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DISSERTATION

FUNCTIONAL CHARACTERIZATION OF *SACCHAROMYCES CEREVISIAE* TFIID AND TFIIA

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RYAN T. RANALLO ENTITLED FUNCTIONAL CHARACTERIZATION OF *SACCHAROMYCES CEREVISIAE* TFIID AND TFIIA BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

FUNCTIONAL CHARACTERIZATION OF *SACCHAROMYCES CEREVISIAE* TFIID AND TFIIA

In eukaryotes, regulation of RNA polymerase II (pol II) transcription is mediated at many stages. Pol II transcription initiation involves the coordinate interactions of many general and gene-specific transcription factors. The general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH) function as factors necessary for accurate transcription initiation *in vitro*.

One of the first steps in transcription initiation is the sequence-specific binding of general transcription factor TFIID to the core TATA element. TFIID is a multiprotein complex consisting of TATA-binding protein (TBP) and 14 TBP-associated factors (TAFs). As a potential rate-limiting step in initiation, TFIID binding the core element is regulated in many different ways via numerous protein-protein and protein-DNA interactions.

Using a TBP mutant partially defective for TFIID formation *in vivo* we show that an intact TFIID complex is not required for transcription of all pol II promoters. In fact, a partially disrupted TFIID complex results in promoter specific transcriptional defects. Furthermore, our results support the notion that TAFs serve as potential, but not obligatory targets for transcriptional activators.

Crystallographic and numerous other studies of TBP have led to the view that TBP-DNA interactions are regulated by TBP dimerization. Therefore, we hypothesized that TFIID oligomerization was a point of transcriptional regulation in yeast. Using biophysical techniques and immunological methods we show no evidence of TFIID dimers in yeast. Our findings challenge the hypothesis that

TBP dimerization regulates DNA binding and therefore transcription initiation in yeast.

The association of TFIID with promoter DNA *in vivo* is stimulated by another general transcription factor TFIIA. We hypothesized that TFIIA also makes contacts with TAFs in the TFIID complex. Using a variety of techniques we show that TAF40 makes direct contacts with TFIIA and TBP. Furthermore we show that the interaction between TFIIA and TAF40 is important for transcription *in vivo*. Functional analysis of TFIIA confirmed the TAF40-TFIIA interaction and perhaps identified a new TFIIA-TAF interaction. These studies highlight the importance of the TFIID-TFIIA interaction in transcription initiation.

Finally, a more in-depth study of TAF40 identified multiple interaction surfaces, one of which is important for essential functions in yeast. The continuation of these studies may reveal novel regulatory mechanisms of transcription initiation in yeast.

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CHAPTER 1

BACKGROUND ON GENERAL TRANSCRIPTION FACTORS TFIID AND TFIIA

1.1 Transcription initiation in yeast *Saccharomyces cerevisiae.*

In eukaryotes, coordinate regulation of gene expression is a fundamental biological process necessary for cellular homeostasis. This regulation is mediated at many stages; however, much of it occurs at the level of transcription initiation. Initiation of transcription on yeast promoters involves the orchestration of many of general and gene-specific factors. The end result is a highly complex gene circuitry that allows cells to grow and respond to a changing environment. Precisely how these and other factors collaborate to control this circuitry is the subject of considerable debate in the field of eukaryotic gene expression.

In yeast there are approximately 6,200 genes regulated by over 200 activators, initiation factors, at least 5 or 6 chromatin remodeling

complexes, and chromatin structure itself (38). Factors that participate in transcription of eukaryotic structural genes (class II) can be classified into two groups. The first class is the general transcription factors (GTFs), which are necessary for accurate transcription initiation *in vitro*. They include RNA polymerase II itself and at least six (GTFs): TFIID, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH (reviewed in 33). These factors assemble on the promoter and form the pre-initiation complex (PIC). PIC formation and reformation is stimulated by the second class of factors, the activator proteins (activators). Activators typically contain at least two separable domains, a DNA-binding domain and an activation domain. DNA-binding domains are sequence-specific binding domains that interact with DNA sequences located near the target gene. The activation domain is responsible for protein-protein interactions with the various initiation components allowing for communication with the general transcription machinery. A summary of some of the many protein complexes involved in transcription initiation in yeast cells is shown in Figure 1.1. The work described within this thesis is focused on functional studies of two general transcription factors, TFIID and TFIIA. This chapter emphasizes the yeast *Saccharomyces cerevisiae* system; however, where appropriate I integrate studies from higher eukaryotes.

1.2 TBP is the universal transcription factor.

In eukaryotic transcription there are three distinct RNA polymerase systems (pol I, II and III) responsible for the transcription of nuclear genes.

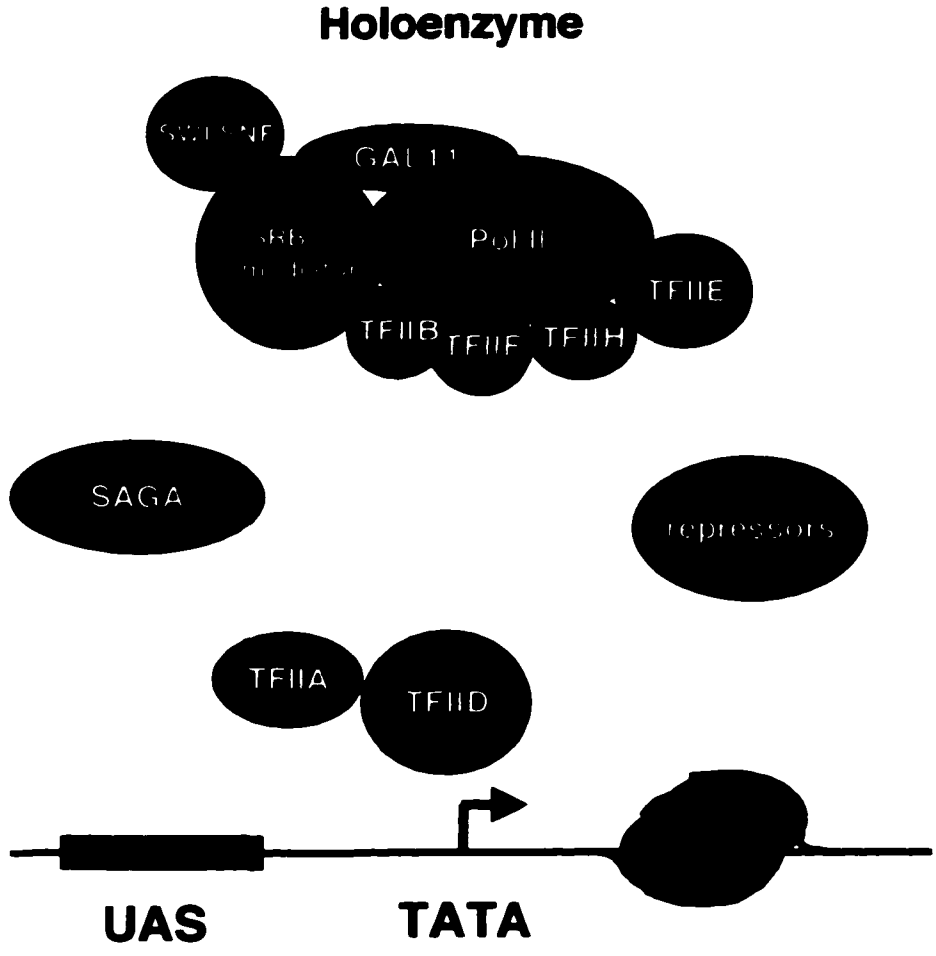

Figure 1.1 Schematic of the many protein complexes involved in RNA polymersase II transcription initiation

Each polymerase requires a set of factors to enable correct recruitment, positioning and catalysis of transcription from the appropriate class of promoter. The TATA-binding protein (TBP) was first identified as a factor required for pol II transcription, but has since been found to play a central role in all three polymerase systems (reviewed in 35). It does so by associating with different sets of proteins (TBP-associated factors or TAFs) depending on the polymerase involved. In RNA pol II transcription TBP is the central component of the TFIID complex (reviewed in 12, 33). In yeast, TFIID is made up of TBP and approximately 14 TBP-associated factors (TAFs) (67, 74, 78, 80). TBP was found to be important for transcription by RNA pol I as part of the SL1 complex (23). RNA pol III transcription was found to require TBP as part of the TFIIIB complex (43, 61, 90). TBP is therefore referred to as the universal transcription factor because it functions in all three polymerase systems as an integral part of the SL1, TFIID and TFIIIB complexes.

1.3 RNA polymerase class II promoters.

Class II promoters contain a variety of elements which modulate their activity. These elements include core elements (TATA box and Initiator), activator binding elements and distal enhancers. The regulation of pol II transcription primarily occurs through the proximal and distal promoter elements. The best-characterized and most universal element is the TATA element, which is located 25 bp (human) to 40-120 bp (yeast) upstream of the

transcription start site (reviewed in 72, 87) and is recognized by TFIID. Mutational analysis of the yeast TATA element has indicated that the consensus sequence is TATAAA(A/T)(A/T) (17, 82, 98). "TATA-containing" and "TATA-less" are terms frequently used to describe yeast promoters; they refer to the degree of deviation from the consensus sequence and thus core promoter strength. Two distinct mechanisms have been proposed for assembly of the preinitiation complex, depending on the strength of the promoter (12). Initiation from a TATA-containing promoter is dependent on strong TBP-DNA contacts for recognition and binding of TFIID to the TATA box. However, for TATA-less promoters, sequence-specific recognition of the core promoter by TBP does not occur, suggesting that other interactions play an important role (12). It has been proposed that TAFs play an important role in making these additional contacts with DNA.

1.4 TBP identification and initial characterization.

In yeast, the gene for TBP (*SPT15*) was originally identified in a genetic screen looking for suppressors of the retrotransposon Ty insertion in the *HIS4* promoter (Suppressor of TY) (27, 32). This insertion caused a shift in the *HIS4* transcription, producing an abnormally long transcript rendering the cells Histidine auxotrophic (His^-). Mutations in TBP and other proteins that altered its binding resulted in normal *HIS4* transcription and a His^+ phenotype. TBP was subsequently purified and characterized to as a 27 kDa protein that is highly conserved from yeast to humans (reviewed in 35).

Furthermore, TBP was found to be functionally interchangeable with human TBP in *in vitro* transcription systems (10, 14).

Sequence analysis of TBP revealed two direct repeats found in the conserved C-terminal region of the protein. The conserved C-terminal region of TBP binds DNA and is 80% conserved from yeast to humans. The N-terminal region is more divergent in length and composition (35) (Figure1.2A). The exact function of the N-terminal region is currently unknown, though studies suggest it is involved in regulated TBP binding to DNA (13, 55, 58).

Crystal structures of TBP have been solved for many different organisms including yeast. In each case only the C-terminal region was crystallized (44, 45 and reviewed in 69). TBP has a unique overall architecture that resembles a molecular saddle (Figure 1.2B). The structure has a two-fold symmetry, each half corresponds to one of the indirect repeats found in the linear amino acid sequence. Each structural domain contains two perpendicular α-helices and 5 antiparallel β-sheets (Figure 1.2B). Two loops at the bottom of the structure are important for DNA intercalation. Upon binding to the minor groove of DNA (Figure 1.2B), TBP bends the DNA 80° toward the major groove (44, 45). The concave undersurface of TBP makes sequence-specific hydrophobic contacts with the minor groove of DNA while the upper surface is exposed for participation in numerous protein-protein interactions (Figure1.2B).

Recruitment and binding of TBP to a promoter is undoubtedly a rate-limiting event in transcription initiation (16, 22, 47, 48, 54, 57) and thus is

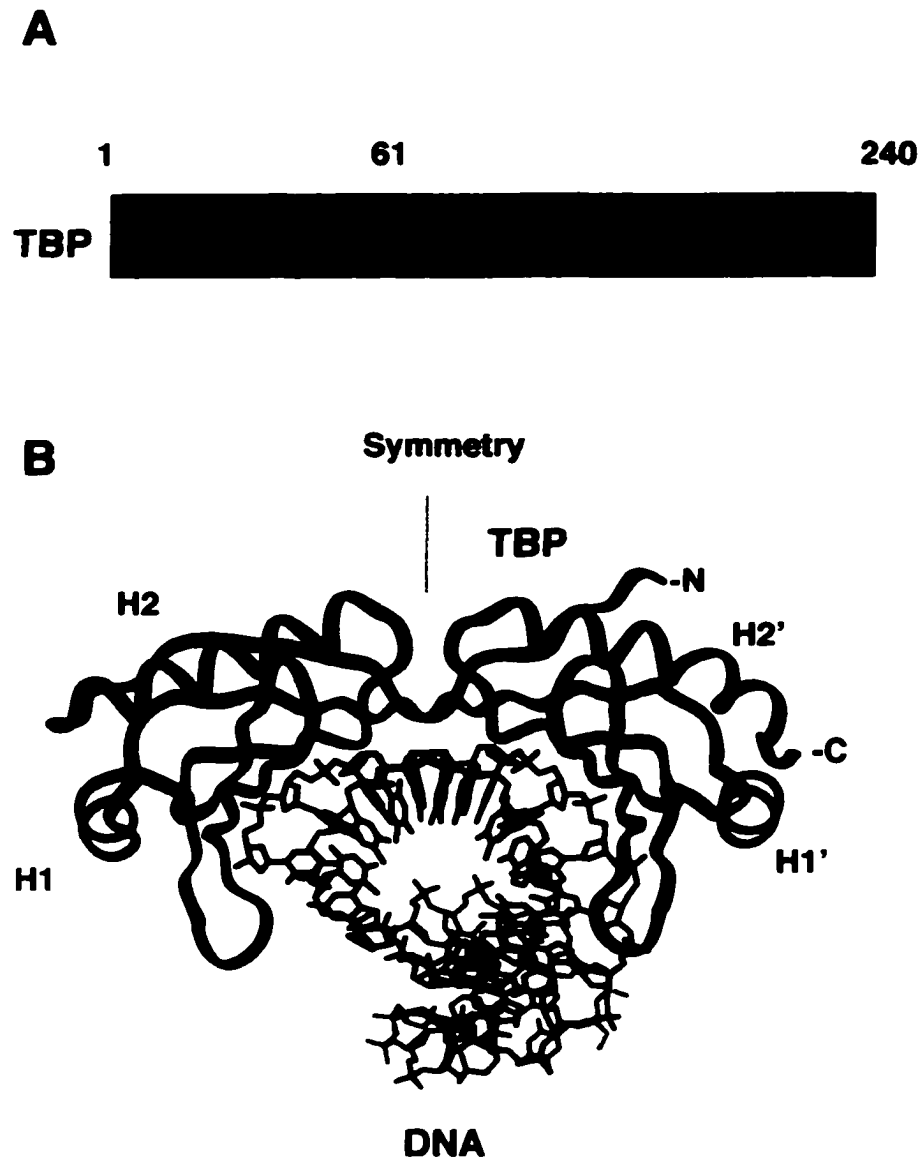

Figure 1.2A and 1.2B (A) schematic of TBP indicating the two separate domains of TBP. (B) Crystal structure of the conserved C-terminal domain of TBP. TBP is in red and DNA is in black.

an important step in regulation. Regulation of this step is thought to occur via many different mechanisms. For example, the dissociation of TBP-TBP dimers has been suggested as a way of regulating TBP-DNA interactions (21 and reviewed in 33). TBP from three different organisms was crystallized as dimer in the absence of DNA (12, 15, 45). The dimerization and DNA binding surfaces are nearly identical, and so dimerization and DNA binding are mutually exclusive on structural grounds. Some studies have concentrated on the kinetic properties of TBP dimers, suggesting that slow dimer dissociation dictates TBP-DNA interactions (20). In addition, chemical cross-linking has been used to suggest that even TFIID forms dimers in the cell (89). Using a combination of biophysical and genetic techniques, we have shown that TBP oligomerization is not a likely point of transcription regulation (13). We used sedimentation equilibrium to show that, at estimated nuclear concentrations full-length TBP would not dimerize. Furthermore, immunoprecipitation of TBP showed no evidence for TFIID dimerization. Thus it appears that TBP dimerization is an unlikely point of transcriptional regulation in yeast.

1.5 Mutational studies have defined multiple binding surfaces on TBP.

TBP interacts with numerous proteins both *in vivo* and *in vitro*. Several mutational studies have been successful in mapping the domains of TBP responsible for interaction with DNA, TFIIA, TFIIB, TAFs and SPT

proteins (summarized in refs 33, 35); however, I will focus on TBP interactions with DNA and TFIIA.

The concave undersurface of TBP is responsible for protein-DNA interactions, while the upper surface of TBP is important for protein-protein interactions. Mutations in the concave undersurface have been found that decrease TBP's affinity for DNA (3, 56, 77). These and numerous other studies have been important in formulating ideas suggesting that transcription activators function at least in part by stabilizing TBP binding to the TATA element. Furthermore, a TBP mutant with altered DNA-binding specificity (TGTA) for the TATA element has also been described (86).

Interaction with TFIIA stabilizes TBP on promoter DNA *in vitro* (29, 41, 91, 96). This stabilization occurs through contacts with TBP and DNA as seen in the yeast TBP-TFIIA-DNA crystal structure (44). Furthermore, mutations in TBP that disrupt the interaction with TFIIA show a nonspecific defect in activated transcription (83, 84). These and numerous other studies have indicated the TBP-TFIIA interaction is important for response to transcriptional activators.

1.6 TBP is associated with transcriptional repressors *in vivo*.

Part of regulating the genome circuitry is down-regulation of genes by transcriptional repressors. Two classes of repressors have been identified, ones that function through core promoter elements and those that are associated with chromatin (reviewed in 33). The latter includes histones,

histone acetylase and deacetylase proteins. The former generally affects TBP function by inhibiting TBP-DNA interactions. The Mot1(TAF170)-TBP complex is an example of a factor that affects TBP's DNA binding activity. Mot1(ADI) was identified both genetically and biochemically as an ATP-dependent inhibitor of TBP binding to DNA (4, 5, 25).

Mot1 was also described as a 170 kDa TBP-associated factor that bound TBP, forming a complex that is distinct from TFIID both in yeast and humans (18, 74). Genetically, Mot1 was isolated in a screen looking for factors that are synthetically lethal with Spt3 (62). These genetic interactions led to the proposal that TFIIA and Spt3 function to stabilize TBP on the promoter, counteracting the effects of Mot1, which functions to remove TBP from DNA. Other transcriptional repressors in yeast include Cc4-NOT complex, BUR proteins, Dr1-DRAP/NC2, Spt4-Spt6, histone deacetylases, and Ssn6-Tup1 (reviewed in 33).

1.7 Historical perspective of the TBP associated factors.

TFIID was originally purified as a fraction required for RNA polymerase II transcription *in vitro* (12). TFIID purified from human cells is a multi-protein complex containing 8-12 proteins (12, 93). TFIID was subsequently identified in Drosophila and yeast. In yeast however, TFIID purified as a single polypeptide TBP (40), which was very similar to its counterparts in Drosophila and humans with regards to DNA binding and transcriptional activity (35). Further characterization of TBP from yeast

extracts indicated that yeast TBP is associated with a number of proteins *in vivo*. Immunoprecipitations and TBP affinity experiments indicated that yeast TBP is complexed with at least seven TAFs *in vivo* (74, 78). Five more TAFs were subsequently identified based on homology to TAFs found in higher eukaryotes (67). Recently however, yeast TFIID was purified to near homogeneity and found to contain two more TAFs, TAF48 and TAF65 (80). Both these TAFs are required for cell viability, but only TAF48 has homologs in human and Drosophila. The apparent lag in identifying TAFs in yeast is due to the instability of the TFIID complex, as well as its sensitivity to chloride ions present in many biochemical purifications (50). The function of the TAF proteins *in vivo* has been the subject of many studies in the past four years; the highlights of these studies are discussed in the following section.

1.8 TAFs are highly conserved from yeast to humans.

In almost every case, TAFs found in yeast have a metazoan counterpart (80) (Figure 1.3). Functional conservation between human and yeast TAFs has been more difficult to show. Almost all yeast TAFs are required for cell viability, indicating at least one critical function in transcription (Figure 1.3). Homology searches and structural studies have indicated that some TAFs resemble certain nonlinker histone proteins. In yeast, TAF17, TAF60 and TAF61, share sequence similarity to histones H3, H4, and H2B respectively (64) (Figure 1.3). Crystallographic studies using human and Drosophila TAFs have confirmed that some TAFs do use histone motifs to

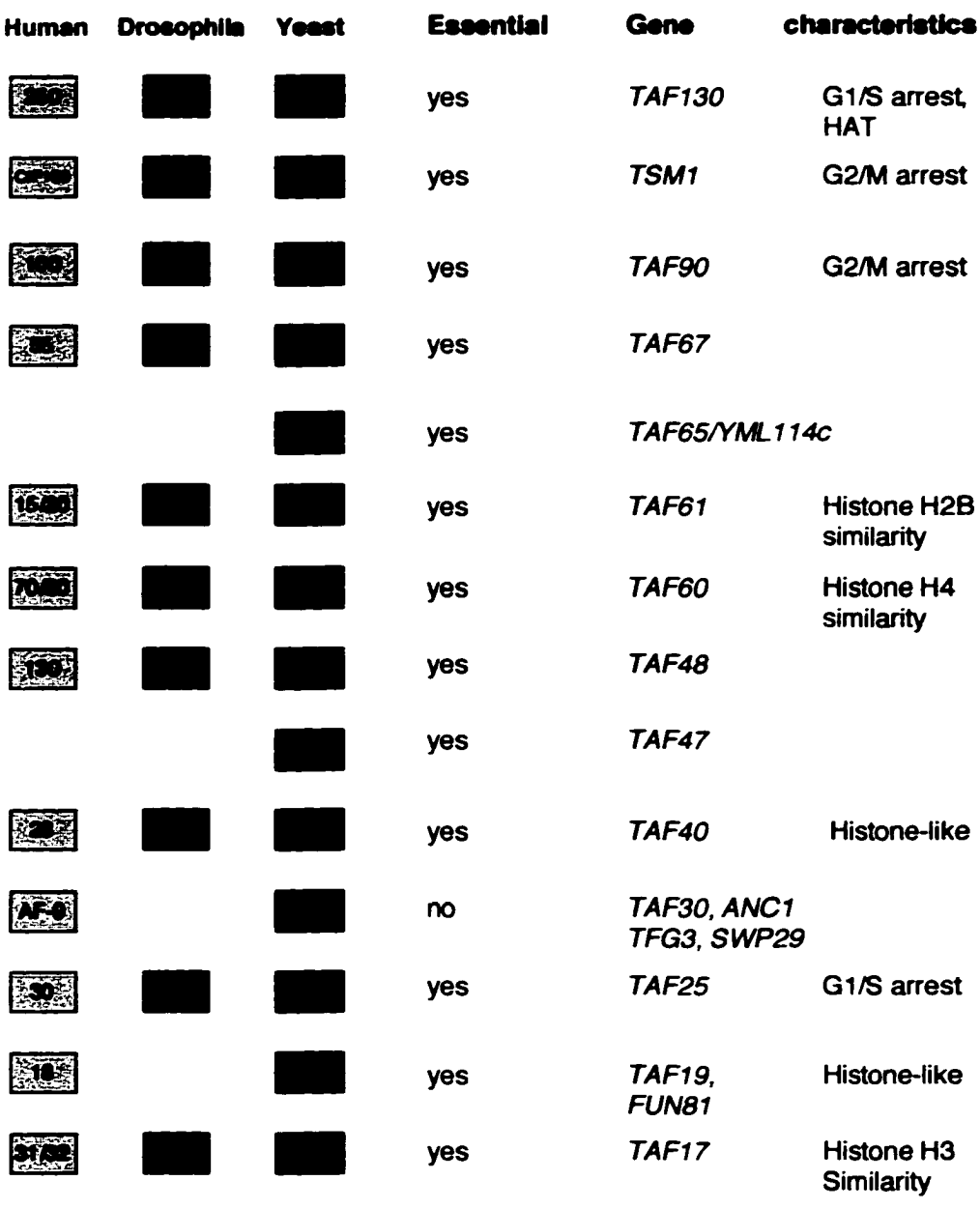

Figure 1.3 Summary of TBP-associated factors (TAFs). Conservation of yeast TAFs through sequence alignments is indicated on the left. Characteristics of each yeast TAF is indicated on the right.

interact with one another (8, 99). Recently, hTAF28 and hTAF18 were crystallized and shown to interact using an atypical histone fold (8). This was unexpected since neither TAF shares sequence similarity to the histones proteins. These and other findings have lead to the hypothesis that TAF-TAF interactions are mediated by evolutionarily conserved histone fold motifs and hint at the existence of a histone-like octamer at the core promoter (36, 99). However, the relevance of this finding has yet to be shown.

1.9 TAFs as coactivators.

Initial biochemical studies using metazoan factors suggested that TAFs function as coactivators required for activator-dependent recruitment of TBP, since TFIID but not TBP could support high levels of transcription in the presence of an activator (37, 75). This led to the TAF-coactivator hypothesis in which activators interact with TAFs, which then bridge interactions to GTFs. This notion was supported by the finding that individual TAFs and partial TFIID complexes reconstituted from a subset of TAFs can mediate activator-dependent transcription (reviewed in 12, 93). However, TAFs are not absolutely required for the response to activators *in vitro*, because activation can occur in reactions lacking TAFs (46, 51, 70).

In vivo depletion studies show that transcriptional activation is relatively unaffected by loss of TAFs, suggesting TAFs may serve as potential but not obligatory targets of activator proteins (65, 94). TAF inactivation can also lead to distinct cell-cycle phenotypes. In fact, individual depletion of

three TAFs (TAF150, TAF130, and TAF90) leads to arrest at various stages of the cell cycle (2, 94) (Figure 1.3). A more in-depth study found TAF130 to be important for transcription from G1- and B-type cyclin genes (76, 95). This TAF130 dependence was mapped to the core promoter and was independent of upstream activators (81). These and additional studies suggest that individual TAFs have promoter-specific requirements in transcription initiation.

1.10 TAFs found in transcription complexes other than TFIID.

Recently, it has been shown that TAFs reside in complexes other than TFIID. In yeast, five TAFs (TAF17, -25, -60, -61, -90) are integral components of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex (30). This complex is responsible for nucleosome acetylation *in vivo* and can interact directly with TBP (85). In fact, TAF61 is necessary for efficient recruitment of SAGA to promoters via interactions with Gcn4 protein (68). A complex similar to SAGA is found in humans and is referred to as the SPT3-TAF31-GCN5 (STAGA) complex (63). STAGA is the likely homolog of the SAGA complex since it contains TAFs, SPTs and Gcn5 (63). The PCAF complex in humans also contains histone-like TAFs, hTAF31 and hTAF20/15 (71). Finally, there is a TBP-free TAF containing complex (TFTC), which has been described in humans (9). This complex has been shown to direct pre-initiation complex formation and transcription initiation *in vitro* without TBP (9, 97). All these alternative complexes share a single subunit (Gcn5) that has been shown to have histone acetyltransferase (HAT) activity. This finding

suggests that initiation factors are directly involved in chromatin remodeling and modification. It also points out the extreme diversity of complexes that contain TAF subunits. It remains unclear if TAF function changes depending on the complex in which it resides.

1.11 TAF-DNA interactions are important for transcriptional regulation.

The results from many studies have indicated that TAFs make contacts with promoter DNA *in vitro*. These interactions suggest that TAFs function in part to modulate the binding of TBP to DNA both negatively and positively. *In vitro*, TFIID, but not TBP is able to recognize and support transcription from TATA-less promoters, suggesting that TAFs function to stabilize TBP on promoters (101). Moreover, in Drosophila TFIID, specific TAFs have been implicated in making contacts with DNA in sequences overlapping the transcription start site (11, 12, 92). Consistent with this, depletion of certain individual yeast TAFs *in vivo* causes a reduction in transcription from promoters lacking consensus TATA boxes (65, 66). Thus, it seems that certain TAFs play a direct role in promoter recognition and stabilization of TFIID to the core promoter.

Another subset of TAFs have been shown to inhibit TBP's ability to bind DNA. Studies using both Drosophila (59) and yeast TAFs (7) have indicated that the N-terminal region of TAF130 (dTAF230) can mimic DNA and bind the concave undersurface of TBP thereby blocking the TBP-DNA interaction (50, 59). Furthermore, this inhibition can be competed away by

incubation with TFIIA (50). These results suggest that TAFs and TFIIA have dynamic interplay that is used to regulate TBP binding to certain promoters *in vivo*. The nature of these interactions will be addressed in a later section of this chapter.

1.12 What is the function of TAFs *in vivo*?

Researchers have investigated the role of TAFs using *in vivo* depletion studies. Initial characterization of some histone-like TAFs (TAF17, TAF61, TAF60) found that they were generally required for most pol II mediated transcription (2, 64, 66, 68); however, genome-wide expression data taken from similar TAF inactivation experiments show that TAFs are not as universally required as initially thought (39). Experiments completed for seven of the 12 TAFs indicate genome dependence on TAFs ranges from 3 to 67%, with TAF17 being the highest (reviewed in 31). Interpretation of these data are complicated by the fact that some TAFs are integral components of the SAGA complex (30, 88). Thus any effect seen after TAF inactivation could be the result of either loss of TFIID or SAGA functions. In fact, genome-wide transcriptional analysis of TFIID and SAGA components suggests that expression of most genes is dependent on the functions of either TFIID or SAGA complex (reviewed in 31).

Targeting TAFs specific to the TFIID complex has been used to study functions of TFIID that are distinct from the SAGA complex. One such study analyzed a temperature-sensitive allele of TAF40 (52). Analysis of this

allele revealed that high temperature causes degradation of TAF40 and subsequent loss of some TFIID components. Analysis of poly $(A)^+$ mRNA levels found a general cessation of RNA pol II transcription, implying that TFIID is required for all RNA pol II transcription (52). This is inconsistent with studies that show TFIID disruption has only promoter-specific defects *in vivo* (76). Genome-wide expression profiles have not yet been completed for TAFs specific to TFIID; thus the requirement of TFIID in genome expression is still undetermined.

1.13 General Transcription factor TFIIA.

TFIIA was originally identified as a general transcription factor necessary for accurate transcription initiation *in vitro* (79). Since then, TFIIA has been shown to be involved in multiple roles in RNA pol II transcription. It has since been redefined as a coactivator since it only stimulates transcription when TAFs are present in transcription reactions (24, 26, 34, 73). In agreement with its general role in transcription, TFIIA can bind and stabilize TFIID on TATA elements *in vitro* (49). This stabilization has also been shown to be important for gene activation *in vivo* (60).

In yeast, TFIIA is composed of two subunits encoded by the *TOA1* and *TOA2* genes. *TOA1* encodes the larger 32-kDa subunit and *TOA2* encodes the smaller 13.5-kDa subunit. Both proteins share sequence similarity with their higher eukaryotic counterparts in addition to being required for cell viability. The Toa1 subunit of TFIIA is highly conserved at

both its N- and C- terminus. These regions are separated by a large unconserved central domain that extends from amino acids 51-215 (42). Toa2 is highly conserved throughout its sequence.

The crystal structure of yeast TFIIA-DNA-TBP complex reveals that the Toa1 and Toa2 subunits come together and form a heterodimeric structure (29, 91) (Figure 1.4). The N- and C-terminal sequences of each subunit make up two distinct structural domains, a four-helix bundle and a ß-domain, respectively (Figure 1.4). The structure reveals that TFIIA makes extensive contacts with TBP, and DNA upstream of the core promoter. Mutations in these binding surfaces verify the importance of these interactions in transcription initiation (42, 60).

TFIIA has been shown to act as an antirepressor and it can counteract the effects of several negative regulators of transcription. Factors like Mot1 and TAF130, which inhibit TBP-DNA interactions, can be displaced by TFIIA. In fact, TFIIA has been shown to counteract the inhibitory effects of the TAF130 N-terminal domain. Recently, a study using full-length yeast TFIIA and dTAF230 revealed that both proteins have overlapping binding sites on yeast TBP (6). This could explain why TFIIA is required in transcription reactions that contain TAFs. The precise nature of these interactions and how they affect transcription *in vivo* remain to be elucidated.

Aside from its role in antirepression, TFIIA plays a significant role in activated transcription *in vivo*. Many direct interactions between TFIIA and

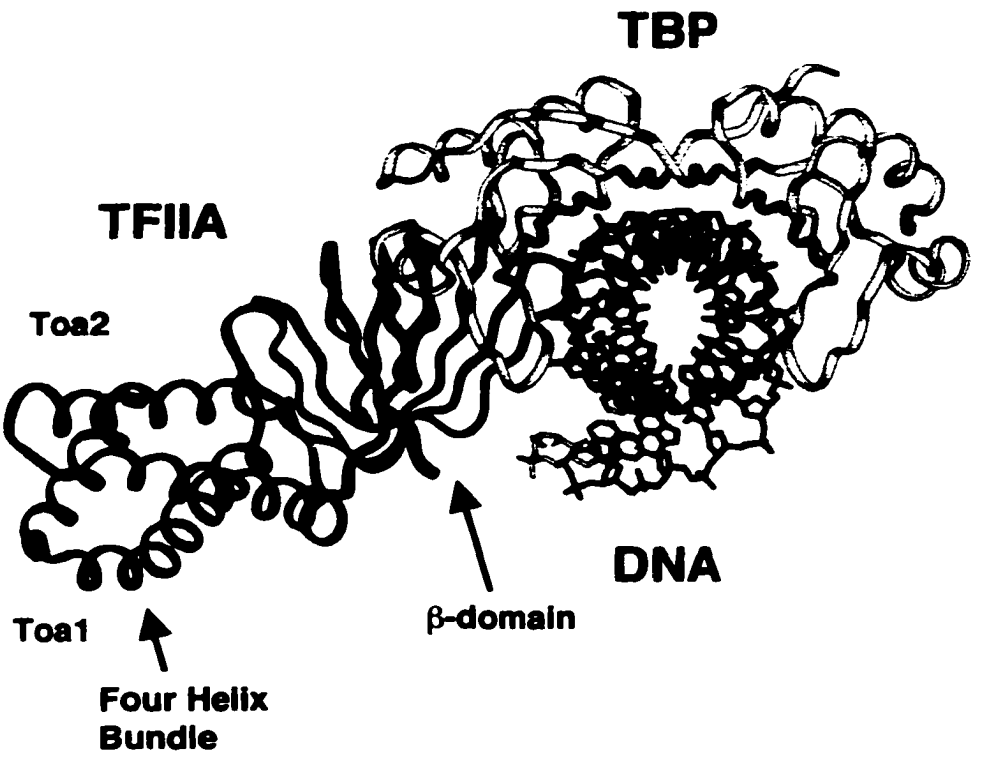

Figure 1.4 *Crystal structure of the TFIIA-TBP-DNA complex.* (A) TBP is shown in yellow ribbons and DNA is in black, the two subunits of TFIIA Toa1 and Toa2 are shown in blue and red respectively. The four-helix bundle domain (4HB) and ß-domain are indicated.

activators have been described *in vitro* (19, 49, 73). Furthermore, mutational studies in both TBP and TFIIA have demonstrated the importance of the TFIIA-TBP interaction for activated transcription *in vivo*. Interestingly, the requirement for TFIIA does not seem to be universal, as only some promoters seem to need TFIIA for activation while others do not (60, 83). This and the above findings suggest that TFIIA may play a critical role in activation by functioning as either an antirepressor or a coactivator.

1.14 TFIIA and TFIID interactions.

Many studies indicate that TFIIA influences PIC formation through interactions with TFIID. TFIIA can bind and stabilize TFIID at the core promoter by making contacts with TBP, DNA and perhaps TAFs (100). In humans these contacts can induce conformational changes in TFIID that cause enhanced binding and cause additional contacts on TATA-Inr containing promoters (28). In fact, our laboratory has recently described a direct interaction between TFIIA and TAF40 (53). This interaction seems to be important for transcription initiation at certain promoters in yeast. This finding is not surprising considering the three-dimensional structure of the human TFIID-TFIIA-TFIIB complex (1). This structure supports TFIIA-TAF interactions since TFIIA maps to a region of the complex that contains TAFs. Thus some experiments suggest a functional link between TAFs and TFIID, although the nature of these interactions is not well understood.

1.15 Statement of significance and thesis layout.

For my dissertation research I have chosen to answer many separate but related questions concerning factors involved in transcription initiation in the yeast *Saccharomyces cerevisiae*. First, I characterized a TBP mutant partially defective for TFIID formation *in vivo* (Chapter 2, and ref 76). I found that loss of multiple TAF subunits from TFIID causes only promoter specific and not global defects in transcription. The results from this work identify a novel tool that can be used to probe the function of TFIID in RNA pol II transcription. A future direction with this project has been initiated using genome-wide expression analysis. The results from this analysis will be critical in understanding the genome requirement for an intact TFIID complex in RNA pol II transcription.

In addition to analyzing the transcriptional requirements of TFIID, I studied a potential regulatory mechanism involving the oligomerization of the TBP subunit of TFIID. Reports of transcriptional regulation through TBP oligomerization have been published using human and yeast TBP (reviewed in 33). Thus I was interested in studying this aspect of regulation in yeast. Initially, I worked on developing assays designed to identify TBP dimers in yeast. Using these assays I was unable to identify TBP dimers from yeast whole-cell extracts. During the same time, Dr. Kevin Lumb and Kathleen Campbell were studying yeast TBP using biophysical techniques. The results from their analysis coincided with my findings in yeast. Thus, through a collaborative effort, we published a manuscript describing the oligomerization

properties of yeast TBP from both biophysical and genetic approaches (Chapter 3, and Ref 13). We concluded that TBP oligomerization is not a primary aspect of transcriptional regulation in yeast. This manuscript is significant in the field of transcription in that it provides an alternative conclusion concerning transcriptional regulation through TBP oligomerization.

The work described in chapters 4 and 5 are related in that they describe work aimed at characterizing the relationship between TFIIA and TFIID. There is a large body of work that suggests TFIID-TFIIA interactions are important for PIC assembly; however, there is little evidence on the precise nature of these interactions. In fact, the work described in Chapter 4 is the first report describing an interaction between TFIIA and TAFs in yeast (53). Furthermore, we show that this interaction has functional consequences for transcription. The experiments described in chapter 5 provide additional support and suggest future research concerning interactions between TFIIA and TAFs. In addition,it provides evidence for an additional TFIIA-TAF interaction.

Chapter 6 of this thesis describes a project designed to analyze TAF40, a TFIID-specific TAF (52). I have used a variety of techniques designed to map interaction surfaces of TAF40. The results from this analysis further define TAF40 and its role in RNA pol II transcription.

Finally, instead of dedicating an entire chapter of this thesis to future directions and perspectives, I chose to include these as separate sections at the ends of chapters 5 and 6.

1.16 References

1. Andell III, F., A. G. Ladurner, C. Inouye, R. Tjian and E. Nogales. 1999. Three-dimensional structures of the human TFIID-IIA-IIB complex. *Science* 286:2153-2156.

2. Apone, L. M., C.-A. Virbasius, F. C. Holstege, J. Wang, R. A. Young and M. R. Green. 1998. Broad, but not universal, transcriptional requirement for yTAF$_{II}$17, a histone H3-like TAF$_{II}$ present in TFIID and SAGA. *Molecular Cell* 2:653-661.

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

4. Auble, D. T. and S. Hahn. 1993. An ATP-dependent inhibitor of TBP binding to DNA. *Genes & Dev.* 7:844-856.

5. Auble, D. T., K. E. Hansen, C. G. F. Mueller, W. S. Lane, J. Thorner and S. Hahn. 1994. Mot1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism. *Genes & Dev.* 8:1920-1934.

6. Bagby, S., T. K. Mal, D. Liu, E. Raddatz, Y. Nakatani and M. Ikura. 2000. TFIIA-TAF Regulatory Interplay: NMR Evidence for Overlapping Binding Sites on TBP. *FEBS* 468:149-154.

7. Bai, Y., G. M. Perez, J. M. Beechem and P. A. Weil. 1997. Structure-function analysis of *TAF130*: Identification and characterization of a high-affinity TATA-Binding Protein interaction domain in the N terminus of yeast TAF$_{II}$130. *Molecular and Cellular Biology* 17:3081-3093.

8. Birck, C., O. Poch, C. Romier, M. Ruff, G. Mengus, A. Lavigne, I. Davidson and D. Moras. 1998. Human TAF(II)28 and TAF(II)18 interact through a histone fold encoded by atypical evolutionary conserved motifs also found in the SPT3 family. *Cell* 94:239-249.

9. Brand, M., C. Leurent, V. Mallouh, L. Tora and P. Schultz. 1999. Three-dimensional structures of the TAF$_{II}$-containing complexes TFIID and TFTC. *Science* 286:2151-2153.

10. Buratowski, S., S. Hahn, P. A. Sharp and L. Guarente. 1988. Function of a yeast TATA element-binding protein in a mammalian transcription system. *Nature* 334:37-42.

11. Burke, T. and J. Kadonaga. 1997. The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by $TAF_{II}60$ of Drosophila. *Genes Dev* 11:3020-3031.

12. Burley, S. K. and R. G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* 65:769-799.

13. Campbell, K. M., R. T. Ranallo, L. A. Stargell and K. J. Lumb. 2000. Reevaluation of Transcriptional Regulation by TATA-binding Protein Oligomerization: Predominance of Monomers. *Biochemistry* 39:2633-2638.

14. Cavallini, B., J. Huet, J. L. Plassat, A. Sentenac, J.-M. Egly and P. Chambon. 1988. A yeast activity can substitute for the HeLa TATA box factor. *Nature* 334:77-80.

15. Chasman, D. I., K. M. Flaherty, P. A. Sharp and R. D. Kornberg. 1993. Crystal structure of yeast TATA-binding protein and model for interaction with DNA. *Proc. Natl. Acad. Sci. U.S.A.* 90:8174-8178.

16. Chatterjee, S. and K. Struhl. 1995. Connecting a promoter-bound protein to the TATA-binding protein overrides the need for a transcriptional activation region. *Nature* 374:820-822.

17. Chen, W. and K. Struhl. 1988. Saturation mutagenesis of a yeast *his3* TATA element: genetic evidence for a specific TATA-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 85:2691-2695.

18. Chicca II, J., D. Auble and F. Pugh. 1998. Cloning and Biochemical Characterization of TAF-172, a Human Homolog of Yeast Mot1. *Molecular and Cellular Biology* 18:1701-1710.

19. Clemens, K. E., G. Piras, M. F. Radonovich, K. Sook Choi, J. F. Duvall, J. DeJong, R. Roeder and J. N. Brady. 1996. Interaction of the Human T-Cell Lymphtropic Virus Type 1 Tax Transactivator with Transcription Factor IIA. *Molecular and Cellular Biology* 16:4656-4664.

20. Coleman, R. A. and B. F. Pugh. 1997. Slow dimer dissociation of the TATA binding protein dictates the kinetics of DNA binding. *Proc. Natl. Acad. Sci. USA* 94:7221-7226.

21. Coleman, R. A., A. K. P. Taggart, L. R. Benjamin and B. F. Pugh. 1995. Dimerization of the TATA Binding Protein. *The Journal of Biological Chemistry* 270:13842-13849.

22. Colgan, J. and J. L. Manley. 1992. TFIID can be rate limiting in vivo for TATA-containing, but not TATA-lacking, RNA polymerase II promoters. *Genes & Dev.* 6:304-315.

23. Comai, L., N. Tanese and R. Tjian. 1992. The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. *Cell* 68:965-976.

24. Cortes, P., O. Flores and D. Reinberg. 1992. Factors involved in specific transcription by mammalian RNA polymerase II: purification and analysis of transcription factor IIA and identification of transcription factor IIJ. *M.C.B.* 12:413-421.

25. Davis, J. L., R. Kunisawa and J. Thorner. 1992. A presumptive helicase (*MOT1* gene product) affects gene expression and is required for viability in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 12:1879-1892.

26. DeJong, J., R. Bernstein and R. Roeder. 1995. Human general transcription factor TFIIA: characterization of a cDNA encoding the small subunit and requirement for basal and activated transcription. *Proc Natl Acad Sci U S A* 92:3313-3317.

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

28. Emami, K., A. Jain and S. Smale. 1997. Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization. *Genes Dev* 11:3007-19.

29. Geiger, J. H., S. Hahn, S. Lee and P. B. Sigler. 1996. Crystal structure of the yeast TFIIA/TBP/DNA complex. *Science* 272:830-836.

30. Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. Steger, J. C. Reese, J. R. Yates III and J. L. Workman. 1998. A subset of TAF_{II}s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. *Cell* 94:45-53.

31. Green, M. 2000. TBP-associated Factors (TAF_{II}s): Multiple, Selective Transcriptional Mediators in Common Complexes. *TIBS* 25:59-63.

32. Hahn, S., S. Buratowski, P. A. Sharp and L. Guarente. 1989. Isolation of the gene encoding the yeast TATA binding protein TFIID: A gene identical to the *SPT15* suppressor of Ty element insertions. *Cell* 58:1173-1181.

33. Hampsey, M. 1998. Molecular genetics of the RNA polymerase II genreal transcriptional machinery. *Microbiology and Molecular Biology Reviews* 62:465-503.

34. Hansen, S. and R. Tjian. 1995. TAFs and TFIIA mediate differential utilization of the tandem Adh promoters. *Cell* 82:565-75.

35. Hernandez, N. 1993. TBP, a universal eukaryotic transcription factor? *Genes & Dev.* 7:1291-1308.

36. Hoffmann, A., C. Chiang, T. Oelgeschlager, X. Xie, S. K. Burley, Y. Nakatani and R. G. Roeder. 1996. A histone octamer-like structure within TFIID. *Nature* 380:356-359.

37. Hoffmann, A., E. Sinn, Y. T. J. Wang, A. Roy, M. Horikoshi and R. G. Roeder. 1990. Highly conserved core domain and unique N-terminus with presumptive regulatory motifs in a human TATA factor (TFIID). *Nature* 346:387-390.

38. Holstege, F. and R. Young. 1999. Transcriptional regulation: Contending with complexity. *Procedings from the National Academy of Sciences* 96:2-4.

39. Holstege, F. C. P., E. G. Jennings, C. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander and R. A. Young. 1998. Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* 95:717-728.

40. Horikoshi, M., C. K. Wang, H. Fujii, J. A. Cromlish, P. A. Weil and R. G. Roeder. 1989. Purification of a yeast TATA box-binding protein that exhibits human transcription factor IID activity. *Proceedings of the National Academy of Sciences of the United States of America* 86:4843-7.

41. Imbalzano, A. N., K. S. Zaret and R. E. Kingston. 1994. Transcription factor (TF) IIB and TFIIA can independently increase the affinity of the TATA-binding protein for DNA. *J. Biol. Chem.* 269:8280-8286.

42. Kang, J. J., D. T. Auble, J. A. Ranish and S. Hahn. 1995. Analysis of yeast transcription factor TFIIA: Distinct functional regions and a Polymerase II-specific role in basal and activated transcription. *Mol. Cell. Biol.* 15:1234-1243.

43. Kassavetis, G. A., C. A. P. Joazeiro, M. Pisano, E. P. Geiduschek, T. Colbert, S. Hahn and J. A. Blanco. 1992. The role of the TATA-binding protein in the assembly and function of the multisubunit yeast RNA polymerase transcription factor, TFIIIB. *Cell* 71:1055-1064.

44. Kim, J. L., D. B. Nikolov and S. K. Burley. 1993. Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* 365:520-527.

45. Kim, Y., J. H. Geiger, S. Hahn and P. B. Sigler. 1993. Crystal structure of a yeast TBP-TATA box complex. *Nature* 365:512-520.

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

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

48. Klein, C. and K. Struhl. 1994. Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. *Science* 266:280-282.

49. Kobayashi, N., T. G. Boyer and A. J. Berk. 1995. A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly. *Mol. Cell. Biol.* 15:6465-6473.

50. Kokubo, T., M. J. Swanson, J.-I. Nishikawa, A. G. Hinnebusch and Y. Nakatani. 1998. The yeast TAF145 inhibitory domain and TFIIA competitively bind to TATA-Binding Protein. *Molecular and Cellular Biology* 18:1003-1012.

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

52. Komarnitsky, P. B., B. Michel and S. Buratowski. 1999. TFIID-specific yeast TAF40 is essential for the majority of RNA polymerase II-mediated transcription in vivo. *Genes and Dev.* 13:2484-2489.

53. Kraemer, S., R. T. Ranallo, R. Ogg and L. A. Stargell. 2000. TFIIA Interacts with TFIID via association with TBP and TAF40. *Submitted*

54. Kuras, L. and K. Struhl. 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. *Nature* 399:609-613.

55. Lee, D. K., J. Dejong, S. Hashimoto, M. Horikoshi and R. G. Roeder. 1992. TFIIA induces conformational changes in TFIID via interactions with the basic repeat. *Mol. Cell. Biol.* 12:5189-5196.

56. Lee, M. and K. Struhl. 1995. Mutations on the DNA-binding surface of TBP can specifically impair the response to acidic activators *in vivo*. *Mol. Cell. Biol.* 15:5461-5469.

57. Li, X. Y., A. Virbasius, X. Zhu and M. R. Green. 1999. Enhancement of TBP binding by activators and general transcription factors. *Nature* 399:605-609.

58. Lieberman, P. M., M. C. Schmidt, C. C. Kao and A. J. Berk. 1991. Two distinct domains in the yeast transcription factor IID and evidence for a TATA box-induced conformational change. *Mol. Cell. Biol.* 11:63-74.

59. Liu, D., R. Ishima, K. I. Tong, S. Bagby, T. Kokubo, D. R. Muhandiram, L. E. Kay, Y. Nakatani and M. Ikura. 1998. Solution structure of a TBP-$TAF_{II}230$ complex: Protein mimicry of the minor groove surface of the TATA box unwound by TBP. *Cell* 94:573-583.

60. Liu, Q., S. E. Gabriel, K. L. Roinick, R. D. Ward and K. M. Arndt. 1999. Analysis of TFIIA function in vivo: Evidence for a role in TATA-binding Protein recruitment and gene-specific activation. *Mol. Cell. Biol.* 19:8673-8685.

61. Lobo, S. M., M. Tanaka, M. L. Sullivan and N. Hernandez. 1992. A TBP complex essential for transcription from TATA-less but not TATA-containing RNA polymerase III promoters is part of the TFIIIB fraction. *Cell* 71:1029-1040.

62. Madison, J. M. and F. Winston. 1997. Evidence that Spt3 Functionally Interacts with Mot1, TFIIA, and TATA-Binding Protein To Confer Promotor-Specific Transcriptional Control in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* 17:287-295.

63. Martinez, E., T. Kundu, J. Fu and R. Roeder. 1998. A Human SPT3-$TAF_{II}31$-GCN5-L Acetylase Complex Distinct from Transcription Factor IID. *Journal of Biological Chemistry* 273:23781-23785.

64. Michel, B., P. Komarnitsky and S. Buratowski. 1998. Histone-like TAFs are essential for transcription in vivo. *Molecular Cell* 2:663-672.

65. Moqtaderi, Z., Y. Bai, D. Poon, P. A. Weil and K. Struhl. 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* 383:188-191.

66. Moqtaderi, Z., M. Keaveney and K. Struhl. 1998. The histone H3-like TAF is broadly required for transcription in yeast. *Mol. Cell.* 2:

67. Moqtaderi, Z., J. D. Yale, K. Struhl and S. Buratowski. 1996. Yeast homologues of higher eukaryotic TFIID subunits. *Proc. Natl. Acad. Sci. USA* 93:14654-14658.

68. Natarajan, K., B. Jackson, E. Rhee and A. Hinnebusch. 1998. $yTAF_{II}61$ has a general role in RNA polymerase II transcription and is required by Gcn4p to recruit the SAGA coactivator complex. *Mol Cell* 2:683-692.

69. Nikolov, D. B. and S. K. Burley. 1997. RNA polymerase II transcription initiation: A structural view. *Proc. Natl. Acad. Sci. USA* 94:15-22.

70. Oelgeschlager, T., Y. Tao, Y. K. Kang and R. G. Roeder. 1998. Transcription activation via enhanced preinitiation complex assembly in a human cell-free system lacking TAF_{II}s. *Mol. Cell.* 1:925-931.

71. Ogryzko, V. V., T. Kotani, X. Zhang, R. L. Schiltz, T. Howard, X.-J. Yang, B. H. Howard, J. Qin and Y. Nakatani. 1998. Histone-like TAFs within the PCAF histone acetylase complex. *Cell* 94:35-44.

72. Orphanides, G., T. Lagrange and D. Reinberg. 1996. The general transcription factors of RNA polymerase II. *Genes and Development* 10:2657-2683.

73. Ozer, J., P. A. Moore, A. H. Bolden, A. Lee, C. A. Rosen and P. M. Lieberman. 1994. Molecular cloning of the small (g) subunit of human TFIIA reveals functions critical for activated transcription. *Genes & Dev.* 8:2324-2335.

74. Poon, D., Y. Bai, A. M. Campbell, S. Bjorklund, Y. J. Kim, S. Zhou, R. D. Kornberg and P. A. Weil. 1995. Identification and characterization of a TFIID-like multiprotein complex from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* 92:8224-8228.

75. Pugh, B. F. and R. Tjian. 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* 61:1187-1197.

76. Ranallo, R., K. Struhl and L. Stargell. 1999. A TATA-binding protein mutant defective for TFIID complex formation in vivo. *Mol Cell Biol* 19:3951-3957.

77. Reddy, P. and S. Hahn. 1991. Dominant negative mutations in yeast TFIID define a bipartite DNA-binding region. *Cell* 65:349-357.

78. Reese, J. C., L. Apone, S. S. Walker, L. A. Griffin and M. R. Green. 1994. Yeast TAF_{II}s in a multisubunit complex required for activated transcription. *Nature* 371:523-527.

79. Reinberg, D. and R. G. Roeder. 1987. Factors involved in specific transcription by mammalian RNA polymerase II: Purification and functional analysis of initiation factors IIB and IIE. *J. Biol. Chem.* 262:3310-3321.

80. Sanders, L. S. and A. P. Weil. 2000. Identification of Two Novel TAF Subunits of the Yeast *Saccharomyces cerevisiae* TFIID Complex. *Journal of Biological Chemistry* 275:13895-13900.

81. Shen, W.-C. and M. R. Green. 1997. Yeast $TAF_{II}145$ functions as a core promoter selectivity factor, not a general coactivator. *Cell* 90:615-624.

82. Singer, V. L., C. R. Wobbe and K. Struhl. 1990. A wide variety of DNA sequences can functionally replace a yeast TATA element for transcriptional activation. *Genes & Dev.* 4:636-645.

83. Stargell, L. A., Z. Moqtaderi, D. Dorris, R. Ogg and K. Struhl. 2000. TFIIA Has Activator and Core Promoter Functions *in Vivo*. *In press*

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

85. Sterner, D., P. Grant, S. Roberts, L. Duggan, B. R., L. Pacella, F. Winston, J. Workman and S. Berger. 1999. Functional Oraganization of the Yeast SAGA Complex: Distinct Components Involved in Structural Integrity, Nucleosome Acetylation, TATA-binding Protein Interaction. *Molecular and Cellular Biology* 19:86-98.

86. Strubin, M. and K. Struhl. 1992. Yeast TFIID with altered DNA-binding specificity for TATA elements. *Cell* 68:721-730.

87. Struhl, K. 1995. Yeast transcriptional regulatory mechanisms. *Ann. Rev. Genet.* 29:651-674.

88. Struhl, K. and Z. Moqtaderi. 1998. The TAFs in the HAT. *Cell* 94:1-4.

89. Taggart, A. K. and B. F. Pugh. 1996. Dimerization of TFIID when not bound to DNA. *Science* 272:1331-1333.

90. Taggart, A. K. P., T. S. Fisher and B. F. Pugh. 1992. The TATA-binding protein and associated factors are components of Pol III transcription factor TFIIIB. *Cell* 71:1015-1028.

91. Tan, S., Y. Hunziker, D. F. Sargent and T. J. Richmond. 1996. Crystal structure of a yeast TFIIA/TBP/DNA complex. *Nature* 381:127-134.

92. Verrijzer, C. P., J.-L. Chen, K. Yokomori and R. Tjian. 1995. Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II. *Cell* 81:1115-1125.

93. Verrijzer, C. P. and R. Tijan. 1996. TAFs mediate transcriptional activation and promoter selectivity. *Trends in Biochemical Science* 21:338-342.

94. Walker, S. S., J. C. Reese, L. M. Apone and M. R. Green. 1996. Transcription activation in cells lacking TAF_{II}s. *Nature* 382:185-188.

95. Walker, S. S., W.-C. Shen, J. C. Reese, L. M. Apone and M. R. Green. 1997. Yeast $TAF_{II}145$ required for transcription of G1/S cyclin genes and regulated by the cellular growth state. *Cell* 90:607-614.

96. Weideman, C. A., R. C. Netter, L. R. Benjamin, J. J. McAllister, L. A. Schmiedekamp, R. A. Coleman and B. F. Pugh. 1997. Dynamic interplay of TFIIA, TBP and TATA DNA. *J Mol Biol* 271:61-75.

97. Wieczorek, E., M. Brand, X. Jacq and L. Tora. 1998. Function of TAF(II)-containing complex without TBP in transcription by RNA polymerase II. *Nature* 393:187-91.

98. Wobbe, C. R. and K. Struhl. 1990. Yeast and human TATA-binding proteins have nearly identical DNA sequence requirements for transcription *in vitro*. *Mol. Cell. Biol.* 10:3859-3867.

99. Xie, X., T. Kokubo, S. L. Cohen, U. A. Mirza, A. Hoffmann, B. T. Chait, R. G. Roeder, Y. Nakatani and S. K. Burley. 1996. Structural similarity between TAFs and the heterotetrameric core of the histone octamer. *Nature* 380:316-322.

100. Yokomori, K., A. Admon, J. A. Goodrich, J. L. Chen and R. Tjian. 1993. Drosophila TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex. *Genes Dev* 7:2235-45.

101. Zhou, Q., P. M. Lieberman, T. G. Boyer and A. J. Berk. 1992. Holo TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. *Genes & Dev.* 6:1964-1974.

CHAPTER 2

A TATA-BINDING PROTEIN MUTANT DEFECTIVE FOR TFIID COMPLEX FORMATION IN VIVO

This chapter was published in *Molecular and Cellular Biology*. The text of the manuscript is presented exactly as it appeared in the Journal. All figures that appeared in the publication are included. In addition, all experiments labeled as "data not shown" are included as supplemental figures. I performed all experiments in this chapter, except for the initial isolation of the TBP mutant, which was done by Dr. Stargell in Dr. Struhl's laboratory. I also wrote the manuscript, which was subsequently edited by Drs. Stargell and Struhl. The literature citation for this chapter is:

> Ranallo, R.T., Struhl, K., and L.A. Stargell, L.A. 1999. A TATA-Binding Protein Mutant Defective for TFIID Complex Formation In Vivo. *Mol. Cell. Biol.* 19 (6):3951-3957.

2.1 Abstract

Using an intragenic complementation screen, we have identified a temperature-sensitive TATA-binding protein (TBP) mutant (K151L,K156Y) that is defective for interaction with certain yeast TBP-associated factors (TAFs) at the restrictive temperature. The K151L,K156Y mutant appears to be functional for RNA polymerase I (Pol I) and Pol III transcription, and it is capable of supporting Gal4-activated and Gcn4-activated transcription by Pol II. However, transcription from certain TATA-containing and TATA-less Pol II promoters is reduced at the restrictive temperature. Immunoprecipitation analysis of extracts prepared after culturing cells at the restrictive temperature for 1 h indicates that the K151L,K156Y derivative is severely compromised in its ability to interact with TAF130, TAF90, TAF68/61, and TAF25 while remaining functional for interaction with TAF60 and TAF30. Thus, a TBP mutant that is compromised in its ability to form TFIID can support the response to Gcn4 but is defective for transcription from specific promoters *in vivo*.

2.2 Introduction

Transcriptional initiation by eukaryotic RNA polymerase II (Pol II) requires general transcription factors (TFIID, -A, -B, -E, -F, and -H) that

nucleate on the TATA promoter element to form the preinitiation complex that is necessary for accurate positioning and initiation by Pol II (for a review, see reference 34). This process begins with the sequence-specific binding of TFIID, a multiprotein complex composed of TATA-binding protein (TBP) and approximately 10 RNA Pol II-specific TBP-associated factors (TAFs) (reviewed in reference 5). TAFs are a set of phylogenetically conserved proteins found in humans, flies, and yeast, ranging in molecular mass from 15 to 250 kDa (for a recent review, see reference 49). Recruitment of TBP, presumably in the TFIID complex, has been proposed to be a rate-limiting step in formation of the preinitiation complex (6, 8, 25, 26, 60).

Two distinct mechanisms have been proposed for assembly of the preinitiation complex, depending on the presence or absence of a canonical TATA element (5). Initiation from a TATA-containing promoter is dependent on strong TBP-DNA contacts for recognition and binding of TFIID to the TATA box. However, for TATA-less promoters, sequence-specific recognition of the core promoter by TBP does not occur, suggesting that TAF-DNA interactions play an important role (5). *In vitro*, TFIID but not TBP is able to recognize and support transcription from TATA-less promoters (62). Moreover, in Drosophila TFIID, specific TAFs have been implicated in making contacts with DNA in sequences overlapping the transcription start site (4, 5, 52). In addition, mutations that hinder the ability of TBP to recognize a TATA box do not affect transcription from TATA-less promoters even though TFIID is required (29). Consistent with this, depletion of certain individual yeast TAFs

in vivo causes a reduction in transcription from promoters lacking consensus TATA boxes (31, 32). Thus, it seems that transcription from TATA-less promoters does not depend on strong TBP-DNA interactions but rather depends on TAF-DNA and TAF-TBP interactions.

In addition to their role in TATA-less transcription, TAFs have been implicated in the response to activators. Transcriptional activators function by increasing recruitment and stabilizing the Pol II machinery at promoters (reviewed in references 40, 48, and 53). *In vitro*, activators can interact with many components of the transcription machinery, including TBP, TAFs, TFIIA, TFIIB, TFIIF, TFIIH, Pol II, and the SAGA complex (12, 15, 17, 19, 21, 28, 39, 47, 61). Initial biochemical studies suggested that TAFs function as coactivators required for activator-dependent recruitment of TBP, since TFIID but not TBP could support high levels of transcription in the presence of an activator. Further, it was hypothesized that TAFs can serve as direct targets for activators, because individual TAFs can interact directly with activator proteins (for reviews, see references 5 and 53), and partial TFIID complexes reconstituted from a subset of TAFs can mediate activator-dependent transcription (7). However, TAFs are not absolutely required for the response to activators *in vitro*, because activation can occur in reactions lacking TAFs (24, 27, 37, 58, 59).

In vivo depletion studies indicate that certain individual TAFs are not generally required for response to activators (31, 54). Instead, depletion of certain TAFs results in specific effects on transcription, depending on the

particular TAF targeted. In the case of depletion of TAF130, a loss of transcription from genes lacking a canonical TATA element (31), from genes encoding small-subunit ribosomal proteins (RPS genes), and from various cyclin genes (43, 55) was observed. The TAF130 dependence of these genes is mediated by the core promoter, not the initiator or the activator binding sites. In mammalian cells, TAF250, the homolog of yeast TAF130, is important for transcription of selected genes, including those involved in cell cycle control (50, 56). Taken together, these data suggest that TAFs play a critical role in gene-specific as well as TATA-less transcription.

Aside from their role in TFIID, certain TAFs are present in the yeast SAGA and mammalian PCAF histone acetyltransferase complexes (16, 38). *In vivo* depletion of TAF61/68, a component of yeast SAGA, indicates that this TAF is necessary for normal enzymatic activity but is dispensable for interaction with TBP or activation domains. Furthermore, depletion of TAF17, which is also present in SAGA, has broad effects on transcription in yeast cells (1, 30, 32). Thus, it seems that TAFs can serve a variety of roles in the transcription process, depending on the complexes in which they reside.

Here we present the characterization of a TBP mutant that is defective for TFIID formation *in vivo*. Our studies differ from previous *in vivo* studies in that we analyzed transcription after TFIID disruption which removes multiple TAFs, not after individual TAF depletion. This approach permits the removal of TAFs from TFIID but not from SAGA or other TAF-containing complexes. We show that TFIID disruption does not prevent the response to

acidic activators, but it causes transcriptional defects at certain cell cycle-dependent and TATA-less promoters. These results support previous findings that the TBP-TAF interactions may not be essential for activated transcription *in vivo* and may instead play a role in gene-specific transcription.

2.3 Material and Methods

2.3a Yeast strains and DNA constructs.

The parental strain of Saccharomyces cerevisiae was BY2 (9), which contains a deletion of the chromosomal TBP locus covered by a URA3-marked 2.4-kb EcoRI-BamHI genomic fragment of the TBP gene locus containing the Pol III-defective allele in which the codon for phenylalanine at position 155 is replaced by that for serine (F155S). Briefly, the parental strain, which is temperature sensitive (ts), was transformed with a set of TBP mutant libraries generated by regional codon randomization on a TRP1-marked plasmid (11). Strains that could grow at the restrictive temperature were isolated, the F155S allele was shuffled out by plating to 5-fluoro-orotic acid, and the resulting strains were tested for a ts phenotype. This last step is necessary because wild-type copies of TBP present in the library would also complement the F155S allele by providing Pol III function. Five TBP mutants generated in this screen have been previously described (45, 46). The sixth mutant is described here and has two substitutions: lysine at position 151 is

replaced with leucine, and lysine at position 156 is replaced with tyrosine (K151L,K156Y). The K151L and K156Y single mutants were generated by site-directed mutagenesis by PCR. PCR products were generated and cloned by using an engineered BamHI site in the TBP-coding sequence. The plasmid shuffle technique was used to introduce the single-substitution TBP molecules into yeast. The reporter construct used for the lacZ assays is YCp86-Sc3801 (44).

2.3b Phenotypic analysis.

The merodiploid strain (containing both the F155S and K151L,K156Y derivatives), along with wild-type TBP and vector (pRS316)-containing strains, were grown in the appropriate media. Cells (10-fold serial dilutions) were spotted onto dropout plates lacking the appropriate amino acids (uracil and tryptophan) and incubated at the indicated temperatures. For the Pol I assay, the plasmid PNOY103 (36) or vector (pRS316) was transformed into both the K151L,K156Y and wild-type TBP-containing strains and colony purified on 2% galactose-containing dropout medium lacking uracil and tryptophan. Cells were then spotted onto galactose-containing medium and incubated at the indicated temperatures. Growth assays of both wild-type TBP-and K151L,K156Y TBP-containing strains were performed as described above.

2.3c Transcriptional analysis.

In most cases RNA analysis was done by quantitative S1 nuclease analysis with approximately 30 to 75 μg of RNA (20). In the temperature shift experiments, cells were heat shocked for 15 min, returned to 30°C for 1 h, and then shifted to 38°C for 1 h. Total RNA prepared by hot-phenol extraction was quantitated by A_{260}. For 3-aminotriazole (AT) induction done at the permissive temperature, strains were grown overnight in synthetic complete medium in the presence or absence of 15 mM AT. Activation competency under the restrictive conditions was investigated by growing the cells overnight in synthetic complete medium, and the restrictive-condition protocol described above was performed. After the 1-h incubation at 38°C, 15 mM AT was added and the cells were incubated for an additional hour at 38°C. The probes used in the RNA analysis are listed in the figure legends. The RNA amounts in each reaction mixture were normalized to the RNA levels obtained from a probe to the intron of the tryptophan tRNA gene ($tRNA^W$).

2.3d Immunoprecipitation and immunoblotting.

Strains were grown to log phase (optical density at 600 nm = 0.1 to 0.2) at 30°C and then subjected to a 1-h heat shock as described above. Cells were harvested before and after the heat shock, and whole-cell extracts were prepared by glass bead lysis in 450 mM Tris-acetate (pH 7.8)-150 mM potassium acetate-60% glycerol, 3 mM EDTA (pH 8.0)-3 mM dithiothreitol-1

mM phenylmethylsulfonyl fluoride (33). Immunoprecipitations were performed as described previously (33), except that 240 µg of extract was used in the immunoprecipitation reaction. Antigen-antibody complexes were recovered by centrifugation and washed four times with 1 ml of buffer A containing 125 mM potassium acetate and 1% Nonidet P-40 (Sigma). Samples were boiled in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer, and proteins were separated on either 7.5 or 12% gels and electroblotted to nitrocellulose. Antibody reactions were performed by standard techniques, and blots were developed by using chemiluminescent detection according to the recommendations of the manufacturer (Pierce). TBP antibodies used in both the immunoprecipitations and Western blots were generated in rabbits by Cocalico Biological (Reamstown, Pa.) with purified recombinant TBP. Yeast TAF antibodies used for immunoblot analysis were kindly provided by Michael Green.

2.4 Results

2.4a Isolation and characterization of a TBP mutant that complements a Pol III defect.

An intragenic complementation screen was used to isolate ts TBP mutants that are functional for Pol III transcription at the restrictive temperature, thereby eliminating any mutants that are structurally

compromised under the restrictive conditions. Complementation depends on the ability of two ts TBP mutants, each of which confers a different functional defect, to support cell viability when they are present in the cell at the same time (10). Six ts mutants were isolated from this screen, five of which have been previously characterized and shown to be activation defective at the permissive temperature (45, 46). Here we describe the final TBP mutant, in which the lysine at position 151 is changed to leucine and the lysine at position 156 is changed to tyrosine (K151L,K156Y).

In keeping with the criteria of the screen, the K151L,K156Y derivative is a ts mutant of TBP that is able to complement the growth defect of TBP-F155S, a TBP mutant that is defective for Pol III transcription at the restrictive temperature (Fig. 2.1A). Substitutions at both lysine 151 and lysine 156 are required to produce the ts phenotype, because the single substitutions support efficient cell growth at the restrictive temperature (Fig. 2.1B). As expected, the K151L,K156Y derivative can also complement, albeit to lower levels than wild-type TBP, the molecular defect (i.e., the loss in transcription by Pol III) caused by the F155S allele (Fig. 2.1C).

To determine if a loss of Pol I transcription was the cause of the ts phenotype conferred by the K151L,K156Y mutant, we utilized a plasmid-based system in which the rRNAs are synthesized from a Pol II promoter (36). Briefly, a construct containing the 35S rRNA gene under the control of a Pol II, galactose-inducible promoter is able to rescue the growth defect

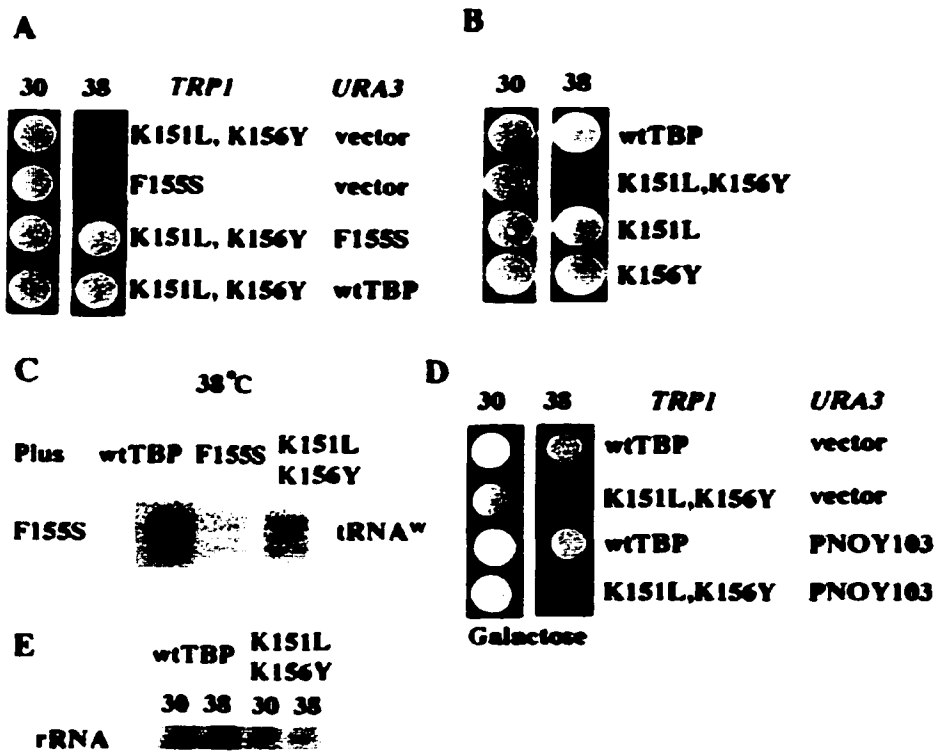

Figure 2.1 Isolation and characterization of a TBP mutant that complements a TBP mutant with a Pol III defect. (A) Growth conferred by the indicated TBP derivatives and wild-type (wt) TBP at both 30 and 38°C. Cells were grown overnight in liquid medium, and approximately 105 cells were spotted onto the medium lacking the appropriate markers. (B) Growth conferred by TBP derivatives singly substituted at position151 or 156. (C) S1 nuclease analysis of $tRNA^W$ transcription with 30 µg of RNA from the indicated TBP derivative after shifting cultures to 38°C for 1 h. (D) Mutant and wild-type strains containing PNOY103 (see text) were grown overnight in liquid medium (2% galactose), spotted to galactose medium, and incubated at 30 and 38°C. (E) S1 nuclease analysis of an unstable portion of the rRNA transcript with 70 µg of RNA from the indicated strains after incubation at 30 or 38°C for one hour.

(inviability) of a mutant that is lacking the largest subunit of Pol I. If the K151L,K156Y derivative is strictly defective for Pol I transcription, then this Pol II-driven rRNA transcript should rescue the ts phenotype when the cells are cultured in galactose-containing medium. However, the presence of the construct causes no change in viability at the restrictive temperature (Fig. 2.1D). Transcription of rRNA by Pol I was also examined directly (Fig. 2.1E). Steady-state levels of an unstable portion of the rRNA transcript were twofold lower under the permissive conditions in the K151L,K156Y strain than in the wild type. It should be noted that under these conditions (permissive), the K151L,K156Y strain grows indistinguishably from the wild-type strain. A slight decrease (1.5-fold) in the level of the rRNA transcript was observed in the K151L,K156Y strain after the 1-h temperature shift. Since these changes were relatively minor (especially compared to the more dramatic changes described below), and taken together with the results with the Pol II-driven rRNA construct, this suggests that the ts phenotype is not due strictly to a defect in Pol I transcription.

Molecular modeling of lysine 151 and lysine 156 on the TBP-TATA cocrystal structure indicates that both residues are located on the upper surface of TBP and exposed to the solvent (Fig. 2.2). Consistent with this, gel shift experiments indicate that the K151L,K156Y derivative and wild-type TBP bind the TATA element with comparable affinities (data not shown). In addition, the mutations map to a surface that is distinct from the surfaces that directly interact with TFIIA and TFIIB (13, 35, 51).

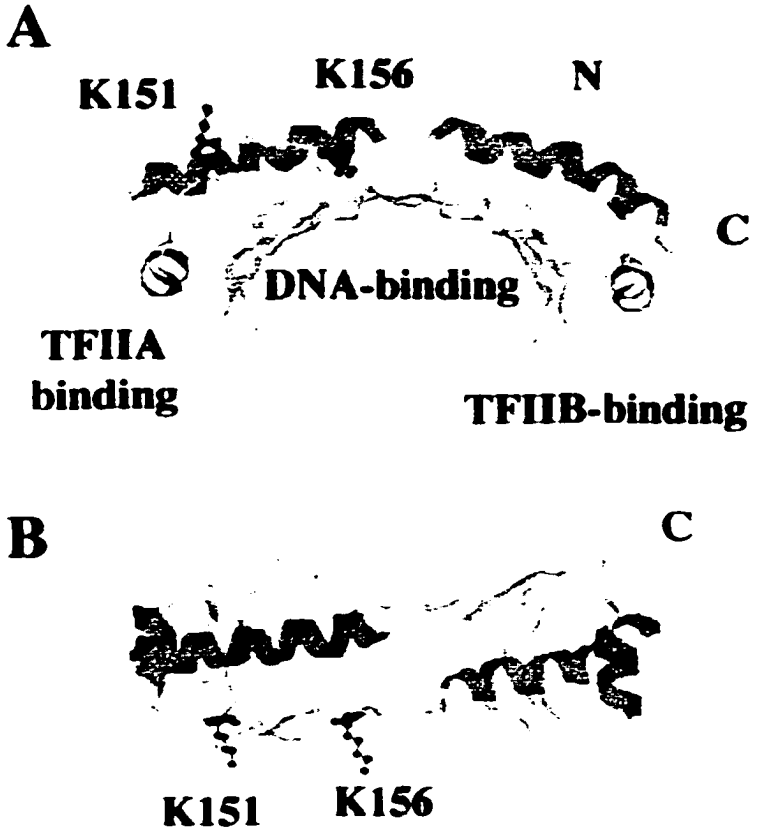

Figure 2.2 Molecular modeling of the K151L,K156Y derivative by using the TBP-DNA cocrystal structure (23) (Rasmol 2.6). (A) TBP is shown as a ribbon drawing, with residues K151 and K156 shown in ball-and-stick format. Known binding sites for DNA, TFIIA, and TFIIB and the C and N termini are indicated (13, 35, 51). (B) Top view of TBP (view in panel A rotated 90° forward)

2.4b The K151L,K156Y mutant is functional for the response to acidic activators at the permissive temperature.

Since the five TBP mutants previously characterized from this complementation screen exhibit activation defects at the permissive temperature (45, 46), we determined whether the K151L,K156Y derivative could respond to acidic activators *in vivo*. The K151L,K156Y mutant strain grows robustly under conditions that require functional interactions with a number of different acidic activators (Fig. 2.3A), suggesting that the mutant allele is competent for activated transcription. The response to Gal4, which activates transcription in the presence of galactose, was assayed by using the lacZ reporter YCp86-Sc3801 (44). In the presence of galactose, the wild-type strain produced 430 ± 30 U (mean ± standard deviation) of β-galactosidase activity. Similarly, the K151L,K156Y strain produced 425 ± 20 U of activity (Activities for culturing in glucose were <1 U for both strains.). Thus, the response to Gal4 conferred by the K151L,K156Y mutant is indistinguishable from the response conferred by wild-type TBP. We also observed that the K151L,K156Y derivative is responsive to the activator Gcn4. Cells grown in the presence of AT, a competitive inhibitor of the *HIS3* gene product that results in activation of the *HIS3* gene, showed wild-type levels of *HIS3* transcription (Fig. 2.3B). Thus, the K151L,K156Y mutant is functional for activated transcription at the permissive temperature.

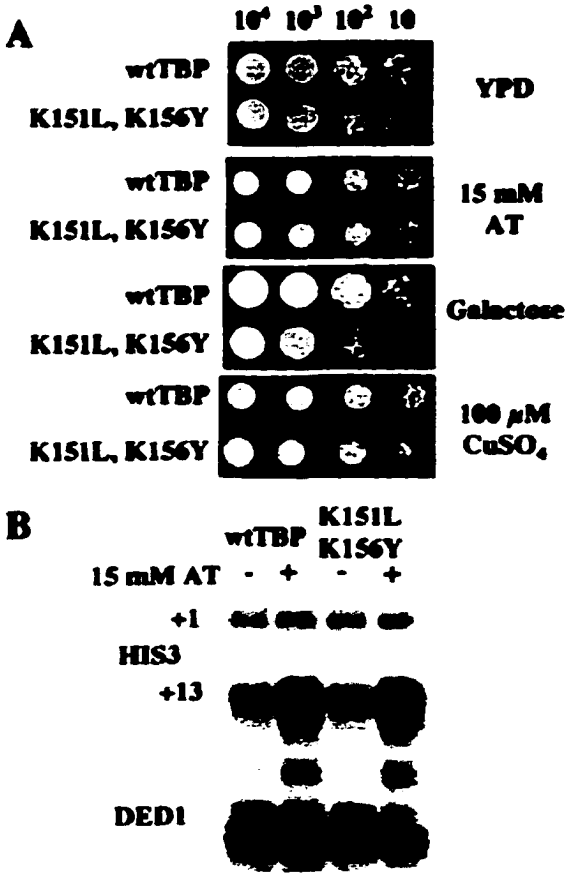

Figure 2.3 The K151L,K156Y mutant is functional for activated transcription at the permissive temperature. wt, wild type. (A) Strains were spotted on plates containing the media indicated at the density shown, followed by incubation at 30°C. Yeast extract-peptone-dextrose (YPD) is used as a growth rate indicator on rich media. Growth on 15 mM AT, galactose, and $CuSO_4$ requires functional interactions with the Gcn4, Gal4, and Ace1 transcription factors, respectively. (B) Analysis of Gcn4-dependent activation of HIS3 transcription. Constitutive HIS3 expression initiates equally from both the +1 and +13 start sites, whereas Gcn4-activated transcription is mediated primarily through the +13 initiation site. Strains were grown to log phase in synthetic complete medium in either the presence (+) or absence (-) of 15 mM AT. Total RNA (30 μg) was hybridized with 100-fold excesses of *HIS3* and *DED1* probes and then subjected to S1 nuclease digestion. The *DED1* transcript is not affected by AT and is used as a loading control in this experiment.

2.4c The K151L,K156Y mutant is defective for transcription from certain Pol II promoters.

We next compared the transcriptional profiles of wild-type TBP and the K151L,K156Y derivative at the restrictive temperature. After a 1-h incubation at the restrictive temperature, there was a significant reduction in message accumulation of the *HIS3* (both the +1 and +13 transcripts), *CMD1*, *MOT1*, and *RPS4* genes (Fig. 2.4A). *DED1* mRNA levels also decreased (slightly less than twofold). In contrast, levels of the *ADH1*, *PGK1*, *ENO2*, *HTA2*, and *FUR1* messages were not affected at all. It should be noted that the apparent half-life of each of these messages is in the range of 10 to 22 min (18), with the exception of the *CMD1* message, which has an apparent half-life of 41 min. Since *CMD1* was one of the transcripts that decreased in the mutant strain after the 1-h temperature shift, this longer half-life is evidently not an issue.

Can we discern a pattern in the subset of the genes that are very sensitive to the K151L,K156Y substitution under the restrictive conditions? Interestingly, the promoters that are sensitive appear to have transcription rates prior to the temperature shift that are lower than those of promoters that are unaffected. Based on relative RNA levels from our quantitative S1 analyses, the *HIS3*, *MOT1*, and *CMD1* genes are fairly weakly transcribed. As a frame of reference, the absolute number of *HIS3* messages has been calculated to be around seven messages per cell (20). Also, this set of promoters that are sensitive to the K151L,K156Y substitutions includes a

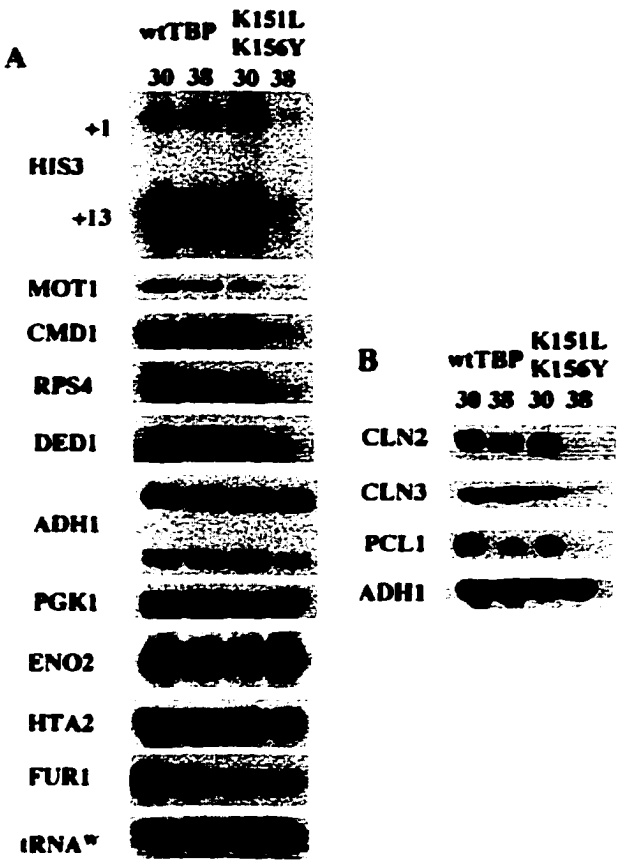

Figure 2.4 Transcriptional analysis of the K151L,K156Y strain under restrictive conditions indicates defects for a collection of Pol II-transcribed genes. (A) Mutant and wild-type (wt) TBP-containing strains were grown to log phase and shifted to the restrictive temperature for 1 h. Total RNA isolated before and after the temperature shift was hybridized with a 100-fold excess of the indicated probe and treated with S1 nuclease; $tRNA^W$ served as a loading control. The promoter for the +1 transcript from the HIS3 gene is considered TATA-less, while the promoter for the +13 transcript from the *HIS3* gene contains a canonical TATA element. The promoters of the other genes tested (*MOT1, CMD1, RPS4, ADH1, PGK1, DED1, ENO2, HTA2,* and *FUR1*) have recognizable TATA elements within 250 bp of their translation start sites. The functional relevance of many of these elements is not currently known. (B) Analysis of cyclin genes at the restrictive temperature. Total RNAs from the indicated strains grown at 30°C and for 1 h at 38°C were blotted to nylon membranes and subsequently hybridized with the indicated cyclin probes. *ADH1* served as a loading and transfer control.

TATA-less promoter, in that the *HIS3* +1 transcript is decreased in the mutant background under the restrictive conditions. Thus, it may be that weaker promoters have a greater requirement for the factor(s) that is defined by the K151L,K156Y mutant. In contrast, the promoters that are insensitive to the K151L,K156Y mutant exhibit relatively high levels of transcription. For example, *ADH1*, *ENO2*, and *PGK1* messages are estimated to be present at approximately 50 copies per cell (18). The only exception is the *RPS4* gene, which is a highly active gene yet shows a loss of transcription in the K151L,K156L strain under the restrictive condition.

Defects in the transcription of RPS genes and in +1 transcription of the *HIS3* gene have been observed after *in vivo* depletion or inactivation of TAF130 (31, 43). This suggested that the K151L,K156Y mutant may be defective for interaction with TAF130. To determine how closely the molecular phenotype of the K151L,K156Y derivative corresponds to that of depletion of TAF130, we analyzed other genes known to be dependent on TAF130 for transcription.

2.4d The K151L,K156Y derivative is defective for transcription of cyclin genes.

Previous TAF depletion experiments demonstrated that certain cell-cycle genes require TAF130 for transcription. We analyzed two cell-cycle-regulated genes (*CLN2* and *PCL1*), previously shown to be dependent on TAF130, and a non-cell-cycle-regulated gene (*CLN3*), shown to be

independent of TAF130 inactivation (55). Northern blot analysis revealed that messages for all three genes (*CLN2*, *CLN3*, and *PCL1*) were not detectable at the restrictive temperature in RNA isolated from the K151L,K156Y strain (Fig. 2.4B). In contrast, *ADH1* message amounts remained constant after the temperature shift. Thus, the fact that *CLN3* message amounts were affected in the K151L,K156Y strain but not by inactivation of TAF130 indicates that the transcriptional defects are overlapping but not identical. The *CLN3*, *CLN1*, and *PCL1* messages are each expressed at fairly low levels (predicted message abundance of around one message per cell (reference 18 and citations therein). Due to the similarities to the TAF130 depletion transcription profile, we next ascertained the ability of the K151L,K156Y TBP mutant to form TFIID.

2.4e The K151L,K156Y mutant is defective for TFIID complex formation.

Immunoprecipitation experiments were done to check the integrity of TFIID in the strain harboring the K151L,K156Y mutant. Whole-cell extracts were prepared from mutant and wild-type TBP-containing strains at both the permissive and restrictive temperatures, and antibodies against TBP were used to immunoprecipitate TFIID. Immunoblot analyses of the precipitated complexes with various TAF antibodies indicate that the K151L,K156Y derivative is competent for interaction with all of the TAFs tested at the permissive temperature (Fig. 2.5). In contrast, after incubation for only 1 h at the restrictive temperature, TFIID isolated from the K151L,K156Y strain lacks

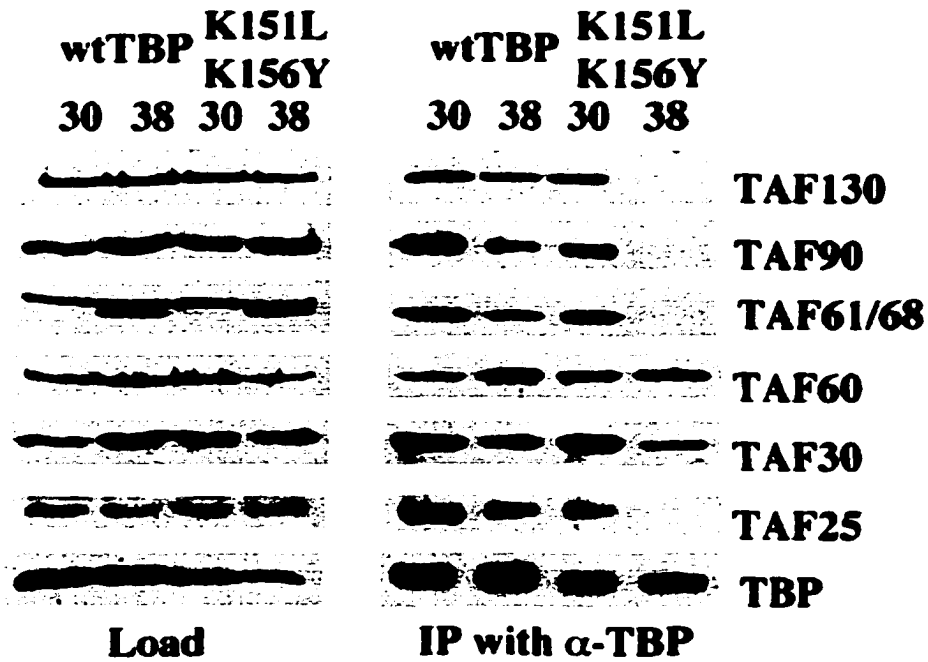

Figure 2.5 TFIID is disrupted in the K151L,K156Y strain. Coimmunoprecipitation of TFIID from both mutant (K151L,K156Y) and wild-type (wt) TBP-containing strains is shown. Strains were grown to log phase and shifted to the restrictive temperature for 1 h. Whole-cell extracts were prepared before and after the temperature shift, and TFIID was immunoprecipitated with anti-TBP antibodies (α–TBP) coupled to protein A-Sepharose beads. The immunoprecipitated (IP) complexes were separated with either a 7.5 or 12% acrylamide gel and electroblotted to nitrocellulose. Blots were first probed with anti-TBP antibodies to serve as an internal control. Blots were then stripped and reprobed for the indicated TAF antibodies. The load represents 1/10 of the input in the IP lanes.

detectable amounts of TAF130, TAF90, TAF68/61, and TAF25. It should be noted that although these TAFs are no longer associated with TBP, they remain detectable in the whole-cell extract and hence are likely to be present in SAGA and other TAF complexes. Surprisingly, TAF60 and TAF30 are still associated with TBP even in the absence of the other TAFs. Thus, at the restrictive temperature, the K151L,K156Y TBP mutant exhibits impaired interactions with some TAFs, while interaction with other TAFs remains intact. To our knowledge, this is the first yeast TBP mutant described that is defective for selective TAF interactions and that is capable of forming partial TFIID complexes *in vivo*. It should be noted that immunoblot analysis with other TAF-directed antibodies (TAF40, TAF17, and TAF170) was attempted, however, these antibodies lacked sufficient titers to detect the corresponding yeast proteins in either the whole-cell extract or the enriched fraction after immunoprecipitation.

2.4f The K151L,K156Y mutant supports activated transcription under conditions in which TBP-TAF interactions are compromised.

Depletion or inactivation of certain individual TAFs does not affect activated transcription in yeast cells (31, 54). We wished to test whether the K151L,K156Y derivative, which is defective for multiple TAF interactions, is capable of Gcn4-dependent activation of *HIS3* transcription at the restrictive temperature. Thus, cells were incubated for 1 h at the restrictive temperature, and then AT was added and the cultures were allowed to incubate for an

additional hour. In the absence of AT, *HIS3* transcription is not detected at the restrictive temperature (Fig. 2.4A and 2.6). However, in the presence of AT, the mutant TBP is able to activate transcription to significant levels (Fig. 2.6). Thus, although the K151L,K156Y mutant is significantly compromised for interactions with TAF130, TAF90, TAF68/61, and TAF25, this TBP mutant is still competent for the response to the Gcn4 acidic activator *in vivo*.

3.5 Discussion

The K151L,K156Y derivative of TBP is defective for TFIID formation. We have characterized a ts TBP mutant (K151L,K156Y) defective for TFIID formation *in vivo*. Substitutions at both lysine 151 and 156 are required for the ts mutant phenotype. Unlike other TBP mutants isolated in this screen (45, 46), the K151L,K156Y derivative is responsive to acidic activators at the permissive temperature. Analysis of transcription from Pol II promoters reveals that a subset of TATA-containing genes, as well as several TATA-less genes, are affected at the restrictive temperature. However, the mutant TBP is capable of Gcn4-dependent activation of *HIS3* transcription. The transcriptional defects overlap with, but are not identical to, the defects observed for depletion of TAF130 (31, 43, 55). Immunoprecipitation of TBP and its associated factors under the restrictive conditions indicates that although the K151L,K156Y protein is functional for interaction with TAF60 and

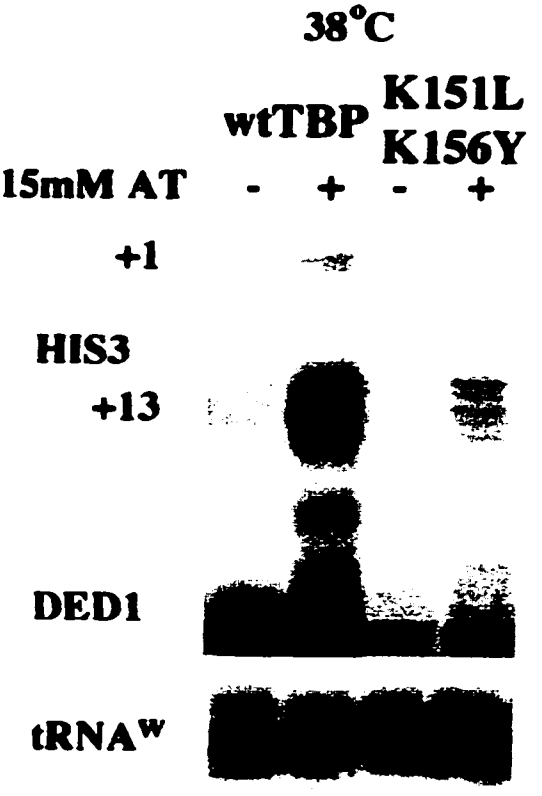

Figure 2.6 Maintenance of activated transcription by Gcn4 under the restrictive conditions in the TFIID-disrupted mutant TBP strain. Strains were grown to log phase in synthetic complete medium, and cultures were shifted to the restrictive temperature for 1 h. Cells were then grown for an additional hour in the presence (+) or absence (-) of 15 mM AT, and total RNA was analyzed for *HIS3*, *DED1*, and *tRNA* expression by S1 analysis.

TAF30, interactions with TAF130, TAF90, TAF68/61, and TAF25 are severely compromised. Because this mutant disrupts only TFIID, leaving SAGA and other potential TAF complexes intact, it provides new information about how TAFs may function *in vivo*.

2.5a Loss of certain TAFs results in promoter-specific defects.

Transcriptional analysis of the K151L,K156Y mutant reveals specific gene defects under conditions of partial TFIID disruption, suggesting that the corresponding promoters are dependent on the TAFs in TFIID for transcription. A subset of these genes (*PCL1*, *CLN2*, and the *RPS* genes) have already been shown to be dependent on TAF130 for transcription (43, 55). Therefore, we would expect them to be affected, as the mutant TBP is defective for interaction with TAF130 at the restrictive temperature. The other promoters affected (*CMD1*, *MOT1*, *HIS3* +1, and *CLN3*) exhibit fairly low levels of transcription, providing evidence for a correlation between the requirement for certain TAFs and the transcription of weaker promoters. In addition, the *HIS3* +1 promoter exhibits TATA-less features. In higher eukaryotes, TAFs and/or TFIID are critical for *in vitro* TATA-less transcription (62). Presumably, weak TATA elements need the additional promoter-TAF contacts, as TBP alone is unable to recognize and support transcription from TATA-less promoters. This notion has also been supported by previous *in vivo* studies, in which depletion of certain individual TAFs causes a decrease in transcription from two genes transcribed from TATA-less promoters (31,

32). We have found a strong positive correlation between the loss of interaction with multiple TAFs and transcription from weaker promoters, in that the K151L,K156Y mutant is defective for the TATA-less promoters tested as well as other weak promoters at the restrictive temperature.

2.5b Certain genes are unaffected in the TFIID-disrupted mutant strain.

There are some genes that are not affected in the K151L,K156Y mutant strain under the restrictive conditions (*ADH1*, *PGK*, *ENO2*, *HTA2*, and *FUR1*). There are at least two possible mechanisms for transcription from these promoters. The first is that transcription from these genes is independent of the loss of the particular TAFs in the context of TFIID. It may be that the unaffected promoters are dependent on the TAFs that remain associated with the TBP mutant or that TBP is sufficient for expression. The second possible mechanism, based on the premise that TAFs are necessary for transcription, is that unaffected promoters can engage the TAFs in an alternative manner that does not involve TFIID. This model is supported by the observation that TAFs exist in at least one other complex (16, 38).

Interestingly, we have observed one promoter that switches from being defective under TFIID disruption conditions to being functional. At the restrictive temperature, the K151L,K156Y mutant is defective for transcription from the *HIS3* promoter, indicating dependence on the missing TAFs. Yet, under conditions of activated transcription by Gcn4, the activity of this promoter is largely independent of the loss of the particular TBP-TAF

interactions. It may be that Gcn4 is able to recruit TBP to the promoter, consequently bypassing the dependence on the TAF-TBP interactions observed under the nonactivated conditions. In this scenario, the TAFs in TFIID are not required for activated transcription. Recruitment of TBP by Gcn4 could be direct or could be indirect via interactions with the holoenzyme mediator complex or the SAGA complex. In accord with this model, Gcn4 is able to interact with some of the holoenzyme mediator complex components, as well as with the SAGA complex (12). Moreover, artificial recruitment of members of the holoenzyme mediator complex can activate transcription, indicating that this complex is able to target TBP to a promoter (40). An alternative hypothesis is that the missing TAFs are required for transcription and that by interaction with several redundant targets, the effect of Gcn4 is to stabilize the mutant TFIID complex on the promoter. In a related model, these TAFs are required and necessary, although not as components of the TFIID complex. Thus, the critical TAFs could be supplied to the promoter via an alternate TAF-containing complex. The finding that Gcn4 is unable to interact with purified TFIID (12) suggests that if TAFs are a target of Gcn4, then this activator is likely to interact with the TAFs in the context of the SAGA complex and not in the context of the TFIID complex.

2.5c Multiple TFIID complexes versus partial TFIID complexes.

TAF130 is the only yeast TAF that has been shown to contact TBP directly (3, 41), and it is thought to serve as a scaffold for TFIID complex

formation. In addition, a ts allele of TAF130 that is rapidly degraded when shifted to the restrictive temperature causes the subsequent degradation of two other yeast TAFs (TAF90 and TAF61/68), while the levels of TAF60 and TAF47 remain unchanged (54). This concomitant degradation suggests that some TAFs are regulated by being part of a TFIID complex, and it is consistent in part with the idea that TAF130 is needed for the overall stability of TFIID. It also suggests the possibility that the TAFs not degraded may exist in a separate TFIID complex not dependent on TAF130 for stability. It should be noted that similar patterns of degradation are seen when other yeast TAFs are depleted (54).

The TBP mutant described in this paper is defective for interaction with TAF130, TAF90, TAF68/61, and TAF25 but is functional for interaction with TAF60 and TAF30. Although many TBP-TAF interactions have been reported for both human and Drosophila TFIIDs, little is known about the TBP-TAF interactions in yeast. Both human TAF70 and Drosophila TAF60/62, the homologs of yeast TAF60, interact with TBP directly (57); thus, it seems likely that TAF60 does contact TBP. More directed experiments, however, are needed to determine if the immunoprecipitated complex represents the remnants of a partially disrupted TFIID complex or a subcomplex that is not affected by the temperature shift. Nevertheless, our studies suggest that these particular TAFs are not dependent on the scaffolding function provided by TAF130 for TFIID formation, perhaps due to direct interactions between TBP and TAF60 and/or TAF30.

2.5d Role of TAFs in activated transcription.

Interactions between isolated TAFs and activator proteins occur *in vitro*, suggesting that TAFs might function as coactivators (53). Furthermore, artificial recruitment of TAFs can bypass the need for an activation domain *in vivo* (2, 14, 22), and reconstituted TFIID subcomplexes containing only a few TAFs can mediate activated transcription *in vitro* (42). However, the view that TAFs are required for activated transcription has been challenged by the finding that inactivation (depletion) of single TAFs does not affect the response to many acidic activators in yeast (31, 54). We have expanded these results by demonstrating that a TBP mutant defective for interaction with multiple TAFs remains responsive to the acidic activator Gcn4. The discrepancy between the *in vivo* and *in vitro* observations may reflect the redundancy of activator targets *in vivo* (48).

It appears that *in vivo* yeast TAFs have developed specialized roles at promoters, perhaps in addition to serving as potential targets for activators. Interestingly, lysines at position 151 and 156 are conserved from yeast to higher eukaryotes. This sequence conservation is likely to reflect functional conservation. This strongly suggests that these residues may also contribute to important TBP-TAF interactions in human TFIID.

2.6 Acknowledgements

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Supplemental Figure For Chapter 2

The following figure (2.7) was addressed as "data not shown" in the manuscript. It is referred to within the text of chapter 2.

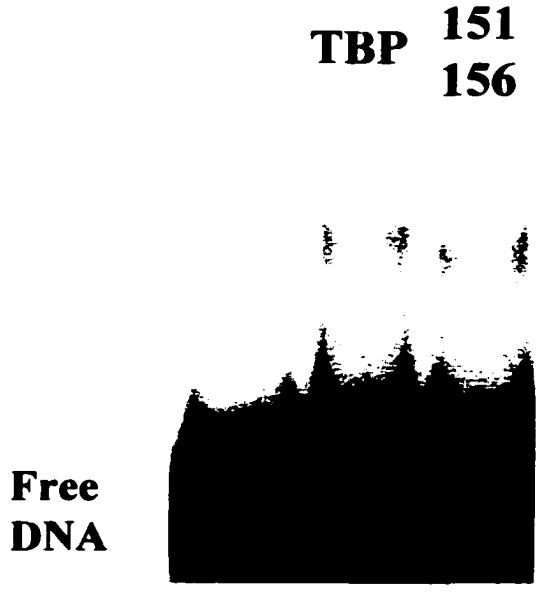


Figure 2.7 Wild-type TBP and K151L,K156Y bind DNA with similar affinity. Proteins were expressed as 6XHis fusion proteins and purified using NTA affinity resin. Proteins were quantited using SDS-PAGE followed by Coomassie brillant blue R-250 staining. Electrophoretic mobility-shift assays were performed using a ^{32}P-labeled 45-base-pair fragment containing the adenovirus early 1B TATA box as described previously (46). 30 ng TBP and 20 pmol DNA were incubated at 25 °C for 30 min in 15 ml of 20 mM Tris (pH 7.5), 40 mM HEPES (pH 7.9), 100 mM KCl, 1 mM DTT, 0.5 mM PMSF, 5 mM $MgCl_2$, 10% glycerol. TBP-DNA complexes were separated from unbound DNA by 6% nondenaturing acrylamide gel electrophoresis and quantified by phosphorimaging.

2.7 References

1. Apone, L. M., C.-A. Virbasius, F. C. Holstege, J. Wang, R. A. Young, and M. R. Green. 1998. Broad, but not universal, transcriptional requirement for yTAFII17, a histone H3-like TAFII present in TFIID and SAGA. *Mol. Cell* 2:653-661.

2. Apone, L. M., C. A. Virbasius, J. C. Reese, and M. R. Green. 1996. Yeast TAFII90 is required for cell-cycle progression through G2/M but not for general transcription activation. *Genes Dev.* 10:2368-2380.

3. Bai, Y., G. M. Perez, J. M. Beechem, and P. A. Weil. 1997. Structure-function analysis of TAF130: identification and characterization of a high-affinity TATA-binding protein interaction domain in the N terminus of yeast TAFII130. *Mol. Cell. Biol.* 17:3081-3093.

4. Burk, T. W., and J. T. Kadonaga. 1997. The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by TAFII60 of Drosophila. *Genes Dev.* 11:3020-3031.

5. Burley, S. K., and R. G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* 65:769-799.

6. Chatterjee, S., and K. Struhl. 1995. Connecting a promoter-bound protein to the TATA-binding protein overrides the need for a transcriptional activation region. *Nature* 374:820-822.

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

8. Colgan, J., and J. L. Manley. 1992. TFIID can be rate limiting *in vivo* for TATA-containing, but not TATA-lacking, RNA polymerase II promoters. *Genes Dev.* 6:304-315.

9. Cormack, B. P., M. Strubin, A. S. Ponticelli, and K. Struhl. 1991. Functional differences between yeast and human TFIID are localized to the highly conserved region. *Cell* 65:341-348.

10. Cormack, B. P., M. Strubin, L. A. Stargell, and K. Struhl. 1994. Conserved and nonconserved functions of yeast and human TATA-binding proteins. *Genes Dev.* 8:1335-1343.

11. Cormack, B. P., and K. Struhl. 1993. Regional codon randomization: defining a TATA-binding protein surface required for RNA polymerase III transcription. *Science* 262:244-248.

12. Drysdale, C. M., B. M. Jackson, R. McVeigh, E. R. Klebanow, Y. Bai, T. Kokubo, M. Swanson, Y. Nakatani, P. A. Weil, and A. G. Hinnebusch. 1998. The Gcn-4p activation domain interacts specifically *in vitro* with RNA polymerase II holoenzyme, TFIID, and the Adap-Gcn5p coactivator complex. *Mol. Cell. Biol.* 18:1711-1724.

13. Geiger, J. H., S. Hahn, S. Lee, and P. B. Sigler. 1996. Crystal structure of the yeast TFIIA/TBP/DNA complex. *Science* 272:830-836.

14. Gonzalez-Couto, E., N. Klages, and M. Strubin. 1997. Synergistic and promoter-selective activation of transcription by recruitment of transcription factors TFIID and TFIIB. *Proc. Natl. Acad. Sci. USA* 94:8036-8041.

15. Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon, and R. Tjian. 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* 75:519-530.

16. Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. Steger, J. C. Reese, J. R. Yates III, and J. L. Workman. 1998. A subset of TAFIIs are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. *Cell* 94:45-53.

17. Hoey, T., R. O. J. Weinzierl, G. Gill, J.-L. Chen, B. D. Dynlacht, and R. Tjian. 1993. Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. *Cell* 72:247-260.

18. Holstege, F. C. P., E. G. Jennings, C. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander, and R. A. Young. 1998. Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* 95:717-728.

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

20. Iyer, V., and K. Struhl. 1996. Absolute mRNA levels and transcriptional initiation rates in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA*. 93:5208-5212.

21. Joliot, V., M. Demma, and R. Prywes. 1995. Interaction with RAP74 subunit of TFIIF is required for transcriptional activation by serum response factor. *Nature* 373:632-635.

22. Keaveney, M., and K. Struhl. 1998. Activator-mediated recruitment of the RNA polymerase II machinery is the predominant mechanism for transcriptional activation in yeast. Mol. Cell 1:917-924.

23. Kim, J. L., D. B. Nikolov, and S. K. Burley. 1993. Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* 365:520-527.

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

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

26. Klein, C., and K. Struhl. 1994. Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. *Science* 266:280-282.

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

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

29. Martinez, E., Q. A. Zhou, N. D. Letoile, T. Oelgeschlager, A. J. Berk, and R. G. Roeder. 1995. Core promoter-specific function of a mutant transcription factor TFIID defective in TATA-box binding. *Proc. Natl. Acad. Sci. USA*. 92:11864-11868.

30. Michel, B., P. Komarnitsky, and S. Buratowski. 1998. Histone-like TAFs are essential for transcription *in vivo*. *Mol. Cell* 2:663-672.

31. Moqtaderi, Z., Y. Bai, D. Poon, P. A. Weil, and K. Struhl. 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* 383:188-191.

32. Moqtaderi, Z., M. Keaveney, and K. Struhl. 1998. The histone H3-like TAF is broadly required for transcription in yeast. *Mol. Cell.* 2:675-682.

33. Moqtaderi, Z., J. D. Yale, K. Struhl, and S. Buratowski. 1996. Yeast homologues of higher eukaryotic TFIID subunits. *Proc. Natl. Acad. Sci. USA.* 93:14654-14658.

34. Nikolov, D. B., and S. K. Burley. 1997. RNA polymerase II transcription initiation: a structural view. *Proc. Natl. Acad. Sci. USA.* 94:15-22.

35. Nikolov, D. B., H. Chen, E. D. Halay, A. A. Usheva, K. Hisatake, D. K. Lee, R. G. Roeder, and S. K. Burley. 1995. Crystal structure of a TFIIB-TBP-TATA-element ternary complex. *Nature* 377:119-128.

36. Nogi, Y., R. Yano, and M. Nomura. 1991. Synthesis of large rRNAs by RNA polymerase II in mutants of Saccharomyces cerevisiae defective in RNA polymerase I. *Proc. Natl. Acad. Sci. USA.* 88:3962-3966.

37. Oelgeschlager, T., Y. Tao, Y. K. Kang, and R. G. Roeder. 1998. Transcription activation via enhanced preinitiation complex assembly in a human cell-free system lacking TAFIIs. *Mol. Cell* 1:925-931.

38. Ogryzko, V. V., T. Kotani, X. Zhang, R. L. Schiltz, T. Howard, X.-J. Yang, B. H. Howard, J. Qin, and Y. Nakatani. 1998. Histone-like TAFs within the PCAF histone acetylase complex. *Cell* 94:35-44.

39. Ozer, J., P. A. Moore, A. H. Bolden, A. Lee, C. A. Rosen, and P. M. Lieberman. 1994. Molecular cloning of the small (g) subunit of human TFIIA reveals functions critical for activated transcription. *Genes Dev.* 8:2324-2335.

40. Ptashne, M., and A. Gann. 1997. Transcriptional activation by recruitment. *Nature* 386:569-577.

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

42. Sauer, F., D. A. Wasserman, G. M. Rubin, and R. Tjian. 1996. TAFIIs mediate activation of transcription in the Drosophila embryo. *Cell* 87:1271-1284.

43. Shen, W.-C., and M. R. Green. 1997. Yeast TAFII145 functions as a core promoter selectivity factor, not a general coactivator. *Cell* 90:615-624.

44. Singer, V. L., C. R. Wobbe, and K. Struhl. 1990. A wide variety of DNA sequences can functionally replace a yeast TATA element for transcriptional activation. *Genes Dev.* 4:636-645.

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

46. Stargell, L. A., and K. Struhl. 1996. A new class of activation-defective TATA-binding protein mutants: evidence for two steps of transcriptional activation *in vivo*. *Mol. Cell. Biol.* 16:4456-4464.

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

48. Struhl, K. 1996. Chromatin structure and RNA polymerase II connection: implications for transcription. *Cell* 84:179-182.

49. Struhl, K., and Z. Moqtaderi. 1998. The TAFs in the HAT. *Cell* 94:1-4.

50. Suzuki-Yagawa, Y., M. Guermah, and R. G. Roeder. 1997. The ts13 mutation in the TAF(II)250 subunit (CCG1) of TFIID directly affects transcription of D-type cyclin genes in cells arrested in G1 at the nonpermissive temperature. *Mol. Cell. Biol.* 17:3284-3294.

51. Tan, S., Y. Hunziker, D. F. Sargent, and T. J. Richmond. 1996. Crystal structure of a yeast TFIIA/TBP/DNA complex. *Nature* 381:127-134.

52. Verrijzer, C. P., J.-L. Chen, K. Yokomori, and R. Tjian. 1995. Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II. *Cell* 81:1115-1125.

53. Verrijzer, C. P., and R. Tijan. 1996. TAFs mediate transcriptional activation and promoter selectivity. Trends Biochem. Sci. 21:338-342.

54. Walker, S. S., J. C. Reese, L. M. Apone, and M. R. Green. 1996. Transcription activation in cells lacking TAFIIs. *Nature* 382:185-188.

55. Walker, S. S., W.-C. Shen, J. C. Reese, L. M. Apone, and M. R. Green. 1997. Yeast TAFII145 required for transcription of G1/S cyclin genes and regulated by the cellular growth state. *Cell* 90:607-614.

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

57. Weinzierl, R. O. J., S. Ruppert, B. D. Dynlacht, N. Tanese, and R. Tjian. 1993. Cloning and expression of Drosophila TAFII60 and human TAFII70 reveal conserved interactions with other subunits of TFIID. *EMBO J.* 12:5303-5309.

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

59. Wu, S.-Y., E. Kershnar, and C.-M. Chiang. 1998. TAFII-independent action mediated by human TBP in the presence of the positive ofactor PC4. *EMBO J.* 17:4478-4490.

60. Xiao, H., J. D. Friesen, and J. T. Lis. 1995. Recruiting TATA-binding protein to a promoter: transcriptional activation without an upstream ctivator. *Mol. Cell. Biol.* 15:5757-5761.

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

62. Zhou, Q., P. M. Lieberman, T. G. Boyer, and A. J. Berk. 1992. Holo TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. *Genes Dev.* 6:1964-1974.

CHAPTER 3

REEVALUATION OF TRANSCRIPTIONAL REGULATION BY TATA-BINDING PROTEIN OLIGOMERIZATION: PREDOMINANCE OF MONOMERS

This chapter was published in *Biochemistry*. The text of this manuscript is presented exactly as it appears in the journal. All the figures that appeared in the manuscript are included. This work is a collaboration with our lab and Dr. Kevin Lumb and Kathleen Campbell. I created figure 3.1 and the data shown in figures 3.6 and 3.8. I also wrote the text describing these figures in the experimental procedures and results sections. The literature citation for this chapter is:

> Campbell, K.M., Ranallo, R.T., Stargell, L.A., and K. J. Lumb. Reevaluation of Transcriptional Regulation by TATA-Binding Protein Oligomerization: Predominance of Monomers, *Biochemistry*; 2000; 39(10); 2633-2638.

3.1 Abstract

The TATA-binding protein (TBP) plays an important role in transcriptional initiation by all three nuclear RNA polymerases. TBP contains a conserved C-terminal domain (cTBP) that binds DNA. Crystallographic studies of cTBP (i.e., TBP without the N-terminal domain) from various species and molecular biology studies of cTBP and mixed cTBP/TBP species have led to the view that DNA binding by TBP is regulated by TBP dimerization. Using sedimentation equilibrium, we show that yeast cTBP forms dimers in solution at 5 °C with a dissociation constant of 7 ± 1 μM. This observation of cTBP dimers in solution is in accord with the dimeric state observed in crystal structures of cTBP. In contrast, physiologically relevant, full-length yeast TBP is monomeric at 5 °C and forms dimers at 30 °C with a dissociation constant of 51 ± 16 μM. This dissociation constant precludes formation of stable full-length TBP dimers at physiological concentrations. In addition, we tested for yeast TBP oligomerization in the presence of TBP-associated factors in the context of TFIID. No evidence for TBP oligomers was found using immunoprecipitation techniques from yeast whole-cell extracts. We conclude that yeast TBP is predominantly monomeric under physiological conditions, arguing against a role for TBP dimerization in the regulation of transcriptional initiation.

3.2 Introduction

Initiation of gene transcription on a TATA-containing RNA polymerase II promoter is a complex process, involving a large number of interactions between multiple transcription factors (1). The RNA polymerase II general transcription factor TFIID consists of both TBP and TAFs. An initial regulatory step in transcription initiation by polymerase II is the binding of the TBP to DNA, which serves to nucleate preinitiation complex assembly (1, 2), and is proposed to be a major rate-limiting step in RNA polymerase II transcription initiation (3-5). In addition, TBP is a central component in transcription by RNA polymerases I and III (1, 2). Accurate prediction of transcription patterns and an understanding of the mechanism of transcription initiation will require a description of the factors that regulate TBP.

The DNA-binding region of TBP is located in the conserved 180-residue C-terminal domain (2). The crystal structures of yeast, human, and plant TBP C-terminal domains have been solved (2, 6, 7). In all cases, the TBP C-terminal domain crystallizes as a dimer (Figure 3.1). However, crystal structures of the TBP C-terminal domain bound to DNA show that the C-terminal domain binds DNA as a monomer (2, 6, 7). The dimerization interface of the C-terminal domain occludes the DNA-binding site, and so dimerization and DNA-binding by the C-terminal domain are mutually exclusive on structural grounds (Figure 3.1). Taken together with cross-linking and pull-down assays of solutions containing the TBP C-terminal

cTBP-DNA

cTBP-cTBP

Figure 3.1 Crystal structures of yeast cTBP and the yeast cTBP-DNA complex (6, 7). The dimerization interface of yeast cTBP occludes the DNA-binding surface. The figure was produced with RASMOL (29).

domain and full-length TBP (8-12), these results have led to the view that DNA-binding by TBP is regulated by dimerization, and that TBP dissociation is a key rate-limiting step in transcriptional initiation (8-12).

We report here the oligomerization properties of cTBP, corresponding to the yeast TBP C-terminal domain, and full-length yeast TBP using sedimentation equilibrium and immunological methods. Sedimentation equilibrium is a thermodynamically rigorous method to characterize protein self-association in solution (13). The technique is sensitive to small amounts of incompetent monomer that are unable to oligomerize, which is detected by a systematic increase in K_d with increasing protein concentration (14). In contrast to chemical cross-linking, sedimentation equilibrium will not generate artificially high levels of an oligomer due to a reactive cross-linking reagent. In addition, problems due to selectivity in pull-down assays and anomalous mobility during gel filtration are avoided. Using sedimentation equilibrium we find that, while cTBP forms dimers, dimerization of full-length TBP is thermodynamically unfavorable at physiological concentrations. In addition, immunological methods provide no evidence for dimerization of TBP in the context of TFIID. Our results challenge the notion that TBP dimerization plays an important role in regulating transcription initiation.

3.3 Materials and methods

3.3a Proteins.

Full-length yeast TBP was expressed in *Escherichia coli* strain BL21 (DE3) with a pET11a vector (called pYTBP) and purified from the soluble fraction with Q, SP, and Heparin HiTrap columns (Pharmacia) equilibrated in buffer A (10 mM sodium phosphate, 100 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM DTT, pH adjusted to 7.5). TBP flowed through the Q column and was eluted from the SP and Heparin columns using linear NaCl gradients in buffer A. Final purification of TBP was by gel filtration with Sephacryl S-100 equilibrated in 10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5. cTBP (residues 49-240) was prepared from purified TBP by trypsin (Sigma T-1426) cleavage of the N-terminal domain (15) and further purified with an SP HiTrap column (Pharmacia) using a linear NaCl gradient in buffer A. Purified TBP and cTBP were stored as aliquots at –70 °C and were not freeze-thawed more than once, since it has been reported that repeated freeze-thaw cycles affect significantly the oligomerization state of yeast TBP (16). N-terminal sequencing indicated that the N-terminal Met of TBP was processed, as observed for authentic TBP from yeast (17). The identities of TBP and cTBP were confirmed with electrospray mass spectrometry, with the expected and observed masses agreeing to within 2 Da.

3.3b DNA-Binding Assays.

Electrophoretic mobility-shift assays were performed using a ^{32}P-labeled 45-base-pair fragment containing the adenovirus early 1B TATA box as described previously (18). TBP (60 pM to 22 nM) and DNA (5.4 nM) were incubated at 25 °C for 30 min in 15 μl of 20 mM Tris (pH 7.5), 40 mM HEPES (pH 7.9), 100 mM KCl, 1 mM DTT, 0.5 mM PMSF, 5 mM $MgCl_2$, 10% glycerol. TBP-DNA complexes were separated from unbound DNA by 6% nondenaturing acrylamide gel electrophoresis and quantified by phosphorimaging.

3.3c Analytical Ultracentrifugation.

Sedimentation equilibrium experiments were performed with a Beckman XL-I and An60-Ti rotor. Data were collected using 12 mm path length six-sector centerpieces at wavelengths of 276 and 280 nm for cTBP and TBP, respectively. Equilibrium was judged to be reached when scans collected 3 h apart were indistinguishable, and was generally attained within 12 h. Solvent densities of 1.060 g mL^{-1} at 5 °C and 1.056 g mL^{-1} at 30°C and partial specific volumes of 0.754 mL g^{-1} for cTBP and 0.746 mL g^{-1} for TBP were calculated as described elsewhere (19).

Data were analyzed with Origin (20) and fit to ideal single-species, monomer-dimer, monomer-trimer, monomer-tetramer, and monomer-octamer models. The mathematical expression used by Origin is described in detail elsewhere (20, 21). Discrimination between the different models was based

on the distribution of residuals and the variance, which approaches 1 as the fit improves (20). Parameters that were allowed to vary during fits to the ideal single-species model were molecular mass and baseline offset, unless stated otherwise. Parameters that were allowed to vary during fits to the self-associating models were the log of the dissociation constant and the baseline offset, with the molecular mass held constant at the known monomeric value (21,279.9 Da for cTBP and 26,871.7 Da for TBP). In all fits the second virial coefficient was held constant at zero, and terms for oligomers not considered in the fit were made insignificant by constraining the association constant to 10^{-20} (21).

Samples were dialyzed at 4 °C against the reference buffer (10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5, unless stated otherwise) for at least 12 h. For cTBP, data were collected at 5 °C using multiple cTBP loading concentrations spanning 6-60 μM, rotor speeds of 20, 30, and 40 krpm, and cTBP from three independent preparations. For TBP, data sets were collected at 5 and 30 °C using multiple TBP loading concentrations spanning 6-60 μM, and rotor speeds of 30, 35, and 40 krpm, and TBP from four independent preparations.

Monomer-dimer association constants ($1/K_d$) were converted from values obtained from the fits in absorbance units (Kabs) to molar values (K_{conc}) using $K_{conc} = K_{abs}\,\varepsilon l/2$, where ε is the extinction coefficient and l is path length (21). Reported errors in K_d values were derived from the standard deviation of the mean K_d value obtained from the fits and the error in the

extinction coefficient. Extinction coefficients for cTBP and TBP were experimentally determined in 10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5, from a linear fit of the change in absorbance with protein concentration to the Beer-Lambert equation. Measurements were made three times to obtain extinction coefficients for cTBP at 276 nm of 10 474 ± 3% M^{-1} cm^{-1} and for TBP at 280 nm of 13058 ± 3% M^{-1} cm^{-1}. Concentrations of the stock solutions were determined by absorbance in 6 M GuHCl, 10 mM sodium phosphate, pH adjusted to 6.5, using extinction coefficients for cTBP at 276 nm of 8700 M^{-1} cm^{-1} and for TBP at 280 nm of 13 370 M^{-1} cm^{-1} (22).

3.3d Analytical Gel Filtration.

Apparent molecular masses were determined at room temperature (approximately 20 °C) by gel filtration with a TosoHaas G2000SW_{XL} column equilibrated in 10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5. A flow rate of 0.5 ml min^{-1} was used. The column was calibrated with lysozyme, chymotrypsinogen A, ovalbumin, albumin, and blue dextran.

3.3e Immunoprecipitations.

Yeast strains expressing TBP and coexpressing TBP and mycTBP were generated from yeast strain BY2 as described previously (23). The mycTBP protein contains an N-terminal triple myc tag (GEQKLISEEDLNx3).

Preparation of whole-cell extracts, immunoprecipitations, and immunoblotting were performed as described previously (24) except that 422 μg of extract was used with monoclonal antibodies generated to the myc tag (Mab 9E10). Complexes were harvested and analyzed by either 10% (TBP and TAF68) or 7.5% (TAF130) SDS-PAGE, electroblotted to nitrocellulose, and probed with polyclonal antibodies specific to TBP, TAF68, or TAF130.

3.4 Results

Sedimentation equilibrium data for cTBP were collected at 5 °C using cTBP from three independent preparations. Twenty-five data sets were fit individually to an ideal, single-species model in which the molecular mass was allowed to vary. In each case, the apparent molecular mass was higher than expected for a monomer but less than expected for a dimer, indicative of a self-associating species (21). Therefore, each data set was fit with the molecular mass held constant at the known monomeric value to single-species, monomer-dimer, monomer-trimer, monomer-tetramer, and monomer-octamer models. In every case the data were fit best by a monomer-dimer model, as indicated by the random distribution of residuals and variances closest to 1 (Figure 3.2). Equivalent results were obtained for cTBP that was either never frozen or freeze-thawed once.

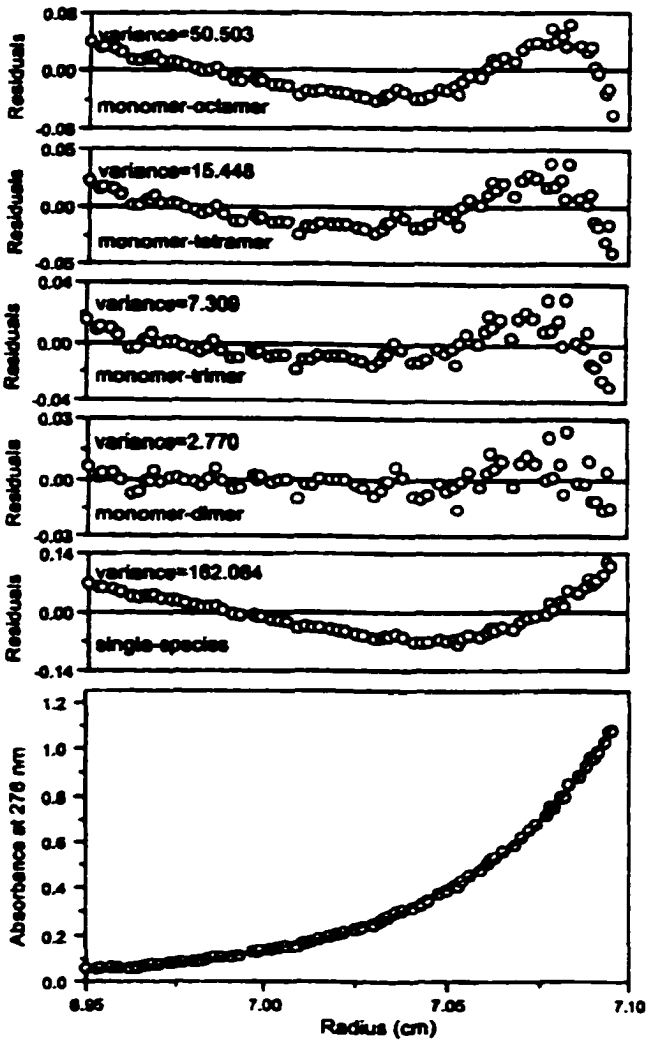


Figure 3.2 Sedimentation equilibrium indicates that cTBP exists in a monomer-dimer equilibrium at 5 °C. Results of the fits of sedimentation equilibrium data for cTBP at 5 °C from a single channel to single-species monomer, monomer-dimer, monomer-trimer, monomer-tetramer, and monomer-octamer models are shown. The residuals are random for the monomer-dimer fit, and exhibit systematic deviations for the other fits. The variance is closest to 1 for the monomer-dimer fit. The residuals for the single-species monomer are indicative of a self-associating system (21).

The mean K_d values for the cTBP monomer-dimer equilibrium obtained from fits of the single data sets is 7 ± 1 μM. Global fits of data collected for each of the three cTBP preparations yielded a mean K_d of 7.9 ± 1 μM. The value of the monomer-dimer K_d for cTBP did not vary systematically in the 25 independent data sets collected over a 10-fold range of total cTBP concentration and at multiple rotor speeds, indicating that cTBP was essentially fully active for dimerization (14). These results indicate that cTBP in solution exists in a monomer-dimer equilibrium at 5 °C at low micromolar concentrations, in accord with the dimeric state of cTBP observed in the crystal structure (Figure 3.1).

In contrast to the behavior observed for cTBP, sedimentation equilibrium measurements indicate that full-length yeast TBP is monomeric at 5 °C. TBP from four independent preparations was used, and 16 data sets were fit to an ideal single-species model in which the molecular mass was allowed to vary. In each case, the residuals were random (Figure 3.3), indicating that TBP behaves as a single ideal species at 5 °C. The average molecular mass of TBP from 16 independent measurements is 27.52 ± 0.79 kDa, which is within experimental error of the expected monomer mass (26.87 kDa). The apparent molecular mass of TBP did not increase systematically with total TBP concentration in the range 6-60 μM, indicating that TBP does not oligomerize significantly at 5 °C (25). Thus, at 5 °C, TBP does not form dimers or higher-order oligomers at micromolar concentrations.

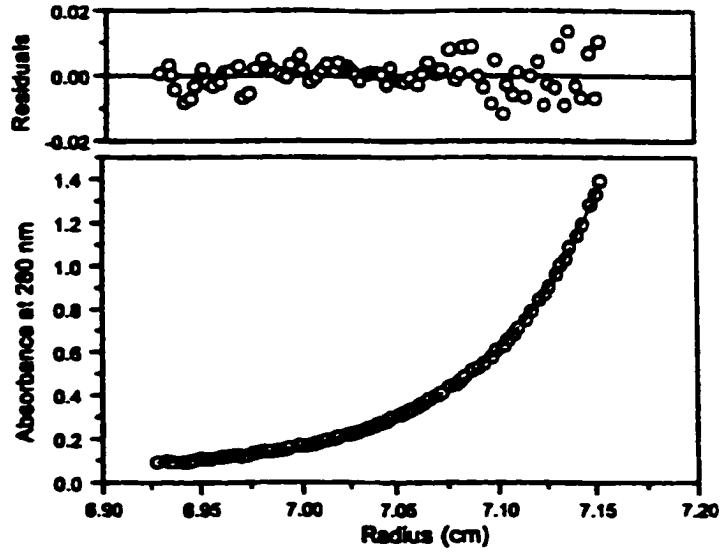


Figure 3.3 Sedimentation equilibrium indicates that TBP is monomeric at 5 °C. The data fit a single-species model, as indicated by the random distribution of residuals, with a molecular weight expected for a monomer.

Sedimentation equilibrium studies of full-length TBP from four independent preparations were also performed at 30 °C (a standard yeast-culturing temperature). Twenty-one data sets were first fit individually to an ideal single-species model in which the molecular mass was allowed to vary. In each case, the apparent molecular mass was higher than expected for a monomer but less than expected for a dimer, indicative of a self-associating species (21). The data were therefore fit to ideal single-species, monomer-dimer, monomer-trimer, monomer-tetramer, and monomer-octamer models in which the molecular mass was held constant at the known value for TBP.

Seventeen data sets exhibited a concentration change of >0.2 absorbance unit across the cell, which is necessary for a reliable discrimination between different models (25). All of these data sets were accounted for best by a monomer-dimer model, as indicated by a random distribution of residuals and variances closest to 1 (Figure 3.4). The four data sets collected at the lowest total concentration (6-15 μM) spanned only a limited concentration range across the cell (approximately 0.1 absorbance unit) and exhibited limited curvature. These data sets could also be accounted for by a monomer-dimer model. Equivalent results were obtained for TBP that was either never frozen or freeze-thawed once.

The mean of the monomer-dimer K_d determinations from individual fits for TBP at 30 °C is 51 ± 16 μM. The K_d did not vary systematically over a 10-fold range of total TBP concentration and at multiple rotor speeds (Figure 3.5), indicating that the TBP was essentially fully active for dimerization (14).

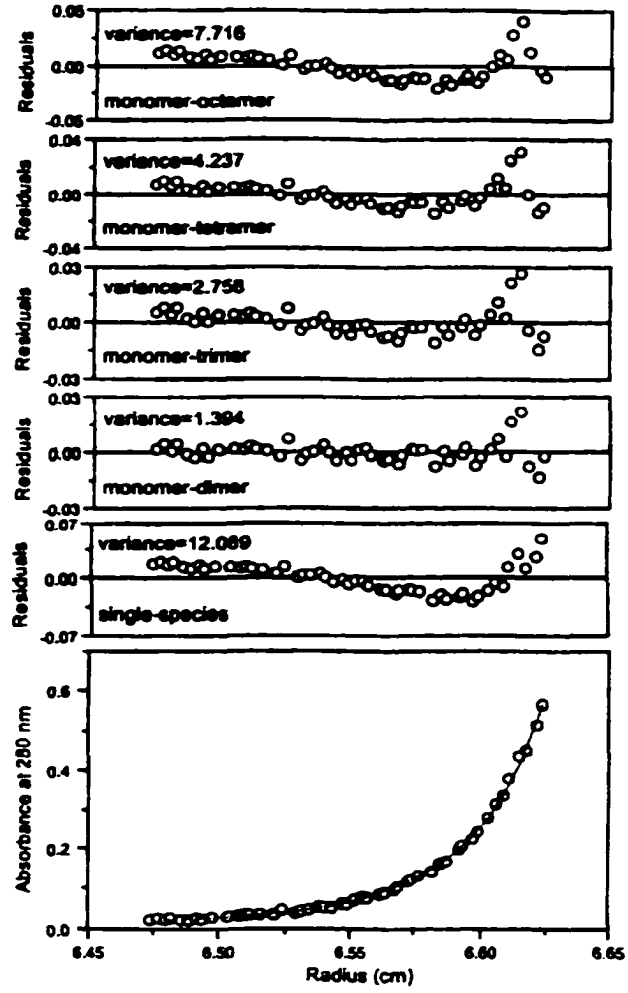


Figure 3.4 Sedimentation equilibrium indicates that TBP exists in a monomer-dimer equilibrium at 30 °C. Results of the fits of sedimentation equilibrium data for TBP at 30 °C to single-species monomer, monomer-dimer, monomer-trimer, monomer-tetramer, and monomer-octamer models are shown. The residuals are random for the monomer-dimer fit, and exhibit systematic deviations for the other fits. The variance is closest to 1 for the monomer-dimer fit. The residuals for the single species monomer are indicative of an associating system (21).

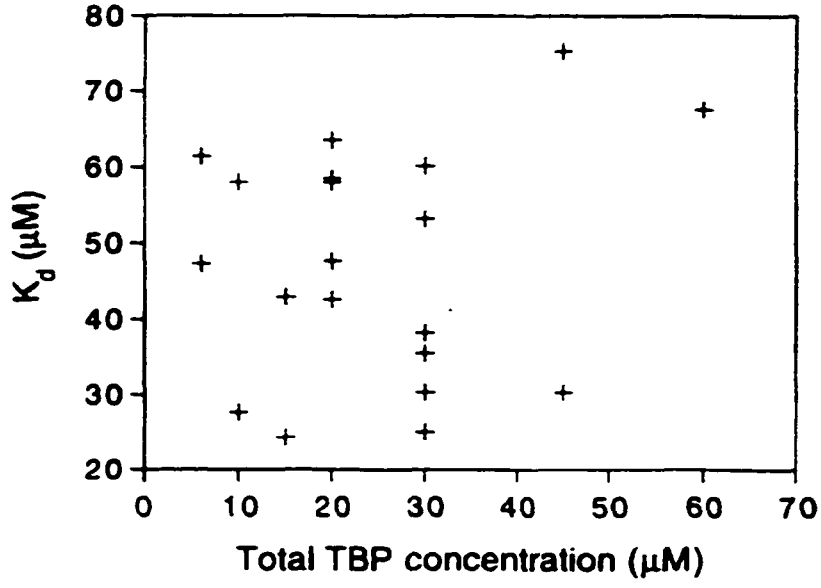


Figure 3.5 The K_d for the TBP monomer-dimer equilibrium obtained from the single-channel fits does not vary systematically with total TBP concentration.

Global fits of data collected for each of the four TBP preparations yielded a mean K_d of 63 ± 16 μM.

A preponderance of TBP monomers was also observed under conditions of different ionic strength (20 mM HEPES, 120 mM KCl, 1 mM DTT, 1 mMEDTA, 10% glycerol, pH adjusted to 7.5, and 10 mM sodium phosphate, 150 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, pH adjusted to 7.5). In addition, the functional activity of TBP was not altered during sedimentation equilibrium, since the TBP was equally active for DNA-binding before and after sedimentation equilibrium (Figure 3.6).

A previous sedimentation equilibrium study reported that yeast TBP forms tetramers and octamers (26). The study was performed at total TBP concentrations of 9 and 15 μM and at two rotor speeds (16 and 24 krpm) (26). The final pH of the buffer used in the study (26) was not reported, and so we repeated the experiments using the same buffer components (20 mM HEPES, 120 mM KCl, 1 mM DTT, 1 mM EDTA) with the pH adjusted to 7.5. The TBP aggregated visibly in this buffer in two independent attempts. The experiments were not pursued further, since others have reported that TBP is rapidly inactivated in the buffer where tetramers and octamers were observed (12), and the physiological relevance of residual, soluble TBP under aggregating conditions is questionable. Inclusion of 10% glycerol in the buffer alleviated the aggregation and gave results that fit a monomer-dimer equilibrium, as described above.

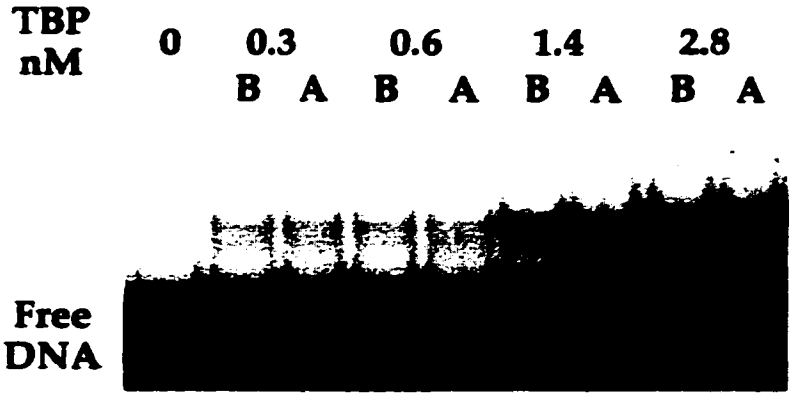


Figure 3.6 TBP exhibited no detectable difference in binding the adenovirus early 1B TATA element (5.4 nM) before (B) or after (A) ultracentrifugation at 30 °C. The intensities of the TBP-DNA bands at a particular TBP concentration are the same to within 3%.

The apparent molecular mass of TBP was also estimated with gel filtration. A single peak was observed, with an apparent molecular mass of 31.6 kDa (Figure 3.7). This mass is within 18% of the known monomeric mass of TBP. The gel filtration results are consistent with the conclusions from the sedimentation equilibrium analysis that TBP is predominantly monomeric at micromolar concentrations.

TBP is associated *in vivo* with TAFs to form the general transcription factor TFIID (2). To test whether the presence of TAFs could favor TBP dimerization, immunological methods were used to evaluate whether TBP dimerizes in the context of TFIID. Since TBP monomers and oligomers cannot be distinguished by immunoprecipitation or immunodetection, TBP was tagged with a myc epitope (mycTBP) to allow isolation and detection of putative mycTBP/TBP heterodimers.

TBP and mycTBP were coexpressed in yeast, and the presence of mycTBP/TBP heterodimers was probed by immunoprecipitation of mycTBP followed by immunoblotting with anti-TBP antibodies. If TBP dimerizes in the context of TFIID, then immunoprecipitation of mycTBP should result in coimmunoprecipitation of TBP. We found that TBP was not present in the mycTBP immunoprecipitations, indicating that TBP does not oligomerize with mycTBP (Figure 3.8). Dilution experiments of the extract containing TBP and mycTBP indicate that 2% dimer formation could have been detected (data not shown). Moreover, TFIID formation is not disrupted by immunopreciptation or the myc tag, since TAF130 and TAF68 are present in the immunoprecipitate

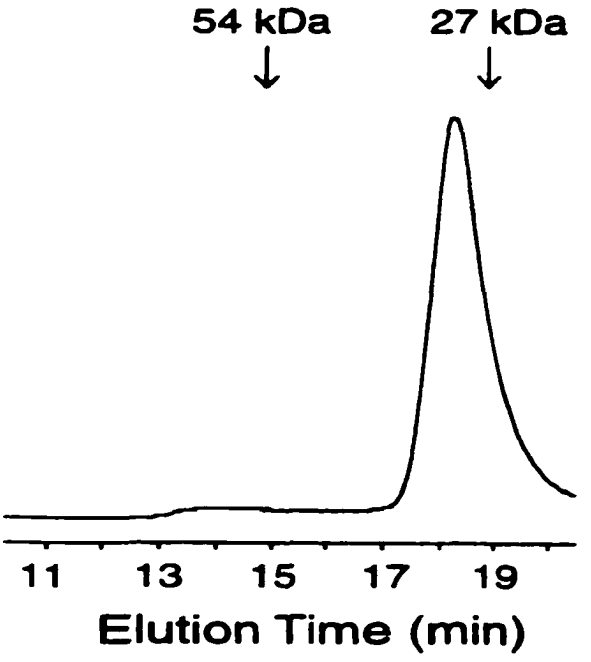


Figure 3.7 TBP elutes during gel filtration as a single peak with an apparent molecular mass of 31.6 kDa. Expected elution times for monomeric and dimeric TBP are indicated.

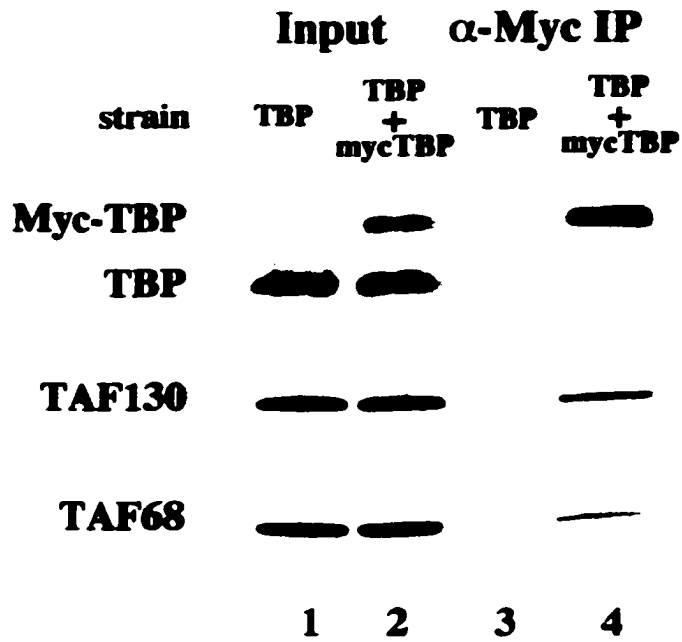


Figure 3.8 Immunoprecipitation assays indicate that TBP is not dimeric in yeast. Whole-cell extracts were prepared from two different strains, one that expresses only TBP (labeled TBP; lanes 1 and 3) and one that expresses both TBP and mycTBP (labeled TBP+mycTBP; lanes 2 and 4). The extract starting material is indicated (labeled Input; lanes 1 and 2). Extracts were immunoprecipitated using myc antibodies (denoted -Myc IP, lanes 3 and 4), and probed with polyclonal antibodies specific to TBP, TAF68, or TAF130. As expected immunoprecipitation with anti-myc antibodies of extracts derived from the strain expressing onlyTBP did not result in isolation of TBP or TFIID (lane 3). Immunoprecipitation of extracts derived from strains coexpressing TBP and mycTBP resulted in isolation of only mycTBP and TAFs and not TBP (lane 4).

(Figure 3.8). Furthermore, the myc tag does not interfere with TBP functions since yeast strains in which TBP or mycTBP were the sole source of TBP were identical for growth at 30 and 37 °C, on alternative carbon sources (raffinose and galactose), in high levels of copper (500 μM) and on 3-aminotriazole (15 mM). Thus, TBP does not self-associate in the context of TFIID.

3.5 Discussion

The C-terminal domains of TBP from several species, including yeast, are dimeric in crystal structures (2, 6). Comparison of the free and DNA-bound yeast cTBP crystal structures shows that the TBP dimerization interface occludes the DNA-binding surface (Figure 3.1). This structural feature raises the possibility that TBP is regulated by dimerization, and that slow dissociation of dimers provides a regulatory step in DNA-binding by TBP and transcription initiation. Indeed, several experimental studies have been interpreted on the basis that TBP is regulated by dimerization (8-12).

In accord with the dimeric state of yeast cTBP observed in the crystal structure (6), we find that, in solution, cTBP forms dimers at 5 °C with a K_d of 7± 1 μM. In marked contrast to the dimerization properties of cTBP at

5 °C, the physiologically relevant, full-length TBP is monomeric at micromolar concentrations. Even at 30 °C, TBP dimerization is thermodynamically unfavorable at physiological concentrations. Since the mean K_d for TBP dimerization is 51 μM at 30 °C, and the nuclear concentration is estimated to be 1-6 μM (9, 27), TBP will be predominantly monomeric at physiological concentrations (Figure 3.9). Moreover, TBP dimers were not observed in immunoprecipitates in the context of TFIID, suggesting that the TAFs or other proteins present *in vivo* do not promote TBP or TFIID oligomerization.

Our results challenge the model for a regulatory role for TBP dimerization in transcription initiation. Our results also demonstrate that TBP dimerization is unfavored by the presence of the N-terminal domain. Since the dimerization interface of cTBP coincides with the DNA-binding surface (Figure 3.1), and the N-terminal domain interferes with TBP dimerization, it is possible that the N-terminal domain occludes the dimerization interface of TBP in solution. Indeed, removal of the N-terminal domain of yeast TBP results in faster binding to DNA and a more stable TBP-DNA complex (15, 28). It is an intriguing possibility that any transcription factor or TBP mutation capable of disrupting the interaction between the N- and C-terminal domains will result in potentially elevated levels of transcription.

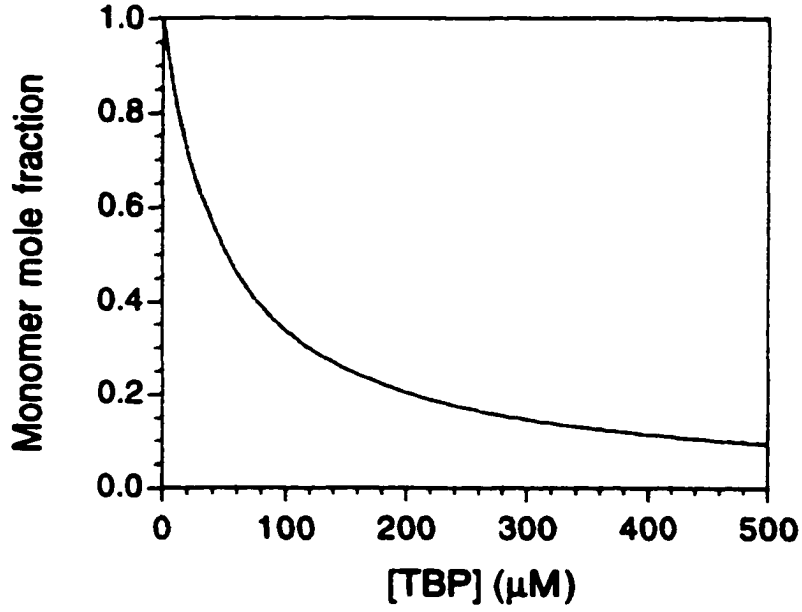


Figure 3.9 Change in mole fraction of monomeric TBP with total TBP concentration. The fraction monomer was calculated for a monomer-dimer equilibrium with a K_d of 51 μM.

3.6 Acknowledgment

We thank M. R. Green for TAF antibodies. Supported by the American Chemical Society Petroleum Research Fund (Grant 32789-G4) and NIH (Grants GM56884 and RR11847). L.A.S. is a March of Dimes Basil O'Connor Starter Scholar.

3.7 References

1. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) Genes Dev. 10, 2657-2683.

2. Burley, S. K., and Roeder, R. G. (1996) Annu. Rev. Biochem. 65, 769-799.

3 Colgan, J., and Manley, J. L. (1992) Genes Dev. 6, 304-315.

4. Kuras, L., and Struhl, K. (1999) Nature 399, 609-613.[CAS][Medline]

5. Li, X., Virbasius, A., Zhu, X., and Green, M. R. (1999) Nature 399, 605-609.

6. Chasman, D. I., Flaherty, K. M., Sharp, P. A., and Kornberg, R. A. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8174-8178.

7. Kim, Y., Geiger, J. H., Hahn, S., and Sigler, P. B. (1993) Nature 365, 512-520.

8. Coleman, R. A., Taggart, A. K. P., Benjamin, L. R., and Pugh, B. F. (1995) J. Biol. Chem. 270, 13842-13849.

9. Taggart, A. K. P., and Pugh, B. F. (1996) Science 272, 1331-1333.

10. Coleman, R. A., and Pugh, B. F. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 7221-7226.

11. Jackson-Fisher, A. J., Chitikila, C., Mitra, M., and Pugh, B. F. (1999) Mol. Cell 3, 717-727.

12. Jackson-Fisher, A. J., Burma, S., Portnoy, M., Schneeweis, L. A., Coleman, R. A., Mitra, M., Chitikila, C., and Pugh, B. F. (1999) Biochemistry 38, 11340-11348.

13. Hensley, P. (1996) Structure 4, 367-373.

14. Yphantis, D. A., Correia, J. J., Johnson, M. L., and Wu, G. (1978) in Physical Aspects of Protein Interactions (Catsimpoolas, N., Ed.) pp 275-303, Elsevier, Amsterdam.

15. Lieberman, P. M., Schmidt, M. C., Kao, C. C., and Berk, A. J. (1991) Mol. Cell Biol. 11, 63-74.

16. Perez-Howard, G. M., Weil, P. A., and Beecham, J. M. (1995) Biochemistry 34, 8005-8017.

17. Schmidt, M. C., Kao, C. C., Pei, R., and Berk, A. J. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7785-7789.

18. Stargell, L. A., and Struhl, K. (1996) Mol. Cell. Biol. 16, 4456-4464.

19. Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E., Rowe, A. J., and Horton, J. C., Eds.) pp 90-125, The Royal Society of Chemistry, Cambridge.

20. Beckman Technical Bulletin LXL/A-TB-009C (1998).

21. McRorie, D. K., and Voelker, P. J. (1993) Self-associating Systems in the Analytical Ultracentrifuge, Beckman Instruments, Inc., Fullerton, CA.

22. Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

23. Cormack, B. P., Strubin, M., Ponticelli, A. S., and Struhl, K. (1991) Cell 65, 341-348.

24. Ranallo, R. T., Struhl, K., and Stargell, L. A. (1999) Mol. Cell. Biol. 19, 3951-3957.

25. Laue, T. M. (1995) Methods Enzymol. 259, 427-452.[CAS][Medline]

26. Daugherty, M. A., Brenowitz, M., and Fried, M. G. (1999) J. Mol. Biol. 285, 1389-1399.

27. Kato, K., Makino, Y., Kishimoto, T., Yamauchi, J., Kato, S., Muramatsu, M., and Tamura, T. (1994) Nucleic Acids Res. 22, 1179-1185.

28. Lee, D. K., Dejong, J., Hashimoto, S., Horikoshi, M., and Roeder, R. G. (1992) Mol. Cell. Biol. 12, 5189-5196.

29. Sayle, R. A., and Milner-White, E. J. (1995) Trends Biochem. Sci. 20, 374-376.

CHAPTER 4

TFIIA INTERACTS WITH TFIID VIA ASSOCIATION WITH TBP AND TAF40

The paper is currently being modified for submission to *Molecular and Cellular Biology*. The work presented in this chapter came about through a collaboration with lab members Ryan Ogg and Dr. Sue Kraemer and myself. I have contributed all the data shown in figures 4.2, 4.3A, 4.3B, 4.7 as well as a portion of figure 4.1. I also wrote sections in the results and experimental procedures describing this work. Figures 4.2 and 4.7 were ongoing work developed and performed by myself and were not included in the original Manuscript. Figure 4.7 is included in the supplemental figure section. The literature citation for this chapter upon acceptance to a professional journal will be:

> Kraemer, S., R. T. Ranallo, R. Ogg, and L. A. Stargell. 2000. TFIIA Interacts with TFIID via association with TBP and TAF40.

4.1 Abstract

TFIIA and TATA-binding protein (TBP) associate directly at the TATA element of genes transcribed by RNA polymerase II. *In vivo*, TBP is complexed with approximately a dozen TBP-associated factors (TAFs) to form the general transcription factor TFIID. How TFIIA and TFIID communicate is not well understood. We show that in addition to making direct contacts with TBP, yeast TAF40 interacts directly and specifically with TFIIA. Mutational analyses of a subunit of TFIIA (Toa2) indicate that loss of functional interaction between TFIIA and TAF40 results in conditional growth phenotypes and defects in transcription. These results demonstrate that the TFIIA-TAF40 interaction is important *in vivo*, and provide a functional role for TAF40 as a bridging factor between TFIIA and TFIID.

4.2 Introduction

Transcription by eukaryotic RNA polymerase II (pol II) involves the assembly of a preinitiation complex consisting of pol II and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (for review see 62). An important step of transcription initiation is binding of TFIID to the core promoter. TFIID, a multisubunit protein complex that is highly conserved among eukaryotes, is composed of the TATA-binding protein (TBP) and

approximately fourteen TBP-associated factors (TAFs) (reviewed in 26). TBP mediates promoter recognition through the sequence-specific binding of the TATA-element found at many promoters. The importance of this event is indicated by studies that show TATA binding by TBP is rate-limiting at a majority of promoters (12, 17, 36, 38, 44, 47).

In yeast, 13 TAFs are required for viability, indicating essential roles for individual TAFs. However, the precise functional requirements for the TAFs remain unresolved. *In vitro* biochemical experiments suggest that TAFs function in higher eukaryotic systems as obligatory coactivators essential for activator response (reviewed in 9, 77). However, functional inactivation and depletion studies with certain TAFs in yeast cells demonstrate that the expression of many genes are unaffected by TAF loss, although TAF inactivation results in distinct cell cycle phenotypes (3, 55, 56, 58, 79). Furthermore, disruption of the TFIID complex with a temperature-sensitive mutation in TBP results in gene-specific transcriptional defects (67). Promoter-specific requirements for certain TAFs are further illustrated by whole-genome transcriptional profiles. For example, inactivation of TAF145/130 has no effect on the expression of a majority of genes, while transcription of a subset of genes is affected (30). This TAF-dependence was mapped to the core promoter (72), indicating important TAF functions in promoter activity *in vivo*. In contrast to these gene-specific effects, inactivation of several other TAFs, namely TAF17 (3, 55, 57), TAF40 (43); TAF60 and TAF61/68 (58) and TAF23/25 (71), results in dramatic effects on

virtually all genes transcribed by Pol II. The requirement for these particular TAFs is not yet understood, but it is clear that certain TAFs may be generally required for transcription while others function at a subset of promoters. To complicate the issue further, it is apparent that certain TAFs in both human and yeast systems can be found in large protein complexes distinct from TFIID, such as the SAGA complex (25) and the SWI/SNF complex (10). Taken together, these studies indicate that different TAFs may have distinct functional roles in transcription, yet the nature of the specific functions for a majority of the TAFs remains to be elucidated.

In addition to TAFs, other transcription factors associate with TBP at the core promoter to mediate transcription. One such factor, TFIIA, has been shown to stabilize the interaction between TBP and DNA at the TATA element (reviewed in 26). Mutational studies of both TBP and TFIIA have demonstrated the importance of the TFIIA-TBP interaction for transcription *in vivo* (34, 50, 64, 73). TFIIA has been described as a coactivator since *in vitro* functions of certain activators are TFIIA-dependent (48, 63), and an anti-repressor because TFIIA can mediate displacement of certain transcriptional inhibitors that act on TBP (4, 5, 22, 32, 41, 49, 52, 54, 65).

A growing body of evidence suggests that the functions of TFIIA and TAFs are connected. DNase I footprinting experiments show that the addition of TFIIA alters the DNA protection pattern of TFIID (14, 15, 48). Consistent with these findings, UV-crosslinking experiments indicate that TFIIA induces a conformational change in TFIID that alters specific TAF

interactions with the core promoter (60). Furthermore, a set of TFIIA mutations that can form a TBP/TFIIA/DNA complex, are defective for forming a complex with TFIID (65). Finally, the three-dimensional structure of the human TFIID-TFIIA-TFIIB complex clearly suggests TFIIA-TAF interactions since TFIIA maps to a large non-central lobe of TFIID with TBP being located more centrally in the structure (1). It is not yet understood how this TFIIA-TAF communication is established or which particular TAFs are involved.

In this report, we investigate the importance of the interactions between TFIIA and yeast TAFs. We demonstrate a direct interaction between TFIIA and TAF40, as well as a direct interaction between TAF40 and TBP. We also find that mutations in TFIIA that diminish the TFIIA-TAF40 interaction result in conditional growth phenotypes and defects in transcription *in vivo*. These results suggest that TAF40 serves as a link between TFIIA and TFIID functions, and reveal a new role for TAF40 in RNA polymerase II transcriptional regulation.

4.3 Experimental Procedures

4.3a DNA constructs.

Activation domain (AD) hybrids were cloned into the 2μ *LEU2* vector pACT2.2 vector (19), which contains the *ADH1* promoter, a nuclear localization sequence, the HA epitope, and the Gal4 activation domain

(residues 768-881). DNA-binding domain (DB) hybrids were created by subcloning from the corresponding AD constructs into the pPC97-*TRP* vector (CEN, *TRP3*), which contains the *ADH1* promoter, a nuclear localization sequence, and the Gal4 DNA-binding domain (residues 1-147). The TAF40 *E. coli* expression plasmid was created by cloning the TAF40 open reading frame into the pET15b vector using the polymerase chain reaction (PCR) and designed oligonucleotides. TOA2-YCP22 contains *TOA2* driven by its native promoter and terminator. It was generated from genomic DNA by PCR. This results in an engineered NcoI site located at the ATG start codon and a BamHI site just downstream of the stop codon. A 99 bp Nco1-Nco1 fragment containing two myc epitopes (GEQKLISEEDLN) was then cloned into TOA2-YCP22 using the engineered Nco1 site, creating myc-TOA2-YCP22. The site-directed *TOA2* mutants were created using olignonucleotide primers containing the desired mutation and PCR. Mutant derivatives were subsequently subcloned into the Gal4-DNA binding domain vector (pPC97-*TRP1*). All cloned PCR products containing the open-reading frame of *TOA2* were completely sequenced. GST-Toa1 was created by subcloning the EcoR1 fragment from TBP-Toa1 (73) into the PGEX-1λT vector.

4.3b Yeast strains.

All yeast strains used in the yeast two-hybrid assay were transformants of MaV103 (78). MaV103 contains the *GAL1* promoter (with four Gal4 binding sites) fused to the *HIS3* promoter and structural gene;

GAL4 and *GAL80* are both deleted in the strain. Viability tests of *TOA2* mutant derivatives were conducted in ROY100. ROY100, a derivative of KY114 (relevant genotype *MATa ade2-101 leu2::PET56 trp1Δ1 ura3-52*), was created using a two-step gene knockout of the complete open reading frame of the*TOA2* gene and contains *TOA2* on a 2μ, *URA3*-marked plasmid. The plasmid shuffle technique was used to introduce the mutant derivatives into ROY100.

4.3c Yeast two-hybrid assays and phenotypic studies.

Both Gal4 DB and Gal4 AD plasmids were transformed into the yeast strain MaV103 using a standard lithium acetate transformation. The resulting strains were grown in the appropriate selection media and 10-fold serial dilutions were performed. Cells were then spotted onto the appropriate plates that either contained or lacked 3-aminotriazole (AT). Cells were grown at 30 °C for 4-7 days. For phenotypic studies, ten-fold serial dilutions of strains were spotted to plates with rich media containing either glucose (YPD) or galactose (YPG), and incubated at either 30 or 38 °C.

4.3d Protein purification.

GST-TFIIA was produced by fusing the Toa1 open reading frame to glutathione S-transferase (GST). Each subunit (GST-Toa1 and Toa2) was expressed separately in *E. coli,* and each insoluble pellet was resolubilized in 8M urea. The subunits were combined and the urea was dialyzed out as

described in (30). After dialysis the soluble supernatant was incubated with glutathione resin. GST-TBP, GST-TFIIB and GST were expressed and purified from bacteria as described (73). TAF40 was purified using the similar denaturing and refolding method used to make recombinant yeast TFIIA. BL21 (DE3) cells containing TAF40 cloned into the bacterial expression plasmid pET15b (His-TAF40) were grown to an OD_{600} of 0.6 and induced for 2 h with 1mM IPTG at 30 °C. Cells were harvested, washed with 25 ml buffer A (20 mM Tris-HCl pH 7.5, 200 mM NaCl), resuspended in 25 ml buffer 1 (20 mM Tris-HCl pH 7.5, 500 mM NaCl), and frozen at –70 °C. Cells were thawed and sonicated. The insoluble fraction was collected by spinning at 10,000 xg for 15 min at 4 °C. Pellets were resuspended in 13 ml Buffer 2 (20 mM Tris-HCl, 500 mM NaCl, 8 M urea, and 1 mM dithiothreitol (DTT). 7.5 ml of Buffer B (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM PMSF, 1 mM DTT, 10% glycerol) was added slowly, and the solution was cleared by spinning at 10,000 g for 15 min at 4 °C. The supernatant was then dialyzed against Buffer B (9 L) for at least 24 h. The soluble fraction was cleared by spinning at 10,000 xg for 15 min at 4 °C. Soluble fraction was bound to Ni-NTA resin (Qiagen) and washed with buffer C (20mM Tris 7.5, 100mM KCl, 10% glycerol, 20mM imidazole, 1mM DTT) and eluted with buffer C containing 200mM imidazole.

4.3e *In vitro* interaction studies.

GST pull-down assay. All proteins used in *in vitro* assays were quantitated using a BSA standard curve, stained with coommassie blue. Approximately 25pmole of GST fusion protein or GST alone were incubated with 40 pmole of His-TAF40 protein in 200 μl of binding buffer (20mM HEPES pH 7.9, 20 mM Tris pH 7.5, 100 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 0.025% NP40, 10% glycerol, 0.5 mM DTT) for 2h at 4 °C. Complexes were recovered by incubation with glutathione sepharose for 1h at 4 °C in binding buffer with 3% BSA. Complexes were washed 2X in binding buffer then incubated with SDS loading buffer, boiled, and 10 μl of sample were separated by SDS-PAGE. Gels were analyzed by immunoblotting with antibodies specific to His-TAF40.

Electrophoretic mobility-shift assays were performed using a ^{32}P-labeled 45-basepair fragment containing the adenovirus early 1B TATA box as described (73). Purified recombinant yeast TBP (5nM), yeast TFIIA (1.5nM), and yeast TAF40 (19nM-142nM) were incubated at 25 ^0C for 30 min in 20μl of 20mM Tris (pH 7.5), 40mM HEPES (pH7.9), 100mM KCl, 1mM DTT, 0.5mM PMSF, 10% Glycerol. Complexes were separated from unbound DNA by 6% nondenaturing acrylamide gel electophoresis in 0.5X TBE and quantified by phosphorimaging.

4.3f Transcriptional Analysis.

Quantitative S1 nuclease analysis was done as described (33) with approximately 30 to 50 μg of RNA. For the temperature shift and AT inductions, cells were grown in synthetic complete medium to an OD600 of 0.5 - 1.0. Cells were pre-heat shocked at 38 °C for 15 minutes, incubated at 30 °C for one hour, followed by 38 °C for one hour. AT was added to a concentration of 20 mM and cells were incubated for an additional hour at 38 °C. Total RNA was prepared by hot-phenol extraction and was quantitated by A_{260}. RNA amounts in each reaction were normalized to the levels obtained from a probe to the intron of the tryptophan tRNA gene ($tRNA^W$).

4.4 Results

4.4a Yeast TAF40 associates with TFIIA *in vivo.*

TFIIA interacts with TBP and DNA at the promoter and stabilizes the TBP-DNA interaction. Yet, within a cell, TBP is most likely bound to DNA in association with TAFs in the TFIID complex. We used a yeast two-hybrid assay to investigate the potential interplay between TFIIA and TAFs. Yeast TFIIA is composed of two subunits encoded by the genes *TOA1* and *TOA2*, both of which are required for viability (69). The DNA-binding domain (DB) of Gal4 was fused in frame with Toa2 (DB-Toa2), creating the bait for the two-

hybrid assay. A panel of TAF proteins, TBP, and TFIIB were fused in frame to the Gal4 activation domain (AD). Fusion proteins were expressed in a yeast strain with the *HIS3* gene under the control of the *GAL1* promoter (which contains four Gal4 binding sites). Interactions between Toa2 and the AD-fusion proteins were determined by examining activation of the *HIS3* gene. *HIS3* gene activation was assayed by growth in the presence of 3-aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product (27). Strains in which the *HIS3* gene is highly expressed, due to interactions between the DB-fusion protein and the AD-fusion protein, will grow on AT.

Expression of DB-Toa2 showed no *HIS3* gene activation (Figure 4.1A), demonstrating that DB-Toa2 does not activate transcription independently. The two subunits of TFIIA, Toa2 and Toa1, exhibited a strong interaction, as expected. This result indicates that DB-Toa2 is not defective for subunit interactions with Toa1 and also suggests that DB-Toa2 interacts with native Toa1 within the cell. We then tested the ability of DB-Toa2 to interact with each of the eleven essential TAFs found in TFIID (for review see 26). A strong interaction was observed between Toa2 and TAF40. The interaction with TAF40 was specific in that Toa2 showed no interaction with the other TAF proteins shown, or with TAF25, TAF60, TAF67, TAF90, TAF130 and TAF150/TSM1 (data not shown). Expression of each of the TAF fusion proteins was confirmed by immunoblotting whole cell extracts with antibodies specific to the HA tag present in the activation-domain vector (data not shown). We also observed no interaction with TBP or TFIIB. Although

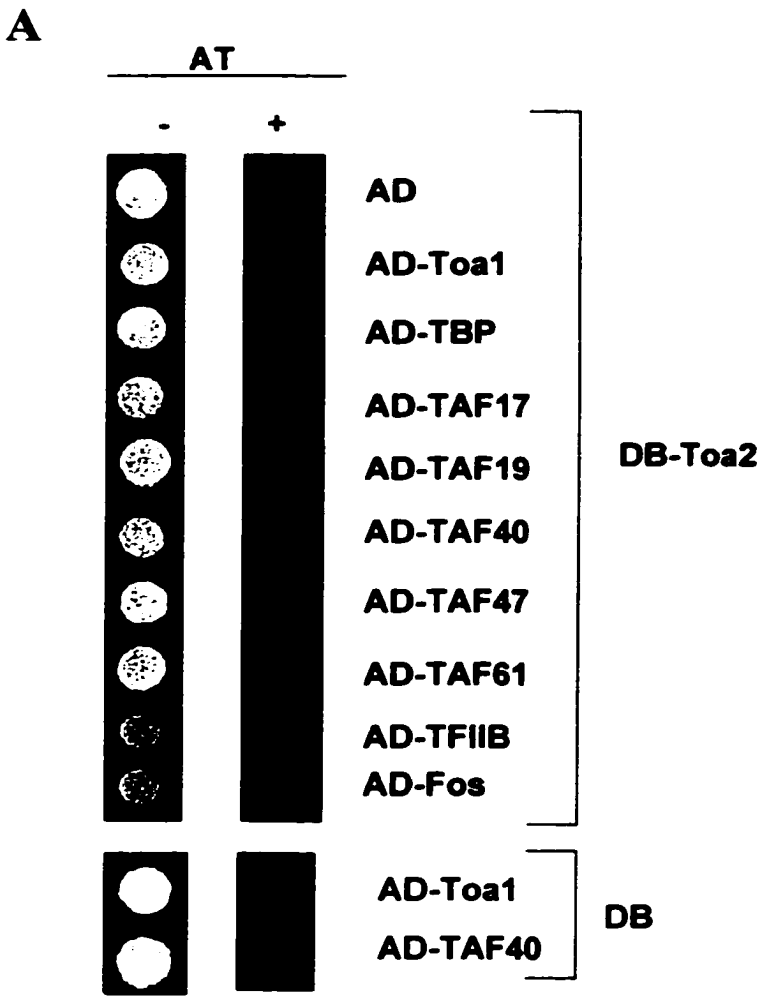


Figure 4.1A TFIIA associates with TAF40 *in vivo*. Two-hybrid assays were used to demonstrate that TFIIA interacts specifically with Toa1 and TAF40. The indicated Gal4 activation-domain (AD) fusion proteins were tested for the ability to interact with a DNA-binding (DB) fusion of Toa2 (DB-Toa2). Approximately 10^4 cells were spotted onto synthetic complete plates containing either 0 mM or 40 mM 3-aminotriazole (AT). Growth on AT is indicative of an interaction between the two hybrid proteins. The bottom panel shows that strains containing AD-Toa1 or AD-TAF40 do not grow on AT with the DB vector alone.

TFIIA and TBP are known to interact in other contexts, evidently the two-hybrid assay precludes detection of this interaction. These results suggest that TAF40 associates with Toa2 within the cell and that the interaction is specific, as expression of each of the other TAFs showed no association with DB-Toa2.

A reciprocal interaction between TAF40 and TFIIA could not be examined because expression of the fusion of TAF40 to the DNA binding domain resulted in high levels of transcriptional stimulation (Figure 4.1B). This indicates that recruitment of TAF40 to a promoter is sufficient to stimulate transcription of that gene. This result is in accord with similar recruitment studies using other TAFs and TBP (2, 7, 12, 21, 24, 35, 36, 83).

TFIIA has been shown to interact with certain activation domains (16, 39, 66). A trivial explanation for the interaction detected is that DB-Toa2 interacts with the AD of AD-TAF40, and the TAF40 domain is stimulating transcription. To examine this possibility, the Fos C-terminal activation domain, which strongly stimulates transcription when bound to the promoter via the Gal4 DB (Figure 4.1B), was cloned into the Gal4 AD vector (AD-Fos). Expression of the AD-Fos hybrid protein, which has two tandem functional activation domains, did not yield a positive two-hybrid interaction with DB-Toa2 (Figure 4.1A). The inability of DB-Toa2 to interact with AD-Fos eliminates the possibility that Toa2 is simply interacting with the Gal4 activation domain of AD-TAF40 and in effect mimicking the activation by DB-TAF40 seen in the artificial recruitment assay.

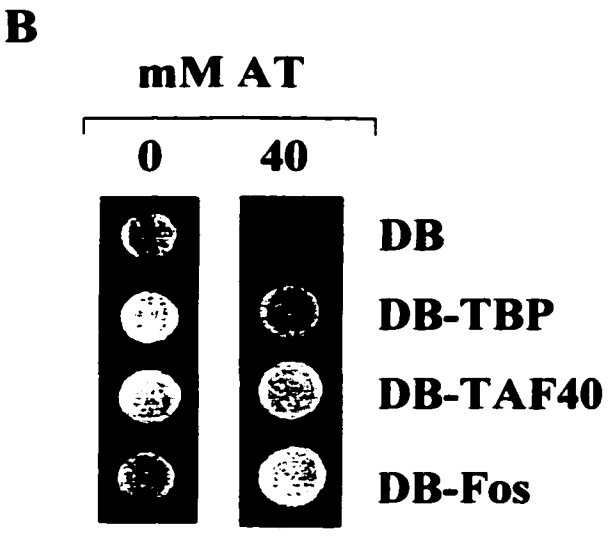


Figure 4.1B TAF40 stimulates transcription in an artificial recruitment assay. DB-TBP, DB-40 and DB-Fos each stimulate high levels of transcription when tethered to a promoter via a heterologous DNA binding domain.

4.4b TAF40 interacts with TFIIA and TBP.

To demonstrate a direct interaction between TAF40 and TFIIA or TBP, proteins were produced *in vitro* and their ability to physically interact was examined using glutathione-affinity pull-down assays (GST pull-down). GST-TFIIA was produced by fusion of glutathione S-transferase (GST) to the Toa1 open reading frame. GST-Toa1 and Toa2 were expressed in *E. coli* and pellets were renatured using 8M urea, then combined and the urea was removed to allow refolding. Activity assays performed on GST-TFIIA using EMSAs showed no loss in activity as compared to untagged TFIIA (data not shown). Proteins were allowed to bind and then incubated with glutathione sepharose. Immuno-blot analysis of the isolated complexes revealed that histidine-tagged TAF40 interacted with either GST-TFIIA or GST-TBP but not with GST or with GST-TFIIB (Figure 4.2A). These results demonstrate that the interaction between TAF40 and TFIIA is direct, and that TAF40 interacts directly with TBP.

An electrophoretic mobility gel shift assay was used to test whether the presence of TAF40 affects the TBP-TFIIA-DNA ternary complex. In the absence of magnesium, TBP does not form a stable complex with DNA and the addition of TFIIA stabilizes the TBP-DNA interaction and shifts the TATA-containing probe (Figure 4.2B). When sub-saturating amounts of TFIIA are added to the reaction, the addition of TAF40 dramatically increased the amount of complex formed. Moreover, the amount of probe shifted increased with increasing TAF40 concentration (Figure 4.2B). Incubation of excess

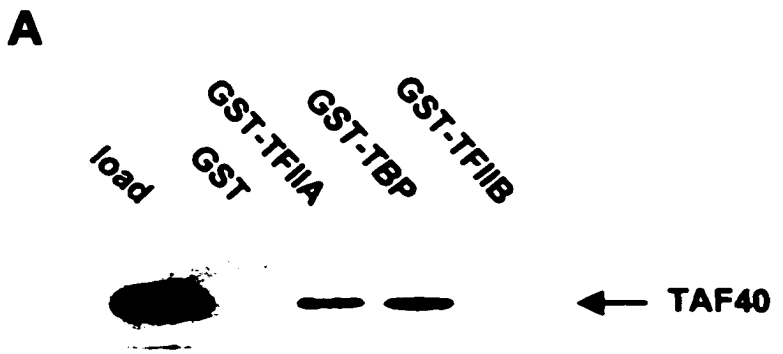


Figure 4.2A TAF40 interacts directly with TFIIA or TBP. To test whether TFIIA interacts directly with TAF40 *in vitro*, a glutathione-affinity chromatography pull-down assay was used. Recombinant GST-TFIIA, GST-TBP, GST-TFIIB or GST were incubated purified histidine-tagged TAF40 (His-TAF40). Complexes were isolated by adding glutathione resin and washing 2X (Pulldowns). Samples were separated by SDS-PAGE and analyzed by immunoblot using antibodies specific to TAF40.

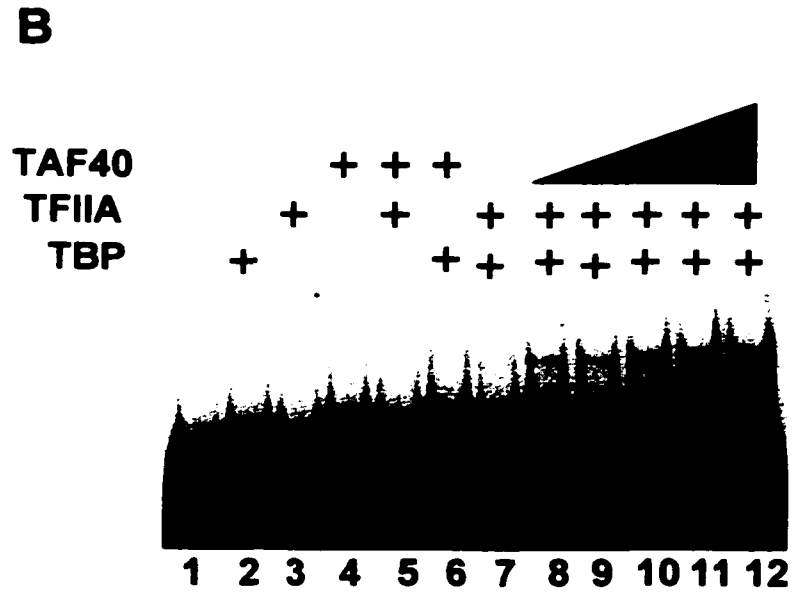


Figure 4.2B TAF40 increases TBP-TFIIA-DNA complex formation on a TATA box. For all reactions, 9nM of radiolabeled adenovirus early 1B TATA box probe was used. Concentrations of TBP and TFIIA were held constant at 5 nM and 1.5 nM, respectively. Lane 1 contains DNA alone. Lane 2 contains TBP, lane 3 contains TFIIA and lane 4 contains TAF40 (142nM). Lane 5 contains TAF40 (142nM) and TFIIA. Lane 6 contains TAF40 (142nM) and TBP. Lane 7 contains TBP and TFIIA, and lanes 8 through 12 contain TBP and TFIIA with increasing amounts of TAF40 (19nM-142nM).

TAF40 with either TBP or TFIIA alone did not result in DNA binding (Figure 4.2B). Thus, TAF40 enhances the formation of a complex containing DNA, TBP, and TFIIA. To demonstrate the specificity of this interaction antibodies specific to TAF40 were titrated into reactions containing TBP, TFIIA and TAF40. The addition of antibodies dramatically reduced the enhancement mediated by TAF40 (supplemental figure 4.7). This effect was not observed when antibodies were added to reactions containing only TBP and TFIIA.

4.4c Mutations in a hydrophobic patch of TFIIA are defective for interacting with TAF40.

Analysis of the crystal structures of the yeast TFIIA-TBP-DNA ternary complex (23, 74), revealed several striking features. TFIIA consists of two domains, a β–domain and a four helix bundle (4HB) domain (Figure 4.3). The β–domain makes all of the contacts with TBP and also binds DNA upstream of the TATA element. The 4HB domain of TFIIA projects away from the TBP-TFIIA-DNA complex into solution. In addition, there are two large solvent-exposed patches of hydrophobic residues on TFIIA: one patch is within the β-domain and the other is within the 4HB domain (Figure 4.3). Hydrophobic interactions are important for many protein-protein interactions. In fact, the hydrophobic region on the β-domain contacts TBP. We hypothesized that the hydrophobic patch on the 4HB domain may contribute to other TFIIA functions, possibly for interactions with TAF40. Therefore, we targeted three residues within this hydrophobic patch for mutational studies:

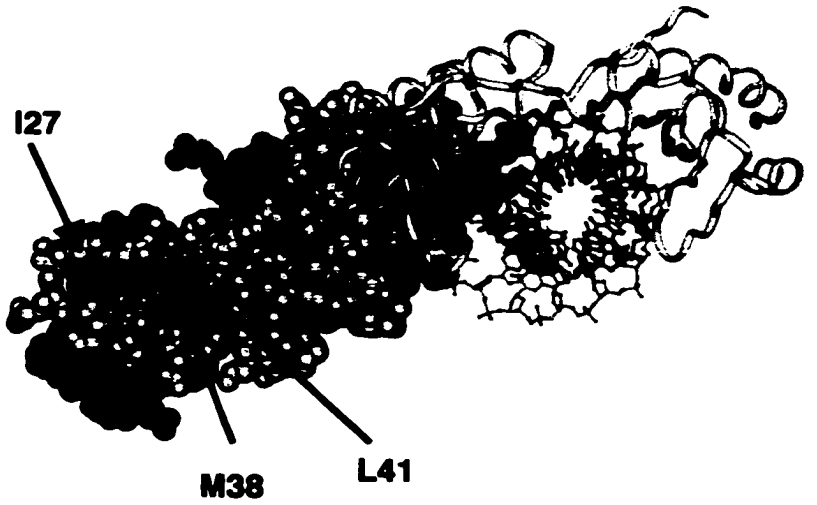


Figure 4.3 Crystal structure of the TFIIA-TBP-DNA complex showing the location of the amino acids replaced in the hydrophobic region of the four helix bundle. TBP is shown in a yellow ribbon, and DNA is black. TFIIA is shown in spacefilling model, with Toa1 in dark blue and Toa2 in light blue. The two hydrophobic patches on TFIIA are shown in gray; one patch contacts TBP. The amino acids in Toa2 selected for replacement by alanine or charged amino acids are shown in magenta and indicated by the arrows and the labels. The figure was created with Insight II, using the coordinates of the TBP-TFIIA-DNA structure (73).

The isoleucine at position 27 of Toa2 was changed to alanine (I27A) or lysine (I27K); methionine at position 38 was changed to alanine (M38A) or lysine (M38K); and leucine at position 41 was substituted with either alanine (L41A) or aspartic acid (L41D).

Each of the mutant derivatives was cloned into the DNA-binding domain vector. To determine whether any of the mutations causes a drastic change in the folding ability of Toa2, the derivatives were tested for the ability to interact with AD-Toa1 (Figure 4.4). DB-L41D was defective for interacting with Toa1, indicating that this Toa2 derivative is compromised for TFIIA formation. The five remaining derivatives were indistinguishable from wild-type Toa2 with regards to their ability to interact with Toa1.

We also tested the Toa2 derivatives for defects in interactions with TAF40. I27A, I27K, and L41D showed significantly weakened interactions with TAF40 in the two-hybrid assay (Figure 4.4). Since L41D was also defective for interaction with Toa1, the loss of the TAF40 interaction may be the result of global defects in the structure of this protein. In contrast, substitutions at I27 are specifically defective for TAF40 interactions in that both I27A and I27K are indistinguishable from wild-type Toa2 for Toa1 interactions. The remaining derivatives exhibited interactions with both TAF40 and Toa1 comparable to wild-type Toa2. These results indicate that the hydrophobic region on the 4HB domain of TFIIA, and in particular residue I27 of Toa2, plays an important role in the interaction between TFIIA and TAF40.

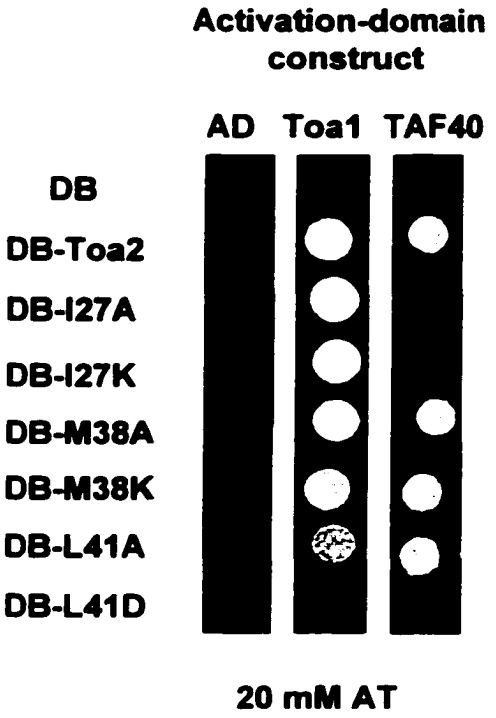


Figure 4.4 Mutations in Toa2 impair interactions with TAF40. The two-hybrid assay was used to identify mutations in the hydrophobic patch of the TFIIA four-helix bundle that are defective for interaction with TAF40. The activation-domain constructs are indicated across the top, the DB-Toa2 derivatives are shown along the left side. Approximately 10^4 cells were spotted onto plates containing 20 mM AT. All strains grew robustly on media lacking AT (data not shown).

4.4d Toa2 derivatives defective for TAF40 interaction impart mutant growth phenotypes.

To examine the physiological relevance of the TFIIA-TAF40 interaction, the I27A and I27K Toa2 mutants (under the control of the *TOA2* promoter and terminator) were expressed in a *TOA2* deletion strain. Both alleles supported cell viability, but each caused a slow-growth phenotype at 30 °C and a temperature-sensitive phenotype at 38 °C (Figure 4.5A). The slow-growth phenotype at 30 °C is consistent with the observation that the TFIIA-TAF40 interaction is disrupted in the two-hybrid assay, which is performed at the same temperature. Furthermore, the I27K mutant was unable to support growth on galactose-containing media, suggesting an inability to respond to the Gal4 activator protein. Mutant phenotypes were not the result of a destablization of Toa2 protein, since the I27A and I27K strains produced amounts of Toa2 protein comparable to wild-type Toa2 at 30 and 38 °C as assayed by immunoblot analyses of whole cell yeast extracts (Figure 4.5B).

4.4e TFIIA mutants compromised for TAF40 interaction are defective for transcription *in vivo*.

To determine the ramifications of a defect in the TFIIA-TAF40 interaction, we compared wild-type, I27A and I27K strains for their transcriptional competency. Constitutive transcription of the *HIS3* gene was examined at 30 and 38 °C (Figure 4.6A). When cultured at 30 °C the I27A

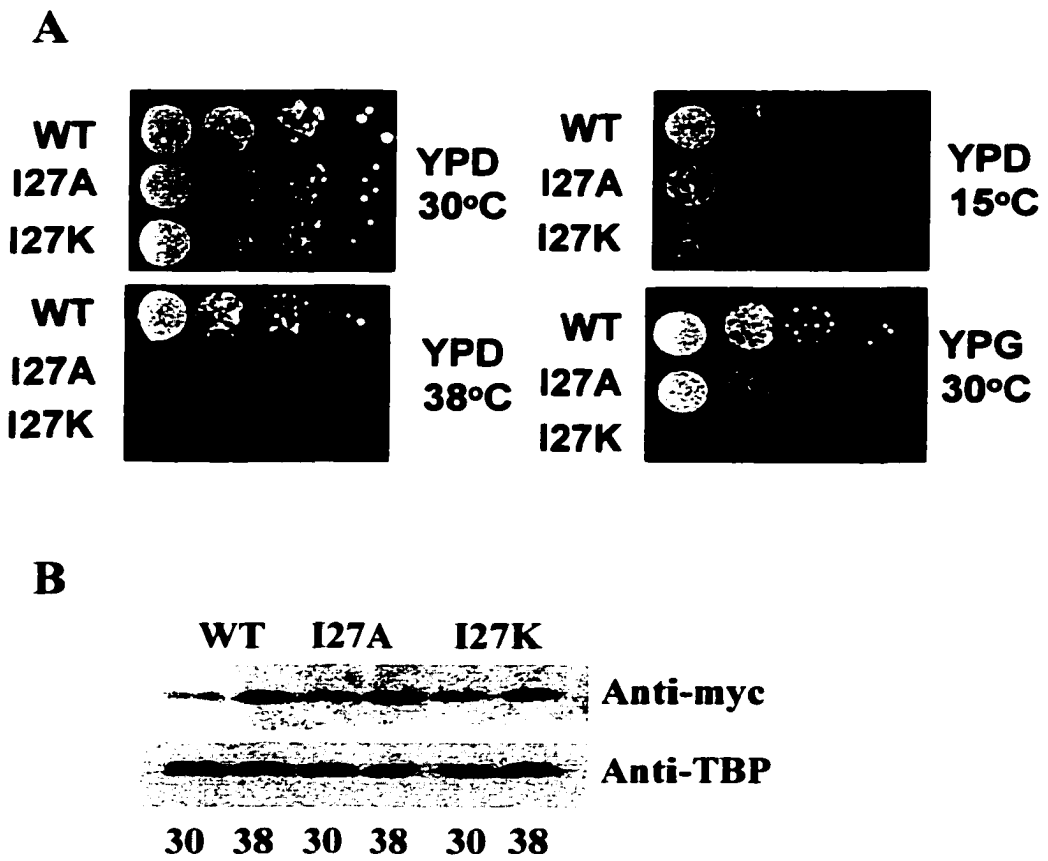
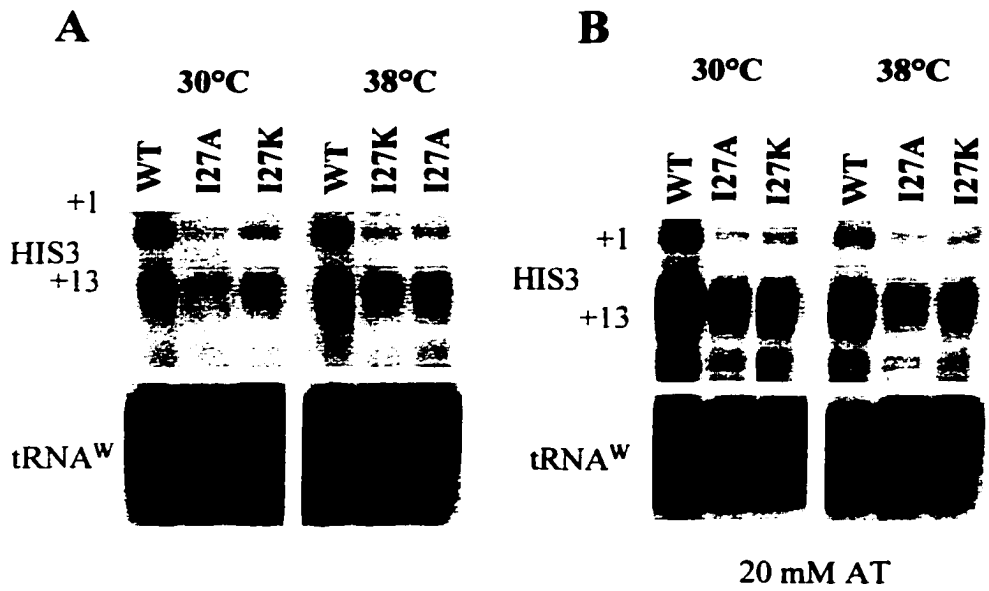


Figure 4.5A and 4.5B TAF40-defective Toa2 derivatives confer mutant phenotypes. (A) Strains containing the I27A and I27K mutations of Toa2 were tested for conditional phenotypes in a *TOA2* deletion strain. The indicated strains were serially diluted (10^4 to 10 cells) and spotted onto rich media plates containing glucose (YPD) or galactose (YPG) and incubated at either 15, 30, or 38 °C. (B) Levels of Toa2, I27A and I27K proteins are indistinguishable. Strains containing wild-type Toa2 in addition to either myc-tagged wild-type Toa2 (WT), myc-tagged I27A (I27A), or myc-tagged I27K (I27K), were harvested after incubation at 30 or 38 °C . Extracts (20 μg) were subjected to SDS-PAGE and immunoblotting with anti-myc antibodies and anti-TBP antibodies (for a load control). The presence of wild-type Toa2 in each of the strains allows for an accurate analysis of the stability of the mutant derivatives, since cell viability is not dependent on their expression.



Figures 4.6A and 4.6B TFIIA mutants defective in the TAF40 interaction have decreased levels of transcription. Analysis of *HIS3* gene transcription in wild-type (WT), I27A or I27K Toa2 strains after culturing at 30 and 38 °C for one hour. Total RNA (30 μg) was hybridized with 100-fold excesses of *HIS3* and *tRNA^w* probes and then subjected to S1 nuclease digestion. The *HIS3* +1 and +13 initiation sites are indicated. The RNA polymerase III-transcribed *tRNA^w* gene serves as a loading control. (B) Analysis of Gcn4-dependent activation of *HIS3* transcription. For assays at 30 °C, 20 mM AT was added for one hour, followed by harvesting the cells. For assays at 38 °C, cells were grown to early log phase, shifted to 38 °C for one hour and then grown an additional hour in the presence of 20 mM AT. Probes and digestion are as above.

and I27K strains exhibit a reduction in *HIS3* gene expression as compared to wild-type cells. Levels of transcription of both the +1 and +13 transcripts of *HIS3* are decreased approximately 3-4 fold. The +1 transcript is generated from a non-canonical promoter element while the +13 transcript is derived from a conventional TATA element (13, 33). This result suggests that the TAF40-TFIIA interaction is important for transcription from both canonical and non-canonical promoters *in vivo*. The decrease in transcription is not exacerbated by a shift to the restrictive temperature, which is consistent with the fact that the TAF40-TFIIA interaction defect is observed at 30 °C.

We also tested the ability of the I27A and I27K strains to respond to acidic activators. Gcn4-dependent activation of *HIS3* transcription was assayed by growing the cells in 3-aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product (Figure 4.6B). To examine the response at the restrictive temperature, cells were incubated at 38 °C for one hour, AT was added, and the cells were incubated for an additional hour before harvesting. For both I27A and I27K mutants, activation of *HIS3* transcription was decreased as compared to wild-type cells. Activation by the acidic activator Gal4 was determined by growing cells in galactose-containing media and assaying for *GAL1* transcript levels (Figure 4.6C). Both I27A and I27K mutants displayed a significant decrease in *GAL1* transcription. Taken together, these defects in transcription for the I27A and I27K derivatives of Toa2 indicate that the TAF40-TFIIA interaction plays a role in transcription *in vivo*.

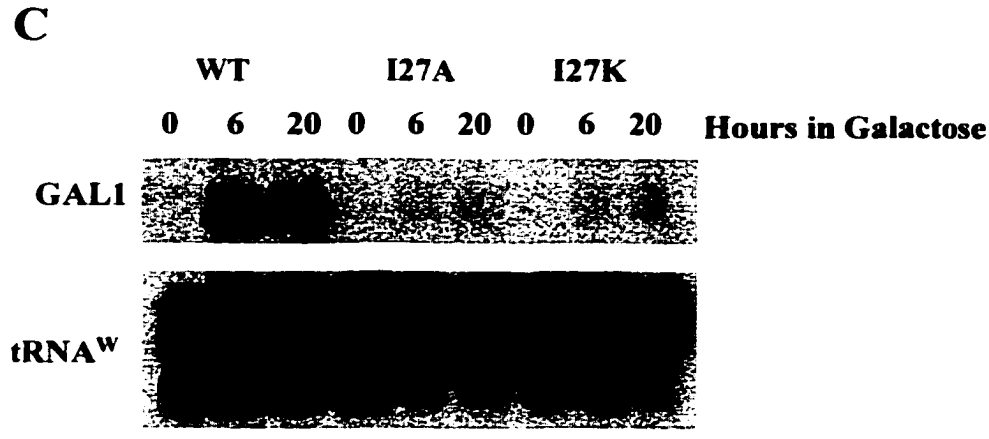


Figure 4.6C Analysis of Gal4-dependent activation of *GAL1* transcription. Strains were grown in raffinose-containing medium to early log phase and then grown for the indicated time in galactose at 30 °C. Total RNA (30 μg) was hybridized with 100-fold excesses of *GAL1* and *tRNA^{w}* probes and then subjected to S1 nuclease digestion.

4.5 Discussion

4.5a TFIIA and TAF40 interact directly.

The ability of TFIIA to interact with TBP and stabilize it on a promoter is well characterized (23, 31, 74, 80), but how TFIIA communicates with TBP in the context of TFIID remains unclear. TFIIA is generally required for transcription in the presence of TAFs, yet it is uncertain how the functions of TFIIA and TAFs are connected. We report here that an important link between TFIIA and TFIID resides in TAF40. The interaction between TFIIA and TAF40 was observed *in vivo* and also shown to be direct with recombinant forms of the proteins. These results suggest that the TFIIA-TAF40 interaction may be directly involved in processes that are TFIIA- and TAF-dependent.

We also identified a direct interaction between TAF40 and TBP. TAF-TBP interactions have been identified in other organisms (28, 29, 40, 53, 59, 75, 81, 82), and a 100-amino acid fragment of yeast TAF130/145 has been shown to interact with TBP (6, 41). However, TAF40 is the first full-length TAF that has been shown to interact directly with yeast TBP in solution. A TBP-TAF40 interaction is consistent with studies that show the human homologue of TAF40, human TAF28, interacts with human TBP (45, 46, 53), and indicates that this interaction is conserved from yeast to humans. Human TAF28 has also been shown to interact with human TAF18, TAF55, TAF100, TAF135, and the viral activator Tax (8, 11, 18, 45, 46, 53). Taken

together with our studies, these results suggest that TAF40 may be a very important player in TAF-TAF, TAF-TBP and TAF-TFIIA interactions.

4.5b Mutations in TFIIA affect the interaction with TAF40.

Functional importance for the TFIIA-TAF40 interaction is supported by mutational studies of the Toa2 subunit of TFIIA. Analysis of the crystal structures of the TFIIA-TBP-DNA complex (23, 74) indicated two hydrophobic patches on the surface of TFIIA. One of the hydrophobic patches is located on the β-domain of TFIIA and is involved with TBP interactions. The second hydrophobic patch is located on the four-helix bundle of TFIIA. Although the four-helix bundle of TFIIA is not involved in either TBP or DNA interactions in the crystal structures, a deletion derivative of human TFIIA lacking two of the helices in this domain is not responsive to activators *in vitro* (51). This result suggests that the four-helix bundle domain of TFIIA is a functionally important target that facilitates formation of an active transcription initiation complex. We constructed mutations within this hydrophobic patch on the four-helix bundle domain of TFIIA and tested them for their ability to interact with TAF40. Mutations at the I27 residue of Toa2 (I27A and I27K) caused a defect in the interaction with TAF40. The loss of interaction is not likely the result of global changes in the structure of the protein, since these Toa2 derivatives interacted normally with the other subunit of TFIIA and were expressed at similar levels to wild-type Toa2. The I27A and I27K mutants also displayed mutant growth phenotypes and defects in transcription *in vivo*.

The isoleucine at position 27 of Toa2 is conserved in yeast, Drosophila and human TFIIA (23), suggesting that this surface may play a critical role in the interaction of the higher eukaryotic homologues of TFIIA and TAF40. It is interesting to speculate that an interaction between TFIIA and TAF40 would orient the TAF40-TBP interaction on the N-terminal repeat of TBP. The surface of TBP situated on the same side of the structure as I27 is the precise location of the altered amino acids in a TBP mutant that has been demonstrated to be defective in TFIID formation *in vivo* (67).

Significant defects in transcription were observed in the I27A and I27K Toa2 mutant strains; however, a complete cessation in transcription was not expected since the mutants support cell viability. In addition, transcriptional defects observed in previous studies in which TFIIA was inactivated (34, 50) were comparable to those reported here. It is clear that inactivation of TFIIA does not typically cause dramatic transcriptional affects. In our TFIIA mutant strains, it is likely that disruption of TFIIA-TAF40 interactions could be compensated by redundancy in other interactions that contribute to formation of the TBP-TFIIA-TAF40 complex. Important interactions for complex stability may arise from TFIIA-TAF40 contacts, TAF40-TBP contacts, TFIIA-TBP contacts, and interactions between DNA and both TFIIA and TBP. Evidence for the importance of compensatory interactions also stems from recent reports that show that mutations in the region of TFIIA that binds DNA can suppress mutant phenotypes of TBP alleles with DNA-binding defects (50). It is interesting to note that mutations

in Toa2 at Glycine 30 (G30) also suppress the TBP mutant defective for DNA binding, and yet G30 mutants have no effect on *in vitro* interactions involving TBP, TFIIA and DNA (50). This result suggests that G30 substitutions compensate *in vivo* by increasing interactions with another component of the complex. G30 is located near the hydrophobic patch of Toa2 and directly abuts I27. It is interesting to speculate that mutations at G30 have the potential to alter the TFIIA-TAF40 interaction.

4.5c TAF40 may act as a communicator between TFIIA and TFIID.

The association between TFIIA and TAF40 is particularly noteworthy because it supports a growing body of evidence that indicates that transcriptional activity mediated by TAFs is dependent on TFIIA. TFIIA is required for *in vitro* transcription reactions when TAFs are present (reviewed in 26). TFIIA has also been shown to induce a conformational change in the TFIID complex bound to a promoter. Specifically, the presence of TFIIA extends the footprint of TFIID downstream of the transcriptional start site, and alters the crosslinking pattern of several TAFs to promoter sequences (14, 15, 20, 60). It has also been shown that an interaction between TFIIA and TFIID results in the generation of a productive form of TFIID that is capable of stably interacting with the promoter (68). Moreover, under certain conditions, TAFs have been shown to inhibit the ability of TBP to bind DNA (41, 42, 59, 76). The addition of TFIIA reverses this TAF inhibition (41, 65). Our discovery that TFIIA interacts directly with TAF40 further supports this functional link

between TFIIA and TFIID. Although we saw no evidence for interactions between TFIIA and the ten other TAFs tested, it remains to be determined if, under different conditions, other yeast TAFs can interact with TFIIA. A direct interaction between TFIIA and Drosophila TAF110 has been reported (84), but a yeast homolog of TAF110 has not been identified.

TAF40 is required for viability in yeast, indicating that it performs an essential and non-redundant function (37), and TAF40 inactivation affects transcription from a majority of RNA polymerase II promoters in yeast (43). The identification and characterization of an interaction between TAF40 and TFIIA is a first step in determining a mechanistic requirement for TAF40 in transcription. Since TAF40 is found only in the TFIID complex (25, 43, 58, 61), our results demonstrate that TAF40 has the potential to serve as a specific link between TFIIA and TFIID functions *in vivo*.

4.6 Acknowledgements.

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Supplemental Figure For Chapter 4

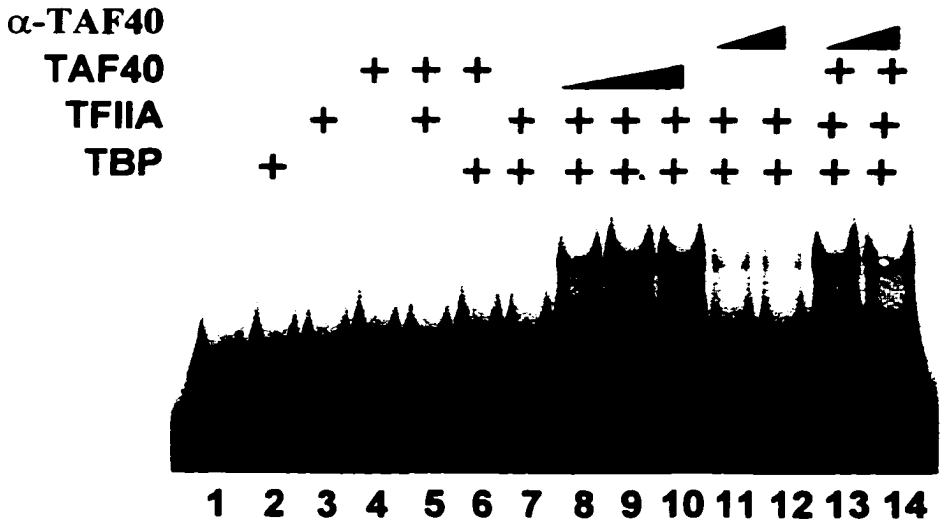

Figure 4.7 TAF40 specifically increases TBP-TFIIA-DNA complex formation on a TATA box. For all reactions, 9nM of radiolabeled adenovirus early 1B TATA box probe was used. Concentrations of TBP and TFIIA were held constant at 5 nM and 1.5 nM, respectively. Lane 1 contains DNA alone. Lane 2 contains TBP, lane 3 contains TFIIA and lane 4 contains TAF40 (142nM). Lane 5 contains TAF40 (142nM) and TFIIA. Lane 6 contains TAF40 (142nM) and TBP. Lane 7 contains TBP and TFIIA, and lanes 8 through 10 contain TBP and TFIIA with increasing amounts of TAF40 (57nM, 72nM, and 142nM respectively). Lanes 11 and 12 contain TBP and TFIIA with increasing TAF40 specific antibody (100-200ng). Lanes 13 and 14 contain TBP, TFIIA and TAF40 (72nM and 142nM) with increasing TAF40 specific antibody.

4.7 References

1. Andell III, F., A. G. Ladurner, C. Inouye, R. Tjian, and E. Nogales. 1999. Three-dimensional structures of the human TFIID-IIA-IIB complex. *Science* 286:2153-2156.

2. Apone, L. M., C. A. Virbasius, J. C. Reese, and M. R. Green. 1996. Yeast $TAF_{II}90$ is required for cell-cycle progression through G_2/M but not for general transcription activation. *Genes & Dev.* 10:2368-2380.

3. Apone, L. M., C.-A. Virbasius, F. C. Holstege, J. Wang, R. A. Young, and M. R. Green. 1998. Broad, but not universal, transcriptional requirement for $yTAF_{II}17$, a histone H3-like TAF_{II} present in TFIID and SAGA. *Molecular Cell* 2:653-661.

4. Auble, D. T., and S. Hahn. 1993. An ATP-dependent inhibitor of TBP binding to DNA. *Genes & Dev.* 7:844-856.

5. Auble, D. T., K. E. Hansen, C. G. F. Mueller, W. S. Lane, J. Thorner, and S. Hahn. 1994. Mot1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism. *Genes & Dev.* 8:1920-1934.

6. Bai, Y., G. M. Perez, J. M. Beechem, and P. A. Weil. 1997. Structure-function analysis of *TAF130*: Identification and characterization of a high-affinity TATA-Binding Protein interaction domain in the N terminus of yeast $TAF_{II}130$. *Molecular and Cellular Biology* 17:3081-3093.

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

8. Birck, C., O. Poch, C. Romier, M. Ruff, G. Mengus, A. Lavigne, I. Davidson, and D. Moras. 1998. Human TAF(II)28 and TAF(II)18 interact through a histone fold encoded by atypical evolutionary conserved motifs also found in the SPT3 family. *Cell* 94:239-249.

9. Burley, S. K., and R. G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* 65:769-799.

10. Cairns, B. R., N. L. Henry, and R. D. Kornberg. 1996. TFG/TAF30/ANC1, a component of the yeast SWI/SNF complex that is similar to the leukemogenic proteins ENL and AF-9. *Mol Cell Biol* 16:3308-16.

11. Caron, C., G. Mengus, V. Dubrowskaya, A. Roisin, I. Davidson, and P. Jalinot. 1997. Human TAF(II)28 interacts with the human T cell leukemia virus type I Tax transactivator and promotes its transcriptional activity. *Proc Natl Acad Sci U S A* 94:3662-3667.

12. Chatterjee, S., and K. Struhl. 1995. Connecting a promoter-bound protein to the TATA-binding protein overrides the need for a transcriptional activation region. *Nature* 374:820-822.

13. Chen, W., and K. Struhl. 1988. Saturation mutagenesis of a yeast *his3* TATA element: genetic evidence for a specific TATA-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 85:2691-2695.

14. Chi, T., and M. Carey. 1996. Assembly of the isomerized TFIIA-TFIID-TATA ternary complex is necessary and sufficient for gene activation. *Genes & Dev.* 10:2540-2550.

15. Chi, T., P. Lieberman, K. Ellwood, and M. Carey. 1995. A general mechanism for transcriptional synergy by eukaryotic activators. *Nature* 377:254-257.

16. Clemens, K. E., G. Piras, M. F. Radonovich, K. Sook Choi, J. F. Duvall, J. DeJong, R. Roeder, and J. N. Brady. 1996. Interaction of the Human T-Cell Lymphtropic Virus Type 1 Tax Transactivator with Transcription Factor IIA. *Molecular and Cellular Biology* 16:4656-4664.

17. Colgan, J., and J. L. Manley. 1992. TFIID can be rate limiting in vivo for TATA-containing, but not TATA-lacking, RNA polymerase II promoters. *Genes & Dev.* 6:304-315.

18. Dubrovskaya, V., A. Lavigne, I. Davidson, J. Acker, A. Staub, and L. Tora. 1996. Distinct domains of hTAF$_{II}$100 are required for functional interaction with transcription factor TFIIF beta (RAP30) and incorporation into the TFIID complex. *EMBO J* 15:3702-3712.

19. Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang, A. E. Kilburn, W.-H. Lee, and S. J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Dev.* 7:555-569.

20. Emami, K., A. Jain, and S. Smale. 1997. Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization. *Genes Dev* 11:3007-19.

21. Farrell, S., N. Simkovich, Y. Wu, A. Barberis, and M. Ptashne. 1996. Gene activation by recruitment of the RNA polymerase II holoenzyme. *Genes and Development* 10:2359-2367.

22. Ge, H., and R. G. Roeder. 1994. Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell* 78:513-523.

23. Geiger, J. H., S. Hahn, S. Lee, and P. B. Sigler. 1996. Crystal structure of the yeast TFIIA/TBP/DNA complex. *Science* 272:830-836.

24. Gonzalez-Couto, E., N. Klages, and M. Strubin. 1997. Synergistic and promoter-selective activation of transcription by recruitment of transcription factors TFIID and TFIIB. *Proc. Natl. Acad. Sci. U.S.A.* 94:8036-8041.

25. Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. Steger, J. C. Reese, J. R. Yates III, and J. L. Workman. 1998. A subset of TAF_{II}s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. *Cell* 94:45-53.

26. Hampsey, M. 1998. Molecular genetics of the RNA polymerase II genreal transcriptional machinery. *Microbiology and Molecular Biology Reviews* 62:465-503.

27. Hill, D. E., I. A. Hope, J. P. Macke, and K. Struhl. 1986. Saturation mutagenesis of the yeast *HIS3* regulatory site: requirements for transcriptional induction and for binding by GCN4 activator protein. *Science* 234:451-457.

28. Hisatake, K., T. Ohta, R. Takada, M. Guermah, T. Yamamoto, M. Horikoshi, and R. G. Roeder. 1995. Evolutionary conservation of human TATA-binding-polypeptide-associated factors $TAF_{II}31$ and $TAF_{II}80$ and interactions of $TAF_{II}80$ with other TAFs and with general transcription factors. *Proc. Natl. Acad. Sci. USA* 92:8195-8199.

29. Hoffmann, A., and R. G. Roeder. 1996. Cloning and characterization of human TAF20/15. *J Biol. Chem.* 271:18194-18202.

30. Holstege, F. C. P., E. G. Jennings, C. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander, and R. A. Young. 1998. Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* 95:717-728.

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

32. Inostroza, J. A., F. H. Mermelstein, I. Ha, W. S. Lane, and D. Reinberg. 1992. Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. *Cell* 70:477-489.

33. Iyer, V., and K. Struhl. 1995. Mechanism of differential utilization of the *his3* T_R and T_C TATA elements. *Mol. Cell. Biol.* 15:7059-7066.

34. Kang, J. J., D. T. Auble, J. A. Ranish, and S. Hahn. 1995. Analysis of yeast transcription factor TFIIA: Distinct functional regions and a Polymerase II-specific role in basal and activated transcription. *Mol. Cell. Biol.* 15:1234-1243.

35. Keaveney, M., and K. Struhl. 1998. Activator-mediated recruitment of the RNA Polymerase II machinery is the predominant mechanism for transcriptional activation in yeast. *Molecular Cell* 1:917-924.

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

37. Klebanow, E. R., D. Poon, S. Zhou, and A. P. Weil. 1997. Cloning and characterization of an essential *Saccharomyces cerevisiae* gene,*TAF40*, an RNA polymerase II-specific TATA-binding protein-associated factor. *J. Biol. Chem.* 272:9436-9442.

38. Klein, C., and K. Struhl. 1994. Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. *Science* 266:280-282.

39. Kobayashi, N., T. G. Boyer, and A. J. Berk. 1995. A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly. *Mol. Cell. Biol.* 15:6465-6473.

40. Kokubo, T., D.-W. Gong, J. C. Wootton, M. Horikoshi, R. G. Roeder, and Y. Nakatani. 1994. Molecular cloning of Drosophila TFIID subunits. *Nature* 367:484-487.

41. Kokubo, T., M. J. Swanson, J.-I. Nishikawa, A. G. Hinnebusch, and Y. Nakatani. 1998. The yeast TAF145 inhibitory domain and TFIIA competitively bind to TATA-Binding Protein. *Molecular and Cellular Biology* 18:1003-1012.

42. Kokubo, T., S. Yamashita, M. Horikoshi, R. G. Roeder, and Y. Nakatani. 1994. Interaction between the N-terminal domain of the 230-kDa subunit and the TATA box-binding subunit of TFIID negatively regulates TATA-box binding. *Proc. Natl. Acad. Sci. USA* 91:3520-3524.

43. Komarnitsky, P. B., B. Michel, and S. Buratowski. 1999. TFIID-specific yeast TAF40 is essential for the majority of RNA polymerase II-mediated transcription in vivo. *Genes and Dev.* 13:2484-2489.

44. Kuras, L., and K. Struhl. 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. *Nature* 399:609-613.

45. Lavigne, A., G. Mengus, M. May, V. Dubrovskaya, L. Tora, P. Chambon, and I. Davidson. 1996. Multiple interactions between hTAF$_{II}$55 and other TFIID subunits. Requirements for the formation of stable ternary complexes between hTAF$_{II}$55 and the TATA-binding protein. *J Biol Chem* 271:19774-19780.

46. Lavigne, A. C., Y.-G. Gangloff, L. Carre, G. Mengus, C. Birck, O. Poch, C. Romier, D. Moras, and I. Davidson. 1999. Synergistic transcriptional activation by TATA-binding protein and hTAF$_{II}$28 requires specific amino acids of hTAF$_{II}$28 histone fold. *Mol. Cell. Biol.* 19:5050-5060.

47. Li, X. Y., A. Virbasius, X. Zhu, and M. R. Green. 1999. Enhancement of TBP binding by activators and general transcription factors. *Nature* 399:605-609.

48. Lieberman, P. M., and A. J. Berk. 1994. A mechanism for TAFs in transcriptional activation: activation domain enhancement of TFIID-TFIIA-promoter DNA complex formation. *Genes & Dev.* 8:995-1006.

49. Liu, D., R. Ishima, K. I. Tong, S. Bagby, T. Kokubo, D. R. Muhandiram, L. E. Kay, Y. Nakatani, and M. Ikura. 1998. Solution structure of a TBP-TAF$_{II}$230 complex: Protein mimicry of the minor groove surface of the TATA box unwound by TBP. *Cell* 94:573-583.

50. Liu, Q., S. E. Gabriel, K. L. Roinick, R. D. Ward, and K. M. Arndt. 1999. Analysis of TFIIA function in vivo: Evidence for a role in TATA-binding Protein recruitment and gene-specific activation. *Mol. Cell. Biol.* 19:8673-8685.

51. Ma, D., I. Olave, A. Merino, and D. Reinberg. 1996. Separation of the transcriptional coactivator and anti-repression functions of transcription factor IIA. *Proc. Natl. Acad. Sci. USA* 93:6583-6588.

52. Meisterernst, M., and R. G. Roeder. 1991. Family of proteins that interact with TFIID and regulate promoter activity. *Cell* 67:557-567.

53. Mengus, G., M. May, X. Jacq, A. Staub, L. Tora, P. Chambon, and I. Davidson. 1995. Cloning and characterization hTAF$_{II}$18, hTAF$_{II}$20 and hTAF$_{II}$28: three subunits of the human transcription factor TFIID. *The EMBO Journal* 14:1520-1531.

54. Merino, A., K. R. Madden, W. S. Lane, J. J. Champoux, and D. Reinberg. 1993. DNA topoisomerase I is involved in both repression and activation of transcription. *Nature* 365:227-232.

55. Michel, B., P. Komarnitsky, and S. Buratowski. 1998. Histone-like TAFs are essential for transcription in vivo. *Molecular Cell* 2:663-672.

56. Moqtaderi, Z., Y. Bai, D. Poon, P. A. Weil, and K. Struhl. 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* 383:188-191.

57. Moqtaderi, Z., M. Keaveney, and K. Struhl. 1998. The histone H3-like TAF is broadly required for transcription in yeast. *Mol. Cell.* 2.

58. Natarajan, K., B. Jackson, E. Rhee, and A. Hinnebusch. 1998. yTAF$_{II}$61 has a general role in RNA polymerase II transcription and is required by Gcn4p to recruit the SAGA coactivator complex. *Mol Cell* 2:683-692.

59. Nishizawa, M., T. Kokubo, M. Horikoshi, R. G. Roeder, and Y. Nakatani. 1997. Drosophila TAF$_{II}$230 and the transcriptional activator VP16 bind competitively to the TATA box-binding domain of the TATA box-binding protein. *Proc. Natl. Acad. Sci. USA* 94:85-90.

60. Oelgeschlager, T., C. Chiang, and R. Roeder. 1996. Topology and reorganization of a human TFIID-promoter complex. *Nature* 382:735-738.

61. Ogryzko, V. V., T. Kotani, X. Zhang, R. L. Schiltz, T. Howard, X.-J. Yang, B. H. Howard, J. Qin, and Y. Nakatani. 1998. Histone-like TAFs within the PCAF histone acetylase complex. *Cell* 94:35-44.

62. Orphanides, G., T. Lagrange, and D. Reinberg. 1996. The general transcription factors of RNA polymerase II. *Genes and Development* 10:2657-2683.

63. Ozer, J., A. H. Bolden, and P. M. Lieberman. 1996. Transcription factor IIA mutations show activator-specific defects and reveal a IIA function distinct from stimulation of TBP-DNA binding. *The Journal of Biological Chemistry* 271:11182-11190.

64. Ozer, J., Lezina LE, Ewing J, Audi S, and L. PM. 1998. Association of transcription factor IIA with TATA binding protein is required for transcriptional activation of a subset of promoters and cell cycle progression in Saccharomyces cerevisiae. *Mol Cell Biol* 18:2559-2570.

65. Ozer, J., K. Mitsouras, D. Zerby, M. Carey, and P. Lieberman. 1998. Transcription factor IIA derepresses TATA-binding protein (TBP)-associated factor inhibition of TBP-DNA binding. *J Biol Chem* 273:14293-300.

66. Ozer, J., P. A. Moore, A. H. Bolden, A. Lee, C. A. Rosen, and P. M. Lieberman. 1994. Molecular cloning of the small (γ) subunit of human TFIIA reveals functions critical for activated transcription. *Genes & Dev.* 8:2324-2335.

67. Ranallo, R., K. Struhl, and L. Stargell. 1999. A TATA-binding protein mutant defective for TFIID complex formation in vivo. *Mol Cell Biol* 19:3951-3957.

68. Ranish, J., N. Yudkovsky, and S. Hahn. 1999. Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. *Genes Dev* 13:49-63.

69. Ranish, J. A., and S. Hahn. 1991. The yeast general transcription factor TFIIA is composed of two polypeptide subunits. *J. Biol. Chem.* 266:19320-7.

70. Ranish, J. A., W. S. Lane, and S. Hahn. 1992. Isolation of two genes that encode subunits of the yeast transcription factor IIA. *Science* 255:1127-1129.

71. Sanders, S. L., E. R. Klebanow, and P. A. Weil. 1999. TAF25p, a non-histone-like subunit of TFIID and SAGA complexes, is essential for total mRNA gene transcription in vivo. *J Biol Chem* 274:18847-50.

72. Shen, W.-C., and M. R. Green. 1997. Yeast $TAF_{II}145$ functions as a core promoter selectivity factor, not a general coactivator. *Cell* 90:615-624.

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

74. Tan, S., Y. Hunziker, D. F. Sargent, and T. J. Richmond. 1996. Crystal structure of a yeast TFIIA/TBP/DNA complex. *Nature* 381:127-134.

75. Tao, Y., M. Guermah, E. Martinez, T. Oelgeschlager, S. Hasegawa, R. Takada, T. Yamamoto, M. Horikoshi, and R. G. Roeder. 1997. Specific interactions and potential functions of human $TAF_{II}100$. *J. Biol. Chem.* 272:6714-6721.

76. Verrijzer, C. P., J.-L. Chen, K. Yokomori, and R. Tjian. 1995. Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II. *Cell* 81:1115-1125.

77. Verrijzer, C. P., and R. Tijan. 1996. TAFs mediate transcriptional activation and promoter selectivity. *Trends in Biochemical Science* 21:338-342.

78. Vidal, M., R. K. Brachmann, A. Fattaey, E. Harlow, and J. D. Boeke. 1996. Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein interactions. *Proc. Natl. Acad. Sci. USA* 93:10315-10320.

79. Walker, S. S., J. C. Reese, L. M. Apone, and M. R. Green. 1996. Transcription activation in cells lacking $TAF_{II}s$. *Nature* 382:185-188.

80. Weideman, C. A., R. C. Netter, L. R. Benjamin, J. J. McAllister, L. A. Schmiedekamp, R. A. Coleman, and B. F. Pugh. 1997. Dynamic interplay of TFIIA, TBP and TATA DNA. *J Mol Biol* 271:61-75.

81. Weinzerl, R. O. J., B. D. Dynlacht, and R. Tjian. 1993. Largest subunit of *Drosophila* transcription factor IID directs assembly of a complex containing TBP and a coactivator. *Nature* 362:511-517.

82. Weinzierl, R. O. J., S. Ruppert, B. D. Dynlacht, N. Tanese, and R. Tjian. 1993. Cloning and expression of *Drosophila* $TAF_{II}60$ and human $TAF_{II}70$ reveal conserved interactions with other subunits of TFIID. *EMBO J* 12:5303-5309.

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

84. Yokomori, K., A. Admon, J. A. Goodrich, J. L. Chen, and R. Tjian. 1993. Drosophila TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex. *Genes Dev* 7:2235-45.

CHAPTER 5

STRUCTURE-FUNCTION ANALYSIS OF GENERAL TRANSCRIPTION FACTOR TFIIA

Chapter five describes a combination of studies designed to isolate mutations in TFIIA that are defective for interaction with the general transcription machinery. These studies involved artificial recruitment experiments followed by a genetic screen designed to search for mutations in Toa1 that caused defects in artificial recruitment activity. I performed all the work in this chapter. This chapter is currently being reformatted into manuscript form for publication. The citation upon acceptance to a professional journal will be:

> Ranallo, R. T., L. A. Stargell. 2000. Structure-Function Analysis of General Transcription Factor TFIIA.

5.1 Abstract

Artificial recruitment experiments have been used as evidence that recruitment of general transcription factors to a promoter is a rate-limiting step of gene activation *in vivo*. In these assays, a DNA-binding domain is connected to a protein in the general transcription complex creating a fusion protein. If functional for artificial recruitment, the fusion protein is able to nucleate preinitiation complex formation and stimulate transcription. The Toa1 subunit of general transcription factor -IIA (TFIIA), when fused to a DNA-binding domain falls into this class of proteins and is able to stimulate high levels of transcription. We have performed a molecular genetic screen designed to isolate mutations in the Toa1 protein that result in the loss of artificial recruitment activity, with the goal of isolating TFIIA mutants with transcriptional defects *in vivo*. After mutagenesis, a series of assays were performed (PCR, restriction digests, two-hybrid analysis, and western blot) to eliminate uninteresting clones. Almost all of the mutations isolated in this screen introduced stop codons, producing Toa1 truncations. Four truncations were chosen for further analysis. Using two-hybrid analysis, all four were found to be positive for interaction with the other subunit of TFIIA (Toa2). Two-hybrid analysis against various TBP-associated factors (TAF) proteins was also performed. Interestingly, some of the truncations interacted with both TAF17 and TAF40, but not with TAF61. These results provide additional support for communication between TFIIA and the TAF subunits of TFIID.

Further characterization of these interactions may yield additional information about their functional significance.

5.2 Introduction

Transcription initiation by eukaryotic RNA polymerase II (Pol II) requires a large assembly of six different general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF) which nucleate on the core promoter to form the preinitiation complex (PIC) (reviewed in 39). This complex is necessary for accurate positioning and initiation by RNA Pol II (reviewed in 3). PIC formation begins with the sequence-specific binding of general transcription factor -IID (TFIID), a multi-protein complex consisting of TATA-binding protein (TBP) and approximately 11 polypeptides referred to as RNA Pol II-specific TBP-associated factors (TAFs) (reviewed in 17). Multiple studies have shown that TFIID binding to the promoter can be a rate-limiting step in initiation (4, 7, 21-23, 26, 29, 53). TFIID binding to promoter DNA *in vivo* is tightly controlled through interactions with many different factors. These factors serve to modulate TFIID recruitment, as well as the rate at which TFIID binds to TATA-Inr promoters (11). In addition to these factors, TAFs themselves have been shown to influence the binding of TFIID to the promoter (24, 32, 40).

The general transcription factor -IIA (TFIIA), a positive regulator of transcription, has been shown to influence PIC formation through interactions with TFIID (1, 5, 6, 11, 31). In yeast, TFIIA is composed of two polypeptides

with molecular mass of 32-kDa and 13.5-kDa, which are encoded by the *TOA1* and *TOA2* genes, respectively. TFIIA was originally described as a factor needed for accurate transcription initiation by RNA polymerase II (34, 43). It has since been shown that TFIIA is dispensable for TBP-mediated transcription; however, it is required in transcription systems containing both TBP and TAFs (TFIID) (8, 9, 18, 41, 44, 48, 54). This is in accord with the finding that TFIIA can bind and stabilize TFIID at the core promoter, most likely by making direct contacts with TBP, DNA, and possibly some TAFs (27, 37, 52). These contacts induce conformational changes that enhance TFIID binding to TATA-Inr promoters, but not to TATA promoters lacking an Inr (11). In fact a direct interaction between TAF40 and TFIIA has recently been described in by our laboratory (25). This interaction seems to be critical for response to the Gal4 activator protein and has moderate effects on *HIS3* gene expression, indicating a functional role for this interaction *in vivo* (25). Thus a number of experiments suggest there is a functional link between TAFs and TFIIA; however, mechanistically the link between TFIIA-induced stabilization of TFIID and interaction with TAFs is not well understood.

The crystal structure of TFIIA reveals that the Toa1 and Toa2 subunits form an intimate heterodimeric complex consisting of two structural domains, a four-helix bundle domain and a ß-sheet domain (Fig. 5.1A) (15, 49). The structure also reveals two hydrophobic regions on the surface of TFIIA. One region is located in the ß-sheet domain and is made up primarily of

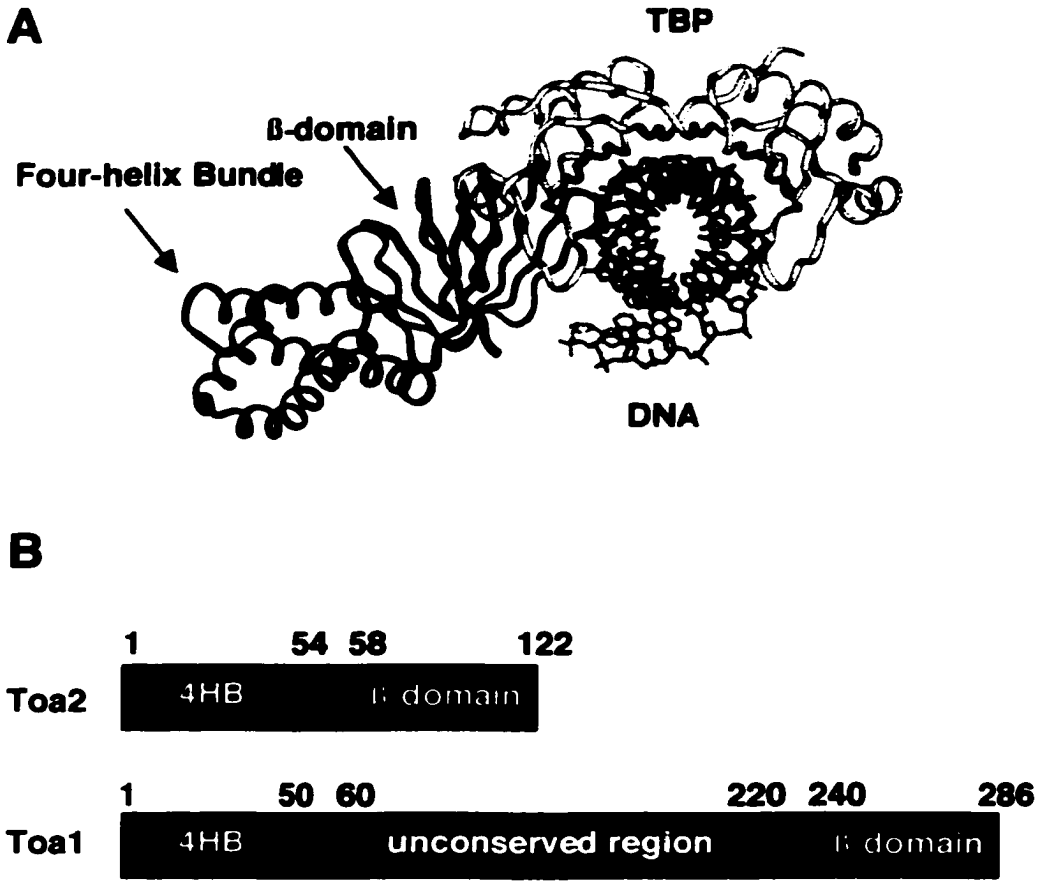

Figure 5.1A and 5.1B Crystal structure of the TFIIA-TBP-DNA complex (15). (A) TBP is shown in yellow ribbons and DNA is in black, the two subunits of TFIIA Toa1 and Toa2 are shown in blue and red respectively. The four-helix bundle domain (4HB) and ß-domain are indicated. (B) Schematic representation of the Toa1 and Toa2 proteins. Darker blue indicates conservation from yeast to human. Toa2 is conserved throughout. Amino acids 1-54 of Toa2 contribute to the 4HB domain and amino acids 58-122 contribute three strands to the ß-domain. Toa1 is 286 amino acids in length. Amino acids 1-60 are conserved as well as amino acids 220-286. The first 50 residues of Toa1 make up two helices of the four-helix bundle (4HB) and residues 240-286 make up the ß-domain.

aromatic residues from Toa2. This region has been shown through numerous studies to be involved in TBP and DNA interactions (reviewed in 17). The other hydrophobic patch is located in the four-helix bundle, which projects away from the TBP-TFIIA-DNA complex. Recently, mutations within this patch have been shown to decrease interaction with TAF40 (25). The conserved C-termini of both Toa1 and Toa2 contribute three strands each to the ß-sheet domain, while the conserved N-termini of each subunit contribute two helices each to the four-helix bundle domain (Fig. 5.1B). The Toa1 subunit of TFIIA is highly conserved at both its N and C-termini, but has a large unconserved central region (Fig. 5.1B). Recently a study using *Arabidopsis* TFIIA found that the equivalent region contained a cryptic activation domain (30).

It is evident that both TFIID and TFIIA play important roles in PIC formation as well as subsequent recruitment of the remaining transcription machinery. In fact, the recruitment and stabilization of transcription factors such as TFIIA and TFIID through interactions with activator proteins has been argued to be a primary mode of activated transcription in eukaryotes (14, 26, 29, 36 and refs therein). In this model, promoter-specific proteins serve to bind and recruit transcription components, thereby localizing the transcription machinery at certain promoters causing elevated levels of transcription. These ideas have been explored through a number of approaches including artificial recruitment or activator-bypass experiments (14, 36 and refs therein). In these experiments, fusion of a transcription component to a heterologous

DNA-binding domain (e.g. Gal4, LexA) creates so-called "nonclassical activators". Nonclassical activators are different from classical activators in that nonclassical activators contain a DNA-binding domain fused to a transcription factor instead of an activation domain. These nonclassical activators, when expressed in the cell, are able to recruit the transcription machinery and activate transcription from designated reporter genes. Artificial recruitment activity has been reported for many transcription components including TBP, TAFs, TFIIB, holoenzyme components, and TFIIA (14, 36, 45 and refs therein).

Two detailed studies of this phenomenon revealed that nonclassical activators are limited in their ability to stimulate transcription from certain genes in both humans and yeast (14, 36). These studies found that nonclassical activators are sensitive to many variables such as the position and number of DNA-binding site(s), the sequence of the promoter region, and the sequence downstream of the start codon (14). These limitations do not apply to classical activators, presumably because they make multiple contacts with the transcription machinery, thereby affecting multiple steps in PIC formation (46). Studies using TBP mutants that do not stimulate transcription when artificially recruited to a promoter have been described as being postrecruitment defective, meaning they are defective for a step after recruitment of TBP to the promoter (47). Mechanistically, this provides evidence for two distinct steps in transcription initiation, one in which TBP is brought or recruited to the promoter followed by a step after TBP is bound to

the TATA element (47). Interestingly, a TBP mutant (N2-1) defective for interaction with TFIIA, was shown to be artificial recruitment defective as well. This is interpreted as evidence that TFIIA plays a role after TFIID is occupying the promoter (45).

The present chapter describes the characterization of yeast TFIIA using *in vivo* assays such as artificial recruitment and two-hybrid analyses. This study was originally designed to isolate mutations in TFIIA that are defective for artificial recruitment, but are not impaired for interaction with TBP. Analysis of these mutants could reveal interactions with TFIIA that are necessary for transcription initiation.

Initially, artificial recruitment assays using a variety of general transcription factors, including both subunits of TFIIA confirmed that non-classical activator proteins differ in their ability to stimulate transcription when artificially recruited to a promoter. The Toa1 subunit of TFIIA was found to activate transcription in an artificial recruitment assay. This finding was then used to develop a genetic screen intended to identify mutations in Toa1 that reduce this artificial recruitment activity. Steps performed in the screen utilized a variety of techniques including random PCR mutagenesis and *in vivo* recombination as a method for localized mutagenesis of TFIIA. Subsequent screening of the TFIIA derivatives isolated revealed most of the mutations resulted in truncated versions of Toa1. Since both the C and N-terminus of Toa1 are necessary for cell viability all other experiments were limited to two-hybrid analysis. Characterization of four truncated derivatives

revealed interesting insights into how Toa1 interacts with Toa2 and certain TAFs within the TFIID complex.

5.3 Materials and Methods

5.3a DNA constructs.

To generate the ADToa1 clone, the Toa1 open reading frame was amplified using PCR and cloned into the 2μ *LEU2* activation domain (pACT2.2) vector (AD) (10), which contains the *ADH1* promoter, a nuclear localization sequence, an HA epitope, and the Gal4 activation domain (residues 768-881). PCR primers were designed to introduce an Nde1 site at the start of the Toa1 ORF and a BamH1 site after the stop codon. The DNA-binding domain construct (DBToa1) was created by subcloning the 800bp Bgl II fragment of ADToa1 into the DNA-binding domain (pPC97) (51) vector (DB) (CEN-*TRP3*) which contains the *ADH1* promoter, a nuclear localization sequence, an HA epitope, the Gal4 DNA-binding domain (residues 1-147). DBmTFIIA is a pPC97 derivative that was created using *in vivo* recombination (38). PCR primers were designed to amplify residues 1-54 and amino acids 216-286 connected by a Gly-Gly-Ser-Gly-Gly linker (20). PCR products were transformed into yeast (MAV103) (51) along with the DB (pPC97) vector cut with Nde1 and EcoR1. The amplified regions of DBmTFIIA were sequenced in their entirety. ADTAF40 was cloned using *in vivo* recombination as

described above (38). DBTI (54-215) was cloned into the pPC97 vector using PCR primers designed to introduce a Sma1 and Bgl II site at amino acids 54 and 215 respectively. DBT1(1-53) was created by placing a stop codon after amino acid 53 using PCR and *in vivo* recombination as described above (38). DBTBP, DBTAF40, ADTAF17, ADToa2 and DBToa2 were described elsewhere (25, 45). ADTAF61 was a gift from Valerie Morris. Clones were sequenced to check junctions.

5.3b Yeast Strains and Media.

All yeast transformations were done using the LiOAc method. MaV103 was used to perform all yeast two-hybrid experiments and *in vivo* recombination (38, 51). Artificial recruitment assays were done in MaV103, CG1945 and Y190 (13,19,51). MaV103 contains the *GAL1* promoter (with four Gal4 binding sites) fused to the *HIS3* promoter and structural gene; *GAL4* and *GAL80* genes are knocked out in this strain. The *URA3* reporter gene in MaV103 is a *SPO13:URA3* fusion in which the first 15 amino acids of *SPO13* are fused to *URA3*. There are 10 GAL4 binding sites cloned upstream in the *SPO13* promoter (51). CG1945 contains a *HIS3* reporter gene consisting of the *GAL1* UAS_G and promoter fused to the *HIS3* structural gene (13). Y190 contains the same promoter architecture for its *HIS3* reporter as MaV103 (19). For artificial recruitment and two-hybrid analysis, strains were grown to the appropriate optical density and cells (10-fold serial dilutions) were spotted onto dropout plates lacking the appropriate amino

acids (leucine, tryptophan or histidine) with or without 3-aminotriazole (AT). Dropout FOA media for selection of *URA3* minus strains was at approximately 15 grams of FOA per liter of media.

5.3c DBToa1 Library construction.

Primers were designed to amplify amino acids 1-93 of the Toa1 open reading frame and allow for recombination into the DBToa1 vector using 50 and 94 base pairs of homology to the DB vector and Toa1 ORF, respectively. Mutagenic PCR reactions contained the following components: (1) 10ng Template; (2) 1X buffer (100mM HEPES 8.4, 15mM $MgCl_2$, 0.125mM $MnCl_2$, 500mM KCl); (3) 200µM of each dGTP, dCTP, dTTP; (4) 40µM-dATP; (5) Taq Polymerse. The vector used in *in vivo* recombination was prepared by digesting 10µg of DBToa1 with Nde1 and Age1, gel purified and quantitated. Yeast cotransformations where done using LiOAc treatment. Cells were added directly to gapped plasmid (360ng) and 11.5µl of PCR reaction (5500ng). These conditions gave approximately 8000 colonies. The efficiency of library transformation was determined by plating a small-scale transformation on dropout plates. The number of colonies per ng of DNA and PCR product was determined to be 3.4 colonies/ng of DNA.

5.3d Rate of mis-incorporation.

Two separate conditions were tested in order to determine the appropriate buffer conditions for the mutagenic PCR. Small scale

transformations were done to allow *in vivo* recombination. Five colonies from each transformation were isolated. Plasmids from each of these strains were recovered by electroporation into *E. coli* using standard techniques. Clones obtained under both mutagenic conditions were sequenced to determine the number of mutations each clone contained in the mutagenized region. This information was used to determine the rate of mis-incorporation for each buffer used. The final buffer used is described above.

5.3e Screening.

DNA was prepared from each strain using glass bead lysis and ethanol precipitation. PCR reactions were performed on 10ng DNA extracted from FOA^+ strains using conventional techniques. Primers were designed to amplify the entire Toa1 open reading frame from the DNA binding domain vector (pPC97). Positive plasmids were recovered by electroporation of extracted DNA into *E. coli* using standard techniques. Plasmid DNA extracted from FOA^+ strains was also digested with Nde1 and BamH1 and checked for the correct size insert (800bp). Plasmid DNA was then transformed into MaV103 harboring both AD and the ADToa2 fusion protein. Strains were colony purified and tested for growth on 3-aminotrialzole (AT) in order to detect a two-hybrid interaction. Western blots were performed on yeast whole-cell extracts prepared by glass bead lysis in 20mM Tris-phosphate pH 6.7, 200mM PMSF. 30µg of extract were separated on a 10%

acrylamide gel and probed using anti-Toa1 antibodies. The blots were then stripped and probed using anti-HA (12CA5) antibodies.

5.4 RESULTS

5.4a Nonclassical activators are limited in their ability to stimulate transcription from certain yeast promoters.

Artificial recruitment of many general transcription factors via fusion to a DNA-binding domain has been shown to stimulate transcription *in vivo* (14, 36 and refs therein). Unlike classical activators, nonclassical activators are limited in their ability to activate transcription from certain genes. This sensitivity has been mapped to the core promoter, as well as sequences downstream of the start codon (14). To investigate the sensitivity of certain transcription factors to promoter architecture, we assayed the artificial recruitment activity of TBP, TFIIA, and TAF40 from three different yeast two-hybrid strains.

The DNA-binding domain (DB) of Gal4 was fused in frame with TBP, Toa1, Toa2, and TAF40, creating the nonclassical activator proteins DBTBP, DBToa1, DBToa2, DBTAF40 respectively. Fusion proteins were expressed in three different yeast strains, each of which contain either a *HIS3* or *URA3* reporter gene (with different numbers of Gal4 binding sites). Activity from the *HIS3* gene can be measured by growth in the presence of 3-

aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product. Thus growth on AT indicates activation of the reporter gene. In contrast, activation of the *URA3* reporter is measured by lack of growth on 5-fluorootic acid (FOA), a compound that is toxic to cells expressing the *URA3* gene. Thus, no growth on FOA indicates reporter gene activation.

Similar to what has been found previously, DBTBP is able to activate transcription on all promoters tested (Table. 5.1) (4, 21, 22, 28, 53). DBTAF40 and DBToa1 showed significant levels of activity from both reporters in MaV103 (*HIS3* and *URA3*) as well as the *HIS3* reporter in Y190 (Table. 5.1). However, both DBTAF40 and DBToa1 failed to stimulate transcription from the *HIS3* reporter in CG1945. In contrast to DBToa1, DBToa2 consistently failed to activate all reporters tested, even though it is functional when recruited to a promoter (25). These results are consistent with artificial recruitment assays performed with both subunits of *Arabidopsis* TFIIA (30).

Interestingly, all of the *HIS3* reporters tested in this analysis are strikingly similar in structure. In fact, MaV103 and Y190 have identical *HIS3* reporters because they were derived from the same parental strain Y153 (10). The regulatory sequences of the *HIS3* reporter gene in CG1945 have been replaced by the *GAL1* UAS_G (which has four Gal4 binding sites) (Fig. 5.2). Expression of this reporter is tightly controlled, thus only stronger interactions can stimulate reporter expression (42). MaV103 contains a very similar *HIS3* reporter gene consisting of the GAL1 UAS_G fused to the *HIS3* promoter and

Table 5.1 Artificial recruitment activity varies between promoters. Nonclassical activators differ in their ability to stimulate transcription from various reporter genes. Three yeast two-hybrid strains each containing integrated reporter genes were tested using nonclassical activators DBTBP, DBTAF40, DBToa1 and DBToa2. Strains were transformed with each construct and tested reporter activity using either 3-aminotriazole (AT) or FOA. As a control for background growth, DB versions of each strain were grown in parallel. Strains from the MaV103 background were grown on 3X FOA to test the *URA3* reporter and 30 mM AT to test the *HIS3* reporter. Y190 strains were grown on 15 mM AT and CG1945 derivatives were grown on 5 mM AT. The number of + marks indicates relative growth on media; ++++ being the highest and --- indicating no growth. (N/A) indicates not applicable.

Strain	Reporters	
Construct	URA3	HIS3
MaV103		
DBTBP	+++	++++
DBTAF40	+++	++++
DBToa1	+++	+++
DBToa2	---	---
Y190		
DBTBP	N/A	++++
DBTAF40	N/A	+++
DBToa1	N/A	++
DBToa2	N/A	---
CG1945		
DBTBP	N/A	+++
DBTAF40	N/A	---
DBToa1	N/A	---
DBToa2	N/A	---

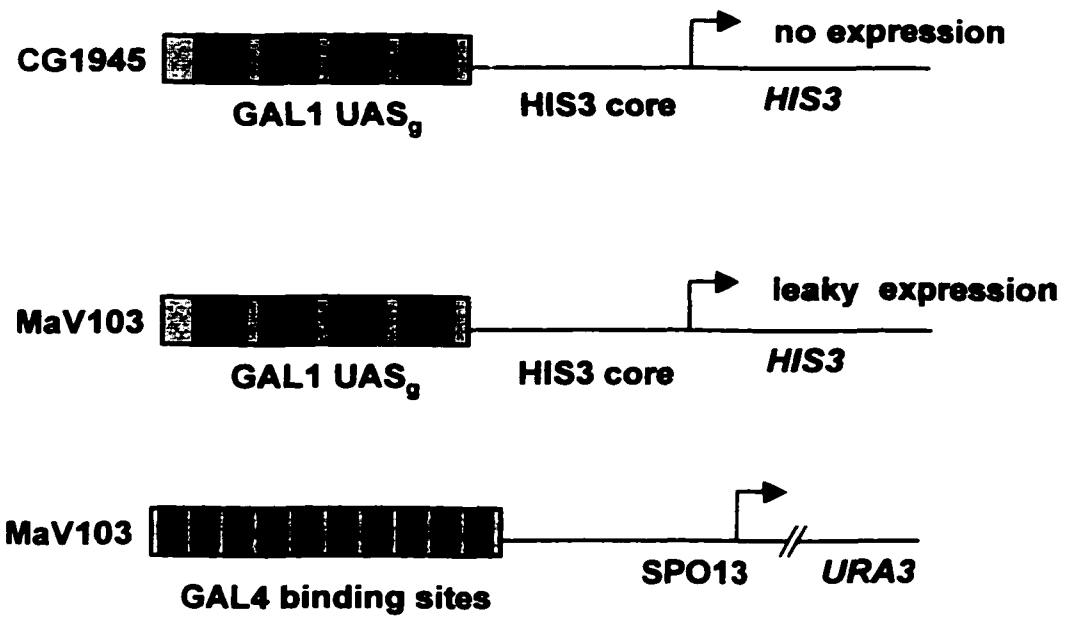

Figure 5.2 Promoter architecture of the *HIS3* and*URA3* reporter genes in CG1945 and MaV103. CG1945 contains a *HIS3* reporter gene, consisting of the *HIS3* promoter in which the regulatory sequences have been replace by the GAL1 UAS_G (with four Gal4 binding sites). There is no basal expression of *HIS3*; therefore CG1945 is phenotypically auxotrophic for histidine. MaV103 contains a very similar *HIS3* reporter gene consisting of the GAL1 UAS_G fused to the *HIS3* promoter and structural gene. Leaky expression of the*HIS3* gene allows for growth without exogenous histidine in the media. Addition of 3-aminotriazole (AT), an antimetabolite of histidine, can suppress this growth as it competitively inhibits the *HIS3* gene product. The *URA3* reporter gene in MaV103 is a *SPO13:URA3* fusion in which the first 15 amino acids of *SPO13* are fused to *URA3*. There are 10 Gal4 binding sites cloned upstream in the *SPO13* promoter.

structural gene. Expression of this reporter; however, is leaky and allows for weaker interaction to be detected. In contrast to the *HIS3* promoters, the *URA3* reporter in MaV103 is a *SPO13:URA3* fusion, in which the first 15 amino acids of *SPO13* are fused to *URA3*. There are 10 *GAL4* binding sites cloned upstream of the *SPO13* promoter (Fig. 5.2) (51).

Thus, through this limited analysis we find that nonclassical activators are unable to activate transcription from certain genes in yeast. However, the reason for these differences is not simply due to promoter architecture, but perhaps the context of the promoter within the genome. To better understand why TFIIA and TAF40 display different reporter gene activation, we used deletion constructs to localize their artificial recruitment activities. Since this study is concerned with TFIIA function, the results from the deletion analysis of Toa1 are presented below. The results for TAF40 are presented in chapter 6. The preceding sections describe the Toa1 mutant screen as well as the characterization of the mutations isolated in this screen.

5.4b Amino acids 1-93 of Toa1 contain most of the artificial recruitment activity as assayed from the *HIS3* reporter.

To learn more about how Toa1 functions at the promoter, we chose to map the region responsible for the artificial recruitment activity. Regions responsible for this activity will then be targeted for localized random mutagenesis. This was done by designing four truncated versions of the Toa1 protein based on both crystallographic and functional studies of TFIIA

(Fig. 5.3A) (15, 20, 50). The DNA constructs encoding the four Toa1 truncations fused to the Gal4 DNA binding domain were tested in MaV103 for artificial recruitment activity, using the full length Toa1 protein as a control (DB-Toa1). As shown in Fig. 5.3B, only the first 93 amino acids of Toa1 were needed for wild type activity, as assayed using the *HIS3* reporter gene. Interestingly, removal of only 40 amino acids (DBT1(1-53) is sufficient to reduce all reporter activity, suggesting that these unconserved amino acids were activating the reporter. This would be consistent with what was found for *Arabidopsis* TFIIA, where similar experiments localized the activity to the central unconserved region of the 50-kDa subunit (30). However the equivalent region in yeast was tested (DBT1(54-215) and failed to activate the *HIS3* reporter gene. Furthermore, this same region when removed from Toa1 almost completely eliminated reporter activity (mDBTFIIA) (Fig. 5.3B).

Regions responsible for TFIIA binding to TBP have been mapped to the conserved C-terminus of Toa1 and Toa2 through numerous studies (for review see 17, 20). Thus random PCR mutagenesis directed at the first 93 amino acids of Toa1 should not generate mutations that affect interactions with TBP, since this region has not been shown to be important for interaction with TBP (Fig. 5.1) (15, 20, 50). Thus mutations in TFIIA that affect artificial recruitment, but not TBP binding, would lend understanding of TFIIA function at the promoter.

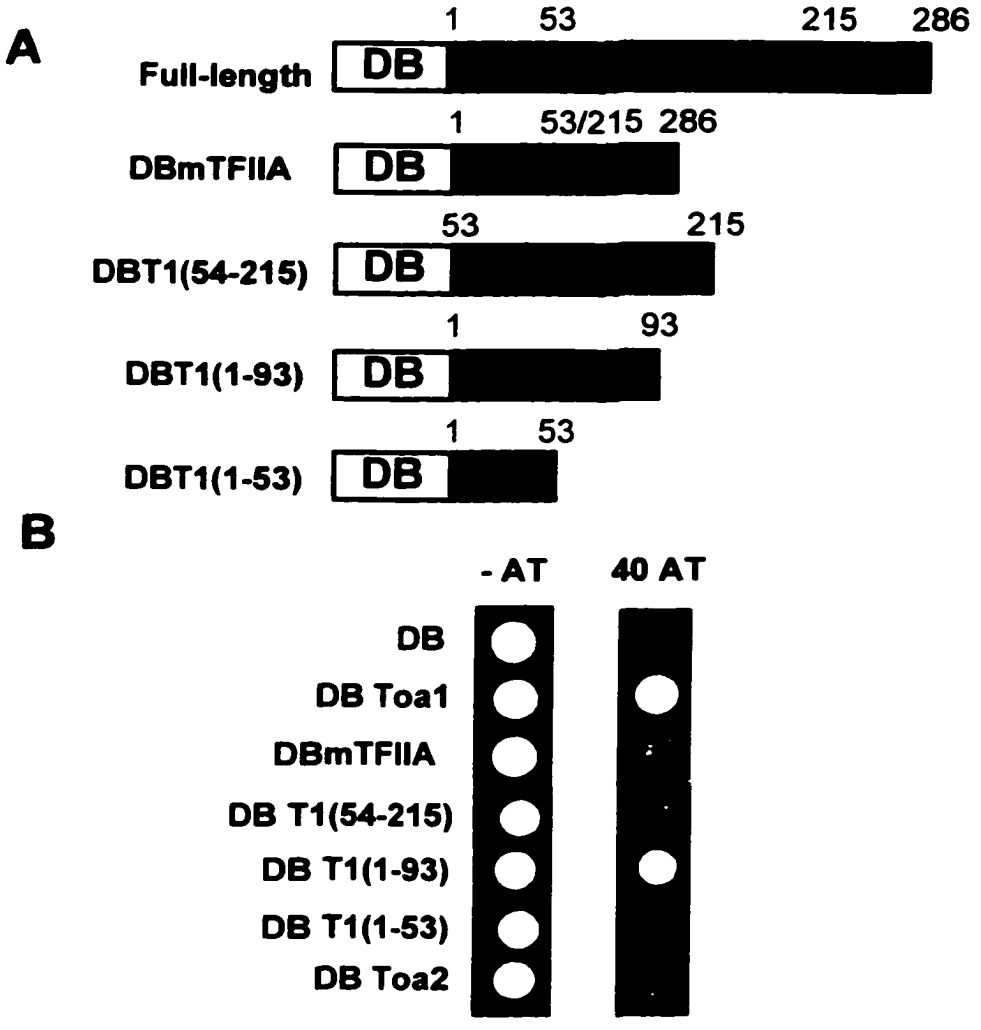

Figure 5.3A and 5.3B The artificial recruitment activity of Toa1 was localized to the first 93 amino acids using a series of deletion mutations. (A) Crystallographic and functional studies were used to design deletions of Toa1. DBmTFIIA contains only the conserved C and N-terminal sequences and was shown to be able to support cell viability. DBT1(54-215), on the other hand, contains only the unconserved region of Toa1. DBT1(1-53) and DBT1(1-93) contain different lengths of N-terminal amino acids. (B) Activity of each construct including DBToa2 was assayed using the *HIS3* reporter of MaV103. Reporter output was measured by growth on 40mM AT using the *HIS3* reporter.

5.4c Steps of the screen implemented to isolate full-length clones defective for reporter activity.

Once the artificial recruitment activity was localized in Toa1, the next step in the screen was to use random PCR mutagenesis to create mutant Toa1 libraries. These libraries were then be screened for the loss of artificial recruitment activity. Clones isolated during this process were then checked for DNA encoding the Toa1 ORF, expression of full-length protein, and the ability to interact with the other subunit of TFIIA (Toa2). The *URA3* reporter gene was used to identify Toa1 derivatives that had lost the ability to activate in the artificial recruitment assay. Recall that the activity of this reporter gene can be assayed by growth on FOA as shown in Fig. 5.4. Toa1 derivatives that have lost the ability to activate the reporter gene are able to grow in the presence of FOA.

Conditions for the random PCR mutagenesis were empirically determined by counting the number of mutations for various mutagenic buffering conditions and nucleotide concentrations (see material and methods). The PCR products generated were cloned into the Gal4 DNA-binding domain vector using *in vivo* recombination (Fig. 5.5). The first 93 amino acids of Toa1 were amplified under mutagenic conditions described previously (35). Primers used generated a 280bp product with 50 and 94 bp of homology to the gapped vector (Fig. 5.5). MaV103 was co-transformed with 360ng gapped vector and 15-fold excess PCR product; transformations were plated directly on dropout FOA media to select for transformants that

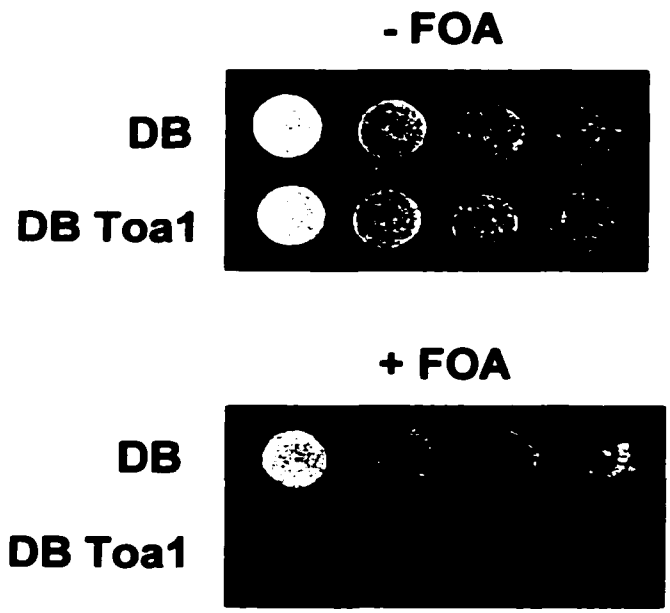

Figure 5.4 The *URA3* gene was used to select for strains that were artificial recruitment defective. The activity of the reporter gene can be measured by growth on FOA. Serial dilutions of both strains were made and spotted to dropout plates both with and without FOA. As shown here strains (DBToa1) that activate the reporter can not grow in the presence of FOA. Toa1 derivatives that have lost the ability to activate will grow on FOA.

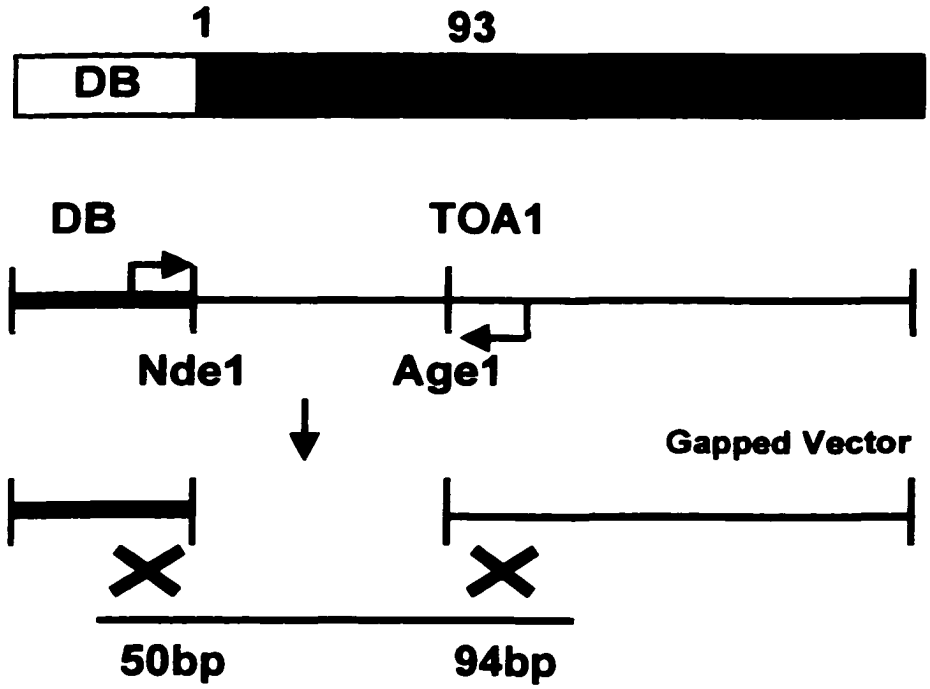

Figure 5.5 Creating a library of Toa1 mutants was done using *in vivo* recombination. Primers were designed to amplify the first 93 amino acids of Toa1 in addition to the indicated homology needed for the recombination. PCR products were generated under mutagenic conditions and co-transformed with 360ng gapped vector and 15-fold excess PCR product. Gapped vector was prepared by digesting DBToa1 with Nde1 and Age 1.

have lost artificial recruitment from the *URA3* reporter gene. Two-hundred and twenty FOA^+ positive transformants were picked and restreaked to FOA containing media to ensure an FOA^+ phenotype. One disadvantage of using *in vivo* recombination is that gapped plasmids can rejoin without using PCR product. The frequency of this event is low; however, plasmids that rejoin without the PCR product will not express DBToa1 and therefore will be FOA^+ positive. In order to remove these strains from the pool, plasmid DNA was prepared from all 220 strains and screened for recombined plasmid using PCR. 145 of the 220 strains picked contained what appeared to be a Toa1 ORF. The plasmid DNA was then digested to eliminate false positive identified in the PCR reaction. 138 of the 145 clones contained a correct size insert indicating 7 false positives.

Constructs verified to contain the Toa1 open reading frame were then used in two-hybrid analysis with a construct encoding the Toa2 open reading frame fused to the Gal4 activation domain (ADToa2). This step was necessary to eliminate mutations that affect subunit association of Toa1 with Toa2. These types of mutations are of no interest to this study. Fifty-five of the 138 strains were positive for interaction with ADToa2. Whole cell extracts were prepared from these 55 trains and screened using both anti-Toa1 and anti-HA antibodies to look for full-length clones. This step is necessary in order to identify any mutations that introduce a stop codon creating a DBToa1 truncation. Unfortunately, 54 of the 55 strains tested using western blot analysis produced a truncated form of DBToa1. The final results after

processing 220 FOA^+ strain are summarized in Fig. 5.6. Only one of the clones does not encode a Toa1 truncation (313A). Further characterization of this clone as well as four DBToa1 truncations was done by sequencing and more two-hybrid analysis. In addition, it was surprising that only a small N-terminal region of Toa1 was positive for interaction with Toa2.

5.4d Clones isolated in the screen encode Toa1 truncations.

A total of five clones isolated in the screen were characterized by sequencing the mutagenized region of Toa1. Results of sequencing the four truncations are summarized in Fig. 5.7. As expected, all four were found to contain mutations that generated, either directly or indirectly, a stop codon. Two of these truncations, 31A and 32B, were found to encode 50 amino acids of Toa1, and the other two truncations, 36C and 311B, were found to encode 60 amino acids of Toa1 (Fig. 5.7). In addition, clones 31A, 32B, and 311B contain multiple amino acid substitutions, while 36C does not contain any additional mutations besides the stop codon (Fig. 5.7). As a condition of the screen, all four truncations, including DBT1(1-53), interacted with ADToa2 (Fig. 5.8). These results show that only the very N-terminus (45 amino acids) of Toa1 is needed for interaction with Toa2, similar to what was found for plant TFIIA (30). The last clone, 313A, had no mutations in the region that was mutagenized. Sequence analysis was not performed on the entire clone. Western blot analysis during the screening found 313A to encode a full-length DBToa1 fusion (Fig. 5.9). Interestingly, it appears that 313A is expressed at

	Number passed	%of Total
Picked 220	220	100%
Restreak to FOA	220	100%
Insert w/ PCR	145	66%
Insert w/ digestion	138	63%
Two-hybrid	55	25%
Truncations w/ western	54	2.2%
Sequenced + mapped	5	0.4%

Figure 5.6 The results from the Toa1 mutant screen are summarized above. The type of assay used to eliminate unwanted derivatives in indicated on the left; the number and % of total of is on the right. 220 stains were initially picked. Approximately 55 strains formed two-hybrid interactions, 54 of which were shown to be a truncation. Five clones were sequenced and their mutations were mapped.

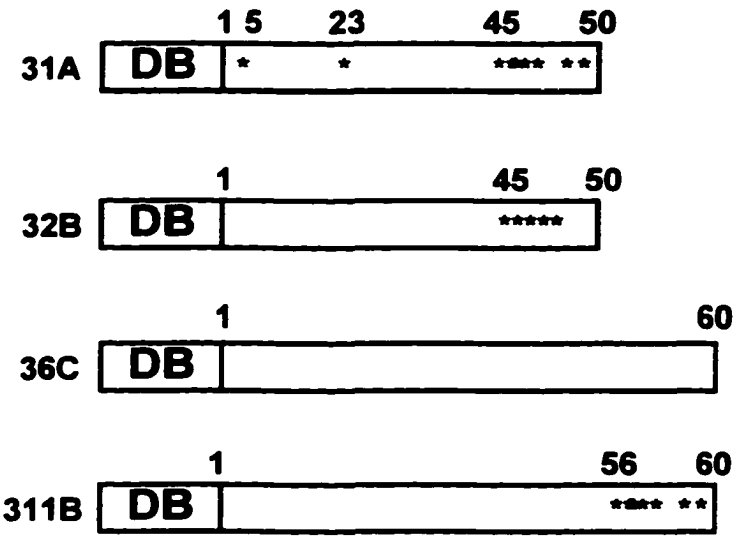

Figure 5.7 Sequencing of 4 clones isolated in the screen . Schematic of the four Toa1 truncations. The clones expressing each Toa1 truncation is indicated. The number of mutations and where they occur is also indicated a top of each schematic. Clone 31A is 50 amino acids long, with 8 mutations starting at residue 5 of Toa1. Clone 32B is also 50 amino acids long; however, it contains 5 mutations starting with residue 45. Clone 36C is 60 amino acids long, with no mutations. Clone 311B is also 60 amino acids long, but it contains 6 mutations starting at residue 56 of Toa1.

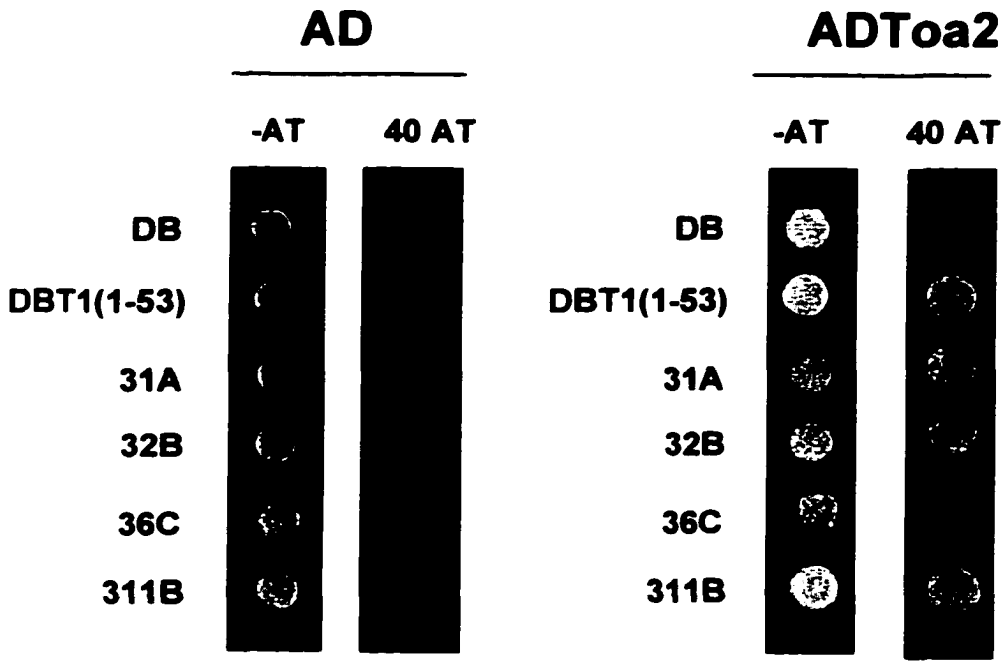

Figure 5.8 Two-hybrid analysis indicates that all four truncations interact with Toa2, the other subunit of TFIIA. The Gal4 activation domain was fused in frame with ADToa2 and tested for interaction with the DBT1(1-53), 31A, 32B, 36C, and 311B. All show a strong interaction with ADToa2. These results indicate that only amino acids 1-45 are needed to interact with ADToa2.

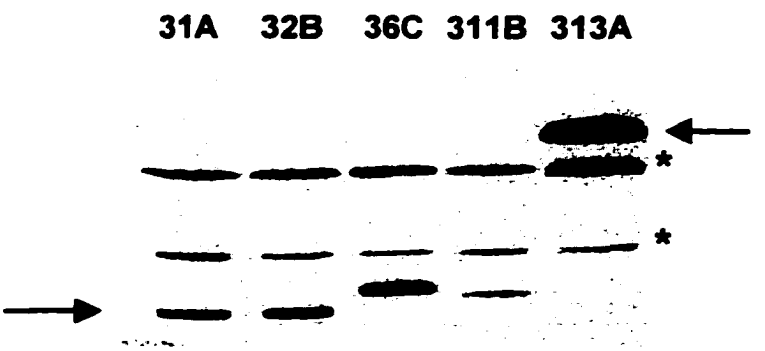

Figure 5.9 Western blot of yeast whole-cell extracts prepared from the indicated strains. 30μgs of extract were separated by SDS-PAGE and transferred to nitrocelluose and probed using anti-HA antibodies. Asterisks indicate a nonspecific band that is present in all lanes. The truncations as well as the full-length clone are indicated.

higher levels than the other clones isolated in addition to DBToa1 (data not shown). Future directions concerning this clone are discussed in the conclusion section.

5.4e Two-hybrid analysis of four Toa1 truncations reveal novel interactions between TAF40 and TAF17 but not TAF61.

TFIIA has been shown to interact with TAF40 *in vivo* and *in vitro* (25). We wanted to determine if these Toa1 truncations could interact with ADTAF40 or various other TAFs. Since the four truncations don't activate transcription they can be used in two-hybrid analysis. All four Toa1 truncations (31A, 32B, 36C, 311B) along with DBT1(1-53) and Toa2 were used in two-hybrid analysis against three different TAF proteins (ADTAF17, ADTAF40, and ADTAF61). Of the clones tested, 31A and 32B interacted strongly with ADTAF17, while DBT1(1-53) showed a weak interaction (Fig 5.10). Interestingly, clones 36C and 311B, which are only 10 amino acids longer, were negative for this interaction. DBToa2 showed an extremely weak interaction with ADTAF17. The panel of truncations was also screened using ADTAF40, yielding very similar results to ADTAF17 (Fig 5.10). DBToa2 in this case showed a strong positive interaction with ADTAF40 as was described previously (25). No positives were obtained when the panel was tested using TAF61 (data not shown), indicating that the ADTAF17 and ADTAF40 interactions are specific. Thus the N-terminal region of Toa1 can interact with TAFs *in vivo*. In addition, the shorter DBToa1 truncations (31A

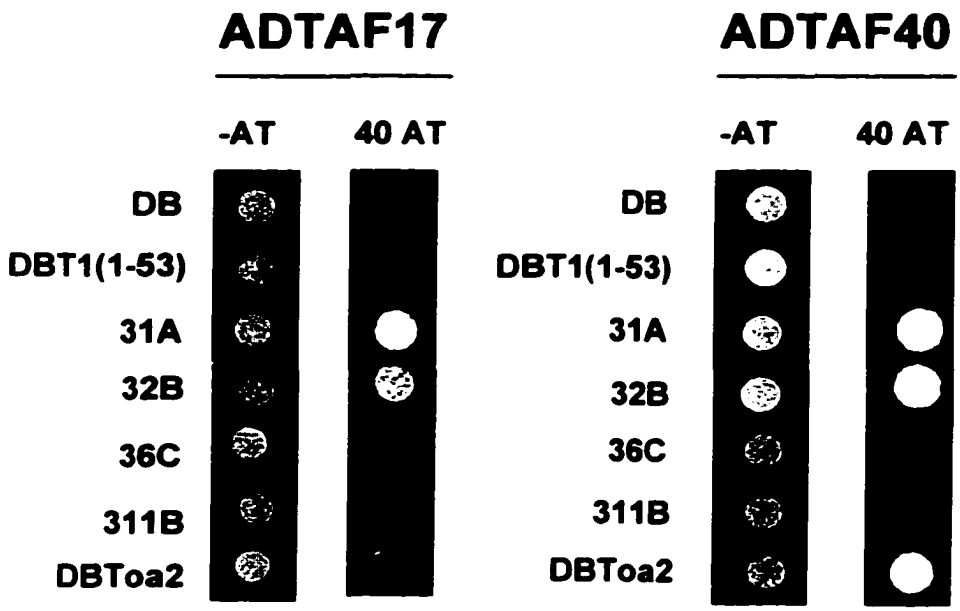

Figure 5.10 Two-hybrid analysis indicates that 31A and 32B interacts with both ADTAF40 and ADTAF17. The Gal4 activation domain was fused in frame with TAF17, TAF40, and TAF61 and tested for interaction with the DBT1(1-53), 31A, 32B, 36C, 311B, and DBToa2. ADTAF17 interacts with 31A, 32B and weakly associates with DBT1(1-53). An extremely weak interaction between ADTAF17 and DBToa2. An identical pattern of interaction was obtain when ADTAF40 was tested. Additionally, DBToa2 was shown to interact strongly with ADTAF40 as previously described (25).

and 32B) interacted better than the longer DBToa1 truncations (36C and 311B) whereas DBT1(1-53) showed an intermediate interaction.

5.5 DISCUSSION

5.5a Evidence that TFIIA makes multiple TAF contacts.

An important mechanism of transcriptional activation is increasing the rate at which TBP binds to the TATA element, since TBP occupancy on the core promoter correlates strongly with transcriptional activity (26, 29). Artificial recruitment experiments with TBP have played a critical role in formulating this idea (14, 36 and refs there within). In these experiments, transcriptional activation occurs in the absence of a classical activator protein such as Gal4 or Gcn4. Instead, a DNA-binding domain (e.g. LexA or Gal4) is fused to a component of the general transcription machinery such as TBP (4, 21, 22, 28, 53), TBP-associated factors (TAFs) (2, 21), TFIIA (45), or SRBs (12). It has been suggested that these fusion proteins work by either setting up PIC formation or by inserting themselves into the holoenzyme itself. However, in contrast to classical activator proteins, fusion of a transcription factor to a DNA-binding domain, a so-called "nonclassical" activator, is extremely limited in its ability to activate transcription as they are sensitive to promoter architecture (14).

This chapter describes artificial recruitment experiments in which general transcription factors TBP, TAF40, and TFIIA were assayed from a

collection of reporter genes. Consistent with what has been previously reported (36,14) we find that some factors (Toa1 and TAF40) are limited in their ability to stimulate when assayed using certain reporter genes. Based on this information a molecular genetic screen was developed aimed at isolating mutations in a specific region of Toa1 that decreases artificial recruitment activity. We mutagenized the region of Toa1 responsible for artificial recruitment activity and then performed a series of assays designed to eliminate uninteresting clones (PCR, restriction digests, two-hybrid analysis, and western blots). Almost all the mutations isolated resulted in a stop codon producing a Toa1 truncation.

Four truncations of varying length were analyzed using two-hybrid screening against TAF17, TAF40, and TAF61. Interestingly some of these truncationed proteins interacted with TAF17 and TAF40 even though some contained multiple mutations. All of the interactions identified mapped to the N-terminal α-helices of Toa1. These results are consistent with the finding that TFIIA and TAF40 interact directly (25), but TFIIA has never been shown to interact with TAF17. These results may have identified a novel interaction between TFIIA and TAF17, further suggesting a functional role for TFIIA-TAF interactions.

5.5b Toa1 and TAF40 are limited in their ability to activate in the artificial recruitment assay.

Nonclassical activators differ in their ability to activate transcription from certain reporter genes. Two detailed studies of this phenomenon have found that these differences are due to promoter architecture. In particular it was found that the upstream activating sequence (UAS), the core promoter and sequences downstream of the start codon can affect the ability of nonclassical activators to nucleate PIC formation (14, 36). The results of this study suggest there may be another factor that affects artificial recruitment activity. This is evident from the fact that DBToa1 and DBTAF40 are able to activate the *HIS3* reporter in MaV103 cells, but not a very similar *HIS3* reporter in CG1945 cells (Table 5.1). Perhaps this difference in activity is simply related the requirement for the individual transcription factor (Toa1 and TAF40) at that particular reporter gene. If the factor is already at the promoter or not required then recruitment of this factor would not result in elevated transcriptional output. Another confounding factor indicating a difference between the two reporters is the basal level of reporter gene expression. The *HIS3* reporter in MaV103 has high levels of basal expression, while the *HIS3* reporter in CG1945 has no basal expression. This difference could be due to the chromosomal locus of the reporter gene. The chromosomal context could affect the ability of nonclassical activator to relieve the repressive effects of chromatin. In either case the results are consistent with the finding that nonclassical activator are limited in their ability

to work at some genes. However, they further suggest that promoter architecture is not the only factor that influences artificial recruitment.

5.5c Mapping of the artificial recruitment activity in TFIIA.

Recently TFIIA was cloned and characterized in *Arabidopsis* (30). This analysis showed that, like yeast TFIIA, *Arabidopsis* TFIIA is composed of two subunits, a smaller 12-kDa subunit which is homologous to yeast Toa2 and a larger 50-kDa subunit which is homologous to yeast Toa1. Artificial recruitment experiments demonstrated that, similar to yeast TFIIA, only the larger 50-kDa subunit stimulates transcription when recruited to a promoter. In addition, it was shown that the central nonconserved region of the 50-kDa subunit contained most of the artificial recruitment activity (30). In contrast, we find that the N-terminal 93 amino acids of Toa1 is responsible for most of the artificial recruitment activity. Furthermore, the removal of 43 amino acids (clone 36C) is sufficient to abolish all reporter activity. This would suggest that these amino acids are responsible for activity. However, mapping studies shown in Fig. 5.3 indicate that the unconserved region alone (DBT1(54-215) does not stimulate transcription. Thus it appears that the combination of both the conserved and unconserved N-terminal amino acids in Toa1 are necessary for activity in yeast.

5.5d Localized random PCR mutagenesis and *in vivo* recombination as a method for library construction in yeast.

The goal of this project was to generate a collection of mutations within the Toa1 subunit of TFIIA and then screen these mutations for a decrease in artificial recruitment activity. A series of techniques was employed that allowed localized random mutagenesis of the first 93 amino acids of Toa1, which encompass the region that was shown to contain the artificial recruitment activity. Once the mutations were generated, a library of these mutagenized constructs was created in yeast directly using *in vivo* recombination. This technique has been described for plasmid construction in yeast (33, 35). This method for mutagenesis has two primary advantages. First, subcloning and amplification in *.E coli* are eliminated. Second, the efficiency of recombination is extremely high, leading to rapid plasmid construction and library generation. This technique, in combination with yeast molecular genetics, creates a powerful tool for understanding how proteins work *in vivo*. Thus the combination of localized PCR mutagenesis and *in vivo* recombination, presents a novel approach to library construction in a suitable screening vector.

5.5e Evidence that TAF40 interacts with TFIIA through the four-helix bundle structural domain of TFIIA.

In yeast, TFIIA is composed of two subunits encoded by the *TOA1* and *TOA2* genes. *TOA1* encodes the larger 32-kDa subunit and *TOA2*

encodes the smaller 13.5-kDa subunit. Both proteins share sequence similarity with their higher eukaryotic counterparts, in addition to being required for cell viability (reviewed in 17). The crystal structure of the yeast TFIIA-DNA-TBP complex reveals two distinct structural domains within TFIIA: the four-helix bundle and ß-sheet domain (Fig. 5.1A) (15, 50). The conserved N-terminus of each subunit contributes two helices to the four-helix bundle, while the conserved C-terminus of each subunit contributes three strands to the ß-domain (Fig. 5.1B). N-terminal fragments of Toa1 (31A and 32B) were shown to interact with TAF40, suggesting that the four-helix bundle structural domain of TFIIA is important for the interaction between TAF40 and TFIIA. In fact, mutations in Toa2 that map within this region of TFIIA, were found to be defective for interaction with TAF40 (25) both *in vivo* and *in vitro*(25). Since all four truncations isolated interacted with Toa2 and only two (31A and 32B) interacted with TAF40 this interaction is probably not mediated solely through Toa2.

Interestingly, a new TFIIA-TAF interaction may have been identified between Toa1 and TAF17. In addition, this interaction maps to the same region of Toa1 as the interaction with TAF40. In fact, it is possible that these two TAFs compete for binding to the same region of Toa1 or that TAF17 interacts with Toa1 via association with TAF40. However before any these possibilities can be addressed further work is needed to determine whether the interaction between Toa1 and TAF17 is direct.

5.5f. Future directions and perspectives.

Only a small population of clones was analyzed using two-hybrid analysis; an obvious next step would be to test more dervatives for interactions with more TAF proteins. The interaction between TFIIA and TAF17 also needs to be examined more closely to determine if it is a direct interaction. Furthermore this interaction needs to be examined using full length TFIIA. This can be done using recombinant forms of the proteins in *in vitro* binding experiments. A full-length Toa1 clone was isolated during the initial stages of the screen. The mutagenized region of the clone was sequenced and no mutations were identified. However there is a possibility that mutations exist elsewhere in the Toa1 open reading frame. Western blot analysis from yeast strains harboring the construct showed that the fusion protein was over-expressed as compared to the other truncations (Fig. 5.9) and full-length DBToa1 (data not shown). A future direction would be to sequence the rest of the open reading frame to determine if any other mutation is present. In addition the Toa1 open reading frame from this clone should be subcloned to a new DB vector to determine if the over expression was due to a mutation in the vector.

The results from this analysis are consistent with previous studies showing an interplay between TFIIA and TAFs (25). In addition, a potential interaction between TFIIA and TFIID via TAF17 and Toa1 has been identified. The characterization of this interaction is an important next step in understanding how TFIIA and TFIID interact via the TAF proteins. Since

TAF17 is also found in the SAGA complex this could lead to a potential interplay between TFIIA and chromatin (16). However, further work is needed to explore these ideas more in depth. In either case it is clear from this and other studies that TFIIA functions by interacting with TFIID *in vivo*. This interaction has been shown to induce conformational changes that modulate TFIID affinity for promoter DNA. The precise nature of these interactions is not well understood. This study was designed to isolate mutations in TFIIA that compromise its function. Even though the goal of this study was not fully realized, useful and significant data were obtained through pursuit of this study.

5.6 ACKNOWLEDGEMENTS

I would like to thank Ryan Ogg for the gift of the clones (DBTBP, ADTAF17, ADToa2, DBToa2). I would also like to thank Dr. Karen VanOrden for critical review of this chapter.

5.7 REFERNCES

1. Andell III, F., A. G. Ladurner, C. Inouye, R. Tjian, and E. Nogales. 1999. Three-dimensional structures of the human TFIID-IIA-IIB complex. *Science* 286:2153-2156.

2. Apone, L. M., C. A. Virbasius, J. C. Reese, and M. R. Green. 1996. Yeast $TAF_{II}90$ is required for cell-cycle progression through G_2/M but not for general transcription activation. *Genes & Dev.* 10:2368-2380.

3. Burley, S. K., and R. G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* 65:769-799.

4. Chatterjee, S., and K. Struhl. 1995. Connecting a promoter-bound protein to the TATA-binding protein overrides the need for a transcriptional activation region. *Nature* 374:820-822.

5. Chi, T., and M. Carey. 1996. Assembly of the isomerized TFIIA-TFIID-TATA ternary complex is necessary and sufficient for gene activation. *Genes & Dev.* 10:2540-2550.

6. Chi, T., P. Lieberman, K. Ellwood, and M. Carey. 1995. A general mechanism for transcriptional synergy by eukaryotic activators. *Nature* 377:254-257.

7. Colgan, J., and J. L. Manley. 1992. TFIID can be rate limiting in vivo for TATA-containing, but not TATA-lacking, RNA polymerase II promoters. *Genes Dev.* 6:304-315.

8. Cortes, P., O. Flores, and D. Reinberg. 1992. Factors involved in specific transcription by mammalian RNA polymerase II: purification and analysis of transcription factor IIA and identification of transcription factor IIJ. *Mol.Cell.Biol.* 12:413-421.

9. DeJong, J., and R. Roeder. 1993. A single cDNA, hTFIIA/alpha, encodes both the p35 and p19 subunits of human TFIIA. *Genes Dev* 7:2220-2234.

10. Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang, A. E. Kilburn, W.-H. Lee, and S. J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* 7:555-569.

11. Emami, K., A. Jain, and S. Smale. 1997. Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization. *Genes Dev.* 11:3007-19.

12. Farrell, S., N. Simkovich, Y. Wu, A. Barberis, and M. Ptashne. 1996. Gene activation by recruitment of the RNA polymerase II holoenzyme. *Genes Dev.* 10:2359-2367.

13. Feilotter, H. E., G. J. Hannon, C. J. Ruddell, and D. Beach. 1994. Construction of an Improved Host For Two Hybrid Screening. *Nucleic Acids Research* 22:1502-1503.

14. Gaudreau, L., M. Keaveney, J. Nevado, Z. Zaman, G. Bryant, K. Struhl, and M. Ptashne. 1999. Transcriptional Activation by Artificial Recruitment in Yeast is Influenced by Promoter Architecture and Downstream Sequences. *Proc. Natl. Acad. Sci. USA* 96:2668-2673.

15. Geiger, J. H., S. Hahn, S. Lee, and P. B. Sigler. 1996. Crystal structure of the yeast TFIIA/TBP/DNA complex. *Science* 272:830-836.

16. Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. Steger, J. C. Reese, J. R. Yates III, and J. L. Workman. 1998. A subset of TAF_{II}s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. *Cell* 94:45-53.

17. Hampsey, M. 1998. Molecular genetics of the RNA polymerase II genreal transcriptional machinery. *Microbiology and Molecular Biology Reviews* 62:465-503.

18. Hansen, S., and R. Tjian. 1995. TAFs and TFIIA mediate differential utilization of the tandem Adh promoters. *Cell* 82:565-75.

19. Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805-816.

20. Kang, J. J., D. T. Auble, J. A. Ranish, and S. Hahn. 1995. Analysis of yeast transcription factor TFIIA: Distinct functional regions and a Polymerase II-specific role in basal and activated transcription. *Mol. Cell. Biol.* 15:1234-1243.

21. Keaveney, M., and K. Struhl. 1998. Activator-mediated recruitment of the RNA Polymerase II machinery is the predominant mechanism for transcriptional activation in yeast. *Molecular Cell* 1:917-924.

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

23. Klein, C., and K. Struhl. 1994. Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. Science 266:280-282.

24. Kokubo, T., M. J. Swanson, J.-I. Nishikawa, A. G. Hinnebusch, and Y. Nakatani. 1998. The yeast TAF145 inhibitory domain and TFIIA competitively bind to TATA-Binding Protein. *Mol. Cell. Biol.* 18:1003-1012.

25. Kraemer, S., R. T. Ranallo, R. Ogg, and L. A. Stargell. 2000. TFIIA Interacts with TFIID via association with TBP and TAF40. Submitted .

26. Kuras, L., and K. Struhl. 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. *Nature* 399:609-613.

27. Lagrange, T., T.-K. Kim, G. Orphanides, Y. W. Ebright, R. H. Ebright, and D. Reinberg. 1996. High-resolution mapping of nucleoprotein complexes by site-specific protein-DNA photocrosslinking: organization of the TBP-TFIIA-TFIIB-DNA quaternary complex. *Proc. Natl. Acad. Sci. U.S.A.* 93:10620-10625.

28. Lee, M., and K. Struhl. 1997. A severely defective TATA-binding protein-TFIIB interaction does not preclude transcriptional activation *in vivo*. *Mol. Cell. Biol.* 17:1336-1345.

29. Li, X. Y., A. Virbasius, X. Zhu, and M. R. Green. 1999. Enhancement of TBP binding by activators and general transcription factors. *Nature* 399:605-609.

30. Li, Y.-F., J. Gourierrec, M. Torki, Y.-J. Kim, F. Guerineau, and D. Zhou. 1999. Characterization and Functional Analysis of *Arabidopsis* TFIIA Reveal that the Evolutionarily Unconserved Region of the Large Subunit has a Transcription Acitvation Domain. *Plant Molecular Biology* 39:515-525.

31. Lieberman, P. M., and A. J. Berk. 1994. A mechanism for TAFs in transcriptional activation: activation domain enhancement of TFIID-TFIIA-promoter DNA complex formation. *Genes Dev.* 8:995-1006.

32. Liu, D., R. Ishima, K. I. Tong, S. Bagby, T. Kokubo, D. R. Muhandiram, L. E. Kay, Y. Nakatani, and M. Ikura. 1998. Solution structure of a TBP-$TAF_{II}230$ complex: Protein mimicry of the minor groove surface of the TATA box unwound by TBP. *Cell* 94:573-583.

33. Ma, H., S. Kunes, P. Schatz, and D. Botstein. 1987. Plasmid Construction by Homologous Recombination in Yeast. *Gene* 58:201-216.

34. Matsui, T., J. Segall, P. A. Weil, and R. G. Roeder. 1980. Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. *J. Biol. Chem.* 255:11992-11996.

35. Muhlrad, D., R. Hunter, and R. Parker. 1992. A Rapid Method for Localized Mutagnenesis of Yeast Genes. *Yeast* 8:79-82.

36. Nevado, J., L. Gaudreau, M. Adam, and M. Ptashne. 1999. Transcriptional Activation by Artificial Recruitment in Mammalian Cells. *Proc. Natl. Acad. Sci. USA* 96:2674-2677.

37. Oelgeschlager, T., C. Chiang, and R. Roeder. 1996. Topology and reorganization of a human TFIID-promoter complex. *Nature* 382:735-738.

38. Oldenburg, K., K. Vo, S. Michaelis, and C. Paddon. 1997. Recombination-mediated PCR-directed Plasmid Construction In Vivo in Yeast. *Nucleic Acids Research* 25:451-452.

39. Orphanides, G., T. Lagrange, and D. Reinberg. 1996. The general transcription factors of RNA polymerase II. *Genes Dev.* 10:2657-2683.

40. Ozer, J., K. Mitsouras, D. Zerby, M. Carey, and P. Lieberman. 1998. Transcription factor IIA derepresses TATA-binding protein (TBP)-associated factor inhibition of TBP-DNA binding. *J Biol Chem* 273:14293-300.

41. Ozer, J., P. A. Moore, A. H. Bolden, A. Lee, C. A. Rosen, and P. M. Lieberman. 1994. Molecular cloning of the small (g) subunit of human TFIIA reveals functions critical for activated transcription. *Genes Dev.* 8:2324-2335.

42. Ranallo, R. T., and L. A. Stargell. unpublished results

43. Reinberg, D., and R. G. Roeder. 1987. Factors involved in specific transcription by mammalian RNA polymerase II: Purification and functional analysis of initiation factors IIB and IIE. *J. Biol. Chem.* 262:3310-3321.

44. Sayre, M. H., H. Tschochner, and R. D. Kornberg. 1992. Reconstitution of transcription with five purified initiation factors and RNA polymerase II from Saccharomyces cerevisiae. *J. Biol. Chem.* 267:23376-23382.

45. Stargell, L. A., Z. Moqtaderi, D. Dorris, R. Ogg, and K. Struhl. 2000. TFIIA Has Activator and Core Promoter Functions *in Vivo*. In press .

46. Stargell, L. A., and K. Struhl. 1996. Mechanisms of transcriptional activation *in vivo*: two steps forward. *Trends Genet.* 12:311-315.

47. Stargell, L. A., and K. Struhl. 1996. A new class of activation-defective TATA-binding protein mutants: Evidence for two steps of transcriptional activation *in vivo*. *Mol. Cell. Biol.* 16:4456-4464.

48. Sun, X., D. Ma, M. Sheldon, K. Yeung, and D. Reinberg. 1994. Reconstitution of human TFIIA activity from recombinant polypeptides: a role in TFIID-mediated transcription. Genes & Dev. 8:2336-2348.

49. Tan, S., G. Ammerer, and T. J. Richmond. 1988. Interactions of purified transcription factors: binding of yeast MAT alpha 1 and PRTF to cell type-specific, upstream activating sequences. *Embo Journal* 7:4255-64.

50. Tan, S., Y. Hunziker, D. F. Sargent, and T. J. Richmond. 1996. Crystal structure of a yeast TFIIA/TBP/DNA complex. Nature 381:127-134.

51. Vidal, M., R. K. Brachmann, A. Fattaey, E. Harlow, and J. D. Boeke. 1996. Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein interactions. *Proc. Natl. Acad. Sci. USA* 93:10315-10320.

52. Weideman, C. A., R. C. Netter, L. R. Benjamin, J. J. McAllister, L. A. Schmiedekamp, R. A. Coleman, and B. F. Pugh. 1997. Dynamic interplay of TFIIA, TBP and TATA DNA. *J Mol Biol* 271:61-75.

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

54. Yokomori, K., A. Admon, J. A. Goodrich, J. L. Chen, and R. Tjian. 1993. Drosophila TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex. *Genes Dev* 7:2235-45.

CHAPTER 6

MAPPING INTERACTION SURFACES OF TAF40

Work presented in this chapter is an extension of work done in Chapter 4. All the experiments presented in this chapter were done by myself. Figures 6.3C and 6.7 were kindly generated by Raji Edayathumangalam. Experiments for this project have recently been completed and the implications of this work are currently being evaluated. I have written this chapter in Molecular and Celluiar Biology format so as to facilitate the manuscript preparation when submitting for publication.

6.1 Abstract

Yeast TAF40 is a TFIID-specific TAF that is required for transcription from most promoters *in vivo*. We have shown that TAF40 makes direct contacts with TBP and TFIIA. Mutations in TFIIA indicate that a functional TAF40-TFIIA interaction is important for transcription *in vivo*. We have used a variety of techniques designed to investigate the interaction surfaces of yeast TAF40. Using the structural information in the hTAF28/hTAf18 crystal structure, we have modeled a histone fold motif onto the primary structure of TAF40 and find that this region specifically associates with TAF19 *in vivo*. In contrast to TAF19, TFIIA needs additional TAF40 sequences in order to maintain interaction with TAF40. We have also identified residues in the modeled histone fold of TAF40 that are important for TBP binding. Phenotypic studies of TAF40 mutants indicate that the TAF40-TBP interaction performs an essential function in transcription initiation *in vivo*. These results further define the importance of TAF40 in transcription by RNA pol II.

6.2 Introduction

RNA polymerase II (pol II) transcription is regulated through numerous protein-protein interactions that regulate the rate at which RNA pol II initiates and reinitiates transcription from promoters *in vivo*. RNA pol II is

recruited and positioned by a set of general transcription factors, in addition to numerous other promoter-specific transcription factors (reviewed in 12, 26). TFIID is the general transcription factor that recognizes and binds the consensus promoter sequence TATAAA (reviewed in 12). This event nucleates formation of the pre-initiation complex (PIC), which is necessary for accurate transcription initiation *in vivo*.

TFIID is a multi-subunit complex consisting of TATA-binding protein (TBP) and a set of 14 TBP-associated factors (TAFs) (reviewed in 9, 11, 12, 28, 31). The TBP subunit of TFIID mediates sequence-specific binding of TFIID to the TATAAA box which is thought to be the initial step in PIC formation. In fact, many different studies show a strong correlation of TFIID recruitment and occupancy with transcriptional output *in vivo* (5, 6, 14, 15, 18, 21). While numerous studies have demonstrated an essential role for TBP in pol II transcription, TAF requirements are more difficult to define.

Originally, highly purified *in vitro* systems indicated that TAFs can function as coactivators serving to bridge activators and general transcription factors. This idea is based on numerous *in vitro* experiments that have identified interactions between activation domains and TAFs (4, 33). In contrast, *in vivo* depletion studies show transcriptional activation is relatively unaffected by loss of TAFs, suggesting TAFs may serve as potential but not obligatory targets of activator proteins (23, 35). TAF inactivation can also lead to distinct cell-cycle phenotypes. In fact, individual depletion of three TAFs (TAF150, TAF130, and TAF90) leads to arrest at various stages of the

cell cycle (1, 35). A more in-depth study found TAF130 to be important for transcription of G1- and B-type cyclins (27, 36). This TAF130 dependence was mapped to the core promoter and was independent of upstream activators (29). These and additional studies suggest that individual TAFs have promoter-specific requirements in transcription initiation.

TAFs are highly conserved from yeast to humans. In almost every case, TAFs found in yeast have a metazoan counterpart (28). Functional conservation between human and yeast TAFs has not been studied directly. In some cases; however, results for humans TAFs have coincided with those in yeast. For example, like yeast TAF130, a temperature-sensitive allele in hTAF250 causes G1/S arrest due to aberrant cell-cycle gene regulation (32).

Initial characterization of some histone-like TAFs (TAF17, TAF61, TAF60) indicated that they were generally required for transcription (2, 22, 24, 25); however, genome-wide expression data taken from TAF inactivation experiments show that TAFs are not as universally required as initially thought (13). Experiments done for 7 of the 12 TAFs indicate genome dependence on TAFs ranges from 3 to 67%, with TAF17 being the highest (reviewed in 9). Interpretation of these data are complicated by the fact that some TAFs are integral components of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex (8, 31). Thus any effect seen after TAF inactivation could be the result of disrupted TFIID or SAGA function or both. The fact that these two complexes share common components suggests that each complex may perform similar or redundant functions *in vivo*. In fact, genome-wide

transcriptional analysis of TFIID and SAGA components suggests that expression of most genes are dependent on the function of either TFIID or SAGA (reviewed in 9).

One way of studying TFIID independently of SAGA, is to deplete TAFs specific to the TFIID. One such study analyzed temperature sensitive alleles of TAF40 (16). Analysis of this allele revealed that high temperature causes degradation of TAF40 and subsequent loss of certain TFIID components. Transcriptional analysis of poly $(A)^+$ mRNA levels found a general cessation of RNA pol II transcription implying TFIID is required for all RNA pol II transcription (16). This is inconsistent with studies that show TFIID disruption causes only promoter-specific defects *in vivo* (27). High copy suppression analysis using individual TAF subunits identified TAF19 as being able to rescue theTAF40 ts phenotype, indicating that the allele is, at least in part, defective for interaction with TAF19 (16). This genetic interaction is also supported by the finding that human homologs of TAF19 and TAF40 (hTAF18 and hTAF28, respectively) were crystallized as a heterodimer (Figure 6.1A) (3). hTAF28 and hTAF18 interacted through a histone motif, even though neither protein shares significant similarity to the histones themselves. The structural information obtained from this study was used to show that helix 2 of the histone fold is important for synergistic activation in transiently transfected human cells. In fact, specific residues on the solvent exposed surface of this helix were identified that decreased TBP-hTAF28 interactions in co- immunoprecipitations.

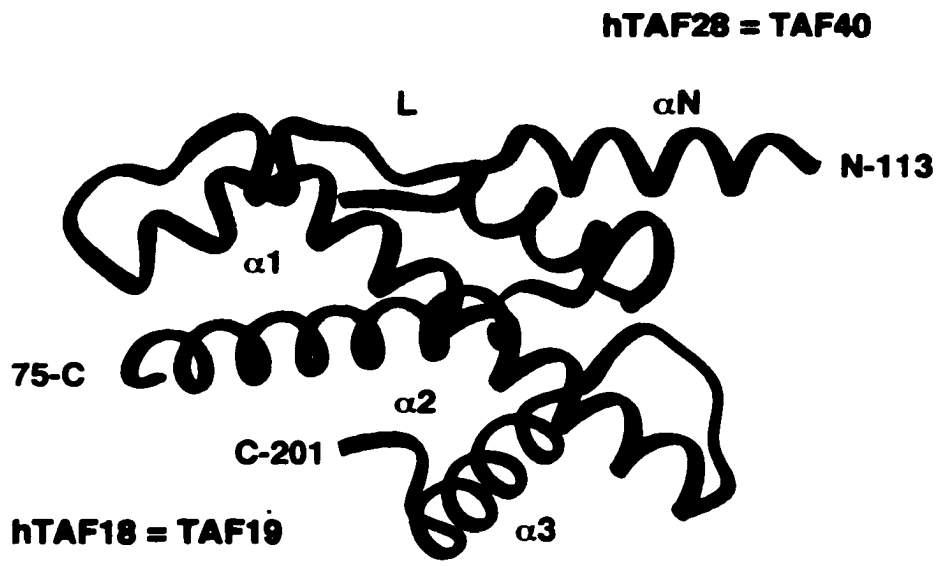

Figure 6.1A Ribbon representation of the crystal structure of the hTAF28/hTAF18 heterodimeric complex. Human TAF28 (hTAF28) is shown in red and human TAF18 (hTAF18) is shown in blue. hTAF28 and hTAF18 interact through a typical histone motif found in the SPT3 family of proteins. The location of the helices and residue numbers corresponding to the full length protein are indicated. This structural information, along with sequence alignments was used to design deletion mutations. The figure was created using Insight II from the coordinates of the hTAF28/hTAF18 structure (3).

These results were interpreted to mean that these residues are important for synergy with TBP *in vivo* (20).

In addition to being an integral component of TFIID, TAF40 has been shown to make direct contacts with both TBP and TFIIA (17). Interestingly, the TBP-TAF40 interaction seems to be conserved through evolution since the human homolog (hTAF28) and human TBP interact *in vitro* and *in vivo* (19, 20). Mutational studies of TFIIA indicate that a functional interaction between TFIIA and TAF40 is required for proper transcription from certain promoters in yeast. The results from this study suggest that TAFs may serve as a communicator between TFIID and TFIIA, and suggest a novel role for TAF40 in transcription by RNA pol II (17).

An important step in understanding why TAF40 is generally required for transcription is to better define interactions with TAF40. To date, TAF40 has been shown to interact directly with TFIIA and TBP, and genetically with TAF19 (16, 17). We sought to get a more accurate description of what regions of TAF40 are responsible for these interactions, as well as test the functional conservation of the hTAF28-hTBP interaction. Using yeast two-hybrid analysis, we have shown that TAF40 specifically associates with TAF19 *in vivo*. Moreover, we show that TAF40 interacts with TAF19 using a modeled histone motif composed primarily of conserved amino acids. In contrast to TAF19, we find that both N- and C-terminal sequences of TAF40 are required for interaction with TFIIA. Similar to what has been found for hTAF28, we find that conserved hydrophobic and

hydrophilic amino acids are important for interaction with TBP. In fact, mutations that affect the interaction with TBP confer temperature-sensitive or lethal phenotypes *in vivo*. These results further define the importance of TAF40 in transcription by RNA polymerase II and suggest that the hTAF28-hTBP interaction is functionally conserved in the yeast TAF40-TBP interaction.

6.3 Materials and Methods

6.3a DNA Constructs.

ADTAF constructs (ADTAF40, ADTAF19) were created using *in vivo* recombination. PCR primers were designed to amplify the open reading frame of each TAF. PCR products were transformed into yeast (MaV103) (34) along with 2μ *LEU2* activation domain (pACT2.2) vector (AD) cut with Nde1 and BamH1. The AD vector contains the *ADH1* promoter, a nuclear localization sequence, an HA epitope, the Gal4 activation domain (residues 768-881). Amplified regions were sequenced in their entirety. ADTAF61 and ADTAF17 are described elsewhere (17). ADTAF40 truncation constructs were created using *in vivo* recombination as described above. Primers used in the PCR reactions were designed to amplify the indicated amino acids of TAF40 (i.e. 99-347) and allow for *in vivo* recombination using Nde1 and BamH1 into the AD vector. Constructs were verified by sequencing junctions. ADTAF40 point mutants were also created using *in vivo* recombination.

Primers were designed to introduce the indicated amino acid substitution. Both PCR products were co-transformed with gapped AD vector as described above. The entire open reading frame was sequenced to verify mutations. These clones were used for subcloning into the pPC97 (DB), pET15b, and YCP22 plasmids described below.

The DNA-binding domain constructs (DBTAF40, DBTAF19, DB-V170K, DB-E185A, DB-E182,E185A) were created by subcloning the appropriate size Bgl II fragment from the correct AD construct into the DNA-binding domain (pPC97) (34) vector (DB). This vector (CEN-*TRP3*) contains the *ADH1* promoter, a nuclear localization sequence, an HA epitope, the Gal4 DNA-binding domain (residues 1-147).

The *E. coli* expression vectors for GST, GST-TBP, GST-TFIIA, and His-TAF40 are described elsewhere (17). *E. coli* expression vectors for the TAF40 point mutants were generated by subcloning the Nde1/BamH1 fragment from the corresponding ADTAF40 vector to pET15b creating a His-TAF40 fusion. Cover plasmids containing mutant TAF40 open reading frames were created by subcloning the Nhe1/BamH1 fragment from the corresponding pET15b-TAF40 expression vector to ZM282. ZM282 is YCP22 (CEN-*TRP*) that contains the TAF40 open reading frame under the control of it own promoter and terminator. This clone was a gift from Zarmick Moqtaderi.

6.3b Yeast Strains.

All yeast transformations were done using the LiOAc method. MaV103 was used for two-hybrid analysis involving DBToa2 and ADTAF40. All other two-hybrid analysis was done in CG1945. Both MaV103 and CG1945 are described in chapter 5. Viability tests were done in ySB366 (a gift from Steve Buratowski), which contains a *TAF40* deletion covered by a *URA3*-marked TAF40 expression plasmid.

6.3c Yeast two-hybrid and phenotypic studies.

Both Gal4 DB and Gal4 AD plasmids were transformed into the appropriate yeast strain using a standard lithium acetate transformation. The resulting strains were grown in the appropriate selection media and ten-fold serial dilutions were performed. Cells were spotted onto the appropriate plates that either contained or lacked 3-aminotriazole (AT). Cells were grown at 30 °C for 4-7 days. For phenotypic studies, ten-fold serial dilutions of strains were spotted to plates with rich media containing either glucose (YPD), ethanol and glycerol (YPEG), glycerol alone (YPG), or galactose (YPGAL), and incubated at 30, 36, 38 or 40 °C (10).

6.3d Protein Purification.

GST-TFIIA was produced by fusing the Toa1 open reading frame to glutathione S-transferase (GST). Each subunit (GST-Toa1 and Toa2) was expressed separately in *E. coli,* each insoluble pellet was

resolublized in 8M urea. The subunits were combined and the urea was dialyzed out as described in (30). After dialysis the soluble supernatant was incubated with glutathione resin. GST-TBP, GST-TFIIB and GST were expressed and purified from bacteria as described (30). TAF40 proteins were purified using a denaturing and refolding method similar to that used to make recombinant yeast TFIIA. BL21 (DE3) cells containing TAF40 cloned into the bacterial expression plasmid pET15b (His-TAF40) were grown to an OD_{600} of 0.6 and induced for 2 h with 1mM IPTG. Cells were harvested, washed with 25 ml buffer A (20 mM Tris-HCl pH 7.5, 200 mM NaCl) and resuspended in 25 ml buffer 1 (20 mM Tris-HCl pH 7.5, 500 mM NaCl), and frozen at –70 °C. Cells were thawed and sonicated. The insoluble fraction was collected by spinning at 10,000 xg for 15 min at 4 °C. Pellets were resuspended in 13 ml Buffer 2 (20 mM Tris-HCl, 500 mM NaCl, 8 M urea, and 1 mM dithiothreitol (DTT). 7.5 ml of Buffer B (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM PMSF, 1 mM DTT, 10% glycerol) were added slowly, and the solution was cleared by spinning at 10,000 g for 15 min at 4 °C. The supernatant was then dialyzed against Buffer B for at least 24 h. The soluble fraction was cleared by spinning at 10,000 xg for 15 min at 4 °C. Soluble fraction was bound to Ni-NTA resin (Qiagen) and washed with buffer C (20mM Tris 7.5, 100mM KCl, 10% glycerol, 20mM imidiazole, 1mM DTT) and eluted with buffer C containing 200mM imidazole.

6.3e *In Vitro* interaction studies.

GST pull-down assay. All proteins used *in vitro* assays were quantitated using BSA standard stained with coommassie blue.

Approximately 25pmole of GST fusion protein or GST alone were incubated with 40 pmole of His-TAF40 protein in 200 μl of binding buffer (20mM HEPES pH 7.9, 20 mM Tris pH 7.5, 100 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 0.025% NP40, 10% glycerol, 0.5 mM DTT) for 2h at 4 °C. Complexes were recovered by incubation with glutathione sepharose for 1h at 4 °C in binding buffer with 3% BSA. Complexes were washed 2X in binding buffer, then incubated with SDS loading buffer, boiled, and 10 μl of sample were separated by SDS-PAGE. Gels were analyzed by immunoblotting with antibodies specific to His-TAF40.

Electrophoretic mobility-shift assays (EMSA). Each binding reaction was performed using a ^{32}P-labeled 45-basepair fragment containing the adenovirus early 1B TATA box as described. Purified recombinant yeast TBP, (5nM), yeast TFIIA (1.5nM), and yeast TAF40 (32 or 64nM) were incubated at 25 ^0C for 30 min in 20μl of 20mM Tris (pH 7.5), 40mM HEPES (pH7.9), 100mM KCl, 1mM DTT, 0.5mM PMSF, 10% Glycerol. Complexes were separated from unbound DNA by 6% nondenaturing acrylamide gel electophoresis in 0.5X TBE and quantified by phosphorimaging.

6.4 Results

6.4a Yeast two-hybrid indicates that TAF40-TAF19 associate in vivo.

A genetic interaction between TAF40 and TAF19 was identified using high copy suppression of a temperature-sensitive allele of TAF40 (16), suggesting that these proteins interact. Furthermore, the human homologs of TAF40 and TAF19 (hTAF28 and hTAF18 respectively) have been shown to interact using an atypically conserved histone motif.

We used yeast two-hybrid analysis to further investigate the potential interaction between TAF40 and TAF19. To test the specificity of the interaction, we included two other TAFs, each of which share sequence similarity to the histone proteins TAF17 and TAF61. All four TAF proteins were fused in frame with either the Gal4 activation domain (AD) or the Gal4 DNA-binding domain (DB) or both. A two-hybrid interaction was obtained between DBTAF40 and ADTAF19 (Figure 6.1B). The reciprocal interaction was also obtained between DBTAF19 and ADTAF40. The interaction is specific in that both ADTAF17 and ADTAF61 were tested and found to be negative even though all fusion proteins are expressed in the cell (data not shown) (17).

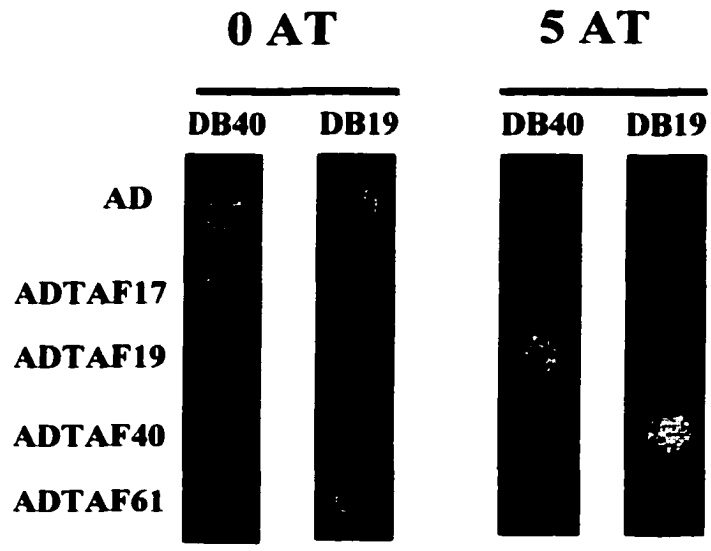

Figure 6.1B Two-hybrid analysis indicates that TAF19 and TAF40 specifically associate *in vivo*. The indicated Gal4 activation-domain (AD) fusion proteins (left) were tested for the ability to interact with the indicated Gal4 DNA-binding (DB) fusion proteins (top). The type of fusion protein is indicated by the addition of either AD for activation domain or DB for a DNA-binding domain. Approximately 10^4 cells were spotted onto synthetic complete plates containing either 0 mM or 5 mM 3-aminotriazole (AT). Growth on AT is indicative of an interaction between the two fusion proteins. A reciprocal two-hybrid interaction was obtained between TAF19 and TAF40.

6.4b Deletion analysis of TAF40 indicates some interactions are mediated through modeled histone-like folds.

A reasonable hypothesis is that TAF40 and TAF19 interact using a histone motif similar to the one found in the hTAF28/hTAF18 crystal structure (Figure 6.1A) (3). To test this hypothesis a potential histone motif was modeled onto TAF40 using sequence alignments. The schematic in Figure 6.2A is a summary of the results from this analysis. One region of similarity consists of amino acids 125-194 of TAF40, which is proposed to contain helices αN, α1, and α2 of the histone fold. A second region of similarity lies within amino acids 313-322, which is proposed to contain helix α3 of the histone fold. A large108-amino acid linker region connects these two regions of homology (Figure 6.2A).

Deletion mutations of TAF40 were designed to test the importance of these modeled helices for interaction with TAF19 (Figure 6.2B). All TAF40 truncations were fused in frame with the Gal4 activation domain vector (AD). Each ADTAF40 truncation was tested in two-hybrid analysis with DBTAF19. Besides the full-length protein, the only positive two-hybrid interaction was observed between DBTAF19 and ADTAF40(99-347) (Figure 6.2C). Small deletions in the conserved C-terminal region of TAF40 disrupts the two-hybrid interaction; however, the unconserved N-terminal 99 amino acids are dispensable for TAF19 interaction. Thus more that just the conserved region of TAF40 was needed to maintain interaction with TAF19 since amino acids 125-347 of TAF40 failed to interact.

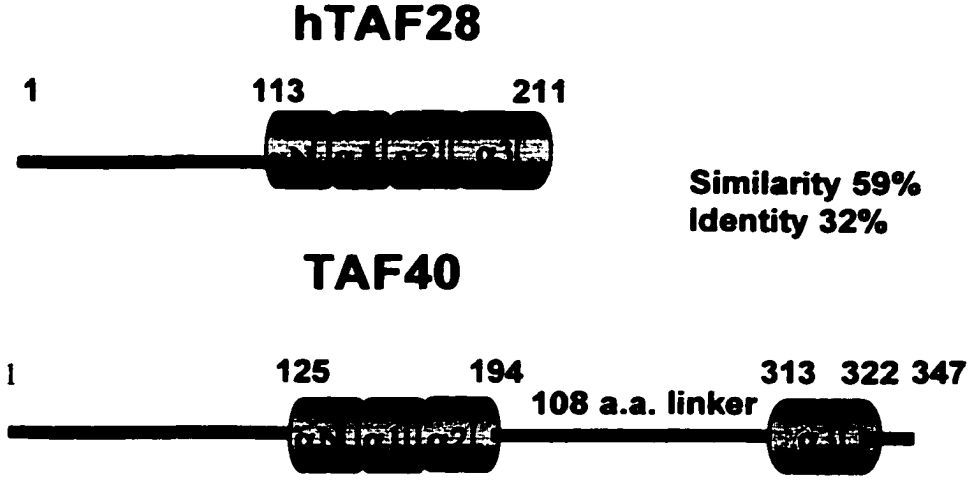

Figure 6.2A Schematic of hTAF28 and TAF40 alingments. Regions of similarity between hTAF28 and TAF40 are shown in the blocked off areas. The percent similarity and identity for hTAF28 and TAF40 is indicated. These alignments were used to model histone helices onto TAF40. These modeled helices were then used to create truncation mutations in order to localize the interaction between TAF40 and various interacting proteins.

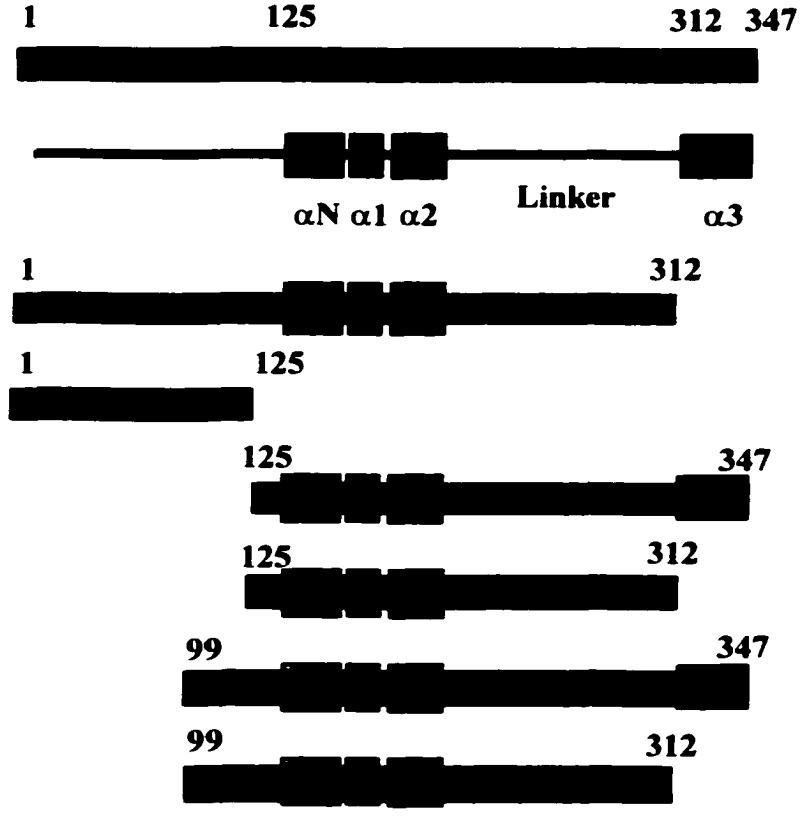

Figure 6.2B Schematic representation of deletion constructs of TAF40. Using both structural and sequence information from hTAF28, histone helices were modeled on TAF40 (top). These modeled helices were then used to create deletion constructs which will be used in specific assays in order to localize TAF40 interactions.

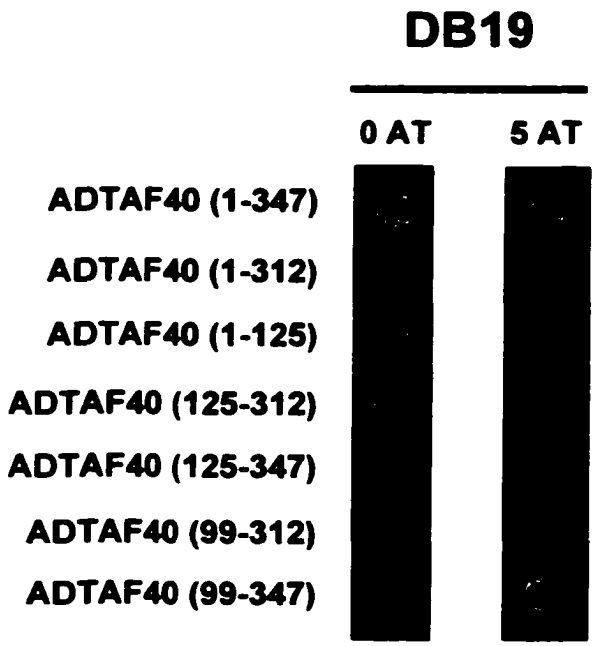

Figure 6.2C ADTAF40 deletion mutations were used in two-hybrid analysis with DBTAF19. The ADTAF40 deletion mutations were fused in frame with the Gal4 activation-domain (ADTAF40) (left) and tested for interaction with DBTAF19 (top). Approximately 10^4 cells were spotted onto synthetic complete plates containing either 0 mM or 5 mM 3-aminotriazole (AT). Growth on AT is indicative of an interaction between the two-hybrid proteins. DBTAF19 specifically associates with amino acids 99-347 of TAF40.

TAF40 fusion protein expression was confirmed by immunoblotting whole-cell extracts with antibodies specific to the HA tag present in the activation-domain vector (Figure 6.2D). Although there was some variability in the levels, we observed no relationship between a positive two-hybrid and expression levels (Figure 6.2D compare lanes 5 and 6).

6.4c Two-hybrid analysis using deletion mutations indicate that both N- and C-terminal sequences are necessary for interaction with TFIIA.

TFIIA and TAF40 also interact in the two-hybrid assay. We have mapped the TFIIA surface involved in this interaction using both mutational (Figure 4.4) and deletion analysis (Figure 5.10). These results suggest that the interaction occurs within the four-helix bundle domain of TFIIA (Figure 4.3). We were interested in mapping the TFIIA interaction domain on TAF40. A two-hybrid assay was performed by fusing Toa2 in frame with the Gal4 DNA-binding domain and testing it for interactions with the series of ADTAF40 deletion mutations. Each strain was tested for growth on 20 and 30 mM AT. Unexpectedly all TAF40 deletions failed to interact with DBToa2 (Table 6.1). Thus, unlike TAF19, DBToa2 (TFIIA) requires both N- and C-terminal amino acids for interaction with TAF40.

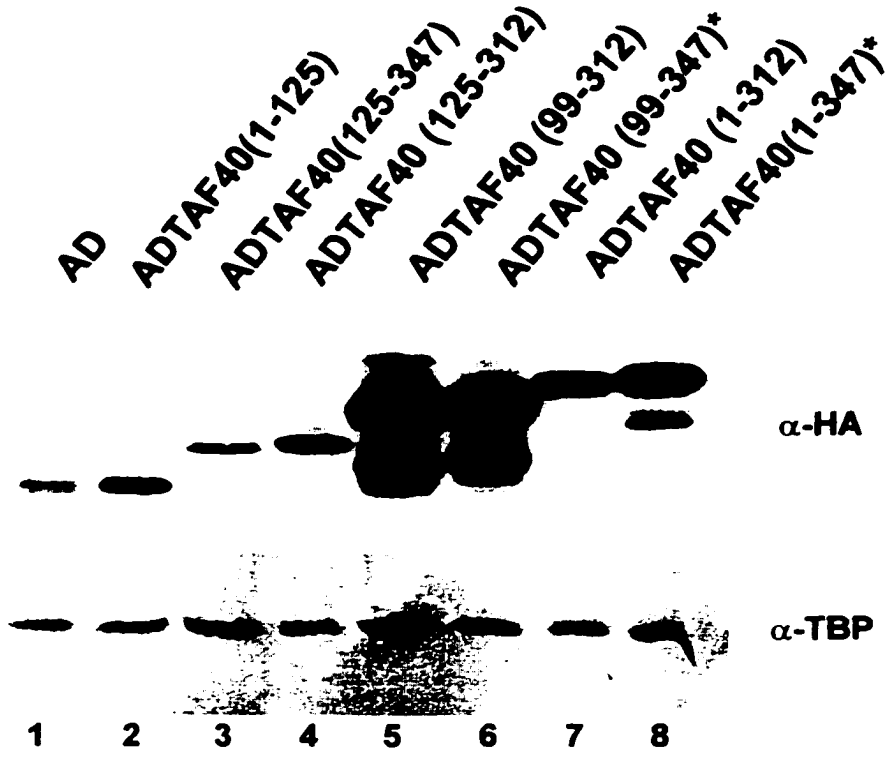

Figure 6.2D Western blot of yeast-whole cell extracts prepared from the indicated strains (top). Two-hybrid strains expressing the indicated deletion construct (top) were grown to log phase and whole-cell extracts were prepared. 25μgs of extract were separated by 10% SDS-PAGE and transferred to nitrocelluose. Blots were probed using anti-HA antibodies (top panel) to detect the ADTAF40 fusion protein. The blot was striped and reprobed using anti-TBP antibodies to serve as a load control (bottom panel).

Table 6.1 A summary of interactions obatined with TAF40 deletions mutations. Two-hybrid interactions with DBToa2 were tested in MaV103. Srains were grown on 20 and 30mM AT to look for reporter gene output.

	DBTAF19	DBToa2
ADTAF40 (1-347)	+	-
ADTAF40 (1-312)	-	-
ADTAF40 (1-125)	-	-
ADTAF40 (125-312)	-	-
ADTAF40 (125-347)	-	-
ADTAF40 (99-312)	-	-
ADTAF40 (99-347)	+	-

6.4d Point mutants in TAF40 do not impair interaction with TAF19 or TFIIA *in vivo*.

Previous studies using hTAF28 showed that helix 2 of the histone fold is important for synergistic activation in transiently transfected human cells. In addition, specific residues (I152, E164 and E168) on the solvent exposed surface of helix 2 were shown to be important for synergy with TBP (Figure 6.3A and B) (20). To identify the TBP-binding region on TAF40, the corresponding residues were chosen for mutational studies, including a Valine at position 170 and two Glutamic acids, one at position 182 and one at 185. The Glutamic acid at position 168 on hTAF28 is not conserved in TAF40 so the glutamic acid at position 185 was chosen. The Valine at position 170 was mutated to a Lysine (V170K) and both Glutamic acids at positions 182 and 185 were mutated to Alanines (E182A and E185A).

We next determined whether the amino acid substitutions (V170K, E182A and E185A) affected the association of TAF40 with either TAF19 or TFIIA. We observed no change in the interaction with TAF19 (Figure 6.3C) or TFIIA (Figure 6.3D) in the yeast two-hybrid assay. AD-V170K could not be assayed for interaction with TAF19 because expression of this construct caused a lethal phenotype in CG1945. Therefore, this mutation was tested as a DB-V170K fusion and was found to interact with ADTAF19 (data not shown). A two-hybrid interaction was attempted for the TAF40-TBP interaction but this interaction cannot be detected using two-hybrid analysis

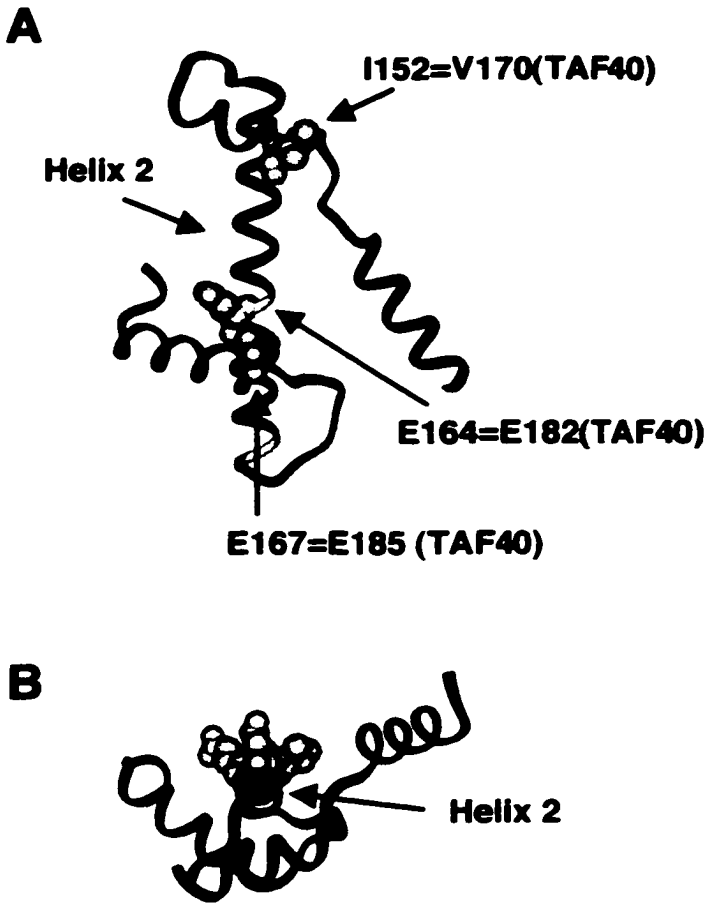

Figure 6.3A and 6.3B Ribbon representation of the crystal structure of human TAF28 (red). (A) Modeling of helix 2 on hTAF28 revealed a hydrophobic region (gray) and hydrophilic region (yellow). Site-directed mutagenesis of helix 2 indicated that both regions are important for interaction with TBP. Specifically, residues (I152, E164, and E167) were found to be important for TBP binding. Thus the corresponding residues in TAF40 (V170, E182, and E185) were choosen for mutational studies. (B) Longitudinal view of hTAF28 (view A rotated 90 °C back into the page). The figure was created using Insight II from the coordinates of the hTAF28/hTAF18 structure (3).

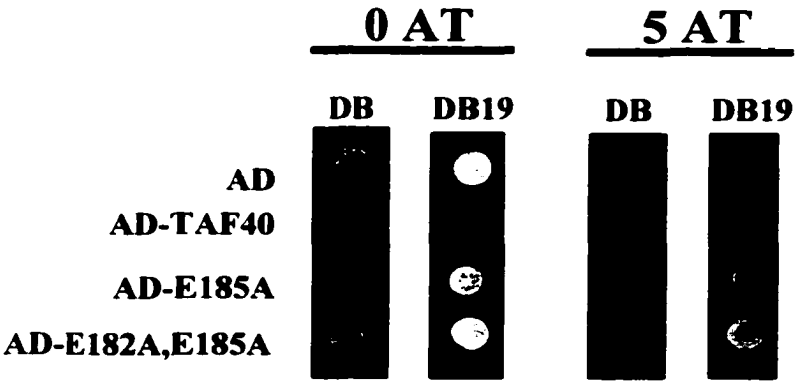

Figure 6.3C Two-hybrid analysis indicates ADTAF40 point mutants are able to associate with DBTAF19 *in vivo*. The three TAF40 point mutations were fused in frame with the Gal4 activation-domain (left) and tested for interaction with DB or DBTAF19 (top). The specific substitution is indicated by the one letter amino acid abbreviation and number. Approximately 10^4 cells were spotted onto synthetic complete plates containing either 0 mM or 5 mM 3-aminotriazole (AT). All strains grow in the absence of AT but only strains with interacting proteins grow on AT. The substitutions don't have an affect on association with DBTAF19. AD-V170K could not be assayed because expression of this construct causes a lethal phenotype in CG1945.

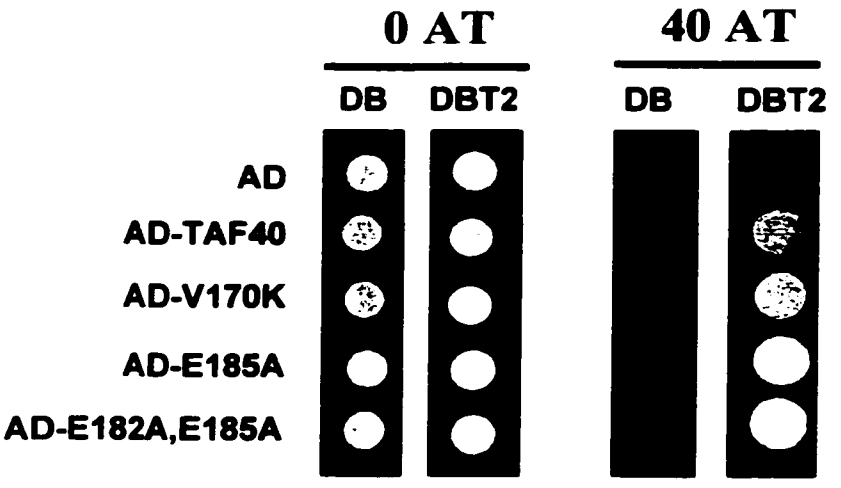

Figure 6.3D Two-hybrid analysis indicates ADTAF40 derivatives are able to associate with DBToa2 *in vivo*. The three ADTAF40 point mutations were tested for interaction with DB or DBToa2 (top). Approximately 10^4 cells were spotted onto synthetic complete plates containing either 0 mM or 40 mM 3-aminotriazole (AT). All strains grow in the absence of AT but only strains with interacting proteins grow on AT. The substitutions don't have an affect on association with DBToa2.

(data not shown). Thus the TAF40 derivatives don't affect association with either TAF19 or TFIIA.

6.4e Point mutations in the modeled helix 2 of TAF40 are defective for interaction with TBP.

Since two-hybrid analysis could not be used to measure TAF40-TBP interactions *in vivo*, a glutathione-affinity chromatography pull-down assay (GST pull-down) was employed *in vitro*. Previously this assay was used to demonstrate that TAF40 makes direct contacts with both TBP and TFIIA (Figure 4.2A). TAF40 derivatives were expressed and purified from *E. coli*. GST fusion proteins were incubated with TAF40 and an equivalent amount of each TAF40 derivative. Immunoblots of the resulting complexes indicate the V170K substitution decreases the TAF40-TBP interaction (Figure 6.4). The E185A mutation shows a slight defect, albeit to a lesser extent. Interestingly, the double mutant E182,E185A (which contains the E185A mutation) binds TBP with wild-type affinity, suggesting a gain of function under the conditions assayed (Figure 6.4). Consistent with the two-hybrid interactions all TAF40 derivatives bound TFIIA with wild-type affinity. Loads indicate equivalent amounts of proteins were used in all reactions.

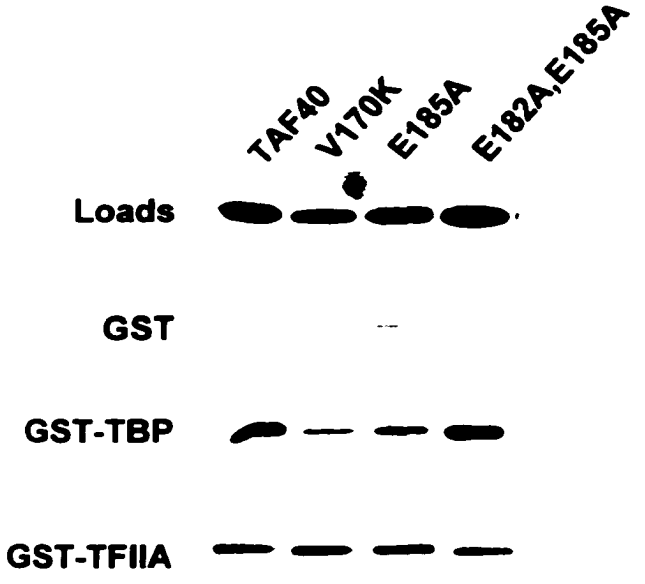

Figure 6.4 GST-pull down using TAF40 derivatives indicates that position 170 and 182 is important for TBP interaction. A Glutathione-affinity chromatography pull-down assay (GST Pull-down) was used to test TAF40 derivatives for interaction with TBP and TFIIA. Recombinant GST, GST-TBP, and GST-TFIIA were incubated with purified histidine-tagged TAF40 derivatives. Complexes were isolated by adding glutathione sepharose and washing 2X. Samples were separated by SDS-PAGE and analyzed by immunoblot using antibodies specific to TAF40. The TAF40 derivative is indicated on top, and the GST fusion used in the reaction is indicated to the left.

6.4f TAF40 derivatives are competent for TBP-TFIIA-DNA complex stabilization.

Previously it was demonstrated that addition of TAF40 to a reaction containing TBP-TFIIA-DNA increases the amount of complex formed in an electrophoretic mobility gel shift assay (EMSA). This result coincides with the notion that certain TAFs can modulate TBP's affinity for promoter DNA. Using the TAF40 derivatives, we examined how a decreased TAF40-TBP interaction affects stabilization of the TBP-TFIIA-DNA complex. In the absence of magnesium, TBP does not form a stable complex with DNA; however, the addition of sub-saturating amounts of TFIIA stabilizes the TBP-DNA interaction and shifts the TATA-containing probe slightly (Figure 6.5). When excess TAF40 is added to the reaction, the amount of probe shifted increases dramatically (Figure 6.5). Equal amounts of each mutant TAF protein were tested in the EMSA assay. Surprisingly, the V170K substitution doesn't affect complex stabilization. Interestingly, E185A and the double mutant E182A,E185A show near wild-type stabilization. If anything perhaps a modest increase (less than 1.5 fold) in complex formation over wild-type TAF40 was observed.

Thus it seems that a defective TAF40-TBP interaction does not translate into defects in TBP-TFIIA-DNA complex stabilization. Perhaps multiple interaction surfaces on TAF40 are important for stabilization.

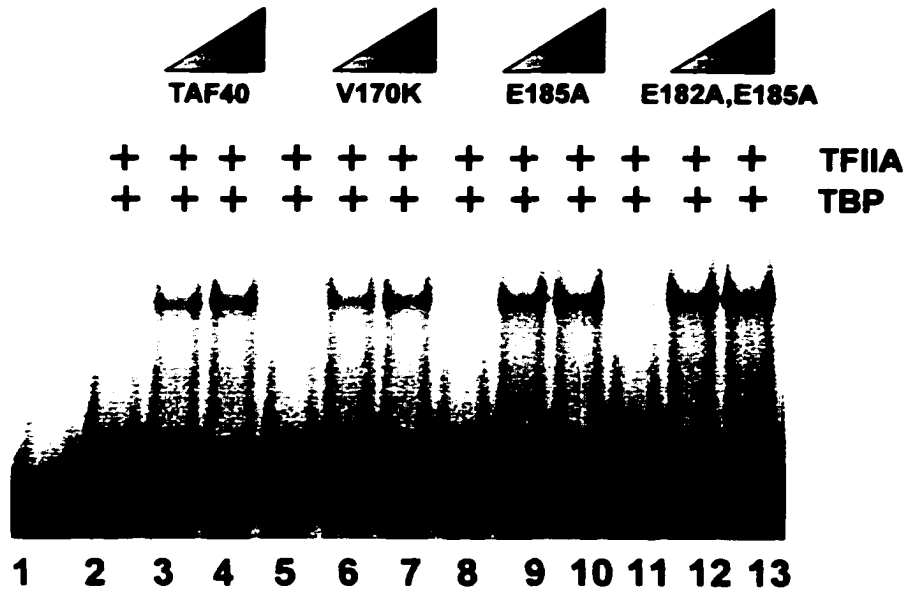

Figure 6.5 Electromobility shift assay using TAF40 derivatives indicates that all mutants are competent for enhancement of the TBP-TFIIA-DNA complex. For all reactions, 9nM of radiolabeled adenovirus early 1B TATA box probe was used. Concentrations of TBP and TFIIA were held constant at 5 nM and 1.5 nM, respectively. Lane 1 contains DNA alone. Lanes 2,-13 contain TBP and TFIIA. Equal amounts (32 and 64 nM each) of wild-type TAF40 (TAF40) and the indicated TAF40 derivatives were added to the appropriate reactions. Complexes were separated from unbound DNA by 6% nondenaturing acrylamide gel electophoresis in 0.5X TBE and quantified by phosphorimaging.

6.4g TAF40 derivatives are compromised for artificial recruitment activity.

Artificial recruitment assays have been used as evidence that recruitment of general transcription factors to a promoter is a rate-limiting step in gene activation *in vivo*. These experiments have been done for many transcription factors, including TBP and TAF40 (Figure 4.1B). The artificial recruitment assay using the *HIS3* reporter in MaV103 was used to further characterize the TAF40 derivatives. Each derivative was expressed as a Gal4 DNA-binding domain fusion (i.e., DB-V170K) and tested for activity from the *HIS3* reporter gene. Growth in the presence of 3-aminotriazole (AT), competitive inhibitor of the *HIS3* gene product indicates gene activation. Consistent with its defect in TBP binding, the V170K (DB-V170K) shows a decrease for artificial recruitment activity (Figure 6.6). Interestingly, the E182,E185A double mutant is completely defective for artificial recruitment (Figure 6.6). This is unexpected because E182,E185A bound TBP, TFIIA and TAF19 with wild type affinities in all assays tested. In fact E182,E185A seemed to enhance TBP-TFIIA-DNA complex formation in the EMSA assay.

A simple explanation for why this derivative is unable to stimulate reporter gene activation is that it is not functional when expressed as a Gal4 DNA-binding domain fusion protein. In order to test this hypothesis, a series of assays were performed on the strain expressing this DB-TAF40 derivative. First, extracts were prepared from strains expressing the DBTAF40 and DB-E182,E185A fusion proteins. Western blots on these extracts show that the

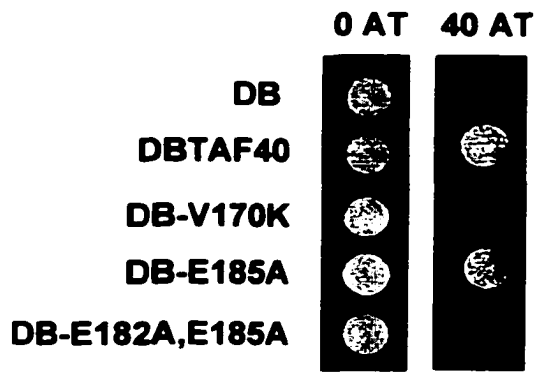

Figure 6.6 Artificial recruitment assay of TAF40 derivatives indicate differences in the ability to stimulate transcription from the *HIS3* reporter. Activity of each DBTAF40 point mutant was tested using the *HIS3* reporter. Strains were transformed with each construct and reporter output was measured by growth on 40mM 3-aminotriazole (40mM AT). DB and DBTAF40 were used as positive and negative controls respectively. Plates without AT were used as a control for growth as they do not require reporter activity for viability.

E182,E185A derivative is expressed in the cell to near wild-type levels. Another possible explanation for the defect is that the fusion is not functional when tethered to the promoter via the DNA-binding domain. Since DB-E182,E185A doesn't have activity when recruited to the *HIS3* reporter gene, it can be used in two-hybrid analysis. DB-E182,E185A was assayed for interaction with the largest subunit of TFIIA (ADToa1). DB-E182,E185A was able to interact with ADToa1, confirming that it is functional when recruited to a promoter (data not shown). Thus it seems that an increase in TBP affinity affects the ability to activate in the artificial recruitment assay. Another possible explanation is that this TAF40 derivative is defective for a yet unidentified activity, which is affecting its ability to activate in this assay.

In addition TAF40 deletion mutations described in section 6.4b were tested in the artificial recruitment assay. Each deletion was fused in frame with the Gal4 DNA-binding domain and tested for activity using the *HIS3* promoter. Each deletion mutation failed to activate transcription from the reporter gene (data not shown). It is interesting to note that even the ADTAF40(99-347) deletion failed to activate indicating that there is no relationship between TAF40/TAF19 interaction and artificial recruitment.

6.4h Phenotypic Analysis of TAF40 mutants strains.

A cell viability assay was performed to better understand how the TAF40 derivatives function *in vivo*. All three mutant TAF40 open reading frames were subcloned into a TAF40 expression vector. This vector allows

for the expression of TAF40 derivatives using the endogenous TAF40 promoter and terminator. Each construct was introduced into a TAF40 deletion strain containing a wild-type *TAF40* gene on a URA3-marked vector. The plasmid shuffle technique was used to test for viability (Figure 6.7). These assays are done by testing for growth on media containing 5-FOA, which kills strains expressing the *URA3* gene. Strains able to grow in the presence of 5-FOA have lost the wild-type copy of TAF40, contain a TAF40 derivative that is able to support cell viability. Strains unable to grow in the presence of 5-FOA contain a TAF40 derivative that is not able to support cell viability (10). Wild-type TAF40 (TAF40) and empty vector (YCP22) were used as positive and negative growth controls. Surprisingly, the V170K derivative is unable to support cell viability (Figure 6.7). This would indicate that the interaction between TAF40 and TBP is critical for TAF40 function *in vivo*. In contrast to V170K, the E185A and E182A,E185A derivatives are able to support cell viability (Figure 6.7). In order to better understand how these derivatives function *in vivo*, strains containing the E185A and E182A,E185A derivatives were tested for conditional phenotypes.

Since both E185A and E182A,E185A are able to support cell viability these derivatives were tested for numerous conditional phenotypes. Wild-type TAF40 (TAF40) was used as a positive growth control in phenotypic assays. Strains were tested for growth on media containing alternate carbon sources. Yeast strains harboring the indicated mutant derivative were spotted to plates containing ethanol and glycerol (YPEG), glycerol alone (YPG), and

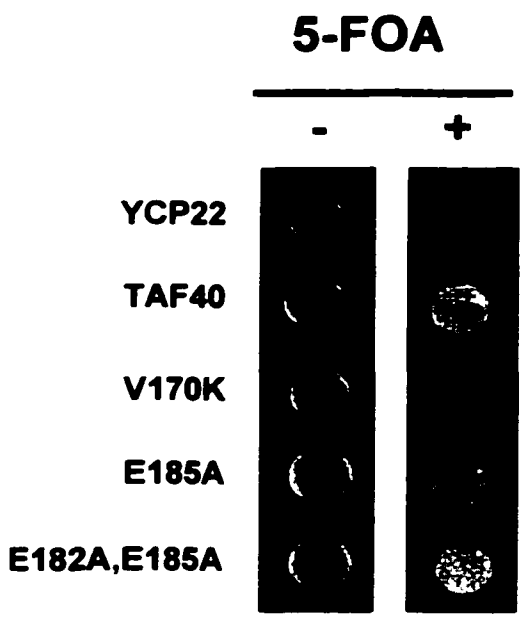

Figure 6.7 Plasmid shuffle technique indicates that the TAF40 derivative V170K is unable to support cell viability. Viability tests of *TAF40* mutant derivatives were conducted in ySB366 which contains a chromosomal deletion of *TAF40* covered by a *URA3*-marked TAF40 plasmid. ySB366 was transformed with YCP22, wild-type TAF40 (TAF40) and the indicated TAF40 derivatives. The resulting strains were spotted to plates containing 5-FOA. As a growth control, strains were also spotted to plates lacking 5-FOA. Strains able to grow in the presence of 5-FOA are able to support cell viability. Strains unable to grow in the presence of 5-FOA are not able to support cell viability.

galactose (YPGAL) and incubated at 30 °C. All strains grew indistinguishably from TAF40 (Figure 6.8). Strains were also tested and found to be wild-type for cold sensitivity (YPD at 15 °C) and inositol auxotrophy (plates lacking inositol) (data not shown). Interestingly, both derivatives display a wild-type growth when incubated at 30 and 36 °C; however, E182A,E185A is unable to support cell viability at 38 °C (Figure 6.8). Thus mutations in the modeled helix 2 of TAF40 cause conditional phenotypes *in vivo*.

6.5 Discussion

Elucidating the function of TAF proteins *in vivo* has proven difficult due to many factors. Among these is the fact that some TAFs are present in both the TFIID and SAGA transcription complexes (7, 8). TAF inactivation experiments therefore becomes hard to interpret for TAFs found in both TFIID and SAGA. Yeast TAF40 appears to be specific to the TFIID complex (16) and has been shown to make numerous contacts with other factors both *in vivo* and *in vitro* (17). Since TAF40 is specific to TFIID it provides analysis of TAF function within TFIID only and not other transcription complexes.

We find that TAF40 specifically associates with TAF19 *in vivo* using a histone motif modeled onto TAF40. Similar mapping studies found that TFIIA needs additional sequences in order to maintain interaction with TAF40. Additionally, we have identified residues in the modeled helix 2 of TAF40 that are important for TBP binding. Mutant studies of these residues indicate that

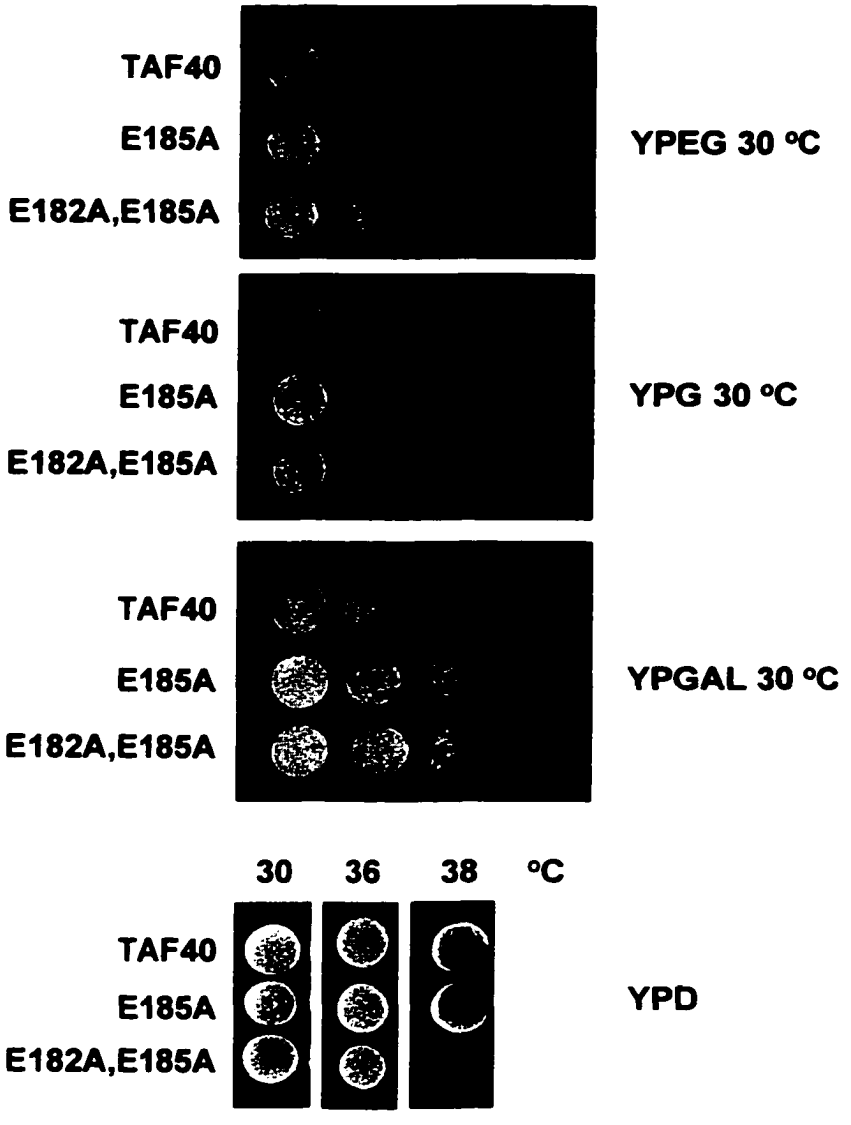

Figure 6.8 Phenotypic analysis of TAF40 derivatives indicate E185A is temperature-sensitive. Strains containing the the indicated TAF40 derivative were tested for conditional phenotypes in a *TAF40* deletion strain. The indicated strains were serially diluted (10^4 to 10 cells) and spotted onto rich media plates containing glucose (YPD) and incubated at either 30, 36 or 38 °C (top). In addition strains were spotted to plates containing ethanol and glycerol (YPEG), glycerol alone (YPG), and galactose (YPGAL).

the TAF40-TBP interaction performs an essential function in transcription initiation *in vivo.*

6.5a Specific association of TAF40 and TAF19 through a modeled histone motif.

TAF40 has been shown to interact directly with TFIIA and TBP (17). In addition to these direct interactions, a genetic interaction between TAF40 and TAF19 has recently been described (16). Support for this genetic interaction comes from the finding that human homologs of TAF19 and TAF40 (hTAF18 and hTAF28 respectively) were crystallized as a heterodimer (Figure 6.1A) (3).

We find that TAF40 and TAF19 specifically interact *in vivo.* Of the four TAFs tested only TAF40 and TAF19 showed a positive two-hybrid interaction. The specificity of this interaction is indicated by the fact that TAF17 and TAF 61 failed to give a positive result in this assay even though they share sequence similarity to histone H3 and H2B respectively (22).

A histone motif was modeled onto TAF40 using structural information and sequence alignments. This motif extends from amino acids 125-194 (helices α-N, α-1, and α-2) and 313 to 322 (helix 3). The motif was used to create deletion mutations to determine if TAF40 and TAF19 interact using a potential histone motif. Amino acids 99-347 of TAF40 were necessary to maintain the interaction with TAF19 as determined by two-hybrid analysis. Thus we show that in addition to the putative histone motif and

linker, TAF40 needs additional N-terminal sequences for interaction with TAF19. Perhaps the N-terminal helix modeled onto TAF40 extends even further than position 125 and deletion to this amino acid disrupts the secondary structure in this region. Interestingly removal of the proposed helix 3 disrupts the interaction further, supporting the model that TAF40 and TAF19 interact using a histone motif.

TAF40 also interacts with TFIIA *in vivo*. This interaction has been shown to be important for transcription from certain genes in yeast (17). Mutational (Figure 4.4) and deletion analysis (Figure 5.10) suggest that this interaction occurs within the four-helix bundle domain of TFIIA (Figure 4.3). We were interested in mapping the TFIIA interaction domain on TAF40. Two-hybrid analysis indicated that both N- and C-terminal regions of TAF40 are required for interaction with TFIIA. In fact, TFIIA failed to interact with all deletion mutations tested; suggesting that TAF19 and TFIIA may have similar but not identical binding sites.

It is interesting that same TAF40 deletion constructs all failed to activate in the artificial recruitment assay. This would suggest that regions required for interaction with TFIIA are similar to those needed for artificial recruitment. In contrast; however, a functional interaction with TAF19 does not correspond to artificial recruitment activity.

6.5b Mutational studies in the modeled helix 2 of TAF40 implicate this region for interaction with TBP.

hTAF28 binds TBP using the solvent expose side of helix 2 within the histone fold (Figure 6.3B). Modeling of this helix indicates both a hydrophobic and hydrophilic region (Figure 6.3A). Mutations within both regions have been shown to affect synergy with TBP (20). We decided to mutate similar residues on TAF40 in order to identify the same TBP binding region. These mutants were then used to test the functional conservation of this interaction. The Valine at position 170 was mutated to a Lysine and both Glutamic acids at positions 182 and 185 of TAF40 were mutated Alanine. All three amino acids substitutions directly affected affinity for TBP without affecting TFIIA and TAF19 interactions. The Valine to Lysine mutation at position 170 caused a decrease in affinity for TBP using a GST pull-down assay. This derivative was subsequently found to confer a lethal phenotype suggesting that interaction with TBP is critical for essential functions in yeast.

Interestingly, double mutant E182A,E185A shows an increase activity in both *in vitro* assays. The GST pull-down assay performed using the E185A mutant shows a modest (less than 2-fold) decrease in interaction with TBP. However the double mutant, which contains the E185A mutation, binds TBP with wild-type affinity suggesting the E182A mutation restores TBP binding. This increase in activity is unique to the TAF40-TBP interaction because it not visible when tested for interaction with TFIIA. This gain of function is also present in the EMSA assay. The double mutant enhances the

TBP-TFIIA-DNA complex above what is seen for wild-type TAF40. In apparent contrast to the *in vitro* studies the double is unable to activate in the artificial recruitment assay, suggesting a defect in PIC formation.

Interestingly, a similar finding was also reported in studies using hTAF28 and hTBP (20). These studies produced mutations in TBP that increased the affinity for hTAF28. This increase in affinity correlated with a defect in the ability of hTAF28 to cause synergistic activation. Thus similar to what was found for the hTAF28-hTBP interaction we find that an increased TAF40-TBP causes defects in artificial recruitment. A simplified model offered by Lavigne et al. is that the hTAF28-hTBP interactions must dissociate to allow hTAF28 to make other interactions. An increase in this interaction would interfere with this event, causing defects in PIC formation (20). Thus similar to what was found for hTAF28, we find that both hydrophobic and hydrophilic residues on TAF40 are important for interaction with TBP, suggesting that the hTAF28-hTBP interaction is functionally conserved in the yeast TAF40-TBP interaction. Furthermore these results suggest that a TAF40-TBP interaction may not be the only interaction that is required for proper transcription initiation.

6.5c The TAF40-TBP interaction is necessary for essential functions in yeast.

A temperature-sensitive (ts) allele of TAF40 has recently been described (16). Analysis of this allele revealed that elevated temperature

causes degradation of TAF40 and a general cessation of RNA pol II transcription. High copy suppression analysis using individual TAF subunits identified TAF19 as being able to rescue the ts phenotype indicating that the allele is, at least in part, defective for interaction with TAF19 (16). This would indicate that this interaction is necessary for cell viability.

Here we present unique TAF40 derivatives that are in some way compromised in binding TBP. In particular a Valine at position 170 causes a decrease in TBP binding. This mutation was subsequently shown to be lethal when assayed for cell viability. In addition a double substitution at positions 182 and 185 which displayed an increase in TBP affinity was found to be temperature-sensitive when assayed *in vivo*.

Thus we have identified residues in TAF40 responsible for interaction with TBP. These mutants display conditional and lethal phenotypes *in vivo*, indicating the interaction between TBP and TAF40 mediates an essential function in yeast. In addition, these results suggest that interactions between TBP, TAF40, and TAF19 are essential for critical aspects of transcription initiation.

6.6 Future Directions and perspectives.

In this report we show that TAF19 and TAF40 interact through a potential histone-fold motif modeled onto TAF40. In addition, deletion analysis determined that amino acids 99-347, which encompass this region, were necessary to maintain this interaction. These same deletion mutants

however failed to interact with TFIIA. An interesting question would be to perform viability studies on the deletion mutations. This analysis could be used to correlate the interaction of both TAF19 and TFIIA and cell viability.

Along those same lines a couple additional deletion mutations might also prove useful to construct. Smaller deletions (less than 99 amino acids) into the N-terminus of TAF40 could better define the minimal domain needed for TFIIA interaction.

A histone fold was modeled onto TAF40. Residues within the modeled helix 2 of TAF40 were mutated and found to be necessary for interaction with TBP. Additional mutations in this region of TAF40 could add insight into the interaction between TAF40 and TBP. Perhaps changing residue 170 to an Alanine would still disrupt interaction with TBP, but be able to support cell viability. In addition, the double mutant E182A,E185A displayed a ts phenotype when assay in a *TAF40* deletion strain. Transcriptional analysis from this mutant before and after temperature shock could reveal transcriptional defects. This would further elucidate the importance of the TAF40-TBP interaction for transcription initiation *in vivo*.

In vitro studies involving TAF19 have recently been initiated. These experiments would attempt to produce TAF40/TAF19 heterodimer using recombinant proteins expressed from *E. coli*. These complexes could be used in numerous biophysical and biochemical studies. Initially, however it would be interesting to test heterodimers of each TAF40 derivative as well as

wild-type TAF40 in the assays described in the results section. Perhaps the activity of a TAF40 TAF19 heterodimer is different for TAF40 alone.

6.7 Acknowledgements.

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6.8 References

1. Apone, L. M., C. A. Virbasius, J. C. Reese, and M. R. Green. 1996. Yeast $TAF_{II}90$ is required for cell-cycle progression through G_2/M but not for general transcription activation. *Genes & Dev.* 10:2368-2380.

2. Apone, L. M., C.-A. Virbasius, F. C. Holstege, J. Wang, R. A. Young, and M. R. Green. 1998. Broad, but not universal, transcriptional requirement for $yTAF_{II}17$, a histone H3-like TAF_{II} present in TFIID and SAGA. *Molecular Cell* 2:653-661.

3. Birck, C., O. Poch, C. Romier, M. Ruff, G. Mengus, A. Lavigne, I. Davidson, and D. Moras. 1998. Human TAF(II)28 and TAF(II)18 interact through a histone fold encoded by atypical evolutionary conserved motifs also found in the SPT3 family. *Cell* 94:239-249.

4. Burley, S. K., and R. G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* 65:769-799.

5. Chatterjee, S., and K. Struhl. 1995. Connecting a promoter-bound protein to the TATA-binding protein overrides the need for a transcriptional activation region. *Nature* 374:820-822.

6. Colgan, J., and J. L. Manley. 1992. TFIID can be rate limiting in vivo for TATA-containing, but not TATA-lacking, RNA polymerase II promoters. *Genes & Dev.* 6:304-315.

7. Grant, P., D. Sterner, L. Duggan, J. Workman, and S. Berger. 1998. The SAGA unfolds: convergence of transcription regulators in chromatin-remodeling complexes. *Trends Cell Biol* 8:193-197.

8. Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. Steger, J. C. Reese, J. R. Yates III, and J. L. Workman. 1998. A subset of TAF_{II}s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. *Cell* 94:45-53.

9. Green, M. 2000. TBP-associated Factors (TAF_{II}s): Multiple, Selective Transcriptional Mediators in Common Complexes. *TIBS* 25:59-63.

10. Guthrie, C., and G. R. Fink. 1991. Guide to Yeast Genetics and Molecular Biology, vol. 194. Academic Press, New York.

11. Hahn, S. 1998. The Role of TAFs in RNA Polymerase II Transcription. *Cell* 95:579-582.

12. Hampsey, M. 1998. Molecular genetics of the RNA polymerase II genreal transcriptional machinery. *Microbiology and Molecular Biology Reviews* 62:465-503.

13. Holstege, F. C. P., E. G. Jennings, C. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander, and R. A. Young. 1998. Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* 95:717-728.

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

15. Klein, C., and K. Struhl. 1994. Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. *Science* 266:280-282.

16. Komarnitsky, P. B., B. Michel, and S. Buratowski. 1999. TFIID-specific yeast TAF40 is essential for the majority of RNA polymerase II-mediated transcription in vivo. *Genes and Dev.* 13:2484-2489.

17. Kraemer, S., R. T. Ranallo, R. Ogg, and L. A. Stargell. 2000. TFIIA Interacts with TFIID via association with TBP and TAF40. Submitted .

18. Kuras, L., and K. Struhl. 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. *Nature* 399:609-613.

19. Lavigne, A., G. Mengus, M. May, V. Dubrovskaya, L. Tora, P. Chambon, and I. Davidson. 1996. Multiple interactions between hTAF$_{II}$55 and other TFIID subunits. Requirements for the formation of stable ternary complexes between hTAF$_{II}$55 and the TATA-binding protein. *J Biol Chem* 271:19774-19780.

20. Lavigne, A. C., Y.-G. Gangloff, L. Carre, G. Mengus, C. Birck, O. Poch, C. Romier, D. Moras, and I. Davidson. 1999. Synergistic transcriptional activation by TATA-binding protein and hTAF$_{II}$28 requires specific amino acids of hTAF$_{II}$28 histone fold. *Mol. Cell. Biol.* 19:5050-5060.

21. Li, X. Y., A. Virbasius, X. Zhu, and M. R. Green. 1999. Enhancement of TBP binding by activators and general transcription factors. *Nature* 399:605-609.

22. Michel, B., P. Komarnitsky, and S. Buratowski. 1998. Histone-like TAFs are essential for transcription in vivo. *Molecular Cell* 2:663-672.

23. Moqtaderi, Z., Y. Bai, D. Poon, P. A. Weil, and K. Struhl. 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* 383:188-191.

24. Moqtaderi, Z., M. Keaveney, and K. Struhl. 1998. The histone H3-like TAF is broadly required for transcription in yeast. *Mol. Cell.* 2.

25. Natarajan, K., B. Jackson, E. Rhee, and A. Hinnebusch. 1998. yTAF$_{II}$61 has a general role in RNA polymerase II transcription and is required by Gcn4p to recruit the SAGA coactivator complex. *Mol. Cell* 2:683-692.

26. Orphanides, G., T. Lagrange, and D. Reinberg. 1996. The general transcription factors of RNA polymerase II. *Genes and Development* 10:2657-2683.

27. Ranallo, R., K. Struhl, and L. Stargell. 1999. A TATA-binding protein mutant defective for TFIID complex formation in vivo. *Mol Cell Biol* 19:3951-3957.

28. Sanders, L. S., and A. P. Weil. 2000. Identification of Two Novel TAF Subunits of the Yeast *Saccharomyces cerevisiae* TFIID Complex. *Journal of Biological Chemistry* 275:13895-13900.

29. Shen, W.-C., and M. R. Green. 1997. Yeast TAF$_{II}$145 functions as a core promoter selectivity factor, not a general coactivator. *Cell* 90:615-624.

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

31. Struhl, K., and Z. Moqtaderi. 1998. The TAFs in the HAT. *Cell* 94:1-4.

32. Suzuki-Yagawa, Y., M. Guermah, and R. G. Roeder. 1997. The ts13 mutation in the TAF(II)250 subunit (CCG1) of TFIID directly affects transcription of D-type cyclin genes in cells arrested in G1 at the nonpermissive temperature. *Mol. Cell. Biol.* 17:3284-3294.

33. Verrijzer, C. P., and R. Tijan. 1996. TAFs mediate transcriptional activation and promoter selectivity. *Trends in Biochemical Science* 21:338-342.

34. Vidal, M., R. K. Brachmann, A. Fattaey, E. Harlow, and J. D. Boeke. 1996. Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein interactions. *Proc. Natl. Acad. Sci. USA* 93:10315-10320.

35. Walker, S. S., J. C. Reese, L. M. Apone, and M. R. Green. 1996. Transcription activation in cells lacking TAF_{II}s. *Nature* 382:185-188.

36. Walker, S. S., W.-C. Shen, J. C. Reese, L. M. Apone, and M. R. Green. 1997. Yeast $TAF_{II}145$ required for transcription of G1/S cyclin genes and regulated by the cellular growth state. *Cell* 90:607-614.