activation domains require coactivators or adaptors in order to stimulate transcription. The *ADA* genes - *ADA1, ADA2, ADA3, ADA5,* and *GCN5* - were identified in a genetic screen designed to isolate mutations in genes which encode transcriptional adaptors. *ada* strains have the phenotypes expected of such adaptor mutants. They are defective in transcriptional activation both *in vivo* and *in vitro.* The activation potentials of both the VP16 and the GCN4 activation domains are greatly reduced in the absence of *ADA2, ADA3,* or *GCN5.* Furthermore, the fusion of *ADA2* or *ADA3* to a heterologous DNA binding domain creates a transcriptional activator that is tightly regulated by the levels of the other *ADA* genes. This suggests that *in vivo* the ADA proteins form a complex which mediates the activation of transcription.

In vitro studies demonstrate that the ADA complex binds the VP16 activation domain. In yeast nuclear extracts, ADA2 and full length VP16 coimmunoprecipitate. However, this interaction is not detected with a truncated version of VP16. Truncated VP16 has very little activity *in vivo.* Furthermore, the VP16 activation domain was shown to directly interact with both ADA5 and ADA2. The biological relevance of these interactions is demonstrated by the finding that neither ADA2 nor ADA5 bind to a variant of VP16 which has a double phenylalanine to alanine substitution. This mutant has very little transcriptional activation potency *in vivo.*

ADA, SRB2 double deletion mutants have a synthetic phenotype, and deletion of an *ADA* gene exacerbates the phenotypes caused by truncations of the CTD. Together these data suggest that the *ADAs* function in parallel to the *SRBs* and that they function with the CTD to mediate the activation of transcription.

All the known ADA proteins cofractionate with RNA polymerase II when purified from yeast extract. Furthermore, *in vitro* the ADA complex can bind the CTD. This physical association between the ADAs and the CTD validates the genetic interactions and suggests that some activation domains function, at least in part, by recruiting the ADA-Pol II complex to promoters.

Thesis Supervisor: Leonard Guarente
Title: Professor of Biology

This work is dedicated both to my grandfather, Irving Silverman, whose life-long fascination with science will always be my inspiration and to my parents, Sue and Gary Silverman, whose support, encouragement, and love made it all possible.

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Adaptors and Their Role in Transcriptional Activation

Part I: Introduction

Transcriptional activation

Transcriptional activation plays an important role in a wide range of biological regulatory programs. Research from numerous labs studying many different biological phenomena has elucidated a variety of signal transduction pathways whose ultimate consequence is the transcriptional activation of target genes. For example, steroid hormone-receptor complexes directly mediate transcriptional activation of target genes (Yamamoto et al., 1992). More complicated multi-step regulatory circuits are often used by environmental and developmental signals to activate transcription of appropriate genes (McKnight and Yamamoto, 1992). Recently transcriptional activation has also been shown to be critical for learning and memory (Bourtchuladze et al., 1994; Huang et al., 1994; Yin et al., 1994). This emphasizes the notion that transcriptional control, a simple idea first discovered in studies of the *E. coli lac* operon (Beckwith and Zipser, 1970), facilitates essential and complex physiological processes.

Two types of DNA elements are necessary for transcriptional activation. One is the promoter element, which encompasses the sequences surrounding the transcription start site. Promoters usually include a TATA-box found upstream from the transcription start site. Those promoters that lack a TATA-box have a conserved element termed the Initiator (Inr) at the transcription start site. These promoter elements, the TATA-box or Inr, serve as the assembly site for the transcription machinery (Zawel and Reinberg, 1993). The other type of element, the UAS or enhancer, is often found at great distances from the promoter element. In yeast, UASs are found hundreds of base pairs upstream from the promoter, while in higher eukaryotes enhancers can be found thousands of base pairs upstream or downstream from the promoter. UAS and

enhancer elements fulfill a similar role; they contain binding sites for transcriptional activators (Guarente, 1988), which then stimulate transcription at the distant promoter.

The TATA-box is sufficient to obtain accurate transcription initiation *in vitro*. This minimal reaction, which has been termed basal transcription, requires the general transcription factors (GTFs) as well as RNA polymerase II (Pol II). The GTFs include the TATA-binding-protein (TBP) which binds to the TATA element nucleating the assembly of the transcription initiation complex. The other general transcription factors, which include TFIIA, TFIIB, Pol II, TFIIF, TFIIE, and TFIIH, bind to the TBP-DNA complex (Conway and Conway, 1993). *In vitro* these factors are able to bind the TBP-DNA complex stepwise to form a preinitiation complex which can initiate transcription after addition of nucleotides (Buratowski et al., 1989). However, it is not clear whether the stepwise assembly of the preinitiation complex or the basal transcription reaction occur *in vivo*.

One of the central problems in molecular biology is how transcriptional activation is mediated over long distances. Activators are often positioned far upstream at UASs and must communicate with their targets, the general transcription factors, at the promoter. This is believed to occur by looping-out the intervening DNA allowing for contact between the activators and the general transcription machinery (Dunaway and Droge, 1989; Müller-Storm et al., 1989). Activated transcription can also be recapitulated *in vitro*; it requires a template with UAS and TATA elements as well as transcriptional activators, the GTFs, and other intermediary factors (see below for more details) (Tjian and Maniatis, 1994). However, activated transcription *in vitro* cannot reproduce the same high level of activation detected *in vivo* nor can activators function over the

same distances that occur *in vivo* (Lue and Kornberg, 1987; Müller-Storm et al., 1989) .

In order to function, transcriptional activators require two domains (Hope and Struhl, 1986). One, the DNA-binding domain, endows activators with specificity to affect only the appropriate target genes by binding to specific UAS elements. The other important domain, the transcriptional activation domain, is responsible for stimulating the initiation of transcription. Activation domains are often characterized by their amino acid composition. Many activation domains are rich in acidic amino acids and these domains are found in all eukaryotes (Triezenberg, 1995). Yeast acidic activation domains function in mammalian cells and vice versa implying a conserved mechanism of action for acidic activation domains (Guarente, 1992). Other types of activation domains have been characterized in mammalian systems including glutamine rich, proline rich, isoleucine rich, or serine/threonine rich. However, these types of activators do not function in yeast (Künzler, 1994; Ponticelli et al., 1995).

Activation domains interact with general transcription factors *in vitro*

The mechanism by which activation domains stimulate transcription has been the focus of much research during the past few years and several models have been proposed. One model of activation argues for direct contact between activation domains and the general transcription factors. The activation domain from the virion protein 16 of Herpes simplex virus (VP16), perhaps the best characterized acidic activation domain (Cress and Triezenberg, 1991; Regier et al., 1993), has been shown to directly interact with several general transcription factors. This concept of a direct interaction between activation domains and the GTFs is appealing because one obvious

mechanism to activate transcription would be to recruit general transcription factors to promoters. Direct interactions have been detected between VP16 and TBP, TFIIB, as well as the p62 subunit of TFIIH (Ingles et al., 1991; Lin and Green, 1991; Lin et al., 1991; Stringer et al., 1990; Xiao et al., 1994). All of these interactions have been shown to depend on a functional VP16 activation domain, arguing for the biological relevance of these interactions. In addition, mutant TBP and TFIIB molecules which no longer bind activation domains also do not support activated transcription *in vitro* (Kim et al., 1994a; Roberts, 1993)

However, the requirement for these *in vitro* interactions for activated transcription is not clear. The mutant TBP referred to above, which does not support activated transcription and does not bind VP16 *in vitro*, also does not interact with a TATA-box nor does it support recruitment of TFIIB. The effects of this mutation on other known TBP interactions, such as with TFIIA or the TBP associated factors (the TAFs, see below) were not tested (Kim et al., 1994a). Thus, it is unclear what contribution the defect in binding VP16 makes to its inability to support activated transcription.

A number of studies have isolated TBP mutants based on their inability to support activated transcription *in vivo*. These mutants were then tested *in vitro* to determine what biochemical defects they possessed. In one study (Tansey et al., 1994), a strong correlation was observed between the inability to support activated transcription *in vivo* and the inability to bind $TAF_{II}250$ (see below for discussion of the TAFs) *in vitro*. Another study isolated a TBP mutant unable to support activated transcription *in vivo* and unable to bind TFIIA *in vitro*. Furthermore, fusion of the smaller subunit of TFIIA to this mutant TBP partially rescues the activation defect, arguing that *in vivo* the stimulation of the TBP-TFIIA complex is (one of) the mechanism(s) of activation (Stargell and Struhl, 1995). Still other TBP mutants, that do not support activated transcription *in*

vivo, show reduced affinity for DNA *in vitro* (Arndt et al., 1995). The study of TBP mutants that are activation defective *in vivo* has not yet uncovered a link between transcriptional activation and the binding of activation domains to TBP. Therefore, while the function of TBP during transcriptional activation appears to be very complex involving interactions with many factors, it is unclear whether direct interactions between activators and TBP play a crucial role. Furthermore, Tansey et. al. have shown that some TBP variants defective in DNA binding and *in vitro* transcription function well *in vivo* indicating that the *in vitro* model system does not completely mimic the *in vivo* situation.

Interactions between TFIIB and the VP16 activation domains have also revealed several interesting findings. Acidic activation domains can bind TFIIB directly and recruit TFIIB to the TBP-DNA complex (Lin and Green, 1991; Lin et al., 1991). Moreover, this interaction causes a conformational change in TFIIB (Roberts and Green, 1994). Choy and Green have shown that the interaction and recruitment of TFIIB are not sufficient for activated transcription *in vitro*. In these experiments, activated transcription requires the presence of the TBP-associated-factors (TAFs, see below) suggesting that activation may involve at least two steps, a TAF independent TFIIB recruitment step and then a TAF dependent step (Choy and Green, 1993). The importance of VP16 mediated TFIIB recruitment for *in vitro* transcription was demonstrated by the isolation of a TFIIB mutant which does not bind VP16 and does not support activated transcription *in vitro* (Roberts, 1993). However, it is not clear that these are the only defects caused by this mutation, especially considering that it is a double amino acid substitution. The effects of this mutation on TFIIB function have not yet been tested *in vivo,* and, given the differences observed with TBP mutants *in vivo* versus *in vitro,* this is a significant caveat.

The discovery of adaptors

Other models for activation posit that intermediary factors, referred to as coactivators, mediators, or adaptors, are necessary to mediate the effects of activation domains (Berger et al., 1990; Kelleher III et al., 1990; Pugh and Tjian, 1990). These intermediary factors are proposed to be necessary for at least two different functions. One type of factor is proposed to bridge the interaction between general transcription factors and activation domains. However, transcriptional activation requires more than a simple bridge between activators and the GTFs, because *in vivo* DNA is packaged into chromatin which is known to inhibit transcription. Therefore another class of factors which counteracts chromatin mediated repression is thought to be necessary for activation *in vivo* (Guarente, 1995). Using a variety of approaches many labs have identified a number of adaptor-type proteins. The remainder of this chapter will consider the role of these adaptor-type molecules in transcriptional activation as well as the multiple interactions that have been documented between activation domains and these potential targets.

Experiments involving *in vitro* transcription reactions first suggested that proteins beyond the defined general transcription factors are necessary for activated transcription. Tjian and colleagues found that recombinant TBP could support basal transcription but not activated transcription *in vitro*, whereas the purified transcription factor IID (TFIID), which contains TBP, could support activation. This result implied that TBP associated factors (TAFs) present in the TFIID fraction are necessary for the activation process (Pugh and Tjian, 1990). In a different approach it was found that *in vitro* transcription could be inhibited, or squelched, by addition of excess exogenous transcriptional activator. This inhibition could be counteracted with the addition of a partially purified factor, termed the mediator, that is proposed to link activation domains with the general

transcription factors (Flanagan et al., 1991; Kelleher III et al., 1990). Likewise, the *in vitro* inhibition resulting from excess activator could be modulated by preventing the exogenous activator from binding DNA. In this case, the exogenous activator inhibited activated transcription but not basal transcription suggesting the activator bound to and sequestered a factor, termed an adaptor, necessary for activated but not basal transcription (Berger et al., 1990).

Part II: Isolation and Characterization of Mammalian Coactivators

The TAFs

Subsequent to finding that TBP was not functionally equivalent to TFIID for activated transcription, TFIID was shown to consist of TBP plus approximately eight tightly associated factors referred to as TAFs (Dynlacht et al., 1991). Reconstitution of the TAFs with TBP is sufficient *in vitro* for activated transcription with a number of different types of transcriptional activators. Many TAFs have been cloned, and some have been shown to bind specific activation domains. For example, dTAF$_{II}$110 interacts with the glutamine rich activation domain of Sp1 (Gill et al., 1994; Hoey et al., 1993), and dTAF$_{II}$40 interacts with the VP16 activation domain and with TFIIB (Goodrich et al., 1993).

The importance of these interactions has been probed by a number of methods. Anti-TAF$_{II}$40 antibodies inhibited activated transcription *in vitro* suggesting that the interaction of TAF$_{II}$40 with activators and basal factors is important for activated transcription (Goodrich et al., 1993). A more compelling result is that activation competent partial TBP-TAF complexes can be assembled with recombinant components. Complexes containing TBP, TAF$_{II}$250 (the core component of the TFIID complex on which the other factors assemble), as well as the particular TAF protein which interacts with the

activation domain being tested (for example TAF$_{II}$110 with Sp1) are sufficient for activated transcription *in vitro* (Chen et al., 1994b).

Additionally, recent experiments with reconstituted TBP-TAF complexes have demonstrated synergistic activation in the presence of two transcriptional activators when the TAF target for each activation domain was present (Sauer et al., 1995a; Sauer et al., 1995b). Synergism occurs when the activation produced by two (or more) activators at one promoter is greater than the sum of activity that occurs with individual activators. In these experiments, synergism was observed at the molecular level by the increased binding of TFIID to the promoter. The TFIID complex has also been shown to be the *in vitro* target of the synergism mediated by multiple VP16 or ZEBRA (an activator from Epstein-Barr virus) activation domains. In this case, synergism was also demonstrated by increased TFIID TATA-box binding (Chi et al., 1995).

Much of the work to date on the role of the TAFs in transcription has utilized *in vitro* transcription reactions, however identification of TAF containing complexes in yeast has enabled their *in vivo* function to be examined. The yeast TAFs (yTAFs) were originally identified by Weil and colleagues (Poon et al., 1994; Poon and Weil, 1993). Other workers have also identified a TFIID-like TAF complex in yeast extracts and have demonstrated that the complex can support transcriptional activation *in vitro* (Reese et al., 1994). All cloned yTAFs are homologous to TAFs from mammals and insects and are essential for viability (Struhl, 1995). Recent experiments using conditional alleles of the various yTAF genes indicate that the yTAFs may not have a specific effect on activated transcription, but rather may be required for transcription in general. In the absence of yTAF$_{II}$130 (which is homologous to human TAF$_{II}$250 the central component of the TFIID complex), the relative level of activated transcription at the *HIS3* and *CUP1* genes remained high. On the other hand,

constitutive transcription of *HIS3* decreased steadily (Struhl, 1996b). These findings cast doubt on the role of the TAFs in activated transcription *in vivo*. Instead, like the general transcription factors, the yTAFs appear to be necessary for all transcription. The lethality of null mutations in yTAF genes is also consistent with a general role in transcription. They do not have the subtle effects on viability and transcription expected of a coactivator.

A role for TAFs in processes other than transcriptional activation has been suggested. TAFs may play a role in repressing basal transcription since, in the absence of activators, TFIID initiates transcription less efficiently than TBP (Chi et al., 1995). In addition, TAF||150 has been demonstrated to be necessary for promoter selection. The Drosophila ADH gene contains two promoters that are utilized differentially during development (Corbin and Maniatis, 1989). It was shown that the selective usage of the distal promoter during early embryogenesis is mediated through its initiator element. Furthermore, the regulation of promoter selection requires TFIID as well as TFIIA (Hansen and Tjian, 1995). TAF||150 is essential for this selectivity which is consistent with other findings demonstrating that it binds to promoter sequences found downstream of the TATA-box (Verrijzer et al., 1994) These findings implicate TAF||150 in the process of promoter selection, but not necessarily in mediating transcriptional activation.

Other Mammalian Coactivators

Other coactivators have been identified both by biochemical fractionation of mammalian *in vitro* transcription systems and by identification of novel proteins which interact with activation domains. PC4/p15 was purified from HeLa cell extracts as an activity necessary for optimal transcriptional activation *in vitro*. The recombinant PC4/p15 protein was shown to bind directly to acidic

activation domains as well as to the TBP-TFIIA-DNA complex. Although these interactions create a direct link between activation domains and the general transcription factors, PC4/p15 mediated activation also requires the TAFs (Ge and Roeder, 1994; Kretzschmar et al., 1994). This result is similar to the finding that TFIIB can directly interact with and also be recruited to promoters by VP16. However, transcriptional activation requires the TAFs. Interestingly, a yeast homolog to PC4/p15 was discovered in a screen for high copy suppressors of conditional alleles of TFIIB. The yeast gene, *SUB1*, shows extensive identity to PC4/p15 in its N-terminal half. In addition the SUB1 protein has been shown to bind to TFIIB, but not to TBP-TFIIA. *In vivo,* deletion of *SUB1* is synthetically lethal with conditional TFIIB mutations, suggesting that *in vivo* the yeast PC4/p15-like protein functions in conjunction with TFIIB during the activation of transcription. An explanation for the differences between the biochemical properties of PC4/p15 and its yeast homolog may lie in their non-homologous C-terminal region (Knaus et al., 1996).

Another set of mammalian coactivators, the p300/CBP class, was identified by two different experimental approaches. The CREB binding protein (CBP) was isolated because it binds the phosphorylated, transcriptionally active, form of CREB (Chrivia, 1993). Furthermore, CBP has been shown to interact with TFIIB *in vitro,* and expression of CBP potentiates CREB mediated activation in tissue culture supporting the hypothesis that CBP is a coactivator (Kwok et al., 1994). p300 is homologous, though not identical, to CBP and can also bind to and potentiate transcriptional activation of CREB (Lundblad et al., 1995). p300 was identified as a factor which is bound by the adenovirus E1A protein during E1A mediated cellular transformation (Eckner et al., 1994). E1A binding to p300 and CBP is believed to cause the E1A mediated inactivation of the SV40 enhancer as well as the transcriptional repression of genes involved

in the control of cellular proliferation. Interestingly, the p300/CBP class of coactivators has several domains homologous to coactivators found in yeast. For example, a bromo domain is found in mammalian coactivators such as $TAF_{II}250$ and CBP as well as in the yeast SWI2, GCN5, and SPT7 proteins. Furthermore, CBP shares a cysteine rich domain with ADA2 that is a possible zinc binding motif, suggesting a conservation of function between the CBP class of coactivators and the ADA complex. (Please see below for more complete information about these yeast coactivators.)

HMG proteins, non-histone components of chromatin, have also been shown to function as coactivators. HMG2 was identified as a coactivator activity required for optimal VP16 mediated activation (Shykind et al., 1995). HMG2 coactivation requires the TAFs as well as TBP, similar to PC4/p15. However, unlike PC4/p15, HMG2 does not appear to interact with either activation domains or the general transcription factors. The conserved HMG-box found in the HMG1 and HMG2 proteins is sufficient for this coactivator activity. HMG1/2 proteins are proposed to function by stabilizing a more active TFIID-TFIIA-DNA complex (Shykind et al., 1995). Another HMG protein, HMG17 which is not homologous to HMG1/2, has also been shown to function as a coactivator but only in reactions using nucleosomal templates (Paranjape et al., 1995). Finally, another class of HMG proteins, HMGI(Y)s, has been demonstrated to play an essential role in the stereospecific assembly of a large cooperative enhancer complex termed the enhanceosome (Tjian and Maniatis, 1994). This complex contains three different transcriptional activators in addition to HMGI(Y) which functions as an architectural factor necessary for forming the proper higher order structure (Thanos and Maniatis, 1995). This model has similarity to the proposal that HMG2 functions by stabilizing the optimal conformation of the TFIID-TFIIA-DNA complex.

Part III: Yeast as a model system for transcription studies, *in vivo* and *in vitro*

The Genetics and Biochemistry of Transcription in Yeast

The conservation of the structure and function of the transcriptional machinery across all eukaryotic species, from yeast to mammals, makes *Saccharomyces cerevisiae* exceptionally well suited for analyzing the *in vivo* mechanism(s) of transcriptional initiation and activation (Guarente, 1992). The conservation of mechanisms involved in yeast and metazoan transcriptional activation was first noted by the similarities between UAS elements and enhancers as well as the conservation of the TATA box (Guarente, 1988). Furthermore, transcriptional activators often function by mechanisms that are conserved throughout all eukaryotes. Mammalian activators, such as steroid receptors, can function in yeast, and conversely yeast activators can function in animal and plant cells.

Moreover, the identification and purification of the yeast TBP was crucial for both mammalian and yeast transcription studies (Hahn et al., 1989). The cloning of yeast TBP was essential for the study of mammalian *in vitro* transcription systems because purification of human TBP had proven to be a formidable task. By screening for sequence homologs of the yeast TBP gene, the genes for TBP from humans, plants, and insects were isolated. Furthermore, the high degree of conservation of TBP across all eukaryotes solidified the notion that the mechanisms involved in transcriptional initiation and activation would be similar in yeast and mammals (Fikes et al., 1990; Hoey et al., 1990). Interestingly, the isolation of the yTBP gene demonstrated that it was identical to a previously cloned gene, *SPT15,* which had been identified in

a screen for mutants that affect promoter selection (Eisenmann et al., 1989). This finding further highlights the notion that yeast genetics can be successfully utilized to identify the factors necessary for transcription *in vivo*.

Subsequent to the isolation of TBP from extracts, the tools of yeast genetics and biochemistry have identified many factors involved in transcription. Not surprisingly, all of the general transcription factors identified in mammalian systems have been identified in yeast. TFIIA, TFIIE, TFIIH, and TFIIF homologs were purified by biochemical fractionation (Sayre et al., 1992). The yeast gene encoding TFIIB, *SUA7*, was identified in a genetic screen for mutants that affect transcriptional start site selection (Pinto et al., 1992). Moreover, the use of yeast genetics has identified several groups of genes whose products are thought to be involved in mediating transcriptional activation. Some of these screens, such as the Ada or Srb selections, were designed specifically to identify potential coactivator molecules (Berger et al., 1990; Nonet and Young, 1989), while other screens, such as those for *ccr, spt* or *swi* mutants, selected for mutants with broad transcription defects (Denis and Malvar, 1990; Herskowitz et al., 1992; Winston, 1992). Subsequent analysis of these mutants has led to interesting findings that are relevant to transcription in all eukaryotes.

The SPTs

The *SPT* genes were isolated in a selection for suppressors of the effects of transposon insertions in the 5' region of yeast genes. The insertions, which contain either a full transposon (a Ty element) or only the long-terminal-repeat of a Ty element (a ∂ element), inhibit the transcription of these neighboring genes (Winston, 1992). This effect is proposed to be due to promoter interference; transcription from the ∂ element promoter out-competes and inhibits transcription from the promoter of the nearby yeast gene (Hirschman et

al., 1988). The net effect of an *spt* mutation is often to lower transcription from the ∂ element promoter and to concomitantly increase transcription from the neighboring gene (Winston, 1992). This implies that the *SPT* gene products are involved in promoter selection.

Most of the *spt* mutants can be divided into two classes depending on which transposon insertions they can suppress (Winston, 1992). As mentioned above, *SPT15* encodes TBP. Several other mutants, *spt3, spt7, spt8,* and *spt20*, have a suppression profile similar to *spt15* mutants. The proteins encoded by this group of genes, referred to as the TBP class, are proposed to function by modulating the affinity of TBP for different promoters or the activity of TBP at different promoters. This model is substantiated by the finding that SPT3 associates with TBP in yeast whole cell extract (Eisenmann et al., 1992). However, it may be premature to conclude that all these gene products function together since there are phenotypic differences among them. For example, *spt7* and *spt20* mutants share several phenotypes, such as inositol auxotrophy and resistance to GAL4-VP16 mediated toxicity, which *spt3* and *spt8* lack (Marcus et al., 1996). (*SPT20* is identical to *ADA5,* see section below about the ADA genes (Roberts and Winston, 1996).) This suggests that SPT20 and SPT7 proteins may function as part of the ADA complex whereas SPT3 and SPT8 may be more closely associated with TBP.

The other group of *SPT* genes, the histone group, derives its classification from the fact that *SPT11* and *SPT12* encode histones H2A and H2B (Winston, 1992). In yeast, the histones, H2A, H2B, H3, and H4, are encoded by four loci: two of which encode H2A and H2B and two of which encode H3 and H4. Addition or reduction in the copy number of any of these loci causes an Spt- phenotype. In addition, deletions of the histone loci have been shown to disrupt the phasing of nucleosomes at promoters. This effect on

chromatin structure by *spt* mutants is consistent with various findings *in vivo* and *in vitro* suggesting that chromatin structure represses transcription (Grunstein, 1990; Workman et al., 1991). The *spt* mutants in the histone class presumably affect promoter selection by destabilizing chromatin structure.

The other members of the histone class, *SPT4*, *SPT5*, and *SPT6* are also thought to modify chromatin structure. As suggested previously, this could be through the processing of histones, the assembly of nucleosomes, or the physical association with histones in structured chromatin (Winston, 1992). Consistent with the latter, recent experiments have shown that the SPT6 protein can mediate nucleosome assembly *in vitro* (Bortvin and Winston, 1996).

The SWI/SNF Complex

The impact of chromatin on transcriptional activation has been further highlighted by two other genetic screens that identified an overlapping set of genes which function to counteract chromatin mediated transcriptional repression. In one screen, the *snf* mutants were isolated based on the fact that they can no longer transcribe the *SUC2* gene (Carlson, 1987). The *SNF* genes fall into two classes: *SNF1* and *SNF4* specifically activate transcription of *SUC2*; while SNF2, *SNF5* and *SNF6* have more pleiotropic effects and genetic interactions between these genes suggest they function as a complex. In a second screen, the *swi* mutants were selected for their inability to transcribe the *HO* endonuclease gene which is necessary for mating type switching. The *swi* mutants also fall into two groups: mutants in the first group, *swi4, swi5*, and *swi6*, have phenotypes specific to *HO* transcription; whereas *swi1*, *swi2*, and *swi3*, have more pleiotropic phenotypes (Herskowitz et al., 1992). Subsequently, it was discovered that the *swi1, 2*, and *3* mutants have phenotypes similar to *snf2, 5* and *6* mutants including defects in transcription of

HO, *SUC2*, and *INO4*. Moreover, cloning studies revealed that *SWI2* and *SNF2* are the same gene (Laurent and Carlson, 1992; Peterson and Herskowitz, 1992).

Suppression analysis led to the first indications that this group of genes might function to counteract chromatin mediated repression. Mutations in *SIN1* and *SIN2*, which encode chromatin proteins, suppress the transcription defects of swi mutants. *SIN2* encodes histone H3 and *SIN1* encodes a HMG1-like protein (Kruger and Herskowitz, 1991). Furthermore, the *swi/snf* mutants can also be suppressed by the deletion of one of the histone H2A-H2B loci (*spt11*) or by mutations in the nucleosome assembly factor *SPT6* (Clark-Adams and Winston, 1987; Hirschhorn et al., 1992; Neigeborn et al., 1987). At the DNA level, the effects of *snf2* or *snf5* mutations can be detected by a change in the chromatin structure at the *SUC2* promoter. The alteration in micrococcal nuclease digestion pattern suggests a change from open to closed nucleosome structure in the mutant strains. *spt11* mutations suppress the change in nucleosomal structure caused by *snf5* mutations (Hirschhorn et al., 1992). Taken together, this suppression analysis argues that the SWI/SNF proteins function by counteracting chromatin mediated repression.

Recently, the SWI/SNF complex has been purified from yeast whole cell extract. This complex contains the SWI1, 2, 3 and the SNF5 and 6 proteins as well as five other unidentified proteins (Cairns et al., 1994; Peterson et al., 1994). One of these unknown proteins, now termed SNF11, was recently cloned by a two-hybrid screen using SWI2/SNF2 as bait (Treich et al., 1995). The SWI/SNF complex contains a DNA-stimulated ATPase activity, consistent with the DNA-stimulated ATPase-like domain found in *SNF2/SWI2*. As predicted by the genetics, the purified SWI/SNF complex is capable of

disrupting nucleosomal structure and allowing transcription factors to bind a chromatin packaged DNA template (Côté et al., 1994).

The cloning of a mammalian *SWI2* homolog (Khavari et al., 1993; Muchardt and Yaniv, 1993) made it possible to purify and characterize a mammalian SWI/SNF complex (Imbalzano et al., 1994; Kwon et al., 1994). The mammalian complex has very similar activities and components as compared to the yeast complex. The human complex has been shown to facilitate the binding of TBP and transcriptional activators, such as GAL4, to nucleosomal templates. The first clue that the SWI/SNF family of genes is conserved throughout evolution came from the discovery that *brahma*, an activator of *Drosophila* homeotic genes, is homologous to *SWI2* (Tamkun et al., 1992).

The mechanism(s) by which the SWI/SNF complex is recruited to promoters is not fully understood. Co-immunoprecipitation experiments indicate that the SWI/SNF complex binds to the DNA binding domain of the glucocorticoid receptor (Yoshinaga et al., 1992). However, the SWI/SNF complex has recently been discovered to be a member of a even bigger complex, the RNA polymerase II holoenzyme (see next section below for details) (Wilson et al., 1996). This complex has been shown to bind to the activation domain of VP16 and is thought to function as a bridge between activation domains and the general transcription machinery. Neither of these two findings, however, explain the observation that the SWI/SNF complex, in isolation, facilitates the binding of TBP to chromatin templates *in vitro* in the absence of activators.

The CCR4 complex

The properties of the *CCR4* gene suggest that it is a glucose regulated transcriptional adaptor (Draper et al., 1994). *CCR4* suppresses the effects of

SPT6 and *SPT10* mutations. In particular, *spt6* and *spt10* strains have a elevated level of *ADH2* transcription when grown in glucose (normally the repressed condition). A *CCR4* mutation reduces this elevated level of transcription (Denis and Malvar, 1990; Malvar et al., 1992). Furthermore, *CCR4* is necessary for induction of *ADH2* expression on ethanol containing media (Malvar et al., 1992). The ethanol induced transcription of *ADH2* is mediated by the transcriptional activator ADR1 (Thukral et al., 1991), and ADR1 activity is dependent on *CCR4* (Draper et al., 1994). Furthermore, fusion of *CCR4* to a DNA-binding domain creates an activator which responds to carbon source regulation (Draper et al., 1994). It is not clear how CCR4 is recruited to the *ADH2* promoter, but it is a necessary coactivator for ADR1. The future characterization of two CCR4 associated proteins should shed light on the molecular mechanism of this interesting coactivator complex (Draper et al., 1994).

The SRBs and the RNA Polymerase II Holoenzyme

The largest subunit of RNA polymerase II has a unique and conserved repetitive structure at its carboxy-terminus. This carboxy-terminal-domain, known as the CTD, consists of multiple repeats of the heptapeptide sequence, Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Allison et al., 1985; Allison et al., 1988; Corden et al., 1985). The number of repeats reflects genome complexity. For example yeast Pol II has twenty-six repeats compared to the mammalian polymerase which has fifty-two repeats (Corden, 1990; Young, 1991). In yeast and mammalian cells these repeats are essential for viability (Allison et al., 1988; Bartolomei et al., 1988; Nonet et al., 1987; Zehring et al., 1988). However, partial truncations of the CTD cause conditional phenotypes. For example, yeast cells expressing an *RBP1* allele encoding only 11 heptapeptide repeats

are viable but exhibit cold and temperature sensitivity as well as inositol auxotrophy (Scafe et al., 1990). The inositol auxotrophy is due to a defect in transcriptional activation of the *INO4* gene. Using an a-amanitin resistant *RBP1* gene, it was recently shown that the CTD is necessary for transcriptional activation in mammalian cells (Gerber et al., 1995).

A selection for suppressors of the cold sensitivity caused by partial truncation of the CTD identified nine *SRB* genes (Hengartner et al., 1995; Liao et al., 1995; Nonet and Young, 1989; Thompson et al., 1993). Subsequently, the nine proteins encoded by the *SRB* genes were shown to co-fractionate in a large complex with the 12 subunits of RNA polymerase II (core RNA polymerase II). This holoenzyme also contains the general transcription factors TFIIF, TFIIH, and sometimes TFIIB (Kim et al., 1994b; Koleske and Young, 1994), but not TBP and TFIIE. Interestingly, the holoenzyme is capable of supporting basal and activated transcription *in vitro* with the addition of TBP and TFIIE. Activated transcription occurs in the absence of the yTAFs (Kim et al., 1994b; Koleske and Young, 1994). Furthermore, an SRB containing complex can be separated from core RNA polymerase by treatment with either monoclonal antibodies directed against the CTD or by fractionation over a CTD column, indicating that the SRB complex binds the CTD as predicted from the genetics (Hengartner et al., 1995; Kim et al., 1994b; Wilson et al., 1996). Either the solo SRB complex (referred to as the mediator) or the entire holoenzyme can bind to the VP16 activation domain, but not to non-functional VP16 variants (Hengartner et al., 1995). The mediator can also support activated transcription when added to an *in vitro* transcription reaction with core RNA polymerase II (Kim et al., 1994b). As mentioned above, this complex (either the mediator or the holoenzyme) also contains the SWI/SNF complex in stoichiometric amounts (Wilson et al., 1996). In addition, the GAL11, SIN4 and RGR1 proteins have been shown to be a

subcomplex that is part of the holoenzyme. These three proteins are of interest because they have all been shown to necessary for the activation of some genes and the repression of other genes (Jiang et al., 1995; Li et al., 1995).

Although all the SRB proteins behave similarly during biochemical fractionation of yeast extracts, their genetic interactions with the CTD indicate that they may play distinct roles in the control of transcription. Dominant gain-of-function mutations in *SRB2, 4 , 5,* and *6* suppress CTD truncations, suggesting that these genes are involved in stimulating transcription (Thompson et al., 1993). However, deletion mutations of these genes cause different phenotypes: *SRB4* and *SRB6* are essential for viability whereas *SRB2* and *SRB5* deletions cause conditional phenotypes which are similar to partial CTD truncations. In fact, deletion of *SRB2* or *5* exacerbates the phenotypes of partial CTD truncations. Together, these results suggest that the SRB4 and 6 proteins play an essential role in conjunction with the CTD in order to activate transcription initiation. On the other hand, SRB2 and SRB5 play a stimulatory role which when hyperactivated can compensate for CTD truncations but when lost exacerbate them. *SRB7, 8, 9, 10,* and *11* were isolated as recessive suppressors of CTD truncations and deletions of *SRB8, 9, 10,* and *11* are viable and suppress CTD truncations (Hengartner et al., 1995; Liao et al., 1995). These data suggest that the recessive group of SRB proteins are responsible for down-regulating the activity of polymerase through their interaction with the CTD. In other words, weakening of the holoenzyme activity through a CTD truncation can be compensated by removing *SRB8, 9,10,* or *11* which are negative regulators of CTD activity. SRB10 and 11, which are a cyclin/CDK pair, presumably mediate their repressive effects by phosphorylating CTD, although this has not yet been shown (Liao et al., 1995).

These genetic findings suggest how other members of the holoenzyme such as GAL11, SIN4 and RGR1 could affect both activation and repression of transcription. These proteins have been shown to form a subcomplex within the holoenzyme. *RGR1* encodes an essential protein whose function is required to retain SIN4 and GAL11 in the holoenzyme. The RGR1/GAL11/SIN4 subcomplex may function in a promoter specific manner to stimulate either the recessive class or the dominant class of SRB proteins, thus activating or repressing transcription (Li et al., 1995).

Mutations in the *GAL11* gene have been isolated in a number of genetic screens. Most interestingly, it was shown that an allele of *GAL11, GAL11P,* could bypass the need for the GAL4 activation domain by creating a new protein-protein connection between the GAL4 DNA-binding domain and the holoenzyme. This finding suggests that activation can be mediated in part by recruitment of the holoenzyme to promoters (Barberis et al., 1995; Himmelfarb et al., 1990).

Not surprisingly, a mammalian equivalent of the holoenzyme has been identified by two groups. Schibler and coworkers have isolated a mammalian holoenzyme-like complex from rat liver extracts. This complex contains, at least, TBP, TAFs, TFIIB, TFIIE, TFIIF, and TFIIH in addition to core RNA polymerase II. This complex is capable of supporting basal transcription, although its response to activators was not examined (Ossipow et al., 1995). Furthermore, a human gene homologous to SRB7 was found in the expressed-sequence-tags (EST) data base. By following this protein during fractionation of calf thymus extract, a mammalian holoenzyme was also characterized. However, this holoenzyme required coactivators such HMG-2 and PC4 to activate transcription *in vitro* (Chao et al., 1996). These findings strongly suggest that in mammals, as in

yeast, RNA polymerase II is associated in large complex containing both general transcription factors and other accessory proteins.

The ADAs

The *ADA* mutants were isolated in a selection designed to identify the target of the VP16 acidic activation domain (Berger et al., 1992; Marcus et al., 1994). Overexpression of the chimeric transcriptional activator GAL4-VP16, which fuses the GAL4 DNA binding domain to the strong activation domain of the Herpes simplex virus VP16, is toxic to yeast cells. Moreover, this toxicity correlates with the ability of the chimera to activate transcription. In other words, point mutations in the DNA binding domain that abolish binding also abolish toxicity and, more importantly, point mutations that weaken the activation domain also lessen toxicity. This correlation between activation potential and toxicity suggests that toxicity is due to the sequestration of the general transcription factors by GAL4-VP16. It was hypothesized that mutations in adaptors, factors that bridge the interaction between the activation domain and the transcriptional machinery, should alleviate this toxicity by disrupting or weakening the connection between activation domains and the general transcription factors (Berger et al., 1992). Strains resistant to GAL4-VP16 mediated toxicity, termed *ada* mutants, were selected. To date, five *ADA* genes have been characterized; *ADA1*, *2*, *3*, *5* and *ADA4*, which is allelic to a previously isolated gene, *GCN5* (Berger et al., 1992; Georgakopoulos and Thireos, 1992; Horiuchi and Guarente, 1996; Marcus et al., 1994; Piña et al., 1993).

The *ADAs* can be grouped into two classes. The best characterized group includes *ADA2*, *ADA3* and *GCN5*. The phenotypes of deletion mutations in any of these genes are very similar. They have a striking slow growth

phenotype on minimal media, are temperature sensitive, and have a marked decrease in GCN4 and VP16 mediated transcriptional activation. Double mutant combinations of any two of these genes have a phenotype no worse than any single mutant. Also, when fused to a heterologous DNA binding domain, ADA2 or ADA3 can activate transcription in a manner that is dependent on the other ADAs. Together, these data suggest that *in vivo* the ADAs form a complex which stimulates transcription (Berger et al., 1992; Horiuchi et al., 1995; Piña et al., 1993; Silverman et al., 1994).

In vitro, these proteins have the properties expected of a transcriptional adaptor complex. ADA2 binds simultaneously to both ADA3 and GCN5 creating a trimeric complex where ADA2 is the linchpin (Horiuchi et al., 1995). Furthermore, the ADA complex from crude nuclear extracts can bind the VP16 activation domain. The interaction occurs with the full length VP16 activation domain, but not with an inactive truncation mutant (Barlev et al., 1995; Silverman et al., 1994). In fact, ADA2 has been shown to be sufficient for this specific interaction with the functional VP16 activation domain and the region of ADA2 necessary for the VP16 interaction has been mapped to the N-terminal one-third of the protein (Barlev et al., 1995).

The other group of ADA genes includes ADA1 and ADA5. The fundamental difference between these two genes and *ADA2, 3* and *GCN5* is that deletion mutants exhibit broader and more severe phenotypes. In particular, *ada1* and *ada5* strains grow slowly on rich media and display defects in transcription at all the promoters tested. Also, mutations in either *ADA1* or *ADA5* cause an Spt$^-$ phenotype (Horiuchi and Guarente, 1996; Marcus et al., 1996). In fact, an allele of *ada5*, *spt20-61*, was isolated in a screen for more *spt* mutants of the TBP class. Additionally, *spt7* mutants have phenotypes similar to

ada5 strains including Ada- and inositol auxotrophy, suggesting that it may function with ADA1 and ADA5 (Roberts and Winston, 1996).

The two groups of *ada* mutants share some phenotypes. GCN4 mediated activation is always severely defective in any of these mutants. Moreover, double mutants combining the two classes, *Δada2Δada5* strains for example, have phenotypes which are no worse than the worst single mutant. This suggests that all of the ADA proteins may function together in a single complex or activation pathway but must play somewhat different roles (Marcus et al., 1996). For example, ADA1 and ADA5 may play a central role in the ADA complex that all (or most) activators utilize while ADA2, ADA3 and GCN5 serve to link only certain activation domains, like GCN4, to the complex. Other members of the complex might bridge the interaction with different activation domains. In fact, consistent with the idea that multiple ADA proteins contact activation domains, ADA5 as well as ADA2 has been shown to interact with the VP16 activation domain (Marcus et al., 1996).

The mechanisms by which the ADA complex mediates its effects on transcription are not fully understood. Barlev and coworkers showed that ADA2 protein is necessary for the binding of TBP (from crude nuclear extract) to VP16 and that ADA2 also binds TBP (Barlev et al., 1995). However, it is not clear how direct the TBP-ADA2 connection is. At the genetic level, *ada5* mutants have some similarity to certain mutations in *SPT15*, the gene encoding TBP. *spt15-21* and *ada5* strains are both inositol auxotrophs, and this allele of *spt15* is partially resistant to GAL4-VP16 mediated toxicity. Consistent with the *in vitro* experiments mentioned above, this suggests that ADA5 may function by interacting with TBP and that the mutation in *spt15-21* disrupts the TBP-ADA5 interaction (Marcus et al., 1996).

On the other hand, Chapter 3 of this thesis describes recent findings arguing that the ADAs mediate their effect on transcription by interacting with the CTD of RNA polymerase II. Briefly, partially purified ADA complex binds to the CTD *in vitro*. Furthermore, RNA polymerase II cofractionates in a large complex with all five ADA proteins. *In vivo*, partial CTD truncations in combination with *ada* mutations cause synthetic lethality. This suggests that the ADA proteins, like SRB2 and SRB5, are necessary for the full functioning of the CTD during transcriptional initiation. Furthermore, double *ada srb2* mutants are lethal, arguing that the ADAs and these SRB proteins function in redundant activation pathways. In all, these *in vivo* and *in vitro* data suggest that the ADAs function by binding to RNA polymerase II in order to stimulate transcription. The interaction between the ADA complex and activation domains further suggests that the ADAs may function by recruiting Pol II to promoters. The inherent contradiction between this model and the TBP interaction described above could be resolved if the ADA-polymerase complex mediates and/or stabilizes a link between Pol II and the TBP complex bound at the TATA box.

Recently, a GCN5 homolog was purified from *Tetrahymena* as a histone H3 specific acetyltransferase. This suggests that the ADAs function by acetylating histones, which is believed to "loosen" chromatin structure, counteracting chromatin mediated transcriptional repression (Brownell et al., 1996). This finding in conjunction with the fact that the ADAs are found in a complex with Pol II may explain why there is a strong correlation between acetylated histones and actively transcribed genes.

By screening the expressed-sequence-tags (EST) data base Berger and colleagues have recently identified human homologs of both *ADA2* and *GCN5.* These human genes show extensive homology to their yeast counterparts, 31% identity for hADA2 and 43% identity for hGCN5. The human genes do not

complement in *ada2* or *gcn5* mutations yeast, but they do share a number of properties with the yeast genes, including direct protein-protein interactions between hADA2 and hGCN5 (Candau et al., 1996). The isolation of these human genes opens an exciting avenue of research - the identification and characterization of the mammalian ADA complex. Moreover, it highlights the conservation of the mechanism(s) of transcriptional activation and implies that what is learned about the yeast ADAs will directly apply to transcriptional activation in mammals.

Part IV: Conclusions and Future Directions

The purification and cloning of TBP was a catalyst for a tremendous leap in the understanding of transcriptional activation. It emphasized the similarities between the yeast and metazoan transcription apparatuses as well as allowed for the discovery of a new type of transcription factor, the coactivator or adaptor.

Numerous factors have been identified which are thought to mediate the effects of activation domains. Some research, such as the characterization of the TAFs, has focused on the purification of factors necessary for activated transcription *in vitro* (Tjian and Maniatis, 1994). This direction has lead to the identification of a plethora of factors as well as the detection of many direct protein-protein interactions. Sometimes all of these findings seem contradictory. For example, does VP16 interact with TAF$_{II}$40, PC4, TBP, TFIIH or TFIIB? Certainly, explanations consistent with all of these results are possible; transcriptional activation probably involves multiple steps, all of which may be targets of activation domains. However, many of these *in vitro* studies suffer from a lack *in vivo* support. This is not a trivial concern as shown by Tansey et. al. (as well as other workers with a more genetic approach, such as F. Winston and colleagues) who demonstrated that TBP mutants which do not

support transcription *in vitro* were fully functional *in vivo* for basal and activated transcription (Arndt et al., 1995; Tansey et al., 1994). The importance of *in vivo* experiments was also recently demonstrated by studies with conditional yTAF mutants which suggest that TAFs are not required for activated transcription *in vivo* (Struhl, 1996b).

In vivo approaches have focused on exploiting the powerful advantages available in yeast to isolate genes whose products are involved in transcriptional activation. These screens have isolated the *SRB*, *ADA*, *SWI/SNF*, and *SPT* genes (Guarente, 1995). The role of the proteins encoded by these different genes is not yet fully clear. However, biochemical characterization of the SRB proteins has uncovered the existence of a new type of complex, the RNA polymerase II holoenzyme, which contains core polymerase plus various accessory proteins such as the SRB, GAL11, and SWI/SNF proteins (Koleske and Young, 1995). The holoenzyme can support activated transcription *in vitro* in the absence of the TAFs.

These findings have led K. Struhl to propose the Triad Model for Transcriptional Activation (Struhl, 1996a). This model proposes that activation occurs through activator recruitment of both TFIID and the holoenzyme. The three-way interactions between the components involved (activator, TFIID, and holoenzyme) will mutually reinforce each other. Thus recruitment of TFIID and holoenzyme by the activator, which binds first by virtue of its high affinity for its cognate binding site, will lead to significant transcriptional activation. Furthermore, through the action of the holoenzyme associated SWI/SNF complex the entire complex of activator, TFIID, and Pol II will be stabilized on chromatin packaged promoters. This is a very appealing model because of its simplicity in synthesizing the different coactivator studies. However, it does not account for the involvement of other factors such as the ADAs, SPT3 and 8, and

CCR4 during transcriptional activation. Also, it does not take into account studies that have suggested that at some promoters *in vivo* activation domains stimulate transcription even when TFIID is already bound to the promoter (Chen et al., 1994a). Furthermore, recruitment is not necessarily the only step during transcription initiation which is stimulated by activators. How an initiation complex consisting of all these various factors and stabilized by multiple reinforcing interactions is able to begin transcription, which must lead to the destruction of this complex structure, remains a mystery (Zawel et al., 1995). Future studies will address many of these questions. Especially important is how all these various factors work together. Or, it is possible they do not all function at the same promoters; there certainly may be promoter specificity in terms of which coactivators are used. Much work is also needed to characterize the different interactions which have been studied *in vitro* to determine if they are of significance for activation *in vivo*.

The role of the ADAs in transcriptional activation needs further investigation. The association of the ADA complex with RNA polymerase II suggests that they may indeed bridge the interaction between activation domains and the transcriptional machinery, however their role in transcription needs to be tested *in vitro.* The ADA-Pol II complex may behave similarly to the holoenzyme, or it may need other factors such as the general transcription factors and TAFs to allow for activated transcription. Also, the acetyltransferase activity of GCN5 suggests that the ADAs may function to counter chromatin mediated repression. *In vitro* this activity may best be demonstrated on nucleosomal templates. Further purification of the complex should allow for the identification of all the components of the ADA complex. Moreover, suppression analysis of *ada* mutants should uncover other members of the complex and illuminate the role of the ADAs *in vivo*. Equally important, all these factors

probably have homologs in mammals and studying these factors should be very exciting and lead to further advances for both systems.

In summary, the study of coactivators and their role in transcriptional activation is at a very exciting juncture. Much future work will focus on synthesizing how and if different factors and complexes work together during activation. Also, analysis of all the interactions which have been characterized between activation domains and possible targets should focus on their *in vivo* relevance, as well as the mechanistic consequences these contacts have on the initiation complex. Finally, the entire picture will not be complete until the models explain the mechanism by which all these factors and interactions lead to the very high level of synergistic transcriptional activation that occurs *in vivo*.

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Chapter 2:

Yeast ADA2 binds to the VP16 activation domain and activates transcription

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Summary

Previously it was shown that yeast ADA2 is necessary for the full activity of some activation domains, such as VP16 and GCN4, *in vivo* and *in vitro*. These results suggest that ADA2 functions as a transcriptional coactivator or adaptor which bridges the interaction between certain acidic activation domains and the basal transcription machinery (Berger et al., 1992). Here we present two findings consistent with this model. First, ADA2 interacts with a region of the VP16 acidic activation domain that requires *ADA2* for activity *in vivo*. Second, ADA2, when fused to a heterologous DNA-binding domain, can stimulate the activity of the basal transcription factors *in vivo*. This ability of ADA2 to activate transcription is mediated by *ADA3*, a gene with properties similar to *ADA2* (Piña et al., 1993). These findings suggest that ADA2 has at least some of the properties expected of a transcriptional adaptor.

INTRODUCTION

Several models have been proposed to explain eukaryotic transcriptional activation, including chromatin disruption by activators (Croston and Kadonaga, 1993; Workman and Kingston, 1992), and interaction between activators and basal factors positioned at the TATA box (Ptashne, 1988). Direct interactions have been demonstrated between the basal factors TFIIB or TBP and acidic activation domains (Lin and Green, 1991; Stringer et al., 1990). These interactions are disrupted by mutations in VP16 which prevent activation of transcription (Ingles et al., 1991; Lin et al., 1991). Further, mutations in TFIIB which prevent activation also prevent binding of VP16 (Roberts et al., 1993). However, it has been clearly demonstrated that these interactions are not sufficient for the activation of transcription (Choy and Green, 1993; Dynlacht et al., 1991), and many investigators have proposed that coactivators or adaptors must exist to bridge the interaction between activators and the basal machinery (Berger et al., 1990; Kelleher et al., 1990; Peterson et al., 1990; Pugh and Tjian, 1990).

Tjian and colleagues have shown that TATA-binding-protein (TBP) is associated with a number of auxiliary factors (TAFs) which are necessary for activated transcription *in vitro* (Dynlacht et al., 1991). In fact, it has been shown that $TAF_{II}110$ interacts with the SP1 activation domain (Hoey et al., 1993), and $TAF_{II}40$ interacts with the C-terminus of the VP16 activation domain. The significance of this interaction was demonstrated by the fact that anti-$TAF_{II}40$ antibodies blocked activation by GAL4-VP16 (Goodrich et al., 1993).

Likewise, ADA2 was proposed to encode a transcriptional adaptor or coactivator because it is necessary for certain acidic activation domains to function *in vivo* and *in vitro* (Berger et al., 1992). The *ada2* mutant was originally isolated based upon its ability to grow in the presence of high levels of

the toxic chimeric activator GAL4-VP16. The toxicity caused by overexpression of GAL4-VP16 correlates with its ability to activate transcription and to bind DNA. This result suggested that toxicity is due to the sequestration of essential general transcription factors by the activator at many sites throughout the genome. The collection of mutants resistant to GAL4-VP16 also includes ADA3 (Piña et al., 1993) and GCN5 (G. Marcus and L. G., unpublished), and mutations in these genes affect cell growth and activation of transcription in a manner similar to the *ada2* mutation.

The hypothesis that ADA2, perhaps in concert with ADA3 and GCN5, constitutes a transcriptional adaptor makes two key predictions. First, ADA2 should bind to those activation domains which require the integrity of *ADA2* for full activity. Second, ADA2 should interact with one or more of the basal transcription factors. In this report we perform experiments that test directly whether ADA2, or a complex containing ADA2, binds to the VP16 activation domain. We also begin experiments to show that ADA2 interacts with the basal transcription factors *in vivo*. The results are consistent with the hypothesis that ADA2 is a transcriptional adaptor.

RESULTS AND DISCUSSION

To examine whether ADA2 binds VP16, we expressed ADA2 tagged with the hemagglutinin epitope (HA) from the influenza virus (Kolodziej and Young, 1991), and determined whether this protein would co-precipitate with a chimera containing the DNA-binding domain of GAL4 fused to residues 413-490 of VP16 (Triezenberg et al., 1988). Yeast extracts containing tagged or untagged ADA2 were prepared. The amount of ADA2 protein in both extracts was comparable, as measured by Western analysis with ADA2 antiserum (data not shown). The extracts and purified recombinant GAL4-VP16 were incubated

together, and mixed with protein A-beads covalently coupled to monoclonal antibody against the HA epitope (Harlow and Lane, 1988). After collecting and washing the beads, proteins that bound to the anti-HA antibody were eluted using a peptide consisting of the HA epitope and analyzed by Western blotting (Field et al., 1988; Zhou et al., 1992).

Figure 1A shows a filter probed with the anti-HA antibody. Note a band of 49kD, the predicted size of ADA2HA, that was specifically bound to the beads. This protein was absent in the extract with untagged ADA2, verifying that the indicated band is ADA2HA. The same samples were probed with antibody to VP16 (Figure 1B), revealing that the GAL4-VP16 chimera was bound to and eluted from the beads when mixed with extracts containing tagged ADA2, but not untagged ADA2. This experiment shows that GAL4-VP16 binds to ADA2 or a yeast multi-protein complex containing ADA2.

In order to determine which portion of the GAL4-VP16 chimera interacts with ADA2, we repeated the co-precipitation experiment with a purified recombinant GAL4-VP16 derivative truncated at residue 456 of VP16. This very preparation shows considerable activity in a transcriptional activation assay *in vitro* (Berger et al., 1990). In experiments in which the full length GAL4-VP16 bound to tagged ADA2 (Figure 2A), the truncated protein did not bind to ADA2 at all (Figure 2B). Thus, residues in VP16 carboxyl to position 456 are essential for binding to ADA2.

To compare the binding *in vitro* of ADA2 to full length but not truncated GAL4-VP16, with the ability *in vivo* of VP16 to activate transcription and respond to ADA2, a set of VP16 derivatives was fused to the DNA-binding and dimerization domains of lexA (Piña et al., 1993). Previous experiments have indicated that VP16 could be separated into functional sub-domains (Goodrich et al., 1993; Regier et al., 1993; Seipel et al., 1992). We utilized a set of

derivatives with domains extending from residue 413 to residues 450, 456, 460, or 470. Another derivative contained VP16 residues 452-490. We were unable to use a construct containing lexA fused to residues 413-490 of VP16 because this construct was toxic in *ADA2+* (but not *ada2-*) cells (data not shown). However, previous experiments showed that a GAL4 fusion to this region of VP16 activated transcription *in vivo* and was reduced 5-10-fold in *ada2* mutant cells (Berger et al., 1992). The ability of the above lexA fusions to activate transcription *in vivo* was monitored by a lacZ reporter containing a single lexA operator site, both in *ADA2+* and Δ*ada2* strains.

The 413 to 470 domain of VP16 was active and highly dependent on ADA2 (Figure 3). The activity of the 413-470 moiety was abolished by mutation of the critical Phe442 residue to Ala (Cress and Triezenberg, 1991). Importantly, the 413-456 moiety was inactive *in vivo*, even though the protein was present at the same levels as the 413-470 fusion protein (data not shown). Thus, the inability of the 413-456 moiety to bind to ADA2 *in vitro* correlates with its inability to activate transcription *in vivo*. Other derivatives, truncated at residues 460 or 450, were also inactive. These assays define an ADA2-dependent VP16 activation domain encompassing Phe442 and extending to a boundary between residues 460 and 470.

Interestingly, the carboxyl moiety of VP16 (containing residues 452-490), shown previously to contact the Drosophila TAF40 as well as replication protein A (Goodrich et al., 1993; Li and Botchan, 1993), was also active *in vivo*, but showed a minimal requirement for ADA2 (less than two-fold). This is consistent with earlier findings that some activation domains (e.g. GCN4) show a strong dependence on ADA2, while others (e.g. GAL4 and HAP4) do not (Piña et al., 1993). In all, these data suggest that VP16 contains at least two functional

domains; one is the ADA2-dependent domain delineated above, whereas the other is a very potent, ADA2-independent, activation domain at the C-terminus.

Given that ADA2 binds to VP16 and that this binding appears to be important for activation *in vivo*, we considered two possible mechanisms. First, if VP16 activity were down-regulated by the binding of a repressor or by internal repression, ADA2 might potentiate activation by preventing repression. Second, ADA2 might directly activate the basal factors, for example, by protein-protein contact. By the latter model, ADA2 itself should possess the ability to activate transcription. We, therefore, tested activation by a lexA-ADA2 chimera (containing all of ADA2), which retained the ability to complement an *ada2* deletion mutation. This chimera was expressed from the strong *ADH1* promoter on a 2μ plasmid.

lexA-ADA2 was active, although only about 10% as active as the lexA-VP16:413-470 construct (Figure 4). This activity decreased to background levels when a similar reporter missing the lexA binding site was used (data not shown). Further, the activity depended on the dose of lexA-ADA2 - i.e., activity was reduced 3-fold when the chimera was expressed on a low-copy plasmid (data not shown). A further indication of the significance of the activity of lexA-ADA2 is its unique dependence on *ADA3*, a second gene identified by the selection for GAL4-VP16 resistant mutants (Berger et al., 1992). This dependence was demonstrated in two ways. First, the activity of lexA-ADA2 was reduced in a *Δada3* strain, To show that this effect was specific, we tested a fusion of lexA to the HAP4 activation domain. As shown in Figure 4 and previously demonstrated, the activity of lexA-HAP4 was only slightly reduced in the Δ*ada3* strain (Piña et al., 1993).

However, the above requirement of lexA-ADA2 for ADA3 is not unique, since certain activators, such as GCN4, also require ADA3 for full activity (Piña

et al., 1993). Therefore, we carried out a second test of ADA3-dependence by over-expressing ADA3 from a 2μ plasmid. In this case the activity of lexA-ADA2 was augmented about 5-fold above the levels observed in a wild type strain, but the activity of lexA-HAP4 (Fig. 4) and lexA-GCN4 (data not shown) was not affected at all.

Because the activity of lexA-ADA2 was sensitive both to a decrease and increase in ADA3 levels, we infer that it reflects a true aspect of ADA2 function. Further our findings suggest that ADA2 and ADA3 are part of a heteromeric complex. We imagine that only a small percentage of the highly expressed lexA-ADA2 is complexed with the other ADAs and that this fraction is preferentially diverted away from the lexA site to *bona fide* yeast promoters. Expression of high levels of ADA3 would increase the number of ADA complexes available to bind to the lexA site. If the ADA complex is required for stimulating transcription, then the observed stimulation of lexA-ADA2 activity by over-expression of ADA3 would be expected.

In summary, our findings show that ADA2, or a complex containing ADA2, binds to VP16, and that this binding is important for activation *in vivo*. Further, they show that ADA2 itself can activate transcription, and that this activity is highly regulated by the level of ADA3 in cells. These findings are consistent with the hypothesis that ADA2, perhaps as a part of a complex, functions as a transcriptional adaptor linking activators and basal factors at promoters. Interestingly, the Cys-rich region at the amino-terminus of ADA2 shares significant similarity to a mammalian protein, CBP, which binds to the active, phosphorylated form of CREB and may itself be a co-activator (Chrivia et al., 1993). It will be important to determine the identity of all of the components

in a probable ADA complex, and to ascertain how ADA2 stimulates the basal factors.

MATERIALS AND METHODS

Plasmids. pADA2HA was constructed by using PCR to engineer an in-frame *Bgl*II site at the carboxy terminus of *ADA2* as well as flanking *Hind*III sites. This PCR product was then ligated into the *Hind*III site of pDB20 (Becker et al., 1991). From this plasmid a *Sma*I-*Bgl*II fragment containing the ADH1 promoter and *ADA2* was isolated and ligated into pHAH4 (Sugiono, 1993), which had been digested with *Sal*I, filled in with *Klenow fragment*, and then digested with *Bgl*II; this results in the fusion of one copy of the nine amino acid 12CA5 epitope from the influenza hemagglutinin protein to the carboxy-terminus of ADA2 (Kolodziej and Young, 1991). Likewise the pADA2-6HisL was created by using PCR to engineer six histidine codons fused to the carboxy-terminus of *ADA2* and flanking *Hind*III sites; this gene was then cloned as a *Hind*III fragment into a version of pDB20LBglII (Berger et al., 1992) with a unique *Hind*III site.

To create the LexA-VP16 fusion constructs, the *Not*I site in pRS316 (Sikorski and Hieter, 1989) was destroyed by cutting with *Not*I, filling-in with *Klenow fragment*, and religating, creating pRS316-Not. The P_{ADH}-lexA202-T_{ADH} cassette was then purified from pADH-lexA202 (Piña et al., 1993) as a *Sma*I-*Sal*I fragment and ligated into pRS316-Not creating pARS/cen-lexA202. Fragments encoding the various VP16 regions with flanking *Not*I sites were generated using PCR and cloned into the *Not*I site at the C-terminus of lexA202.

LexA-ADA2 contains lexA residues 1-202 fused to the entire coding sequence of ADA2 (Marcus et al., 1994). pADH-lexA202-HAP4 was described earlier (Piña et al., 1993) The 2μ ADA3 overexpression plasmid was constructed by using PCR to generate an ADA3 gene with flanking *Bam*HI sites

which was then cloned into the *Bgl*II site of the ADH expression plasmid pDB20LBglII (Berger et al., 1992).

Co-immunoprecipitations *Δada2* strains carrying a 2μ plasmid expressing either ADA2HA (pADA2HA) or ADA2-6His (pADA2-6HisL) from the constitutive ADH1 promoter were used to prepare nuclear extracts (Lue and Kornberg, 1987). Nuclear extracts were diluted to approximately 2mg/ml in IP buffer (10% glycerol, 50mM Hepes KOH pH7.3, 100mM Kglutamate pH7.3, 0.5mM DTT, 6mM MgOAc, 1mM EGTA, 0.1% NP40 and protease inhibitors(Berger et al., 1990)). 6μg of purified recombinant GAL4(1-147)-VP16((413-490) or (413-456)) was then added to 1ml of extract and incubated for 3 hours at 4°C. After two centrifugations to clear the reactions, the extract was mixed with 25μl of a slurry of protein A-agarose beads (Sigma) crosslinked to purified anti-HA monoclonal antibody (Berkeley Antibody Co.) (Harlow and Lane, 1988). After an overnight incubation at 4°C the beads were pelleted by centrifugation and washed six times with IP buffer. Then bound proteins were eluted from the antibody by incubation with 1mg/ml HA peptide (Berkeley Antibody Co.) (Field et al., 1988). Eluants were precipitated with 10% trichloroacetic acid before SDS polyacrylamide gel electrophoresis and transfer to Immobilon-P membranes (Millipore). Western blots were probed with anti-HA or anti-VP16 antibody and visualized using the Lumi-Phos chemi-luminescent system (Boeringher Mannheim).

Yeast Manipulations. The lexA reporter plasmid is YEp21-Sc3423 (Hope and Struhl, 1986) or pRBHIS (Marcus et al., 1994) . Yeast manipulations and ß-galactosidase assays were performed as in Piña et. al (Piña et al., 1993). All strains were BWG1-7a and its derivatives or PSy316 (Berger et al., 1992; Piña et al., 1993).

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Fig 1 GAL4-VP16 specifically co-immunoprecipitates with ADA2. In brief, extracts containing either HA tagged or untagged ADA2 were mixed with recombinant GAL4-VP16, and incubated with agarose beads covalently coupled to the anti-HA monoclonal antibody. After the beads were collected and washed, proteins bound to the antibody were eluted with HA peptide, subjected to gel electrophoresis, and analyzed on the Western blots shown. Panel *a.* shows a Western blot probed with anti-HA antibody (Berkeley Antibody Co.). The lanes labeled '+' contain ADA2HA extract while the lanes labeled '-' contain untagged ADA2 extract. Lanes labeled 'start' contain material prior to the immunoprecipitation and the lanes marked 'super' contain supernatant that did not bind to the beads. The material eluted with the HA peptide was loaded in the lanes marked 'eluant'. The faint band in the eluant '-' lane that runs slightly slower than ADA2 is a small amount of IgG heavy chain that has leached off the agarose beads. Panel *b.* shows a Western blot from the same experiment that was probed with anti-VP16 antibody (generous gift of S. Berger, J. Culp and Brian Hellmig). The lanes are marked as in part *a.*

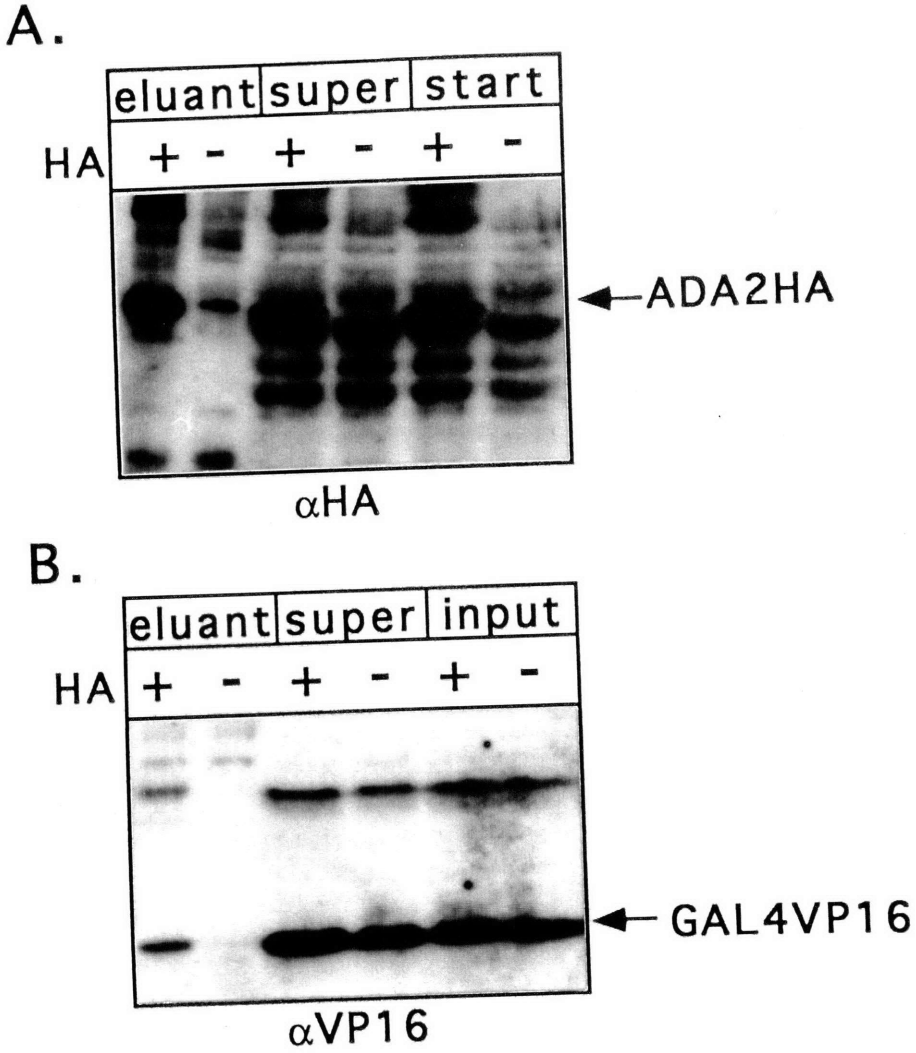
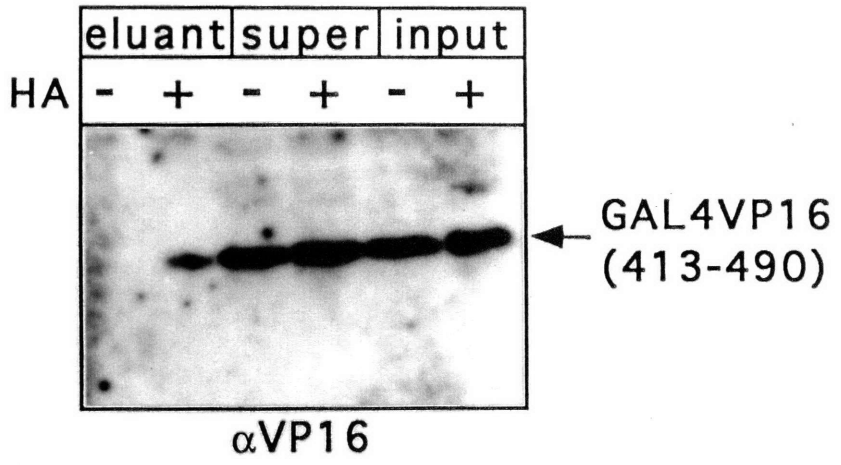

A.
eluant
super
start
HA
+ - + - + -
ADA2HA
αHA
B.
eluant
super
input
HA
+ - + - + -
GAL4VP16
αVP16

Fig. 2 The full VP16 activation domain but not a version truncated at residue 456 co-immunoprecipitates with ADA2. a. The results of a co-immunoprecipitation experiment with ADA2 and the full VP16 activation domain (residues 413 to 490) were analyzed by Western blot probed with anti-VP16 antibody. *b.* A Western blot from an experiment identical to that in part *a* except ADA2 was co-immunoprecipitated with a version of VP16 truncated at residue 456 and then analyzed with anti-VP16 antibody. The high-molecular-weight band in *b* represents GAL4-VP16 dimers that, presumably, have become cross-linked in that preparation. The lanes are marked as in figure 1.

A.


eluant
super
input
HA
- + - + - +
GAL4VP16
(413-490)
αVP16

B.

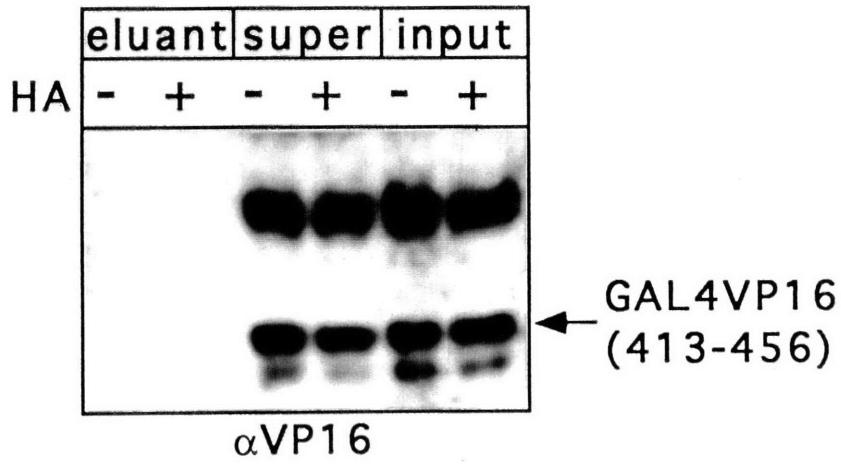

eluant
super
input
HA
- + - + - +
GAL4VP16
(413-456)
αVP16

Fig 3. VP16 residues 413-470 comprise a potent, and ADA2-dependent, activation domain. (*Upper*) A wild type strain (BWG1-7a) and an isogenic *Δada2* strain were transformed with the indicated lexA-VP16 fusion plasmid and a lacZ reporter with one lexA binding site upstream of the CYC1 promoter (Berger et al., 1992; Hope and Struhl, 1986). Fusions contain lexA residues 1 to 202 and the indicated residues of VP16. The entry labeled 413-470FA has the substitution of an alanine for a phenylalanine at residue 442 (Cress and Triezenberg, 1991). (*Lower*) Activity of a lexA fusion with the C-terminal portion of VP16 is ADA2 independent. '-' indicates that no part of VP16 was fused to the DNA binding domain. ß-galactosidase activity was determined and the specific activity is indicated on the x-axis. Mean values are shown; 3-6 independent experiments were performed for each fusion construct and the standard deviations were less than 25%.

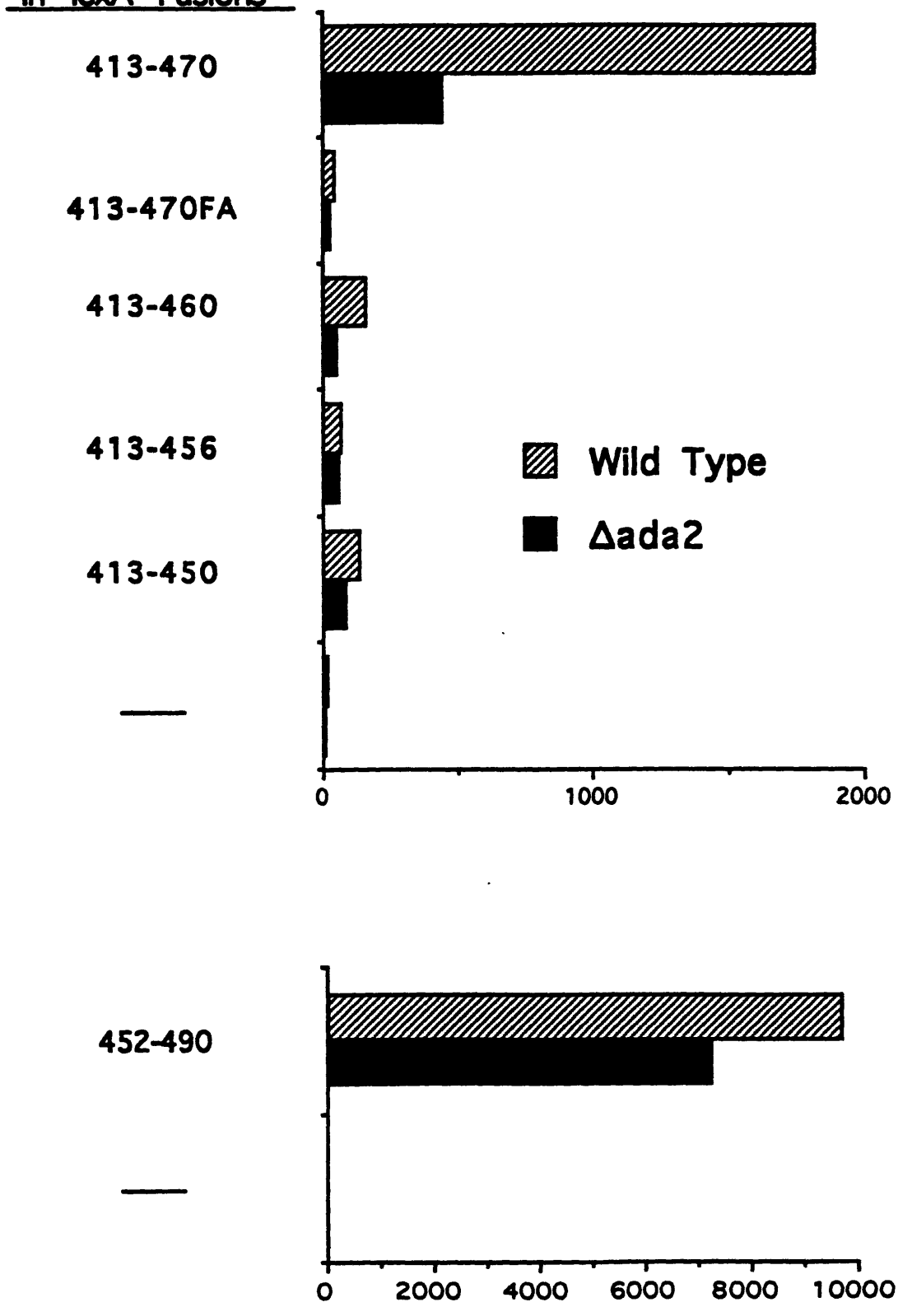
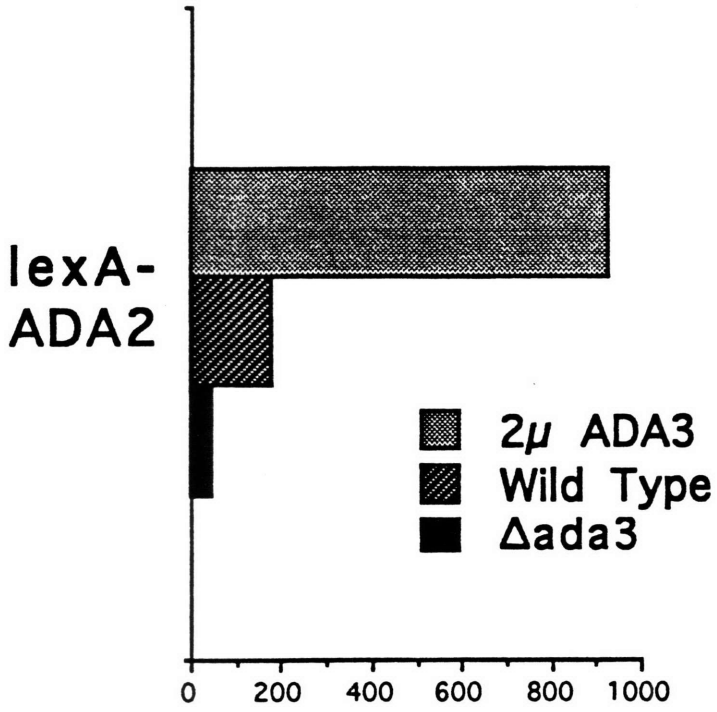
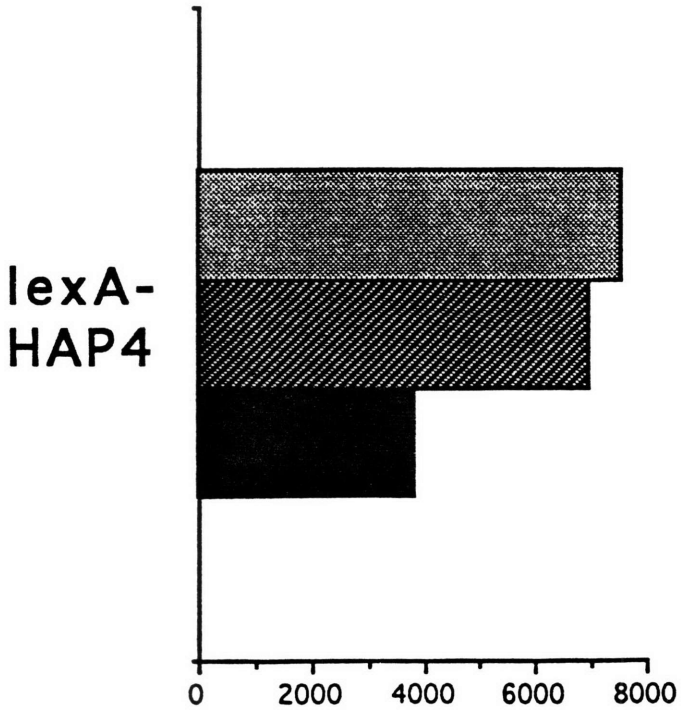

VP16 Residues
in lexA Fusions
413-470
413-470FA
413-460
413-456
413-450
452-490
Wild Type
Δada2
0
1000
2000
0
2000
4000
6000
8000
10000

Fig. 4 LexA-ADA2 is an ADA3 dependent transcriptional activator. The top panel shows that ADA3 regulates the activity of the lexA-ADA2 fusion. The lower panel shows that the activity of lexA-HAP4 is relatively insensitive to the levels of ADA3. The x-axis indicates ß-galactosidase specific activity. The mean of 3-5 independent experiments is shown; the standard deviations were less than 25%.


lexA-
ADA2
2μ ADA3
Wild Type
Δada3
0
200
400
600
800
1000


lexA-
HAP4
0
2000
4000
6000
8000

Chapter 3:

Genetic and Physical Interactions Between the ADAs and RNA Polymerase II

ABSTRACT

The *ADAs - ADA1, ADA2, ADA3, ADA5, and GCN5 -* were identified in a screen designed to isolate the target of transcriptional activation domains. Further studies have shown that the *ADAs* have the properties expected of genes encoding transcriptional adaptors, which are factors proposed to mediate the interaction between transcriptional activators and the general transcription machinery. ADA2 is shown to directly bind to the VP16 activation domain but not to a double Phe-->Ala substitution mutant. The *ADAs* interact genetically with the *SRBs* and the CTD of RNA polymerase II in a manner which suggests that the *ADAs* and the *SRBs* function in parallel and that both mediate their effect by interacting with the CTD. Furthermore, a complex containing all of the known ADA proteins was partially purified from yeast extract, and most of the ADA complex was found associated with RNA Pol II. Moreover, we show that *in vitro* the ADA complex can bind to the CTD specifically. Together these findings are consistent with a model in which the ADAs function by associating with Pol II, and in which this ADA-Pol II complex is a direct target of activation domains.

INTRODUCTION

The expression of protein coding genes, which in eukaryotes are transcribed by RNA polymerase II (Pol II), is often regulated by transcriptional activation (Guarente, 1992). Most Pol II promoters have a TATA box located just upstream of the transcription start site. This sequence element serves as the binding site for the TATA-binding protein (TBP) which then nucleates the assembly of the transcription initiation complex. However, different cis-acting sites, termed UAS or enhancer elements, located hundreds (in yeast) or thousands (in higher eukaryotes) of base pairs away from the TATA box, are necessary for transcriptional activation. These elements serve as the binding sites for transcriptional activators which contain a domain dedicated to binding specific DNA sequences as well as a domain necessary for transcriptional activation (Hope and Struhl, 1986). The mechanism by which these domains activate transcription has been the focus of much research. Transcriptional coactivators (or adaptors) are proposed to function by mediating the interaction between activation domains and the general transcription machinery (Berger et al., 1990; Kelleher III et al., 1990; Pugh and Tjian, 1990).

Factors of the coactivator/adaptor class have been identified by biochemical purification and in genetic screens. The best studied coactivators, the TBP associated factors or TAFs, facilitate the action of numerous activators *in vitro* . *In vivo* studies have focused on genetic screens designed to identify transcriptional coactivators. Coactivators identified in genetic selections include the *SRB* genes (Hengartner et al., 1995 and references therein), the *ADA* genes (Berger et al., 1992), and *SUB1* (Knaus et al., 1996). Other screens, for example the *SPT* (Winston, 1992), *SWI/SNF* (Neigeborn and Carlson, 1984; Stern et al., 1984), and *CCR* (Denis et al., 1994) screens, isolated mutants with

broad pleiotropic transcription defects suggesting that the mutations may lie in genes whose products are also involved in transcriptional activation.

The *SRB* genes were identified as suppressors of the conditional phenotypes caused by truncation of the carboxy-terminal-domain (CTD) of RNA polymerase II. The CTD is a conserved structure consisting of a repeated heptapeptide sequence at the C-terminus of the largest subunit of polymerase (*RPB1*). The CTD is essential for viability in yeast (Scafe et al., 1990), and it is necessary for transcriptional activation in both yeast and mammalian cells (Gerber et al., 1995; Scafe et al., 1990). In yeast, wild type *RBP1* contains twenty-six copies of the CTD heptapeptide repeat. Interestingly, truncation of the CTD leaving only eleven repeats causes a number of conditional phenotypes. These conditional phenotypes, which include temperature and cold sensitivity as well as inositol auxotrophy, are suppressed by mutations in the *SRB* genes (Scafe et al., 1990).

Interestingly, the *SRB* genes do not all have identical phenotypes. Some *SRB* genes were identified as dominant suppressors of CTD truncations. This suggests that they normally stimulate transcription through their interaction with the CTD, and that these dominant alleles have increased stimulatory activity. Two of these genes, *SRB2* and *SRB5*, are not essential for viability, but loss-of-function alleles of these genes exacerbate the phenotypes caused by CTD truncations. In other words, *SRB2* or *SRB5* deletions in combination with CTD truncations lead to phenotypes more severe than either an *SRB* deletion or CTD truncation alone. In contrast, mutations in *SRB 7, 8, 9, 10,* and *11* were isolated as recessive suppressors of CTD truncations suggesting that they normally function to repress transcriptional activity through their interaction with the CTD.

Analysis of the SRB proteins during the fractionation of yeast whole cell extracts has identified a novel form of RNA polymerase II called the holoenzyme. This complex contains the twelve subunits of RNA polymerase II as well as approximately twenty-five other polypeptides including the proteins encoded by the nine *SRB* genes, the components of the SWI/SNF complex, SIN4, RGR1, and GAL11 (Hengartner et al., 1995; Kim et al., 1994; Koleske and Young, 1994; Li et al., 1995; Liao et al., 1995; Thompson et al., 1993; Wilson et al., 1996). The holoenzyme also contains all of the general transcription factors except TBP and TFIIE. The holoenzyme is capable of mediating activated transcription *in vitro* with the addition of only TBP and TFIIE (Koleske and Young, 1994). The TAFs are not required for this activity, which is in contrast to findings from the fractionation of mammalian and *Drosophila in vitro* transcription systems (Tjian and Maniatis, 1994). The isolation of the *SRB* genes and the discovery of the holoenzyme highlights the power of coupling yeast genetics and biochemistry to study transcription. Moreover, recent work has also identified a large holoenzyme-like activity in mammalian extracts and a human homolog of an *SRB* gene has been identified, further emphasizing that the use of yeast genetics to study transcription often uncovers novel but evolutionarily conserved aspects of the transcriptional mechanism (Chao et al., 1996; Ossipow et al., 1995).

The *ADA* genes, *ADA1, ADA2*, *ADA3* , *ADA5* and *GCN5*, were identified in a screen specifically designed to isolate the *in vivo* target of transcriptional activation domains (Berger et al., 1992; Marcus et al., 1996; Marcus et al., 1994; Piña et al., 1993; Horiuchi and Guarente, 1996). In this screen, the chimeric activator GAL4-VP16, containing the GAL4 DNA binding domain fused to the powerful acidic activation domain from the Herpes simplex virus VP16 gene, was overexpressed causing severe toxicity in yeast cells. Since this toxicity

correlates with the potency of transcriptional activation, it was postulated that toxicity was due to the sequestration of the general transcription machinery by GAL4-VP16 (Berger et al., 1992). We further predicted that if adaptors bridged the interaction between activation domains and the general transcription factors, mutations which relieve GAL4-VP16 mediated toxicity would identify genes, termed *ADA,* which encode adaptor proteins.

Further experiments have shown that the ADAs have the properties expected of an adaptor. *ada2, ada3,* and *gcn5* mutants exhibit defects in VP16 and GCN4 mediated transcriptional activation. Furthermore, *ADA2* or *ADA3* themselves can activate transcription when tethered to the DNA at sites near the promoter. Mutations in *ada1* and *ada5* cause defects in transcriptional activation mediated by many transcriptional activators including GCN4. Additionally *ada1* and *ada5* mutants have two other phenotypes, inositol auxotrophy and Spt-, which are commonly found in mutants with transcription defects. In fact, *ADA5* is identical to *SPT20* (Horiuchi and Guarente, 1996; Marcus et al., 1996; Roberts and Winston, 1996).

The adaptor model also predicts that the ADAs should interact with activation domains. Consistent with this prediction, two acidic activation domains which require the ADAs for activity *in vivo* and *in vitro* have been shown to interact with the ADAs (Barlev et al., 1995; Berger et al., 1992; Silverman et al., 1994). In fact, ADA5 directly binds to the VP16 activation domain (Marcus et al., 1996); ADA2 directly binds to VP16 (Barlev et al., 1995) and to an activation domain from ADR1; and GCN5 binds to a different activation domain from ADR1 (C. Denis, personal communication).

The ADAs seem to function together as a complex. Double mutant combinations of any two the *ada* genes possess phenotypes no more severe than the worst single mutant. In addition, the transcriptional activity shown by

ADA2 and *ADA3* when tethered to promoters is dependent on the levels of the other *ADAs*. Furthermore, ADA2, ADA3, and GCN5 can form a complex *in vitro*. Together these data argue that the ADA proteins function as a complex in order to mediated transcriptional activation.

Recent studies have discovered one mechanism by which the ADA complex might function as a coactivator. A GCN5 homolog from *Tetrahymena* was purified because it is a histone specific acetyltransferase. Yeast GCN5 has this same activity (Brownell et al., 1996). This may explain the observation that acetylated histones are preferentially associated with actively transcribed genes. Histone acetylation is believed to weaken the nucleosome-DNA interaction and thus facilitate transcription. In addition to this *Tetrahymena GCN5,* human homologs of both *ADA2* and *GCN5* have recently been isolated, suggesting that the role of the ADAs in transcriptional activation is conserved throughout all eukaryotes (Candau et al., 1996).

To further understand the role of the ADAs in transcription, especially how they relate to other well characterized components of the transcriptional machinery, we have continued the genetic and biochemical analysis of the *ADA* genes. The evidence presented here demonstrates that the ADAs form a complex that is capable of bridging the interaction between activation domains and RNA polymerase II. These findings link Pol II directly to the ADA complex which includes GCN5, a histone acetylating enzyme.

RESULTS

ADA2 does not bind to a double Phe-->Ala VP16 mutant

Previous studies using yeast extracts have shown that the ADA complex binds to the acidic activation domains of VP16 and GCN4 but not to a truncated version of VP16 or the HAP4 activation domain (Barlev et al., 1995; Silverman

et al., 1994). Experiments with recombinant proteins have shown that the N-terminal one-third of ADA2 can directly interact with the VP16 activation domain (Barlev et al., 1995). In order to obtain more decisive evidence for the biological relevance of this interaction, the ADA2-VP16 interaction was further analyzed by testing the ability of ADA2 to bind a double Phe-->Ala VP16 mutant. Three GST-VP16 variants were utilized in this experiment: the full length VP16 activation domain (amino acids 413-490) which depends on ADA2 for its activity; VP16Δ456 (amino acids 413-456), which has very little activity *in vivo* (1% of full length); and VP16FAFA, a mutant which contains two substitutions (Phe442 to Ala and Phe475 to Ala) in the context of the full length activation domain. The double point mutant is important because these two substitutions create an activation domain that has very little activity *in vivo* but maintains its size and charge (Regier et al., 1993). GST-VP16 fusion proteins were purified from *E. coli* and incubated with *in vitro* translated ADA2. Glutathione-Sepharose beads were used to precipitate GST fusions and associated proteins. The beads were washed, and bound proteins were eluted with glutathione, separated by electrophoresis and autoradiographed. In this assay (see figure 1) the full length VP16 protein (lane marked WT) bound ADA2 while the truncated protein (lane marked Δ) did not; moreover the double point mutant (lane marked FF) did not interact with ADA2. This experiment strengthens the claim that the binding of ADA2, and by inference the ADA complex, to activation domains is functionally relevant.

ada mutations exacerbate the phenotypes of CTD truncations

In yeast, sequential truncations of the *RPB1* CTD heptapeptide repeats cause increasingly severe phenotypes (See Introduction). An *RPB1* gene containing only eleven heptapeptide repeats, instead of the wildtype twenty-six,

causes conditional phenotypes (Scafe et al., 1990). However, when the same truncation is combined with *Δsrb2* or *Δsrb5* mutations, the resulting strain is inviable. This synthetic enhancement suggests that the SRB2 and SRB5 proteins, and presumably the proteins encoded by the other dominant *SRB* genes, are necessary to enhance the functioning of Pol II through their interaction with the CTD.

Since the *ada* mutants have similar phenotypes to *srb2* and *srb5* strains, such as slow growth and temperature sensitivity, it was of interest to test whether *ada* mutations also interact with CTD truncations. In order to test this possibility, the strain JAy47, which carries an *RPB1* gene controlled by the GAL promoter, was employed (Archambault et al., 1992). This strain grows only when galactose is used as the carbon source. In the presence of an alternate source of *RPB1*, i.e. from a plasmid, this strain can grow on glucose (Archambault et al., 1992). The genes for *ADA1, ADA2*, and *ADA3* were deleted in JAy47. As shown in Figure 2, these strains (JAy47, JAy47Δada1, JAy47Δada2 and JAy47Δada3) are inviable on glucose media when they do not contain another copy of *RPB1* (see sector marked vector), but grow when a wildtype copy of *RPB1* is expressed from a plasmid (see sector marked wildtype). Also, the Ada+ strains (row marked wildtype) expressing *RPB1* genes encoding thirteen, eleven, nine, and seven heptapeptide repeats exhibit the expected growth phenotypes. A strain that express an *RBP1* allele with eleven heptapeptide repeats grows as well as a strain expressing wildtype *RPB1* at 22°C, but is cold and temperature sensitive as expected (data not shown). At 22°C the strain expressing the nine repeat allele grows slowly as expected in this genetic background (Rick Young personal communication), and the strain with only seven repeats is inviable (See top left panel of Figure 2, sectors marked, 13, 11, 9, and 7 repeats respectively.) (Scafe et al., 1990).

As expected *Δada2* or *Δada3* strains with a full length CTD display no obvious phenotypes on rich media while the *Δada1* strain has a mild growth defect (Berger et al., 1992; Horiuchi and Guarente, 1996; Piña et al., 1993). However, the growth of strains with CTD truncations is greatly slowed when combined with the *Δada* alleles. When combined with an *Δada* mutation, strains with the nine CTD repeats are inviable and strains with eleven or thirteen CTD repeats grow very poorly (or not at all in the case of the *Δada1* strain). (See the left column of Figure 2, rows labeled *Δada1, Δada2*, or *Δada3* compared to top row labeled wildtype.)

Importantly, this synthetic enhancement is specific to CTD truncation mutations. The right hand column of figure 2 shows the phenotypes of six other *rpb1* alleles which have mutations that do not lie in the CTD. All of these mutants are temperature sensitive (data not shown and (Thompson et al., 1993)). However, none of these mutants have a synthetic phenotype when combined with *ada* deletions. In other words, the synthetic phenotype caused by combining *rpb1* mutations and *ada* deletions is specific to those mutations in polymerase which weaken the ability of the CTD to function. This genetic interaction suggests that the ADAs function in a pathway with CTD in order to activate transcription.

ada, srb2 double mutants exhibit synthetic lethality

The genetic data above suggest the possibility that the *ADAs* may function in a manner similar to the dominant class of *SRBs*. Loss-of-function mutations in either the *ADA* genes or the *SRB2* and *SRB5* genes exacerbate the phenotypes caused by CTD truncations. In addition, *srb2* and *srb5* deletion strains have growth phenotypes similar to *ada* deletions. These similarities

suggest that the *ADAs* and the dominant class of *SRB* genes may function in parallel activation pathways.

In order to determine if the *ADAs* function in parallel to *SRB2*, double mutants were constructed. To clearly determine the viability of *ada, srb2* double mutants, a plasmid shuffle system was utilized. First, the deletion strains, *Δada2, Δada3, Δgcn5*, and the isogenic wildtype strain were transformed with a *URA3* marked plasmid carrying the appropriate *ADA* gene (or a similarly marked vector in the wildtype strain) creating four Ada+ Ura+ strains. Subsequently these strains were transformed with a *HIS3* marked *SRB2* disruption construct and *Δsrb2* strains were isolated. When the ADA plasmid (marked pADA in the Figure 3 key) is maintained, these strains grow well at room temperature (see upper plate of Figure 3). As expected, the *Δsrb2* strains are temperature sensitive (data not shown). However, when strains lacking the *ADA* gene are selected, using 5-Fluoroorotic acid (5-FOA) to select against the *URA3* marked *ADA* plasmid, the double mutants show a severe growth defect (see lower plate, sectors marked *Δada Δsrb2*). In contrast, strains containing only the *Δsrb2* mutation or strains containing only an *Δada* mutation grow perfectly well on 5-FOA. The synthetic phenotype caused by a combination of *ADA* and *SRB2* deletions is consistent with the hypothesis that the *ADAs* function in a pathway that is parallel and partially redundant to the dominant *SRBs*. The genetic interactions of these genes with CTD truncations suggest that their gene products both function by interacting with the CTD of Pol II.

The ADAs interact with the carboxy-terminal domain of RNA polymerase II

In order to determine if the ADAs physically interact with the CTD as predicted by the genetic data, we analyzed eluants from a CTD column and

found ADA2 and ADA3 in the 1M Urea elution (data not shown, N.S. Dave Chao, Richard Young and L.G.), suggesting the ADAs interact with the CTD (Thompson et al., 1993). To study this further, a GST-CTD precipitation assay was utilized (Wilson et al., 1996). A GST-CTD fusion, or as a control GST alone, was crosslinked to Sepharose beads. These beads were incubated with partially purified ADA complex (see below for details), and bound proteins were washed extensively, eluted in high salt and analyzed by immunoblot. As shown in Figure 4a, ADA1, ADA2, and ADA3 bound to the GST-CTD beads but not to the GST beads. As displayed, approximately 10% of the onput ADA proteins bound to CTD beads in this assay (compare lanes 2 and 5 of Fig. 4a) which is consistent with other experiments using immobilized CTD proteins (R. Young personal communication). As shown in Figure 4b, a silver stained polyacrylamide gel from the same experiment, the binding of the ADAs to the CTD was specific. Clearly the GST-CTD beads bound far less than 10% of all the proteins in the starting material (compare lane 9 to 6). This specific interaction suggests that the CTD is the binding target of the ADAs.

The ADA proteins cofractionate with core RNA polymerase II

To obtain more definitive evidence that the ADA complex binds to RNA Pol II, yeast extract was fractionated on three successive columns. Whole cell extract was prepared from a strain carrying pPADA26HIS, which expresses a 6-histidine tagged *ADA2* gene from its own promoter. The tagged version of *ADA2* fully complements a deletion of the gene. The extract was first fractionated on a Bio-Rex 70 column and step eluted with increasing concentrations of potassium acetate. Interestingly, the ADAs - ADA1, ADA2, ADA3, GCN5 and ADA5 - cofractionate in two different elutions, the 600mM step which contains about 60% of the ADAs, and the 1200mM elution which contains

about 40% of the ADAs (data not shown). The two ADA enriched fractions were then separately fractionated over a Nickel-NTA agarose column. Following the Nickel column the ADA enriched fractions were fractionated further on a Poros HQ-20 anion exchange column and eluted with a potassium acetate gradient. Figure 5a shows an immunoblot across a portion of this final gradient. The ADAs cofractionate, peaking in fractions containing approximately 1100mM salt. This cofractionation is true for both the material originally from the Bio-Rex 600mM elution (BR600) and the material from the Bio-Rex 1200mM elution (BR1200) (See Figure 5a and 5c). The peak of ADA protein from the HQ-20 column represents at least a 500-fold purification. The cofractionation strongly supports earlier findings that ADA2, ADA3, ADA5 and GCN5 interact *in vitro* and *in vivo* (Candau and Berger, 1996; Horiuchi et al., 1995; Marcus et al., 1996; Marcus et al., 1994; Silverman et al., 1994). In total, this partial purification greatly strengthens the notion that the ADAs (ADA1, ADA2, ADA3, ADA5, and GCN5) function together in a complex to facilitate transcriptional activation.

The partially purified ADA complex was further analyzed for other cofractionating transcription factors. Interestingly, RNA polymerase II, as assayed with anti-RPB1 monoclonal antibodies, cofractionates precisely with the ADAs during the final elution gradient. This is true only for the material originally eluted from the Bio-Rex 70 in 600mM salt (BR600); no RPB1 is detected in the BR1200 material (See figure 5a compared to 5c). It is possible that this complex represents the holoenzyme, as ADA2 is present in the holoenzyme at levels less than one-tenth that of the SRB proteins (data not shown). Therefore, the ADA enriched fractions were analyzed for the presence of holoenzyme components. Interestingly, neither SRB4, SRB5, nor SWI3 cofractionate with the ADAs and polymerase (see figure 5b). In the BR600 material the SRB/SWI complex elutes from the HQ-20 column at a much lower

salt concentration than the ADA/Pol II complex. The BR1200 material does not contain any of these holoenzyme components (data not shown). This result suggests either that the SRB/SWI proteins detected here represent free SRB complex, or that the SRB complex can be stripped from polymerase under conditions where the ADAs are still tightly associated. Similar to the SRB/SWI proteins, TFIIH binds to the HQ-20 column but elutes in lower salt than the ADA/Pol II complex. TBP, TFIIB, and TFIIE do not cofractionate with the ADAs; they flowthrough the final HQ-20 column. None of these general transcription factors are found in the BR1200 material (data not shown). These findings suggest that *in vivo* the ADAs exist in two pools: one of free ADA complex represented by the BR1200 material; and another where the ADAs are tightly associated with RNA polymerase II. This polymerase association represents a novel complex that is different from the previously characterized holoenzyme as it does not cofractionate with known components of the holoenzyme. Moreover, this association with polymerase validates both the *in vivo* and *in vitro* interactions between the ADAs and the CTD of RNA Pol II.

DISCUSSION

ADA2 interacts only with functional activation domains

This study further examines the genetic and biochemical properties of the ADAs. The importance of the ADA2-VP16 interaction is highlighted by two findings. The VP16 activation domain requires ADA2 for its full activation potential *in vivo* and *in vitro* (Barlev et al., 1995; Berger et al., 1992; Silverman et al., 1994). Secondly, this interaction occurs only with variants of VP16 that mediate the activation of transcription. As shown earlier, and demonstrated here, ADA2 does not bind to a truncated form of VP16, VP16Δ456, that has very little activity *in vivo*. Furthermore, the experiments presented here demonstrate

that ADA2 does not bind a VP16 mutant with a double phenylalanine to alanine substitution. This mutation has very little activity *in vivo* but retains the same charge and size characteristics of the wildtype protein. This significantly strengthens the argument that the ADA2-VP16 interaction is biologically relevant.

Interestingly, the ADA2-activation domain interaction is not the only connection between the ADA complex and activation domains. Like ADA2, ADA5 has been shown to directly interact with the full length VP16 activation domain but not with VP16Δ456 or VP16FAFA (Marcus et al., 1996); and GCN5 has also been shown to bind an activation domain from the ADR1 protein (C. Denis, personal communication). Thus, it appears that the ADA complex has multiple interactions with activation domains. In addition, different activation domains may interact with different subunits of the ADA complex. These interactions may recruit the ADAs to promoters during transcriptional activation.

The VP16 interaction was previously shown to be mediated by the N-terminal one-third of ADA2 (Barlev et al., 1995). Interestingly, this region is highly conserved between the yeast *ADA2* and the recently isolated human (Candau et al., 1996), *S. pombe,* and *K. lactis ADA2* homologs (unpublished data, Thomas Oehler, Paul Spellman, N.S. and L.G.). This suggests that the ADA2-VP16 interaction also occurs in mammalian cells. The role of the human ADA2 awaits further study; however, the conservation of many factors involved in transcription suggests that the human ADAs will function in a manner similar to the yeast ADAs.

The ADAs and SRB2-SRB5 act in parallel to stimulate CTD function

Genetic interactions have often led to significant insights into the *in vivo* role of transcription factors. For example, the suppression of *swi* and *snf*

mutants by alterations in chromatin first suggested that the SWI/SNF proteins might counteract chromatin mediated repression, an idea which has subsequently been verified by biochemical studies (Cairns et al., 1994; Côté et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994; Peterson et al., 1994; Peterson and Herskowitz, 1992; Winston and Carlson, 1992). Synthetic lethality between non-null mutations in an essential gene and mutations in other genes provides genetic evidence that the two gene products function in the same pathway (Guarente, 1994). Since the CTD is essential for viability, the synthetic lethality between *ada* mutations and non-lethal CTD truncations suggests that the ADAs and the CTD function in a common pathway. The synthetic lethality between the ADAs and the CTD is allele specific; other mutations in *RPB1* which lie outside the CTD are not synthetically lethal with *ada* deletions. This allele specificity argues for a specific link between the CTD and the ADAs.

The hypothesis that the *ADAs* are necessary for the full functioning of the CTD suggests that they may act in parallel to the dominant *SRBs* (*SRB2*, *4*, *5* and *6*). The SRB proteins are known to physically associate with RNA Pol II as components of the RNA polymerase II holoenzyme. Furthermore, at the genetic level *srb2* and *srb5* loss-of-function mutations interact with the CTD in a manner similar to *ada* deletions. *srb2* and *srb5* deletions exacerbate the effects of CTD truncations, suggesting that SRB2 and SRB5 proteins function by stimulating transcription through their interaction with the CTD (Thompson et al., 1993). Consistent with the model that the *ADAs* and these *SRBs* function in parallel, we found that *ada* deletions have a synthetic phenotype when combined with *srb2* deletions. Synthetic lethality between null alleles of two non-essential genes indicates that these genes function in parallel (Guarente, 1994). This supports the hypothesis that the *ADAs* and *SRB2* function in

parallel activation pathways. A simple model incorporating all the genetic data argues that both the ADA proteins and the SRB2 and SRB5 proteins enhance the ability of the CTD to function during the activation of transcription. Deleting one gene of either class does not cause inviability because the other class of genes can provide the same or similar activity. However, eliminating the function of both groups of genes causes a severe slow growth phenotype because the CTD can no longer function properly.

The ADA complex can associate with RNA polymerase II.

The data presented here strengthens the model that the ADAs function as a complex in order to activate transcription (Horiuchi et al., 1995; Silverman et al., 1994). Earlier studies demonstrated that ADA2, ADA3, and GCN5 can form a trimeric complex *in vitro* (Horiuchi et al., 1995; Marcus et al., 1994). Previous work also suggested that the ADA complex functions *in vivo* to activate transcription (Silverman et al., 1994). In this study an ADA complex containing ADA1, ADA2, ADA3, ADA5 and GCN5 was purified from yeast whole cell extract, supporting earlier suggestions of an ADA complex.

Moreover, the ADA complex specifically cofractionates with RNA polymerase II. The specificity of this cofractionation is demonstrated by the fact that none of the other general transcription factors tested, neither TBP, TFIIB, TFIIE, nor TFIIH cofractionate with the ADAs. In addition, the ADA complex can bind the CTD specifically, which validates the genetic interactions observed between the *ADAs* and the CTD. Furthermore, neither SRB4, SRB5, nor SWI3 fractionate with the ADAs and Pol II arguing that the ADA-Pol II complex is different from the holoenzyme and is consistent with the idea that it functions in parallel to the SRBs.

Taken together, the data presented here support a model whereby some activators interact with the ADA-Pol II complex in order to mediate the activation of transcription. The ADA-activation domain interaction may serve to recruit RNA polymerase II to promoters through the association of the ADA complex with Pol II. But it is also possible that the ADA-CTD connection affects some other step in transcription initiation that is enhanced by activators. For example, it may stabilize a larger complex which also contains TBP, as suggested by two previous studies (Barlev et al., 1995; Marcus et al., 1996). Also, the association of the ADAs with polymerase may allow for both the recruitment of polymerase and the concomitant acetylation of histones, since GCN5 has recently been shown to have histone specific acetyltransferase activity (Brownell et al., 1996). Acetylated histones are found preferentially at actively transcribed genes (Hebbes et al., 1988). The fact that not all of the ADA complex is found complexed with polymerase, i.e. the BR1200 fraction, suggests that there is a pool of free ADA complex available in the cell. The importance of such a free pool is not clear. One possibility is that the association of the ADA complex with polymerase could be regulated in order to modulate the overall level of transcriptional activation.

Future experiments will focus on two important aspects of ADA molecular biology. There is no reason to believe that all the members of the ADA complex have been identified. If the ADA complex is as complicated as the SRB complex, it is not necessarily true that mutations in all the members of the complex will have similar phenotypes. In fact, mutations in the known components of the ADA complex do not all have identical phenotypes; *ada2*, *ada3*, and *gcn5* strains have very similar properties, but *ada1* and *ada5* mutants have additional phenotypes such as Spt- and inositol auxotrophy (Horiuchi and Guarente, 1996; Marcus et al., 1996; Roberts and Winston, 1996). Therefore,

continued purification of the complex is of utmost interest to identify all of its components. One protein that is predicted to be in the ADA complex is SPT7, because *spt7* strains have phenotypes very similar to *ada5* mutants (Roberts and Winston, 1996). Most importantly, future experiments will also focus on the characterization of the biochemical activities found in the ADA complex.

Material and Methods

Yeast strains and plasmids

All strains used have been described previously (Berger et al., 1992; Marcus et al., 1994; Piña et al., 1993). CTD truncations were analyzed in the strain JAy47 (Archambault et al., 1992). *ADA* deletions were constructed in this strain using previously described deletion constructs pADA2KO for deletion of *ADA2* (Berger et al., 1992); pADA3KO for deletion of *ADA3* (Marcus et al., 1994); and pADA1KO for deletion of *ADA1* (Horiuchi and Guarente, 1996). Candidate deletion strains were scored for slow growth on minimal galactose media and mated to their cognate Ada- strain to score for proper integration of the deletion plasmid. Finally, deletions were verified by transforming with the proper *ADA* gene and scoring for complementation.

SRB2 deletions were constructed by transforming PSY316 and isogenic *ADA* deletion strains with a 3.3kb fragment from pTK33 which replaces the *SRB2* gene with *HIS3*. Proper *SRB2* deletions were selected based on their growth phenotypes, non-complementation when mated to a *Δsrb2* strain, and complementation by an *SRB2* plasmid. As described in the text, the *Δada* strains were transformed with the appropriate *URA3* marked *ADA* plasmid prior to disruption of *SRB2*. The *Δada2* strain contained pNS3.8 (Berger et al., 1992); the *Δada3* strain contained pA3P2U (gift of S. Treadway); and the *Δgcn5* strain contained p5-1,2D (Marcus et al., 1994)

To make the plasmid pPADA26HIS, pDB200CADA26HIS was digested with *Spe*I, filled-in with T4 DNA polymerase, and then digested with *Afl*II. The fragment containing the C-terminal one-third of ADA2 fused to six histidines followed by the ADH terminator was purified and cloned into pNS3.8 (Berger et al., 1992) that had been digested with *Afl*II and *Msc*I. pDB200CADA26HIS is an ARS/cen derivative of pADA26HIS (Silverman et al., 1994). This created a plasmid which contains the ADA2 promoter expressing the ADA2-six histidine fusion gene followed by the ADH terminator.

pGVPFAFA, the GST-VP16 expression vector, was made by amplifying the VP16FAFA sequence from the plasmid pMSVP16FA442FA475 (a gift of S. Triezenberg) and ligating into pGVP (Lin and Green, 1991).

The plasmids used for the CTD truncation experiments were pN11 (seven CTD repeats), pN15 (nine repeats), pC6 (eleven repeats), pV17 (thirteen repeats), and pRP114 (wildtype *RPB1*), all gifts from the Young lab.

GST pull down assays

GST-VP16 pull down experiments were performed as described previously (Marcus et al., 1996) except Triton X-100 was not used in the buffer. The ADA2 *in vitro* transcription/translation vector was also described previously (Horiuchi et al., 1995). Briefly, 10µg of purified GST fusion proteins were bound to glutathione-Sepharose (Pharmacia), then 10µl of *in vitro* translated ADA2 and 200µl of S(300) buffer (20mM Hepes 7.6, 300mM potassium acetate, 25mM magnesium acetate, 20% glycerol and protease inhibitors (Hengartner et al., 1995)) were added. The beads were preblocked in 1mg/ml *E. coli* extract and the initial incubation was also done in the presence of this blocking extract. After incubation for one hour, the beads were centrifuged, washed six times in 1ml of S(300) buffer, and then bound proteins were eluted in S(300) buffer

supplemented with 20mM reduced glutathione (Sigma). Eluted proteins were separated by SDS-PAGE and autoradiographed. Following autoradiography, the polyacrylamide gels were rehydrated and stained with Coomassie blue to determine that equal amounts of the GST fusion proteins were bound to and eluted from the beads (data not shown).

GST-CTD pull down experiments utilized either GST or GST-CTD protein beads that were made by crosslinking the proteins to CNBr activated Sepharose per the manufacturers protocol (Pharmacia). The proteins (a gift of D. Chao and R. Young) were crosslinked at equal density, 5 milligrams protein per milliliter of beads, and the crosslinking was equally efficient as determined by Coomassie staining. Equal volumes of GST of GST-CTD beads (10µl) were incubated with 100µl of partially purified ADA complex in a volume of 1ml of S(300)1%T buffer (same as above except Triton X-100 was added to 1% and no blocking agents were necessary). After binding for one hour on ice, beads were pelleted, washed at least six times in 1ml S(300)1%T buffer, and bound proteins were eluted with 1M potassium chloride. Eluted proteins were precipitated with trichloroacetic acid, separated by SDS-PAGE and analyzed by Western blot.

Fractionation of whole cell extracts

Strain 1-7aΔada2 (Berger et al., 1992) carrying pPADA26HIS was grown in minimal media to yield approximately 500g wet weight of cells. Preparation and fractionation of whole cell extract on a Bio-Rex 70 column (5x10cm) was performed as described by others (Sayre et al., 1992) except elutions were performed with 250, 600 and 1200 mM potassium acetate. Elutions were analyzed for the presence of ADA2 and ADA3 proteins by Western blot. The relevant fractions from the 600mM potassium acetate and 1200mM potassium

acetate elutions were pooled and dialyzed against Buffer N (20mM Hepes KOH7.6, 10% glycerol, 300mM potassium acetate, 10mM ß-mercaptoethanol, and protease inhibitors). This material was then bound to an NTA-Ni agarose column (1.5x10cm) and eluted with an imidazole gradient. Fractions enriched in ADA2 and ADA3 were pooled and diluted into Buffer H (20mM Hepes KOH7.6, 10% glycerol, 1mM DTT, 1mM EDTA, and protease inhibitors) to a final salt concentration of 100mM potassium acetate. This material was then fractionated over a Poros HQ-20 column (4.6x100mm) (PerSeptive Biosystems) with a elution gradient from 100mM to 1500mM potassium acetate of 10 column volumes using the BioCAD Sprint apparatus (PerSeptive Biosystems). Fractions from this column are used in the analysis shown in Figures 2 and 5.

Western blots and anti-sera

ADA1, ADA2, ADA3, ADA5 and GCN5 anti-sera were described elsewhere (Horiuchi and Guarente, 1996; Marcus, 1995; Marcus et al., 1996; Marcus et al., 1994). 8WG16 the anti-CTD monoclonal (Thompson et al., 1989), anti-TFB1, anti-SRB5 (Koleske and Young, 1994), anti-SRB4 (Hengartner et al., 1995) and anti-SWI3 (Peterson et al., 1994) antibodies were all described elsewhere. Western blots were performed by standard techniques. ADA1, ADA2, and ADA3 anti-sera were affinity purified using strip purification (Harlow and Lane, 1988), anti-ADA5 IgG molecules were purified on a Protein-A column. After blocking and incubation with primary antibody, blots were washed and incubated with secondary anti-rabbit (or anti-mouse for 8WG16) antibody crosslinked to Horseradish peroxidase (Amersham) and visualized using the ECL system (Amersham).

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Figure 1: ADA2 interacts with the wildtype VP16 activation domain but not with the VP16FAFA mutant. GST-VP16 fusions were used to precipitate *in vitro* translated ^{35}S-labeled ADA2 (see Materials and Methods for details). The lane marked OP contains 2% of the ADA2 protein that was loaded onto the beads. The other lanes show the ADA2 protein that was precipitated with wildtype VP16 (lane marked WT), or with VP16FAFA (lane marked FF), or with VP16Δ456 (lane marked Δ). Wildtype GST-VP16 contains the full VP16 activation domain, residues 413-490. VP16FAFA is a double Phe to Ala substitution mutant at residues 442 and 475 within the full length VP16 activation domain. VP16Δ456 is a truncation mutant of VP16 that contains residues 413-456 of VP16. VP16FAFA and VP16Δ456 have very little activity *in vivo* (Regier et al., 1993; Silverman et al., 1994). As demonstrated here, they also do not bind ADA2.

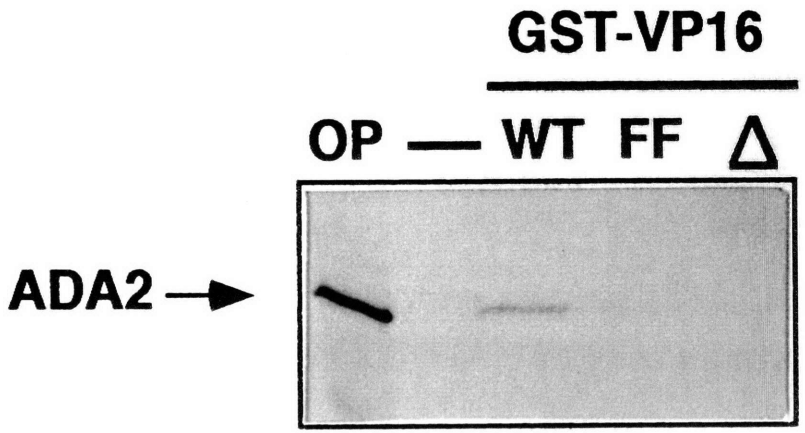

GST-VP16
OP
—
WT
FF
Δ
ADA2

Figure 2: CTD truncations and ADA deletions show synthetic enhancement. The top row is a key detailing which *RBP1* allele each sector contains. Vector indicates that the strain does not contain an *RBP1* expressing plasmid. WT indicates that a wildtype allele of *RBP1* is expressed; 13 repeats, 11 repeats, 9 repeats, or 7 repeats indicates that *RBP1* alleles with 13, 11, 9, or 7 heptapeptide repeats, respectively, are expressed. In the right hand column, other *rbp1* alleles are expressed as indicated. The far left column indicates the *ADA* genotype of the strains in each row. As is clearly exhibited, deletion of the *ADA* genes makes the phenotypes caused by CTD truncations much more severe. For example, compare the growth of strains expressing *RBP1* alleles with 9 repeats. Also, the effects were specific to CTD truncation mutations. Compare the synthetic effects in the left column to the normal growth seen in the column on the right. All strains were grown on glucose so that the endogenous *RBP1* allele in this strain was not expressed.

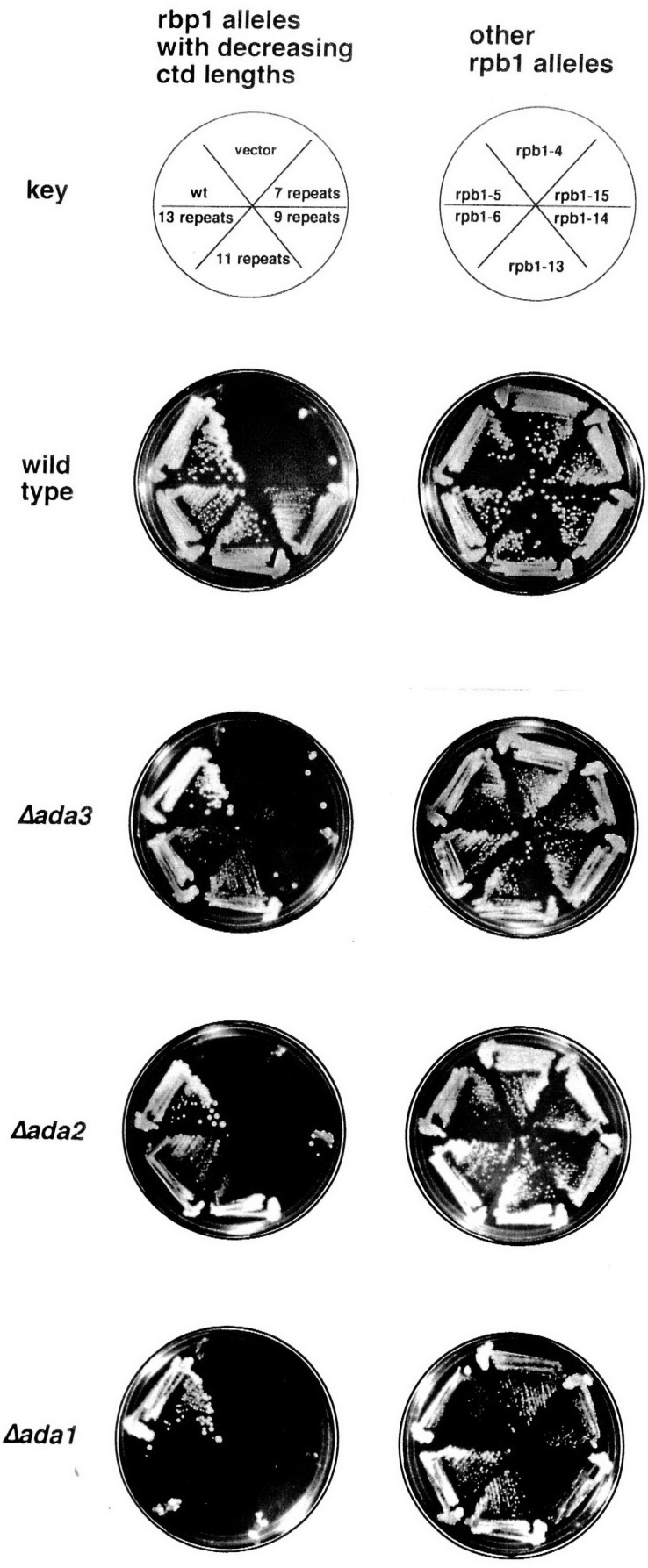
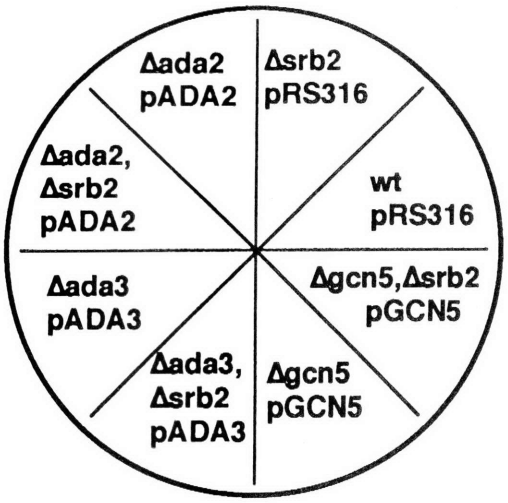

rbp1 alleles
with decreasing
ctd lengths
other
rpb1 alleles
key
vector
wt
7 repeats
13 repeats
9 repeats
11 repeats
rpb1-4
rpb1-5
rpb1-15
rpb1-6
rpb1-14
rpb1-13
wild
type
Δada3
Δada2
Δada1

Figure 3: *SRB2, ADA* double deletion mutations display synthetic phenotypes. The top panel is a key for the lower two panels. Four strains, wildtype, Δada2, Δada3, and Δgcn5, were transformed with URA3 marked plasmids encoding, respectively, either no *ADA* gene (pRS316), *ADA2* (pADA2), *ADA3*(pADA3), or *GCN5* (pGCN5). This created four Ura+ Ada+ strains. Then the *SRB2* gene was replaced with the *HIS3* gene. The middle panel shows growth of all strains on rich media selecting for the URA3 marked ADA plasmids. The lower panel, a plate containing 5-FOA, shows the phenotype of double *ada, srb2* mutants created after the loss of the ADA plasmid. As is clear in the lower panel, deletion of *ADA2, ADA3,* or *GCN5* in combination with the *SRB2* deletion causes a severe slow growth phenotype. However, in strains with only one gene deleted, either the control wildtype strain that is always Ada+ or the strains with an intact *SRB2* gene, growth on 5-FOA occurs normally. Note, the double mutants will form colonies after more than six days on 5-FOA plates, whereas the control strains grow in less than two days. When these double mutant strains are restreaked to rich non-selective media they also exhibit a severe slow growth phenotype.



Key

Media selecting
for plasmid

Media selecting
against plasmid

Figure 4: The ADA complex binds to the CTD of RNA Pol II. Either GST or GST-CTD beads were used to precipitate proteins from ADA enriched fractions (see Material and Methods). (**A**) An immunoblot from this experiment that was probed with antibodies to ADA1, ADA2 or ADA3 (as labeled). Lanes 1 and 2 were loaded with 40% and 10%, respectively, of the material which was added to the protein-beads. Lanes 4 and 5 show the material that was eluted from the GST or GST-CTD beads, respectively. By comparing lane 2 to lane 5 it is clear that ~10% of the ADA proteins which were loaded on the GST-CTD beads were bound and eluted. However, no ADA proteins bound the control GST beads. (**B**) Moreover, a silver stained polyacrylamide gel from the same experiment shows that 10% of the total protein in the sample did not bind the GST-CTD beads (compare lane 6 to lane 9). Note, that ADA2 protein is difficult to detect because the antibody is not very sensitive (data not shown), but on close inspection it is clearly detected in the GST-CTD elution.

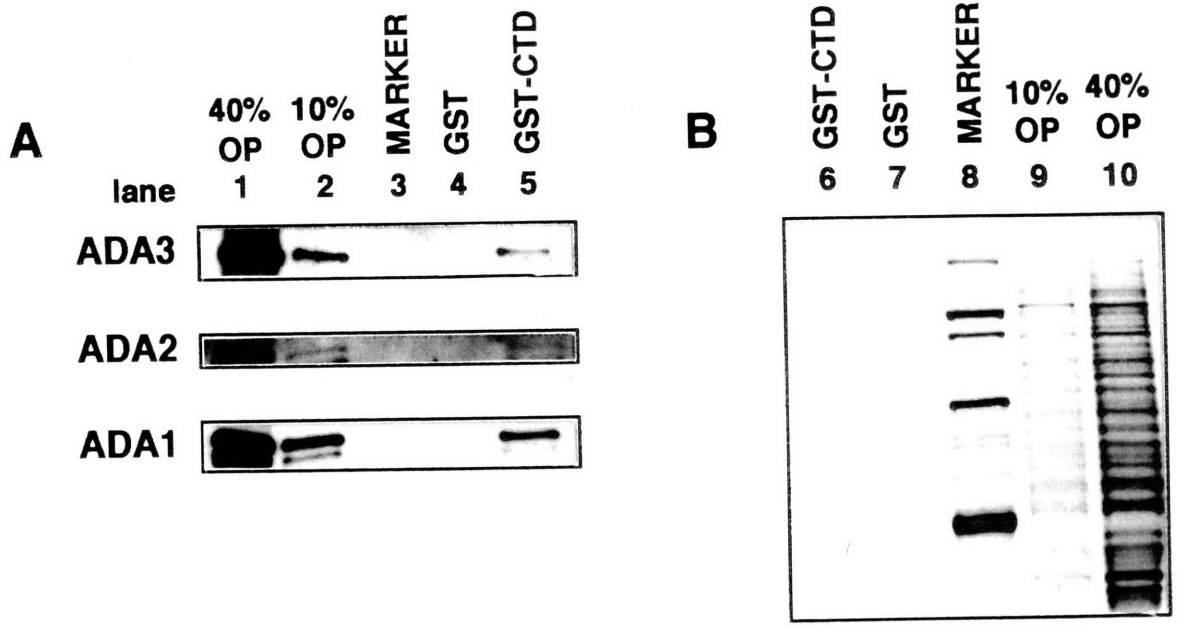
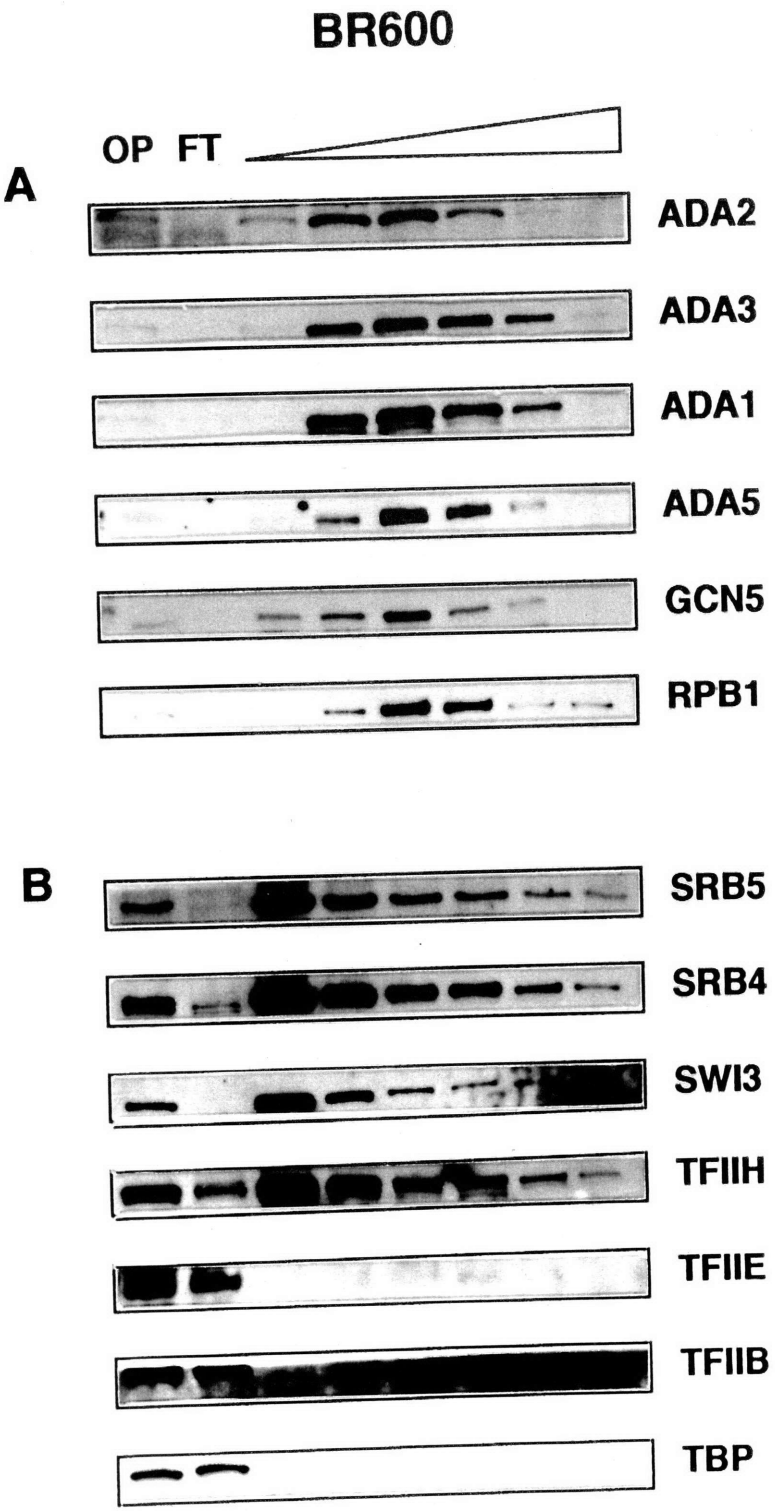
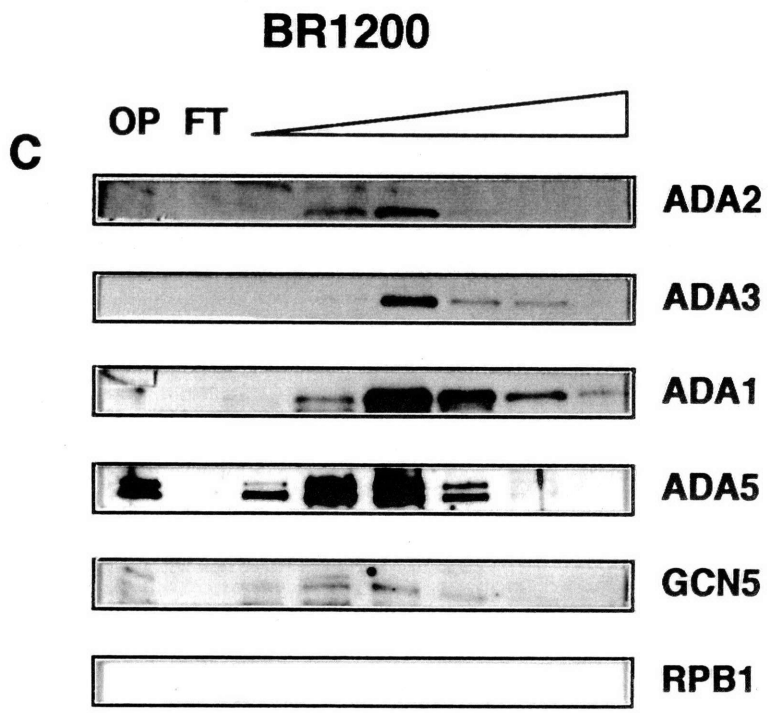

A
40% OP
10% OP
MARKER
GST
GST-CTD
lane
1
2
3
4
5
ADA3
ADA2
ADA1
B
GST-CTD
GST
MARKER
10% OP
40% OP
6
7
8
9
10

Figure 5: ADA1, ADA2, ADA3, ADA5, GCN5 and RNA Polymerase II cofractionate. All panels display immunoblots probed with the indicated antisera. Panels A and B show immunoblots of fractions from the HQ-20 column that was loaded with material originally from the Bio-Rex 70 600mM potassium acetate elution (BR600). The onput, flowthrough, and every other fraction, between fraction 14 and fraction 24, were probed as indicated at the top of panel A. (**A**) ADA1, ADA2, ADA3, ADA5, GCN5, and RBP1 cofractionate in a single peak near fraction 18. (**B**) These blots contain the same samples and were loaded in the same order as panel A; they show that SRB4, SRB5, SWI3, and TFIIH (the p62 subunit encoded by *TFB1*) elute much earlier than the ADA-Pol II complex . Other general transcription factors, TBP, TFIIB, and TFIIE (the small subunit encoded by *TFA2*) do no bind this column and are found in the flowthrough. (**C**) The material originating from the Bio-Rex 70 1200mM KOAc elution (BR1200) was fractionated on a HQ-20 column as in panels A and B and analyzed by immunoblot. ADA1, ADA2, ADA3, ADA5, and GCN5 cofractionate but no RBP1 is detected. (A control lane indicates that the a-RBP1 antibody would have detected RBP1 protein present in these fractions (data not shown).) No other general transcriptional factors were found in the BR1200 material (data not shown).


BR600
OP FT
A
ADA2
ADA3
ADA1
ADA5
GCN5
RPB1
B
SRB5
SRB4
SWI3
TFIIH
TFIIE
TFIIB
TBP


BR1200
C
OP FT
ADA2
ADA3
ADA1
ADA5
GCN5
RPB1