DISSERTATION

THE TFIIA-TAF11 INTERACTION IN RNA POLYMERASE II TRANSCRIPTION

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 MARY M. ROBINSON ENTITLED THE TFIIA-TAF11 INTERACTION IN RNA POLYMERASE II TRANSCRIPTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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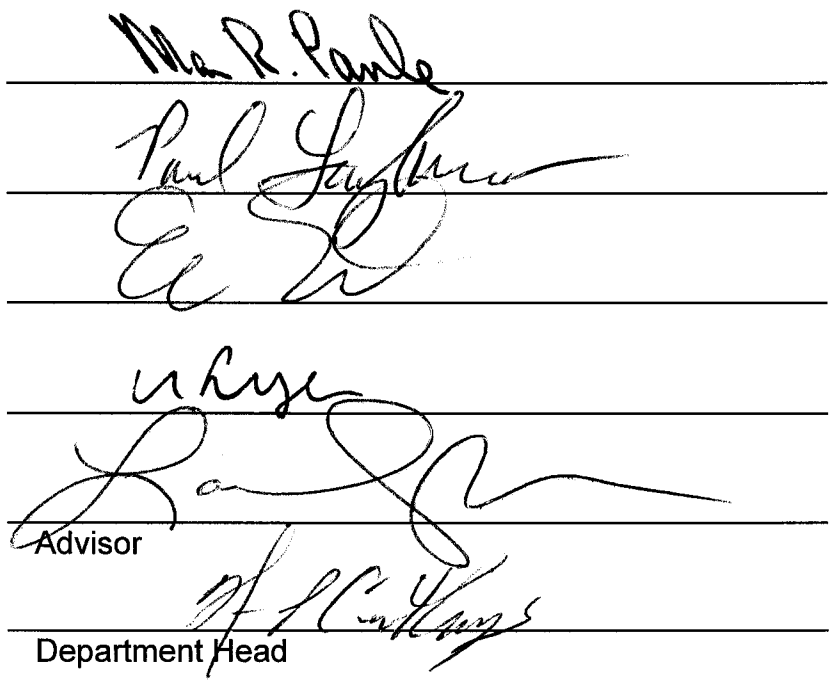

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ABSTRACT OF THE DISSERTATON

THE TFIIA-TAF11 INTERACTION IN RNA POLYMERASE II TRANSCRIPTION

In eukaryotes, RNA polymerase II transcription is a highly regulated process that requires the cooperative interaction of multiple proteins and protein complexes. Transcription is predominantly regulated at the initiation where a large complement of proteins must assemble to form a preinitiation complex (PIC), comprised of RNA polymerase II and general transcription factors, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. TFIID is required to nucleate the assembly of the PIC and its association to promoters is the rate-limiting step for initiation. TFIID is a multiprotein complex comprised of the TATA binding protein (TBP) and 14 TBP associated factors (TAFs). Both TBP and TAFs make significant contributions to TFIID association with promoters. TBP binds via sequence specific contacts with the TATA element. TAFs facilitate the TFIID affinity and specificity for core promoters through specific interactions with promoter sequences and other components of the transcription machinery.

The general transcription factor TFIIA influences TFIID association with promoters through associations with TBP and TAF11. The importance of TFIIA interaction with TBP for transcription has been demonstrated by numerous studies, but TFIIA interaction with TAF11 has not been well defined. To examine the functional significance of the TFIIA-TAF11 interaction, we employed

biochemical and genetic analyses to refine the intramolecular contacts between these proteins, and to identify the mechanistic requirements for their association.

Our studies determined that interaction with TFIIA requires two distinct regions of TAF11. The structurally conserved histone fold domain (HFD) can mediate interaction with both TFIIA and TAF13. In addition to this region, the N-terminal region of TAF11 is also required for interaction with TFIIA. In vitro studies determine that association between TAF11 and TFIIA is important for interactions at the core promoter. TAF11 imparts changes to TFIIA and TBP interactions with DNA, leading to stimulation of overall complex formation. Moreover, TFIIA-TAF11 interactions at weak or non-consensus TATA elements can be stabilized by TAF11. Finally, our studies demonstrate that TAF11 interaction with TFIIA facilitates core promoter functions that are important for RNA polymerase II transcription.

Taken together, the work presented provides a solid foundation for advancing our understanding of RNA polymerase II regulation, with a particular emphasis on the interaction between TFIIA and TAF11 and provides further insights into the functional requirement for this interaction in vivo.

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Sometimes the unexpected twists and turns in life present us with opportunities and challenge us to leave behind the comfort of our routines. Moving forward means exploring possibilities, changing our perspectives and demanding more from ourselves. It has been my experience, that despite the challenges, each new venture enriches our lives in many unexpected ways. The past five years here at CSU have held true to that, and I am tremendously grateful to have had the opportunity at this juncture of my life.

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My graduate studies have updated my skills and equipped me with the essentials necessary to explore new possibilities in the field of science and I look forward to participating in these new adventures.

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CHAPTER 1

INTRODUCTION TO RNA POLYMERASE II TRANSCRIPTION

1.1 RNA polymerase II transcription is an essential and complex biochemical process.

Eukaryotic cells contain an enormous amount of genetic material to encode proteins that are necessary for sustaining the life of an organism. Thousands of protein coding genes must be differentially expressed during development, in specific cell types, and in response to a wide variety of extracellular signals. Transcription by RNA polymerase II is a fundamental biochemical process by which genetic information encoded in the DNA is expressed. This complex process, involving multiple proteins, must be coordinately regulated to ensure proper patterns of gene expression. Failure to properly regulate transcription leads to severe developmental abnormalities or disease. The precise mechanisms that determine how gene expression is adjusted to ensure that specific gene products are generated at the correct location and time is not yet understood.

Over the last two decades, a plethora of biochemical and genetic analyses in model organisms have identified a diverse number of protein factors that are required for transcription control. Despite this wealth of data, the functional roles of only a fraction of the transcription machinery components are well characterized. Moreover, protein factors rarely function alone, but rather associate with other proteins, forming large multiprotein complexes to perform a

concerted function. Recently, large-scale approaches have been employed to identify novel protein-protein interactions and purify whole protein complexes. (35, 124). These analyses have provided valuable insight with regard to identifying protein subunits and cataloging proteins into common functional complexes. It is clear from these studies that certain proteins participate in multiple interactions and are components of multiple complexes. Just how specific protein subunits communicate with each other to contribute to overall complex function has not been well defined. Therefore, to fully understand the complex regulatory circuitry of RNA polymerase II transcription, it is imperative that the molecular organization and the mechanistic requirements for specific protein interactions be determined.

1.2 RNA polymerase II transcription initiation requires the assembly of a pre-initiation complex.

The process of RNA polymerase II transcription can be regulated at any stage; initiation, elongation, or termination; however, most control mechanisms target the initiation step. The core transcription machinery is comprised of more than 40 protein subunits (Fig 1.1). These proteins function in a cooperative manner via interactions with each other and DNA to precisely regulate transcription initiation. The focal point for control is the formation of the preinitiation complex (PIC), a large multiprotein assembly containing RNA polymerase II and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (reviewed in 43, 97). TFIID is the only general transcription

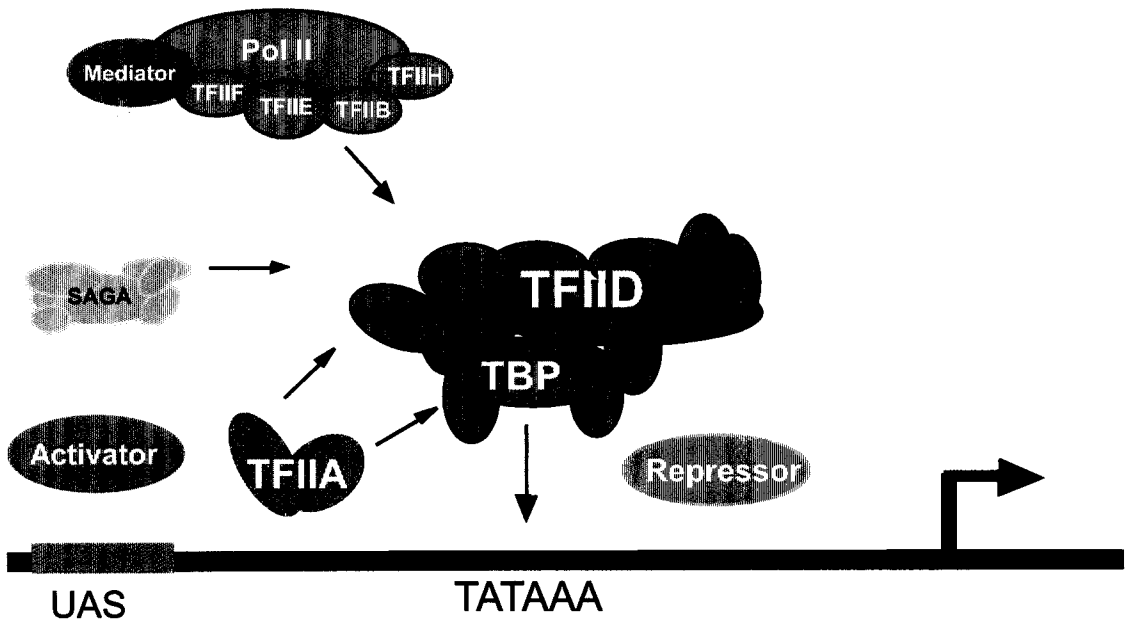

Figure 1.1 RNA polymerase II transcription complexes

factor that binds specifically and independently to the core promoter. TFIID is a multiprotein complex comprised of the TATA binding protein (TBP) and fourteen TBP associated factors (TAFs), (1, 14, 38, 41, 127). TBP associates with the core promoter through sequence specific contacts with the TATA element. TAF subunits serve to direct core promoter selectivity and integrate activation signals from enhancer bound regulators. The rate and efficiency of PIC assembly is highly dependent on TFIID promoter association.

1.3 Transcription regulation requires a balance between positive and negative regulators.

In purified in vitro systems, the PIC is the requisite complex sufficient for initiating basal levels of transcription. In vivo however, a balance of positive and negative regulators is necessary to support the full spectrum of transcription activity. The assembly of transcription complexes is highly dependent on the physical state of chromatin. The packaging of DNA into nucleosomes and the assembly of high order chromatin structure repress gene expression. Chromatin remodeling complexes are required to allow transcription factor access to the DNA and there is an excellent correlation between the disruption of high order chromatin structure and transcription activity (reviewed in (69).

Transcription control mechanisms employ gene repressors or activators to limit or rapidly induce the expression of specific genes respectively. General repressors of transcription function by inhibiting the binding of TBP to the TATA element or by blocking subsequent steps in PIC assembly (reviewed in 34 and

69). Conversely, activators counteract the negative effects of repressors and accelerate formation of the PIC. The predominant function of activators is to stimulate transcription factor recruitment. This is achieved by direct interactions with general transcription factors, (reviewed in 105, 123, 141) additional coactivators, (27, 125) or by associating with chromatin remodeling activities (90-92). Thus, a combination of multiple protein-protein and protein-DNA interactions ensures that transcription initiation is highly controlled to permit diverse patterns of gene expression.

1.4 TBP-TATA interactions

Since PIC assembly is predicated on the binding of TFIID to the promoter, activators and repressors modulate PIC assembly through changes to TFIID promoter association. The sequence specific binding of TBP to the consensus sequence TATAAA is required to nucleate the assembly of the PIC. The rate and efficiency of this step is highly dependent on the TBP-TATA element interaction. Numerous studies demonstrate both a qualitative and quantitative correlation between TBP association with promoters and transcription activity (62, 73). Mutations that reduce the binding of TBP to DNA affect the ability of TBP to activate transcription (4, 68). Moreover, inefficient TBP-TATA element interactions can only support low levels of transcription (51).

A number of studies demonstrate that TBP association with the TATA element is the rate limiting step in transcription and suggest that the PIC assembles inefficiently in the absence of activators (17, 21, 53, 55, 136).

Activators bind to regions upstream of the TATA element, and through direct or indirect interaction with TBP, modulate the level of initiation by either enhancing TBP recruitment or increasing the stability of TBP interaction with DNA (17). As such, TBP occupancy with promoter DNA is augmented in the presence of activators (62, 73). Other studies demonstrate that activator interactions induce conformational changes that are required to trigger subsequent steps in PIC assembly (19, 108)

In contrast to stimulating TBP association with DNA by activators, repressors minimize unregulated TBP binding by blocking its association with the DNA. Other repressors bind to TBP-DNA complexes and induce TBP dissociation from the DNA or prevent TBP interactions that are required to form productive transcription complexes (reviewed in 104).

1.5 TAF contributions to transcription regulation

Although there is a strong correlation between TBP promoter occupancy and transcription activity (62, 73), TAFs also contribute to transcription regulation on multiple levels. Certain TAFs orient and stabilize TFIID promoter association by direct contacts with specific promoter elements (13, 16). These specific interactions are especially important for stable TFIID association with promoters lacking a TATA box. However, through a variety of other activities, TAFs can modulate TFIID promoter association without direct contacts with the DNA.

In early studies using reconstituted in vitro systems, it was observed that TFIID supported activated transcription, whereas TBP did not (14). Subsequent

experiments demonstrated that addition of TAFs to TBP restored transcription activation (88, 102, 119, 121, 142, 143). This suggested that one or more TAFs might function as coactivators of transcription. Since then, a number of TAF-activator interactions have been identified suggesting that certain activators target specific TAFs to couple activator mediated signals to the transcription machinery (reviewed in 14 and 127). Moreover, in vitro studies in *Drosophila* indicated that multiple TAF-activator interactions promote the cooperative binding of TFIID to the promoter, leading to synergistic activation (111).

The transcriptional requirements for TAFs assessed in early experiments used in vitro transcription systems with highly purified components. The discovery of yeast TAFs provided the opportunity to examine the role of TAFs in living cells. Although TAFs are essential for cell viability in yeast, global defects in transcription upon TAF inactivation were not observed (3, 86, 113, 131). In addition, in vitro studies indicate that transcription activation can occur in mammalian cellular extracts devoid of TAFs (95). These studies suggest there is functional redundancy between TAFs and other components of the transcription machinery. Recent studies using in vivo chromatin crosslinking assays determined that promoters exhibit differential requirements for TAFs. Some promoters require only TBP for function (TAF independent), whereas other promoters require both TBP and TAFs (TAF dependent) (61, 72). Moreover, differential TAF recruitment to these distinct classes of promoters correlates with their transcription requirement (72), suggesting promoter specific requirements for certain TAFs. Promoter specific functions are also demonstrated by TAF1,

which is required for the expression of genes involved in cell cycle progression in yeast and human systems (46, 132, 133). Furthermore, TAF1 dependence was mapped to sequences that surround the promoter in yeast (113).

Specific enzymatic activities have been associated with TAFs. Human TAF1 possesses histone acetyltransferase (HAT) activity (85) and a subset of TAFs are associated with the HAT complexes, Spt-Ada-Gcn5-acetyltransferase (SAGA) in yeast (40) and p300/CBP associated factor (PCAF) in human cells (96). HAT complexes have the capacity to acetylate and neutralize the positively charged lysine residues within the N-terminal tails of histones. This results in chromatin decondensation and facilitates the binding of TBP to the promoter. In yeast, the presence of TAF5, TAF6, TAF9, TAF10, and TAF12 in SAGA implicates a mechanistic connection between the general transcription machinery and chromatin modifying activities. The function of TAFs in SAGA is unknown, but has substantially complicated our interpretation of TAF function, since any particular activity could be exerted in the context of TFIID or SAGA.

TAFs can also act as repressors of transcription and inhibit the formation of transcription complexes. While yeast TAF1 is required for transcription activation of certain genes, in vitro, the N-terminal domain dissociates TBP from the DNA (8, 58). A similar function has been described for the N-terminal domain of *Drosophila* TAF1, which binds to the concave DNA binding surface of TBP and competes with DNA for TBP binding (75).

1.6 TFIID structure

Cross species sequence comparisons reveal strong conservation among TAFs and subsequently aided the identification of these TFIID subunits in yeast, mammals, *Drosophila* and *C. elegans* (33, 110, 130). As such, a unified nomenclature has recently been adopted to aid in cross species comparisons and to clarify the relationships between TAF homologues (Table 1.1) (122). Yeast TFIID is comprised of fourteen TAFs and nearly all are essential for cell viability.

The initial characterization of TAFs revealed remarkable sequence similarity to core histones H3, H4, and H2B with homologous amino acid sequences coincident with a histone fold domain (HFD) (45, 57). In yeast, nine TAFs contain a HFD and five histone-like TAF-TAF interactions have been identified through a combination of structural, biochemical and genetic interaction studies in yeast, humans, and *Drosophila* (71, 109, 112, 134, 137). The predominance of histone-like TAF-TAF interactions led to the proposal that a nucleosome-like octamer structure could be an architectural element in the organization of TFIID (33, 45, 112).

Recently, the molecular organization of TFIID has been examined and three-dimensional structures of yeast and human TFIID have been determined by electron microscopy and image analysis. These images reveal a tri-lobed clamp like structure and immunolabeling experiments have localized TBP, TFIIA, TFIIB, and specific TAF subunits to regions within this structure (2, 10, 71). As

TABLE 1.1

New Pol II TAF Nomenclature

New Name[1]	Yeast	Human	Drosophila	Characteristics[2]
TAF1	TAF145	TAF250	TAF230	Involved in promoter binding, G1/S progression, HAT, kinase (human)
TAF2	TAF150	TAF150	TAF150	Involved in promoter binding; G2/M arrest (yeast)
TAF3	TAF47	TAF140	TAF155	Interacts with TAF10 via histone fold domain (yeast)
TAF4	TAF48	TAF130	TAF110	Involved in interaction with activators
TAF5	TAF90	TAF100	TAF80	G2/M arrest (yeast) Present in SAGA
TAF6	TAF60	TAF80	TAF60	Similar to histone H4; Present in SAGA Binds DPE (Drosophila)
TAF7	TAF67	TAF55		Interacts with activators and TAF1 to inhibit HAT activity
TAF8	TAF65	BAB71460	Prodos	None known
TAF9	TAF17	TAF32	TAF40	Similar to histone H3; Present in SAGA Interacts with p53
TAF10	TAF25	TAF30	TAF24	G1/S arrest (human) Present in SAGA
TAF11	TAF40	TAF28	TAF30β	Similar to histone H3; atypical histone fold similar to Spt3
TAF12	TAF61	TAF20	TAF30α	Similar to histone H2A Present in SAGA
TAF13	TAF19	TAF18		Similar to histone H4; atypical histone fold similar to Spt3
TAF14	TAF30			Shared with TFIIF Non homologue in metazoans
TAF15		TAF68		Binds RNA and ss DNA

[1] New Pol II TAF nomenclature including the corresponding known homologues(Tora et al Genes Dev 2002)
[2] Green, M.R. Trends Biochem. Sci. 2000 and Lee et al, Annu. Rev. Genetics 2000

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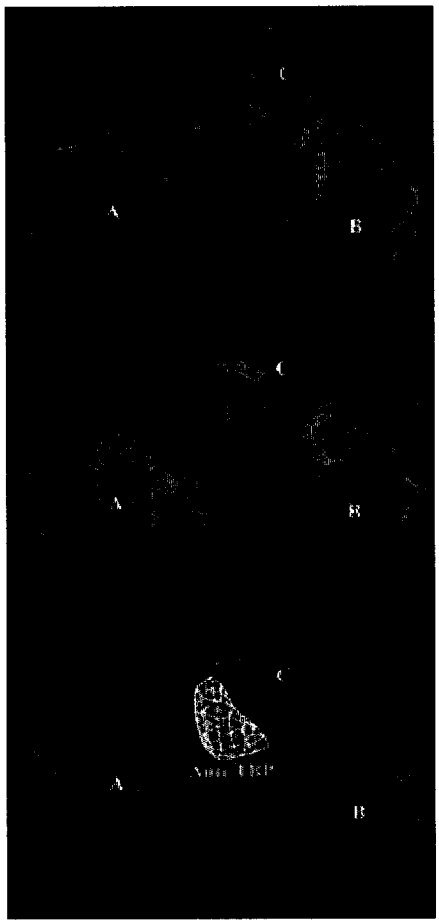

Andel et al, Science 1999

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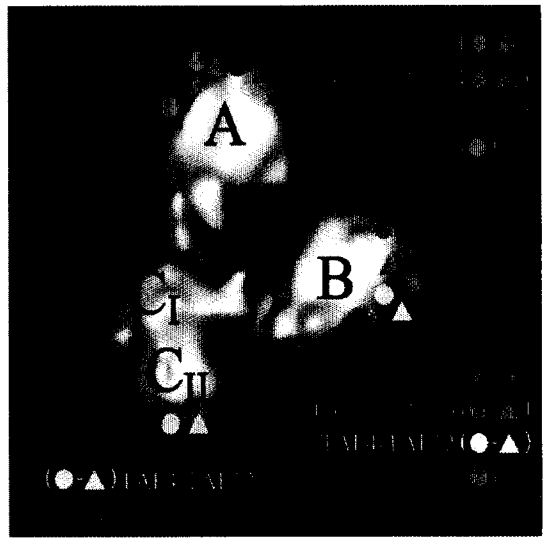


Leurent et al, EMBO J 2002

Figure 1.2 Three dimensional structure of human (A) and yeast (B) TFIID determined by electron microscopy and image analysis.
(A) Tri-lobed human TFIID structure represented by blue mesh with A, B, and C lobes as indicated. The positions and mapping of TFIIB and TFIIA were determined by electron density differences between holo-TFIID and the corresponding complexes. TFIIB is indicated by green mesh and TFIIA by magenta mesh. The postion for TBP was determined using antibodies to TBP and is indicated by yellow mesh. (B) 3-D yeast TFIID structure represented in yellow with lobes A, B, C_I and C_{II} as indicated. The position and mapping of TAFs was determined by immunolabeling experiments using yeast TAF antibodies. The location of each HFD-containing yeast TAF pair is shown in a different colour, and each partner of a pair is identified by a circle or a triangle as indicated.

one might expect, TBP is located in proximity to the central cavity supporting the hypothesis that this is the major site for DNA binding. TFIIA and TFIIB are located in opposing lobes of the structure and the nine histone-like TAF are present in distinct regions within the three lobes. Some TAF pairs are located in two distinct sites, suggesting they may be present in more than one copy in yeast TFIID; however, the exact stoichiometry of TAF subunits within TFIID has not been consistently reported. These and other structural determinations provide the context for understanding the functional interactions between TFIID and other transcription factors.

1.7 TAF11 function

Unlike certain TAFs, which are shared between TFIID and SAGA complexes, TAF11 appears to be a component specific to TFIID (40, 59, 89). The interaction between TAF11 and TAF13 has been defined by structural (9), biochemical (81), and genetic (59, 138) data. This suggests that the TAF11/TAF13 heterodimer is the biologically relevant form of these proteins. TAF11 uses a histone-like domain to interact with TAF13 (9). In contrast to other TAF heterodimers, which share sequence similarity to histone proteins, the conserved regions corresponding to the histone folds in both TAF11 and TAF13 are similar to SPT3 (a component of the SAGA complex) family of histone fold motifs (9). In addition to the histone-like interaction with TAF13, TAF11 interacts with TAF6, TAF10, and TAF 12 (138), indicating that TAF11 mediates important

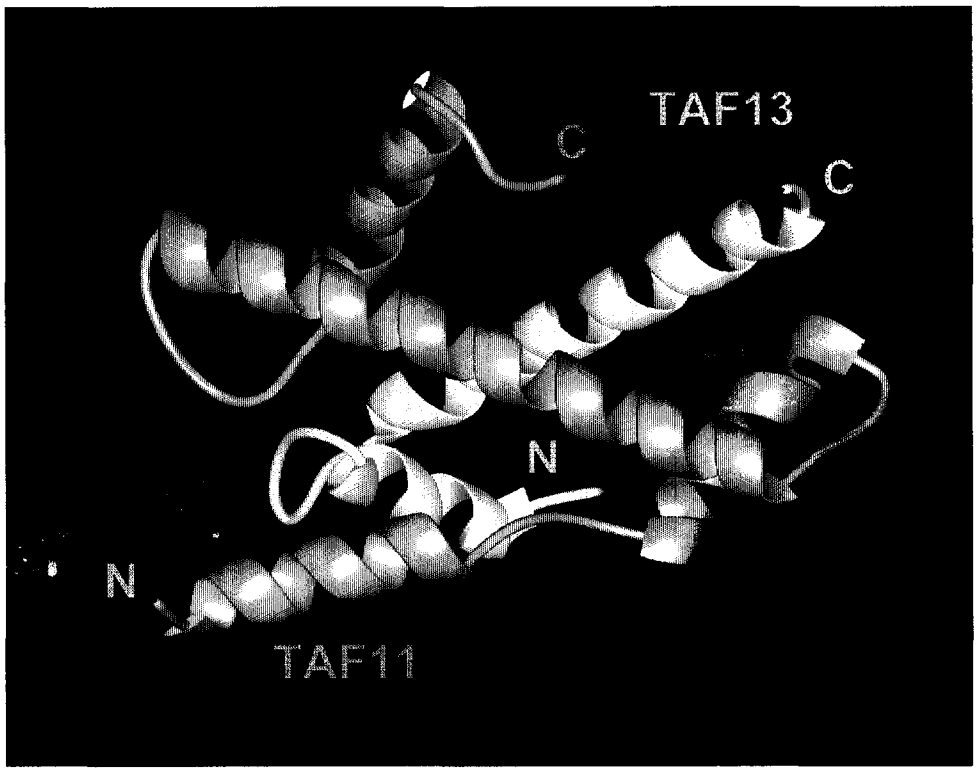


Figure 1.3 Human TAF11-TAF13 co-crystal structure. Shown is a ribbon diagram of the crystal structure of TAF11 (aa.113-201) in gray and TAF13 (aa. 14-74) in yellow. Helices comprising the TAF11 histone fold domain, αN, α1, α2, and α3 are labeled in red.

contacts within TFIID. Interestingly, TAF6, TAF10, and TAF12 are also present in the SAGA complex and it is possible these TAF interactions might facilitate recruitment of the SAGA complex to the promoters. In addition, our studies also show that TAF11 interacts directly with TBP and TFIIA (60). It is not known whether TAF11 uses unique or overlapping regions to mediate these multiple interactions.

TAF11 must perform at least one essential non-redundant function in transcription since it is required for cell viability (54). Transcription analysis in yeast using conditional TAF11 alleles indicates that TAF11 inactivation causes diminished transcription for a number of RNA polymerase II transcribed genes, suggesting a broad transcriptional requirement for TAF11 function (59). In addition, inactivation of TAF11 or TAF13 preferentially affects transcription derived from non-consensus promoters suggesting a mechanistic requirement for these TAFs in transcription of genes lacking consensus TATA elements (70, 86, 87). Transcription defects have also been observed when TAF11 interaction with TFIIA has been disrupted by mutations in TFIIA (60). Studies in human systems reveal that TAF11 can act as a coactivator for viral and nuclear receptor mediated transcriptional activity (15). Moreover, mutation analysis implicates the importance of specific residues within the HFD of TAF11 for synergistic activation, suggesting interactions via this domain are important for this function (66).

1.8 The general transcription factor TFIIA

Productive associations between TFIID and promoters can be also be aided by interactions with the general transcription factor TFIIA. Yeast TFIIA is comprised of two subunits encoded by the essential genes, *TOA1* and *TOA2* (106, 107). The Toa1 and Toa2 protein subunits interact forming two domains, a six stranded beta domain and a four-helix bundle domain (4HB) (37, 120). Mutation analysis determined that residues within the beta domain of TFIIA facilitate interaction with TBP (52). The four-helix bundle domain, however, projects away from the TBP-DNA complex, providing a surface for protein-protein interaction. The utilization of this domain for mediating protein interactions was first indicated by the observation that mutations in specific residues within the four helix bundle do not affect TFIIA-TBP interactions but result in conditional phenotypes and defects in transcription (52, 98). Furthermore, the work described in Chapter 2 demonstrates that the four-helix bundle domain is able to activate transcription in vivo, when artificially recruited to a promoter via a heterologous DNA binding domain (115). In accord with this evidence, our studies localized an interaction with TAF11 to this region (60). Moreover, we observed that TFIIA mutants, with substitutions at 4HB domain residues, exhibit defects in transcription in vivo, yet remain functional for TBP and Toa1-Toa2 interaction in vitro (60).

TFIIA interacts with TBP and promoter DNA upstream of the TATA element to form the TFIIA-TBP-DNA complex (37, 120). The interactions with TBP and DNA stabilize and increase the affinity of TBP for the TATA element

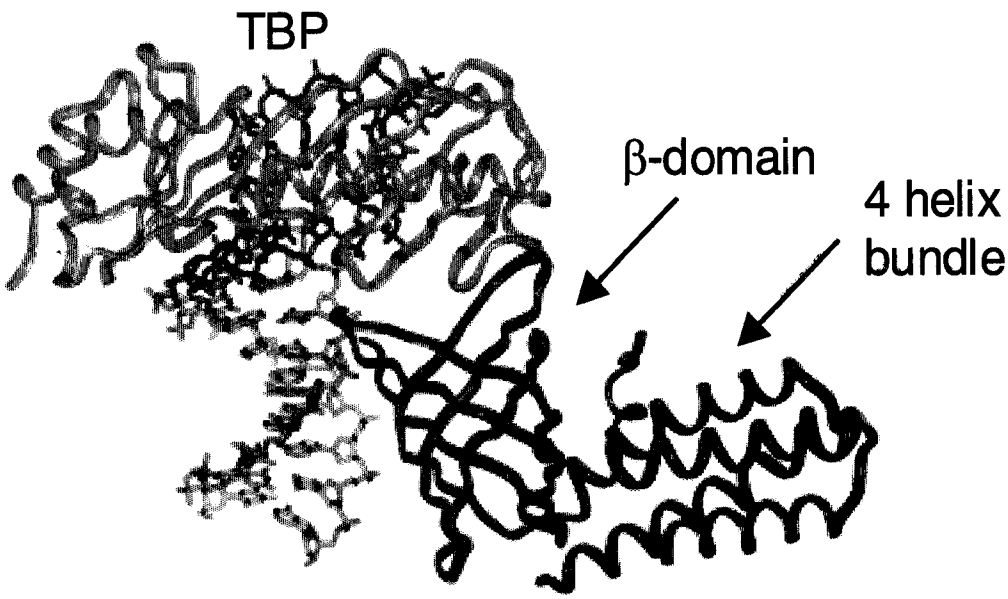


Figure 1.4 Structure of the TFIIA-TBP-DNA complex (10, 32). Shown are TBP (gold ribbon), Toa1 (blue ribbon), Toa2 (red ribbon), and DNA (black ball and stick). The ternary complex viewed from the upstream region of DNA, with the TATA element perpendicular to the plane of the paper. The two structural domains of TFIIA, the β-domain and the 4 helix bundle, are indicated. (Figures were created using Insight II.)

(47). The importance of the TFIIA-TBP interaction for transcription has been demonstrated by a number of studies. For example, over expression of TFIIA can suppress conditional phenotypes of TBP mutants with defects in DNA binding (76). TBP mutants defective for interaction with TFIIA exhibit an impaired response to acidic activators, and conditional phenotypes can be suppressed by fusing the Toa2 subunit of TFIIA to TBP (116). In addition, TFIIA can act as an anti-repressor, by inhibiting the ability of certain repressors to dissociate TBP from the promoter (5, 6, 39, 83) or counteracting repressors that inhibit subsequent steps in PIC assembly (36, 49, 83).

1.9 The functions of TFIIA and TFIID are linked

In addition to direct interaction with TBP, there are a number of studies that link the functions of TFIIA and TFIID in a broader context. TFIIA is required to stimulate transcription in reaction mixtures containing TFIID (25, 100, 118, 140) and TFIID association with promoters is TFIIA dependent (109). TFIIA has been described as a coactivator, since the function of certain activators is TFIIA dependent. Through interactions with activators, TFIIA facilitates rapid recruitment and stable formation of the TFIIA-TFIID-DNA complex (24, 56, 74). Moreover, studies show that simple recruitment of TFIID is not sufficient to activate transcription, but activator induced rearrangement of the TFIID-TFIIA-DNA complex on the promoter is required for TFIID to be a target for the remainder of the transcription machinery (19, 30).

As a coactivator, TFIIA acts as a molecular bridge between activators and the transcription machinery. The dissemination of regulatory signals from activators to TFIID is likely achieved through the dynamic interplay between TFIIA and TAFs. The purpose of these cooperative interactions is to regulate the binding of TFIID to promoter DNA. As such, direct interactions between TFIIA and TAFs have been identified (42, 60, 139). In vitro studies of human TFIIA and TAF4 demonstrate that cooperative interaction between TFIIA and TAF4 can counteract TAF1 mediated repression of TBP binding to the promoter (42). Although a direct interaction between TFIIA and TAF4 has not been reported in yeast, TFIIA blocks the inhibition of TBP binding to the TATA element by competing with the N-terminal region for TAF1 for overlapping binding sites on TBP (7, 58).

Our studies identified a direct interaction between TFIIA and TAF11 (60). In addition, we determined that through interaction with TFIIA, TAF11 enhances TFIIA-TBP-DNA complex formation. Using mutation analysis, the importance of these associations is further demonstrated by TFIIA mutants that are defective for interaction with TAF11. These mutants exhibit conditional growth phenotypes and defects in constitutive and activated transcription (60). In addition, cells expressing TFIIA derivatives with mutations that disrupt both TAF11 and TBP interactions are not viable, indicating the interplay between these proteins is critical for transcription initiation (60).

1.10 Statement of dissertation research significance.

Our previous studies determined that TFIIA interacts directly and specifically with TAF11. For my dissertation research, I have extended the characterization of this association by defining the required interaction surfaces for both proteins. In addition, I have identified a mechanistic role for this association in RNA polymerase II transcription initiation.

The study in Chapter 2 determined that the four-helix bundle domain of TFIIA provides a surface for mediating protein interactions required for transcription. Subsequent to these findings, others in our lab determined that mutations at residues within the hydrophobic surface of the four helix bundle domain disrupt the interaction between TFIIA and TAF11, implicating the involvement of this surface for interaction with TAF11 (60).

Chapter 3 describes several approaches used to extend the characterization of the interaction between TAF11 and TFIIA. In our earlier study, we observed that TAF11 interaction with TFIIA stimulates formation of the TFIIA-TBP-DNA complexes measured by electrophoretic mobility assays. To further examine the nature of this enhancement, I collaborated with Anka Bric in the laboratory of Dr. Marvin Paule to perform protein-DNA photocrosslinking studies. These studies reveal that TAF11-TFIIA-TBP-DNA complex enhancement was not a result of direct interactions between TAF11 and promoter DNA but rather through protein-protein interactions.

To define TAF11 intramolecular contacts between TFIIA and TAF11, I employed an allele of Toa2, toa2^{I27K}, which is defective for interaction TAF11, in

a genetic screen to isolate TAF11 compensatory mutants. Isolation and characterization of compensatory mutants revealed that two surfaces of TAF11 are required for interaction with TFIIA.

The genetic selection I used in this study led to the identification of TAF11 alleles defective for interaction with TFIIA. Phenotypic and transcription characterization of TAF11 alleles revealed the importance of the TAF11-TFIIA interaction for transcription in vivo and determined that TAF11 stabilizes TFIIA-TBP-DNA complexes formed on promoters.

In summary, this research has identified the interaction determinants required for the TFIIA-TAF11 interaction. Integration of this data with previous structural determinations forms a solid foundation for understanding the molecular organization of interaction between TFIIA and TFIID. In addition, this study indicates that the TFIIA-TAF11 interaction provides critical functions for transcription initiation by enhancing the formation of transcription complexes.

CHAPTER 2

TRANSCRIPTIONAL ACTIVITY OF THE TFIIA FOUR-HELIX BUNDLE IN VIVO

This chapter was published in the journal *Proteins*. The text of the manuscript is presented exactly as it appeared in the journal. All the figures that appeared in the manuscript are included. This work was produced in collaboration with the laboratory of Dr. Kevin Lumb with Joshua Adkins. Ryan Ogg, a former graduate student designed the four-helix bundle expression constructs and performed the initial in vivo characterization shown in figure 2.3. I expressed and purified recombinant four-helix bundle protein used for biophysical determinations and performed yeast artificial recruitment and two-hybrid assays shown in figure 2.4. Drs. Stargell and Lumb wrote the manuscript. The literature citation for this chapter is:

Stargell, L.A., Ogg, R.C., Adkins, J.N., Robinson, M.M. and K.J. Lumb, 2001, Transcriptional Activity of the TFIIA Four-Helix Bundle in Vivo. Proteins, 43:227-232.

2.1 Abstract

TFIIA contributes to transcription initiation by stabilizing the TBP-TATA interaction and by mediating the response to transcriptional activators and inhibitors. TFIIA contains a six-stranded β-sheet domain and a four-helix bundle. The β domain makes functional contacts with DNA and TBP. The role of the four-helix bundle was investigated using a structure-based model of this domain (called 4HB). 4HB adopts a highly stable, helical fold, consistent with its structure in the context of TFIIA. Like TBP and other intact transcription factors, 4HB is able to activate transcription in vivo when artificially recruited to a promoter via a heterologous DNA binding domain. Thus, in addition to making important contacts with TBP and DNA via the β domain, TFIIA makes other specific, functional contacts with the transcriptional machinery via the four-helix bundle.

2.2 Introduction

Initiation of transcription on a TATA-containing RNA polymerase II promoter is a complex process, involving a large number of interactions between multiple factors. These factors include TFIID, comprising the TATA-binding protein (TBP) and TBP-associated factors, TFIIA, TFIIB, TFIIE, TFIIF, TFIIH and RNA polymerase II (reviewed in 97). An important step in initiation-complex assembly is the recognition of the TATA element by TBP and the formation of a stable TBP-TATA complex. TFIIA binds both TBP and DNA directly and at least

part of the functional role of TFIIA is related to stabilization of the TBP-DNA interaction (12, 23, 56, 74, 100, 118, 140). TFIIA has also been implicated in mediating the response to transcriptional activators (12, 23, 56, 74, 100, 118, 140), and in relieving the inhibitory effects of transcriptional repressors (5, 49, 58, 75, 79, 80, 82).

TFIIA is highly conserved among eukaryotes both in primary sequence and function (26, 100, 140). Yeast TFIIA is a heterodimer of two proteins encoded by the essential genes, *TOA1* and *TOA2* (107). The crystal structure of the yeast TBP-TFIIA-DNA complex (37, 120) reveals that the Toa1 and Toa2 subunits of TFIIA are tightly associated and contribute equally to a six-stranded β-sheet domain and a left-handed four-helix bundle (Fig 2.1). The β-domain of TFIIA makes all of the TBP and DNA contacts (37, 120). In contrast, the four-helix bundle domain of TFIIA projects away from the remainder of the complex in the crystal structure, and does not contact TBP or DNA.

To investigate the role of the four-helix bundle of TFIIA, a fusion protein (called 4HB) corresponding to the four-helix bundle, but not the β-sheet domain, was characterized. Spectroscopic analyses establish that the 4HB folds as a stable, helical structure. In vivo studies demonstrate that 4HB is sufficient for recruiting the remainder of the initiation complex when fused to a DNA-binding domain. We conclude that interactions with TFIIA involving the four-helix bundle, in addition to those observed with TBP and DNA via the β-domain, play an important functional role in transcription initiation.

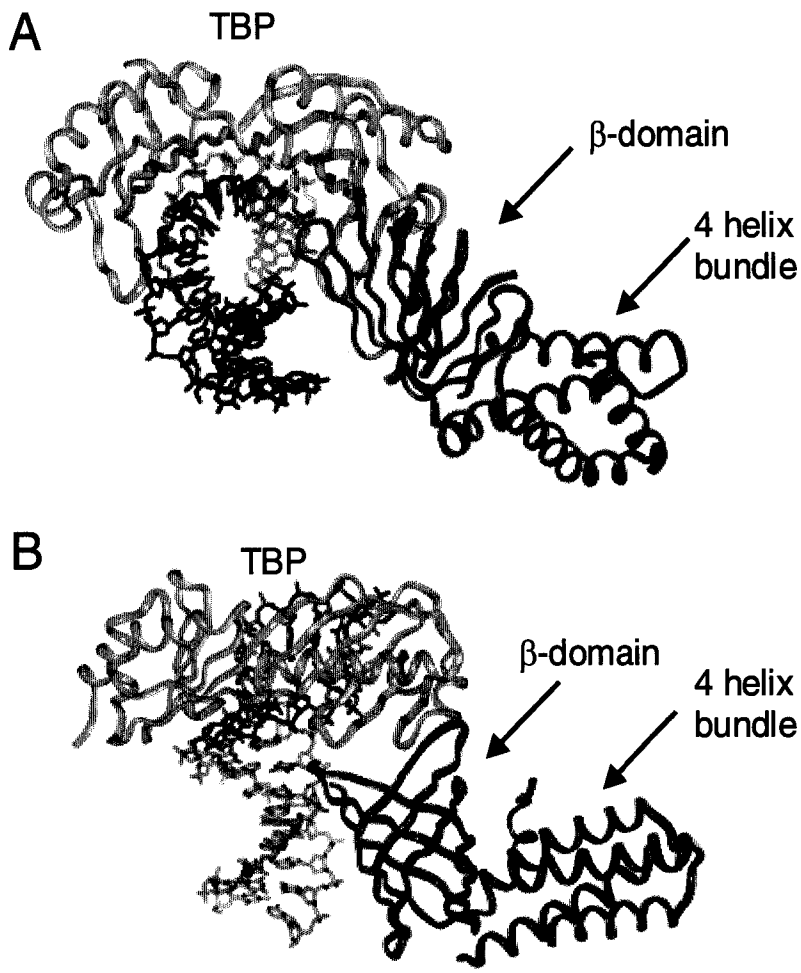


Figure 2.1 Structure of the TFIIA-TBP-DNA complex (10, 32). Shown are TBP (gold ribbon), Toa1 (blue ribbon), Toa2 (red ribbon), and DNA (black ball and stick). (A) The ternary complex viewed from the upstream region of DNA, with the TATA element perpendicular to the plane of the paper. The two structural domains of TFIIA, the β-domain and the 4 helix bundle, are indicated. (B) Image in A rotated 90 degrees out of the plane of the paper.

2.3 Materials and Methods

2.3a Expression and purification of 4HB

4HB was expressed as a GST-fusion protein. An expression vector encoding GST-4HB (4HB with a N-terminal GST-tag) was constructed by ligating PCR products encoding the first 177 base pairs of Toa2, 9 base pairs encoding a Gly linker and the first 285 base pairs of Toa1 (i.e, Toa2-Gly-Gly-Gly-Toa1) into pGEX-2T (Pharmacia). GST-4HB was expressed in *Escherichia coli* strain DH5α, purified using Glutathione Sepharose 4B (Pharmacia) per the manufacturer's protocol and dialyzed into 10 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM DTT, pH 8.5. The GST tag was cleaved with thrombin (Novagen). Final purification of 4HB was by reversed-phase C_{18} high-performance liquid chromatography using a linear water/acetonitrile gradient containing 0.1% trifluoroacetic acid. The identity of 4HB was confirmed with MALDI mass spectrometry (expected mass 18412 Da; observed 18413 Da).

2.3b Circular dichroism spectroscopy

CD spectra were collected with a Jasco J720 spectrometer. Samples contained 10 μM 4HB in 10 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7.0. 4HB concentration was determined by absorbance in 6 M GuHCl assuming an extinction coefficient at 276 nm of 18,050 M^{-1} cm^{-1} (29). GuHCl concentration was determined by refractometry (101). Helix content was estimated from $[\theta]_{222}$ using the equation described by (18).

2.3c Analytical Ultracentrifugation

Sedimentation equilibrium experiments were performed with a Beckman XL-I. Data were collected at 25^0C and 276 nm at rotor speeds of 30 and 40 krpm on samples of total 4HB concentration of approximately 14, 29, and 44nM. Samples were dialyzed against the reference buffer (10 mM sodium phosphate, 150 nM NaCl, 1 mM DTT, pH 7.0). A solvent density of 1.01 g ml^{-1} and a parila molar volume of 0.7216 g ml^{-1} was calculated as described elsewhere (65). Data sets were fit to various models (ie, single species, monomer-dimer, monomer-trimer, monomer-dimer-tetramer, and monomer-tetramer), using ORIGIN (as provided by Beckman Instruments). Discrimination between the various models was based on the distribution of residuals and the variance.

2.3d Artificial recruitment and two-hybrid assays

Activation domain (AD) hybrids were cloned into the 2μ *LEU2* vector pACT2.2 (28), 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-*TRP1* vector (129), which is CEN and *TRP1*-marked, and contains the *ADH1* promoter, a nuclear localization sequence, and the Gal4 DNA-binding domain (residues 1-147). Strains for the artificial recruitment assays were created by standard lithium acetate transformation of the appropriate plasmids into yeast strain MaV103 (129). MaV103 contains the *GAL1* promoter (with four Gal4 binding sites) fused to the

HIS3 TATA element and structural gene; *GAL4* and *GAL80* are both deleted in the strain. Strains for the yeast two-hybrid assays were created by lithium acetate transformation of the Gal4 DB and Gal4 AD plasmids into the yeast strain CG1945 (Clontech). CG1945 contains the *GAL1* promoter and the *GAL1* TATA element fused to the *HIS3* structural gene; *GAL4* and *GAL80* are both deleted in the strain. The resulting strains were diluted and spotted onto appropriate plates that either contain or lack 3-aminotriazole (AT). Cells were grown at 30 ^{0}C for 3-7days.

2.4 Results and Discussion

2.4a Structure-based excision of the TFIIA four-helix bundle

The four-helix bundle of TFIIA in the TFIIA-TBP-DNA crystal projects away from the TBP-DNA binding surface, and does not make intermolecular contacts with TBP or DNA (Fig. 2.1). These suggest that the four-helix bundle of TFIIA could be excised from the remainder of TFIIA and retain the ability to fold. A protein model of the four-helix bundle (called 4HB) was designed by fusing the first 59 amino acids of Toa2 with a flexible triglycine linker to the first 95 amino acids of Toa1. 4HB corresponds to residues 1-59 of Toa2, followed by three Gly residues, followed by residues 1-95 of Toa1, with GlySerProSerAsnSerCysSer at the N-terminus derived from the thrombin cleavage of a GST tag.

2.4b Autonomous folding of the TFIIA four-helix bundle

Circular dichroism (CD) spectroscopy was used to characterize the secondary structure and stability of 4HB. The CD spectrum of 4HB contains double minima at 208 and 222 nm and a positive band at 190 nm (Fig. 2.2A), indicating that 4HB is highly helical (135). The helix content at 0 °C is 37 %, estimated from $[\theta]_{222}$ (18). The helix content corresponds to 61 residues, which compares well with 58 residues observed in helices in the crystal structure of TFIIA (37, 120). The midpoint of thermal denaturation (T_m) of 4HB exceeds 80 °C at pH 7, and is approximately 70 °C in 2 M GuHCl, pH 7.0 (Fig. 2.2B). The data indicate that 4HB folds independently of the remainder of TFIIA to form a thermostable, helical structure.

2.4c The TFIIA four-helix bundle is sufficient to recruit the transcriptional machinery.

4HB was characterized for functional activity in vivo. If the four-helix bundle of TFIIA is in contact with the initiation complex, then 4HB should be capable of recruiting the rest of the transcriptional machinery to a promoter. This hypothesis was tested by fusing 4HB to a Gal4-binding domain and determining whether transcription was observed from a test promoter containing the Gal4 DNA-binding site. This type of artificial recruitment assay has been used to demonstrate that many other members of the initiation complex can recruit a functional Pol II transcription complex (reviewed in 103). The assay described

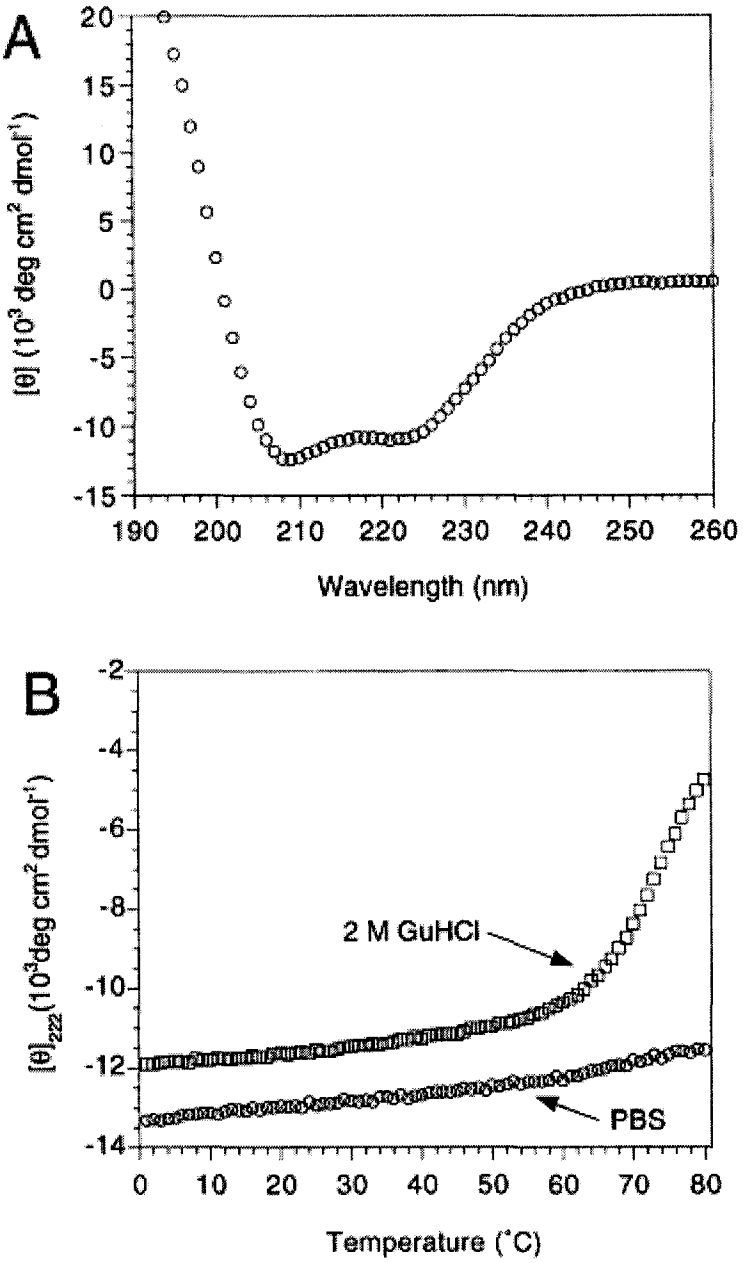


Figure 2.2 4HB folds as a stable, helical monomer. (A) CD spectrum of 4HB (10 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7.0, 0 °C). The minima at 208 and 222 nm indicate that 4HB adopts a helical structure. (B) Temperature dependence of $[\theta]_{222}$ of 4HB (10 mM). The T_m of 4HB exceeds 80 °C in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7.0) and is approximately 70 °C in PBS containing 2 M GuHCl. The change in the folded baseline in the presence of GuHCl is reproducible. Thermal unfolding of 4HB is reversible, with >95% of the starting signal regained on cooling.

here is the first application of artificial recruitment to examine the functional activity of a domain of a transcription factor comprised of two subunits.

4HB was fused to the Gal4 DNA-binding domain (DB; residues 1-147 of Gal4), which confers DNA-binding specificity at a Gal4 promoter controlling expression of the *HIS3* gene. Yeast cells are only viable on 3-aminotriazole (AT) if the *HIS3* gene is transcribed. A strain expressing just the DB domain fails to grow on AT as expected (Fig. 2.3). Recruitment of TBP to the test promoter via fusion with the DB (DB-TBP) results in high levels of transcription of the *HIS3* gene and robust growth on AT, in accord with previous results (17, 53, 136). Yeast transformed with the DB-4HB hybrid also grow robustly on AT, indicating that the 4HB domain of TFIIA is sufficient for recruiting the initiation complex.

An early report on the biochemical characterization of TFIIA suggested that TFIIA might form dimers (106). Since artificial recruitment of TFIIA, via the fusion protein DB-Toa1, results in transcriptional activity from the reporter gene (114), a possible mechanism for transcriptional activation by 4HB is that 4HB recruits endogenous TFIIA via formation of 4HB-TFIIA oligomers. To test whether 4HB interacts with TFIIA in vivo we used a yeast two-hybrid assay. Although DB-4HB activates transcription in the yeast strain MaV103, it is well established that activity in the artificial recruitment assay is sensitive to promoter structure and architecture (34, 93). Thus, we utilized a second strain (CG1945; Clontech) in which expression of DB-4HB fails to activate transcription (Fig. 2.4). Activation-domain fusions to 4HB, Toa1 and Toa2 were then tested for

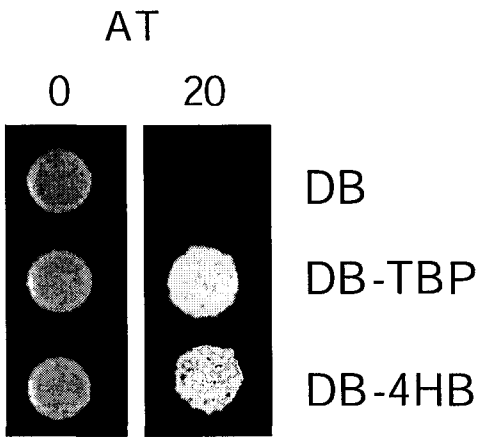


Figure 2.3 The four-helix bundle of TFIIA is sufficient for recruitment of theinitiation complex in vivo. Yeast strain MaV103 was transformed with either the Gal4 DNA binding domain (DB), DB-TBP or DB-4HB. Cells (10^3) were spotted onto media lacking (0) or containing 20 mM aminotriazole (AT) and incubated at 30 °C. All strains grow in the absence of AT. Since AT is a competitive inhibitor of the *HIS3* gene product, the ability to grow robustly on AT requires high levelsof expression of the *HIS3* gene. Thus, growth on AT indicates the hybrid is competent for artificial recruitment of the transcription machinery.

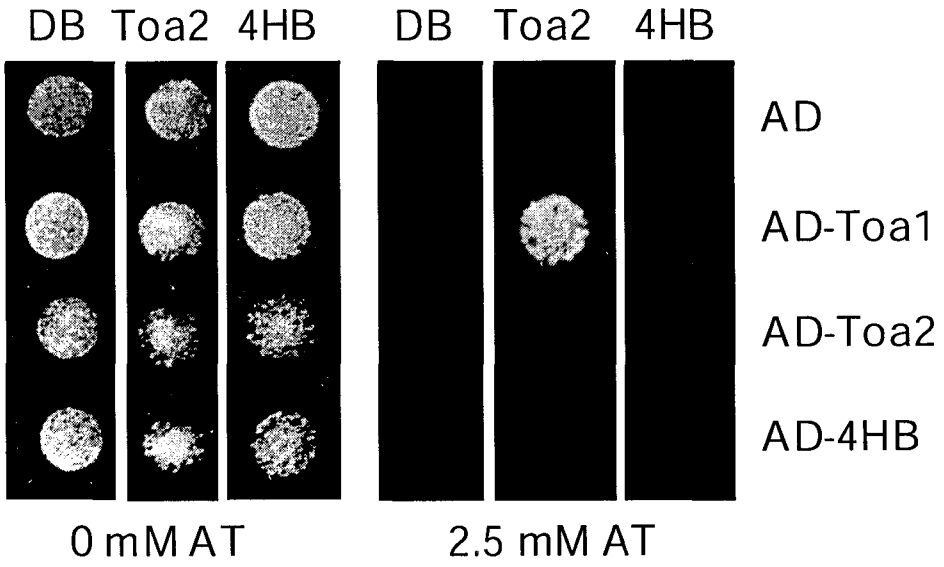


Figure 2.4 The four-helix bundle of TFIIA does not associate with itself or the individual subunits of TFIIA in vivo. The indicated Gal4 activation domain (AD) fusion proteins were tested for their ability to interact with DNA binding domain fusions to Toa 2 (DB-Toa2) or 4HB (DB-4HB) using two-hybrid analysis in yeast strain CG1945. Cells (10^4) were spotted onto media lacking or containing 2.5 mM AT and incubated at 30 °C. All strains grow in the absence of AT. Growth on AT is indicative of interaction between the hybrid proteins.

interaction with DB-4HB. Positive interactions were detected by activation of the *HIS3* gene, which is indicated by growth on AT. As expected, an interaction between DB-Toa2 and AD-Toa1, the two subunits of TFIIA, is easily observed by robust growth on AT (Fig. 2.4). In contrast, no interaction is detected for DB-4HB with either AD-4HB, AD-Toa1 or AD-Toa2. These results indicate that 4HB does not mediate transcription activation by nucleating the oligomerization of TFIIA. We conclude that, in addition to making important contacts with TBP and DNA via the β-domain, TFIIA also makes additional specific contacts with the transcriptional machinery through the four-helix bundle.

2.4d Implications for TFIIA function

Since the four-helix-bundle domain of TFIIA is not involved in interactions with either TBP or DNA, what is the function of the TFIIA four-helix-bundle? Four-helix-bundles are found in functionally diverse proteins such as transcription factors, cytochromes, lymphokines, enzymes and phage-coat proteins (44). Four-helix bundles are observed both independently and as components of larger folding units and they have the potential to mediate protein-protein interactions.

Although the α-helices of the TFIIA four-helix bundle domain cannot be deleted in vivo (52), all of the established interactions with TBP and DNA occur through the β-domain of TFIIA (37, 120). Thus, the four-helix bundle of TFIIA may be required solely for the dimerization of the Toa1 and Toa2 subunits of TFIIA. We demonstrate here, however, that the TFIIA four-helix bundle is

capable of recruiting the remainder of the RNA polymerase II transcription complex in vivo. Our results do not preclude a role of the four-helix bundle in the assembly of the Toa1-Toa2 heterodimer. Nonetheless, the four-helix bundle of TFIIA clearly has a functional role beyond oligomerization in polymerase II transcription initiation. Indeed, substitution of several residues in the four-helix bundle results in temperature-sensitive phenotypes and defects in activated and basal transcription in vitro, while having wild-type affinity for TBP and Toa1-Toa2 interactions (52). In addition, a deletion derivative of human TFIIA equivalent to removal of the Toa1 α–helices is unable to support activated transcription in vitro (78). We conclude that the 4HB domain of TFIIA is a functionally important target in transcriptional regulation that facilitates formation of an active transcription initiation complex.

2.6 Acknowledgments

We thank R. W. Woody for use of the CD spectrometer, and R.T. Ranallo for assistance with Figure 2.1. Supported by National Institutes of Health grant GM56884 (LAS).

CHAPTER 3

TAF11 INTERACTS WITH TFIIA VIA TWO DISTINCT DOMAINS TO PROMOTE STABLE ASSOCIATION OF TFIIA-TBP-DNA COMPLEXES

The work described in this chapter presents biochemical and genetic evidence that serves to extend the characterization of an interaction between TAF11 and TFIIA. This chapter is currently being modified for submission to the journal *Molecular and Cellular Biology*. DNA-photocrosslinking studies shown in figures 3.1B, 3.1C and 3.1D were performed in collaboration with Anka Bric and the laboratory of Dr. Marvin Paule. In addition, lab members Gayatri Yatherajam performed co-immunoprecipitation experiments shown in figure 3.7, and Aaron Borland assisted with two-hybrid assays used to characterize the TAF 11 compensatory mutant collection shown in Table 3.3. Ryan Ranallo constructed TAF11 deletion derivatives and performed the initial characterization shown in figure 3.6. The literature citation for this chapter upon acceptance to a professional journal will be:

Robinson, M.M., A. Bric, G. Yatherajam, R.T. Ranallo, A. Borland, M.R. Paule and L.A. Stargell. TAF11 interacts with TFIIA via two distinct domains to promote stable association of TFIIA-TBP-DNA complexes.

3.1 Abstract

TFIIA interacts with TFIID via association with TATA binding protein (TBP) and TAF11. The importance of the TFIIA-TBP interaction and its contributions to transcription has been well characterized. In addition, TAF11 provides a functional link between TFIIA and TFIID. In this study, we demonstrate that via protein interactions TAF11 imparts changes to TFIIA and TBP-DNA contacts. These alterations enhance the formation and stabilization of TFIIA-TBP-DNA complexes. To map the surfaces of TAF11 required for interaction with TFIIA, we employed the toa2^{I27K} allele, specifically defective for interaction with TAF11, in a genetic screen to isolate TAF11 compensatory mutants. Analysis of compensatory mutations revealed that the interaction with TFIIA requires two distinct regions of TAF11, the conserved histone fold domain (HFD) and the N-terminal region. Further evidence for the functional importance of the TAF11-TFIIA interaction is indicated by cells expressing TAF11 alleles defective for interaction with TFIIA. These alleles exhibit conditional growth phenotypes and defects in transcription. Taken together, this study provides insight into the molecular organization of the TAF11-TFIIA interaction and demonstrates a mechanistic role for this association in vivo.

3.2 Introduction

Transcription by RNA polymerase II requires the cooperative interaction of multiple proteins to facilitate the assembly of a preinitiation complex (PIC) comprised of Pol II and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH at the core promoter (reviewed in 43). An essential step in this highly regulated process is the recognition of the core promoter by the general transcription factor TFIID, a multiprotein complex comprised of TATA binding protein (TBP) and approximately 14 TBP associated factors (TAFs). TBP binds the TATA element through sequence specific contacts. TAFs facilitate TFIID affinity and specificity for core promoters through specific interactions with promoter sequences and other components of the transcription machinery. TAF-promoter interactions can compensate for absent or weak TBP-TATA interactions (13, 128). As coactivators TAFs stimulate general transcription factor recruitment through interactions with gene specific activators (14). As such, there is a strong correlation between TFIID association with promoters and transcription initiation. Thus, through a variety of interactions regulatory proteins and transcription factors modulate TFIID association with promoters by affecting recruitment and/or altering stabilization.

In addition to TFIID, general transcription factor TFIIA influences steps required for transcription initiation. TFIIA enhances PIC assembly by increasing the affinity of TBP for DNA (11, 48, 52, 67) and stabilizes the TBP-TATA association through direct interactions with both DNA and TBP (37, 120). Mutational studies of both TFIIA and TBP clearly demonstrate the importance of

the TFIIA-TBP interaction for transcription in vivo (52, 76, 99, 116). Aside from direct interaction with TBP, TFIIA influences TFIID promoter functions through associations with TAFs. Studies show that TFIID association with promoters is TFIIA dependent (109) and in higher eukaryotes, addition of TFIIA alters the DNA protection pattern of TFIID (19, 20, 74). UV crosslinking experiments show that addition of TFIIA induces a rearrangement within TFIID, enhancing additional DNA contacts downstream of the TATA element (94). These changes likely contribute to the overall affinity and stability of TFIID promoter associations. In addition, TFIIA can relieve TAF mediated repression by competing with the N-terminal region of TAF1 for TBP binding.

Our previous studies determined that TFIIA interacts directly and specifically with TAF11 (60). The importance of this interaction in vivo is demonstrated by the observation that TFIIA strains expressing mutations that disrupt interaction with TAF11 exhibit conditional growth phenotypes and defects in transcription (60). These studies also demonstrated that TAF11 interaction with TFIIA enhances TFIIA-TBP-DNA complex formation (60), suggesting this association is important for core promoter functions (60). The importance of TFIIA-TFIID interactions is further underscored by the observation that TFIIA mutations that disrupt interaction with both TAF11 and TBP are lethal (60). Clearly, the interplay between these proteins has critical functions that are required for transcription in vivo.

In this work, we present biochemical and genetic evidence that serves to extend the characterization of an interaction between TAF11 and TFIIA. Our

studies determine that interaction with TFIIA requires two distinct regions of TAF11. The structurally conserved HFD can mediate interaction with both TFIIA and TAF13. In addition to this region, we found that the N-terminal region of TAF11 is also required for interaction with TFIIA. We determined that the association between TAF11 and TFIIA is important for interactions at the core promoter. TAF11 imparts changes to TFIIA and TBP interactions with DNA, leading to stimulation of overall complex formation. Moreover, TFIIA-TBP interactions at weak or non-consensus TATA elements can be stabilized by TAF11. Finally, we determined that TAF11 interaction with TFIIA facilitates core promoter functions that are important for transcription. Taken together, this study provides insight into the molecular organization and the functional significance of TAF11 interaction with TFIIA in vivo.

3.3 Materials and Methods

3.3a Yeast strains

All *Saccharomyces cerevisiae* strains used in the yeast two-hybrid assays and for genetic selection of compensatory mutants and interaction studies were transformants of either MaV103 (129) or CG1945 (31). Both strains contain the *HIS3* reporter but in different promoter contexts. MaV103 contains the *GAL1* promoter, with four Gal4 binding sites fused to the *HIS3* TATA element. CG1945 contains the *GAL1* UAS_G and the *GAL1* TATA element fused to the *HIS3* structural gene. CG1945 cells exhibit tighter control of background levels of

expression from the *HIS3* gene. GAL4 and GAL80 are deleted in both strains. Viability testing, phenotypic characterization and transcription analysis of TAF11 mutant derivatives were conducted in YSB366 (relevant genotype *MAT**a**, ura3-52, leu2, his3 Δ200, taf11Δ::LEU2)* (59).

3.3b DNA constructs

Activation domain (AD) hybrids of TAF11 mutant derivatives were constructed using primers designed to incorporate changes and subcloned using in vivo recombination to the 2μ *LEU2* marked vector pACT2.2-*LEU2* (28). The AD plasmid contains the *ADH1* promoter, a nuclear localization sequence, the HA epitope and the Gal4 activation domain (AD) (residues 768-881). All PCR derived products were completely sequenced. The DNA-binding domain (DB) hybrid constructs used in this study, DB-toa2^{I27K}, DB-Toa2, and DB-TAF13, were constructed as previously described (60, 138) using the pPC97-*TRP1* vector (129), which contains the ADH1 promoter, a nuclear localization sequence, and the Gal4 DNA-binding domain (residues 1-147). TAF11-YCP22 constructs contain TAF11derivatives driven by the TAF11 native promoter and terminator generated by PCR from genomic DNA. An *EcoR1* site was engineered at the ATG start codon and utilized for inserting three myc epitopes (GEQKLISEEDLN), creating myc-TAF11-YCP22.

3.3c Site Specific Protein-DNA Crosslinking

Oligodeoxyribonucleotides containing a phosphorothiorate at the third nucleotide from the 5' end were derivatized with azidophenacyl bromide as described (64). Derivatized oligodeoxyribonucleotide primers were phosphorylated with [γ^{32}P]-ATP and annealed to a 76 bp complementary oligodeoxyribonucleotide encompassing the *HIS3* promoter. Double stranded templates were prepared by primer extension followed by ligation.

3.3d Isolation of TAF11 compensatory alleles

The TAF11 gene was randomly mutagenized by PCR-based misincorporation as described previously (84). Using the pACT2.2–TAF11 plasmid as the template, primers were used to amplify the 1050 bp of theTAF11 open reading frame. The amplified products contained approximately 50 bp of upstream and downstream sequence homologous to pACT2.2–LEU2 to allow for in vivo recombination. The mutant library was co-transformed with the NdeI-BamHI gapped AD vector into the yeast strain MaV103 expressing the Toa2 derivative, toa2^{I27K}, fused to the Gal4 DNA binding domain (DB- toa2^{I27K}). Transformants were plated to synthetic complete media lacking tryptophan and leucine. Compensatory alleles were selected by replica plating to synthetic media containing 20 and 40 mM 3-aminotriazole (AT). Plasmids from strains exhibiting growth on AT were recovered and retransformed into the strain expressing DB- toa2^{I27K} to confirm linkage of the plasmid to AT resistance.

3.3e Two-hybrid assays

Gal4 DB plasmids and Gal4 AD plasmids were transformed into yeast strains CG1945 or MaV103 using standard lithium acetate transformation. The resulting strains were spotted in 10-fold serial dilutions or streaked onto the appropriate selection media that either contained or lacked 3-aminotriazole (AT) and grown at 30^0 C for 4-7 days.

3.3f Co-immunopreciptiation assays

Co-immunoprecipitation experiments were essentially performed as described previously (32, 88) with a few modifications. The TAF11 deletion strain YSB366 was modified by integration of a PCR-derived HA tag to the C-terminus of the chromosomal copy of TAF1. The HA tag was amplified using the pFA6a – 3HA-TRP1 construct and corresponding primers as previously described (77). Modified TAF11 deletion strains expressing epitope-tagged TAF11 derivatives were grown in rich medium containing 2% glucose to optical density (600 nm) of approximately 1.0. Protein extracts were prepared immediately and precleared using 50 μl of protein A- sepharose beads (Pharmacia) for 1 hr at 4°C. Anti-TBP antibodies were coupled to protein A-sepharose beads, and protein extracts were incubated with 50 μl of antibody-coupled beads at room temperature for 2 hrs. Following six washes, the beads were boiled in loading buffer, and 15 μl was loaded for SDS-PAGE followed by immunoblot.

3.3g Transcription analyses

Quantitative S1 nuclease assays were conducted as described (50). Cells were cultured in rich media containing 2% dextrose at 30^0 C to mid log phase and harvested. When a temperature shift was involved, cells were heat shocked by incubation at 38^0 C for 15 min, then returned to 30^0 C for 1 hour and then incubated at 38^0 C for an additional hour. To activate transcription of the *HIS3* gene, AT was added to a final concentration of 20 mM, and cells were incubated additional hour. RNA was prepared by hot phenol extraction and quantitated spectrophotometrically at 260nm. Approximately 25-30 μg of RNA was hybridized with excess probe overnight at 55^0 C. S1 nuclease digestion of hybridized samples was performed at 37^0 C for 45 min. RNA levels were quantitated for each transcript by phosphoimaging and normalized to levels of $tRNA^w$, which are unaffected in TAF11 derivative strains.

3.3h Electrophoretic mobility shift assays

Binding reactions contained 0.1 pmoles of a 23 base pair ^{32}P-labeled fragment containing the sequence 5'AATTCC**CATAAA**GTAATGTGGAG 3', derived from the *HIS3* promoter as previously described (117) purified recombinant yeast TBP (30.7 nM), GST tagged TFIIA (3 nM) and TAF11/TAF13 (19 nM) and 100ng of poly [dGdC]. Reactions were incubated at 25^0C for 30 minutes in 20 μl of 20 mM Tris (pH 7.5), 40 mM HEPES, 100 mM KCl, 1mM DTT, 0.5mM phenylmethylsulfonyl fluoride and 10% glycerol. Complexes were separated on 5% acrylamide gel containing 50 mM Tris-borate, 1 mM EDTA and

quantified by phosphoimaging. For stability studies, TFIIA-TBP-DNA and TAF11/TAF13-TFIIA-TBP-DNA complexes were allowed to reach equilibrium for 30 minutes, then challenged with 1000-fold molar excess of the specific competitor poly [dAdT] for the specified amount of time. Samples were resolved on 5% acrylamide gels and complexes were examined by phosphoimaging.

3.4 Results

3.4a TAF11 imposes qualitative changes in TFIIA and TBP - DNA contacts through protein-protein interactions.

The protein interactions between TFIIA and TBP at the TATA element are critical for transcription initiation. Our previous studies show that TAF11 contributes to TFIIA and TBP associations at the promoter by specifically enhancing TFIIA-TBP-DNA complex formation (60). It is possible that the observed increase in complex formation could be due to direct interactions between TAF11 and promoter DNA or a result of changes to protein-DNA contacts. To examine the nature of TAF11 complex enhancement, we employed protein-DNA photocrosslinking assays, to map positions of TFIIA, TBP and TAF11 contact at specific sites within the yeast *HIS3* promoter. Our interests were two-fold: first, to determine if TAF11 interacts directly with DNA; and second, to determine whether TAF11 imposes changes to TBP-DNA and TFIIA-DNA contacts at specific sites within the *HIS3* promoter.

Twelve site-specific derivatized promoter fragments were constructed by incorporating azidophenacyl bromide (a photoactivatable crosslinking agent) and an adjacent radioactive label at single defined phosphates within the *HIS3* promoter (Fig 3.1A). We used recombinantly expressed TBP, TFIIA and the TAF11/TAF13 heterodimer to examine protein-DNA interactions. The TAF11/TAF13 heterodimer was used because structural (9) and genetic (59) data suggest that the heterodimer is the biologically relevant form of TAF11. For each promoter fragment, we formed protein-DNA complexes with TBP, and TBP with TFIIA, in the absence and presence of TAF11/TAF13. Irreversible DNA crosslinks were induced by exposure to UV radiation. Nucleoprotein complexes were resolved using SDS/PAGE, and protein identities were determined based on molecular masses. TAF11 and Toa1 are similar in molecular weight. To distinguish between Toa1 and TAF11- DNA complexes, we used a mutant form of TFIIA, mini TFIIA (mTFIIA), which lacks amino acids 55-215 located in the unconserved region of Toa1. In keeping with previous studies using electrophoretic mobility shift assays, we found no significant binding of TAF11 or TAF13 to DNA. In addition, we did not observe TAF11 or TAF13 DNA contacts when the heterodimer was in a complex with TFIIA and TBP (Figs 3.1B, 3.1C, 3.1D). This indicates that when the protein complex is formed, neither TAF11 nor TAF13 is within the proximity of the DNA such that protein-DNA contacts occur. These results concur with DNAseI footprinting experiments, which show that TAF11/TAF13 has no dramatic effect on the region of DNA protected by

TBP-TFIIA complexes (Fig 3.2) and suggests that no additional DNA contacts are mediated by TAF11/TAF13.

Despite the absence of TAF11 and TAF13 DNA contacts, we detected alterations in the crosslinking patterns for TBP and TFIIA in binding reactions containing TAF11/TAF13. TFIIA crosslinking was limited to two positions upstream of the TATA element, -3/4 and –6/-7 (Figs 3.1B and 3.1C). TFIIA-DNA contacts at these sites are consistent with crystallographic structure determinations (37, 120) and previous protein-DNA crosslinking studies (64). At these sites, addition of TAF11/TAF13 to TFIIA-TBP complexes altered the intensity of TBP and TFIIA crosslinks. These modest changes could result from conformational changes in TBP and TFIIA upon interaction with TAF11. Interestingly, TAF11/TAF13 has no effect on the crosslinking pattern of mTFIIA at these same sites, suggesting that the unconserved region absent in this mutant protein may be a determinant in TAF11/TAF13 imposed changes to protein – DNA contacts. Furthermore, TAF11/TAF13 induced changes to protein-DNA contacts appear to be site specific, since alterations are not observed for TBP contacts at +1/+2 near the TATA element (Fig 3.1D). Taken together, our data indicate that TAF11/TAF13 induces qualitative changes to TBP-DNA and TFIIA-DNA contacts without direct interactions with the DNA. Therefore, the imposed changes, which enhance TBP-TFIIA complex formation, are likely mediated through protein-protein interactions.

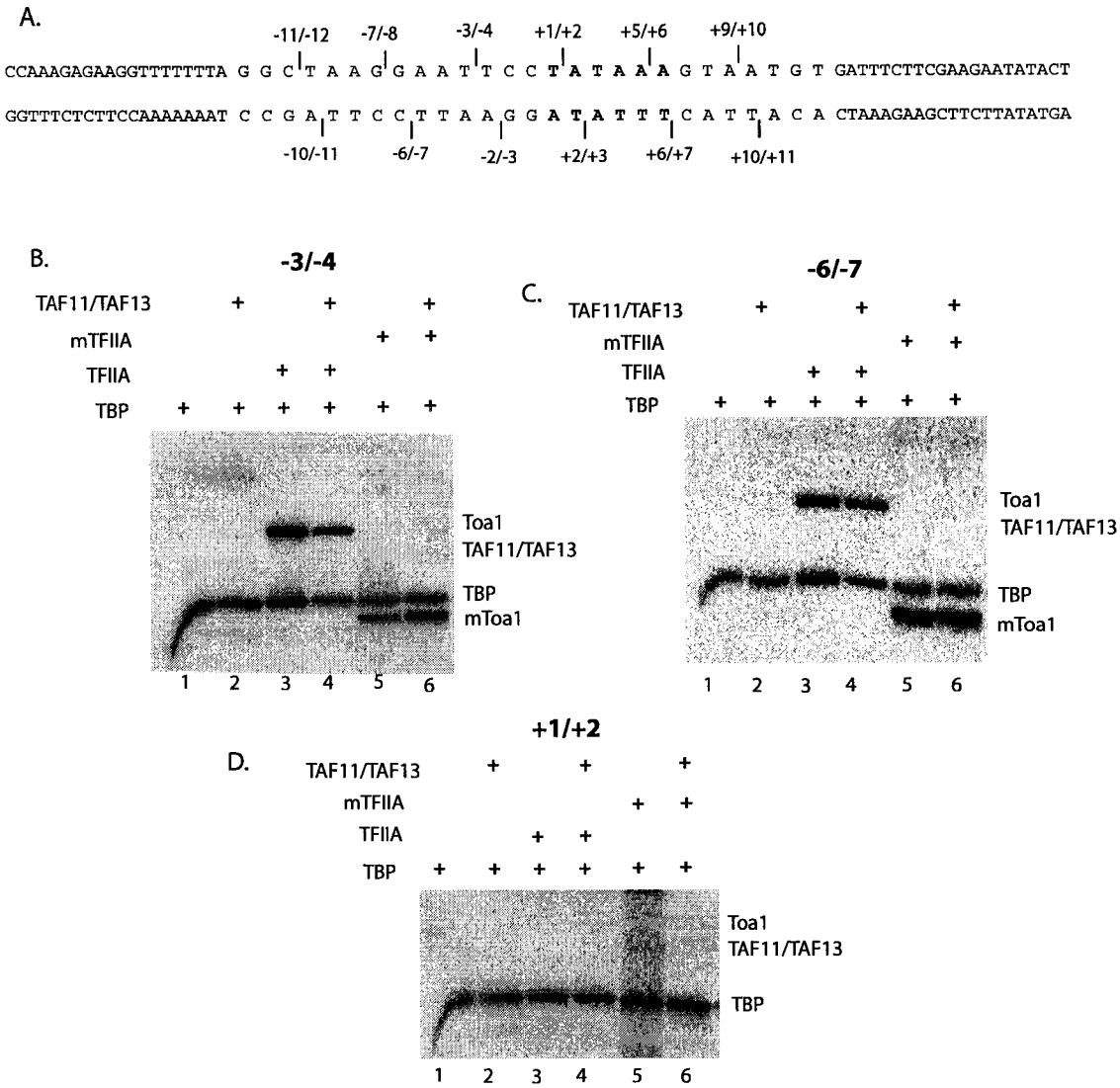


Figure 3.1 TAF11 alters TFIIA and TBP -DNA contacts within the *HIS3* promoter (A) Sequence of the yeast *HIS3* promoter. Phosphates at which the phenylacyl bromide photoactivatable crosslinking agent was incorporated and analyzed for protein interactions are denoted with vertical lines. Sites are numbered with respect to the TATA element. The TATA element is in bold. (B) Site specific photocrosslinking results for TBP, TFIIA, mTFIIA, and TAF11/TAF13 at site -3/-4. Binding reactions contained 30 fmole of derivatized promoter fragment 12.5ng of TBP, 50ng TFIIA, 50 ng of mTFIIA, 62.4 ng of TAF11/TAF13. For each panel, lane 1 contains TBP, lane 2 contains TBP and TAF11/TAF13, lane 3 contains TBP and TFIIA, lane 4 contains TBP, TFIIA and TAF11/TAF13, lane 5 contains TBP and mTFIIA, and lane 6 contains TBP, mTFIIA, and TAF11/TAF13. (C) Protein -DNA photocrosslinking data for site +6/+7. (D) Protein-DNA photocrosslinking data for site +1/+2.

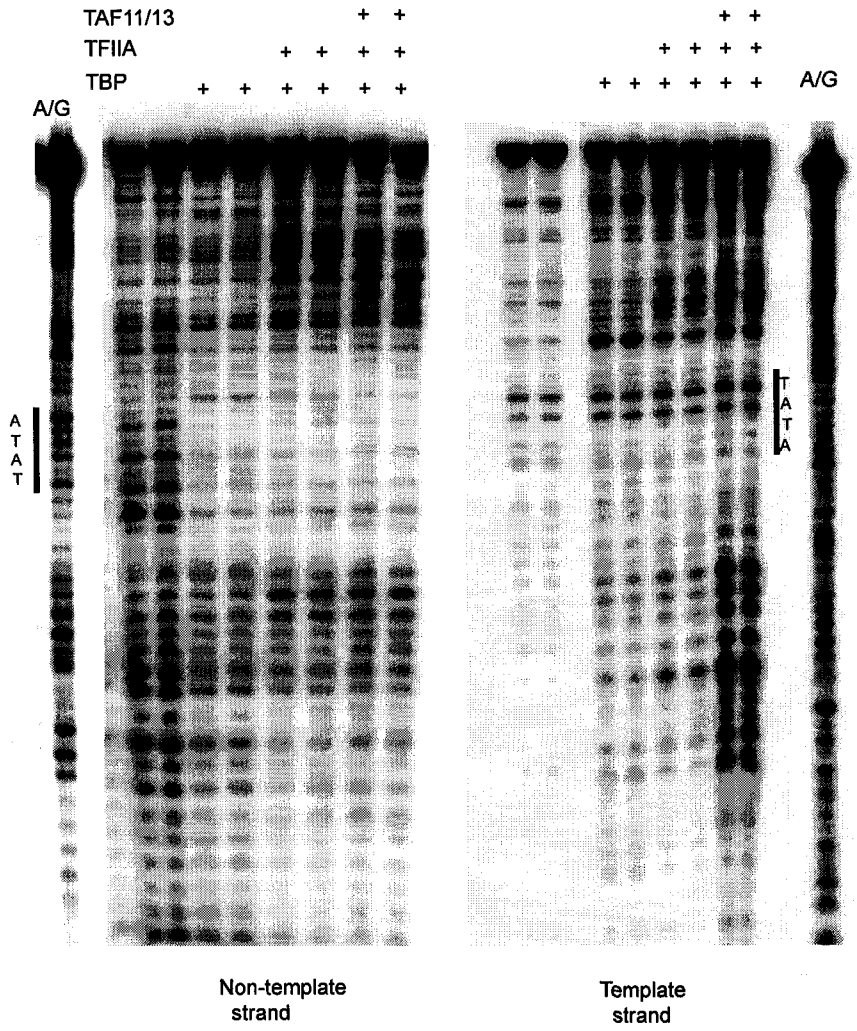


Figure 3.2 TAF11/13 does not effect the region of DNA protection formed by TFIIA-TBP complexes. DNaseI footprinting on a 76 bp fragment of the *HIS3* promoter that has been labeled on the template or RNA-like strands. Binding reactions containing recombinant TBP (370nM), TFIIA (35.5 nM) and TAF11/TAF13 (93 nM) were digested with 1.0U of DNaseI for 1 min at room temperature. DNA was precipitated and resuspended in loading dye and fractionated on10% polyacrylamide sequencing gel. Maxam-Gilbert (A/G) sequencing reactions of probe were simulataneously fractionated with the footprinting reactions to serve as markers.

3.4b Genetic selection for TAF11 compensatory mutants.

To better understand the molecular organization of protein interactions that contribute to TAF11 induced complex enhancement, we extended our characterization of the TAF11-TFIIA interaction. Previously, we identified mutations in the small subunit of TFIIA, Toa2, that affect interaction with TAF11. Specifically, a toa2 allele containing a single lysine substitution at isoleucine 27 ($toa2^{I27K}$) is defective for interaction with TAF11 (60). The I27 residue is located within the four-helix bundle domain of Toa2, a region that projects away from the TBP-TFIIA-DNA complex. Loss of interaction by substitution of lysine at this residue indicates this region is important for interaction with TAF11. In addition to mapping the Toa2 interaction surface, the $toa2^{I27K}$ allele provides a tool to use in a genetic screen to isolate compensatory mutants in TAF11. The goal of this approach was to identify compensatory mutations that would reveal domains of TAF11 important for interaction with TFIIA. In a previous study, the two-hybrid assay was used to demonstrate loss of interaction between $toa2^{I27K}$ and TAF11. For this genetic screen, we use the two-hybrid assay to exploit the loss of interaction exhibited by $toa2^{I27K}$ to isolate TAF11 compensatory alleles that suppress the $toa2^{I27K}$ interaction defect. An advantage for use of the two-hybrid assay in this genetic screen is that it allows direct isolation of alleles, which specifically restore the interaction with $toa2^{I27K}$. In addition, genetic selection does not require compensatory alleles to support cell viability since the two-hybrid assay is performed in strains expressing wild type TAF11 and TFIIA.

However, due to the artificial nature of this assay, two-hybrid interactions may be magnified or diminished in this system. Nevertheless, we used the two-hybrid assay to isolate TAF11 compensatory alleles. PCR-based misincorporation was used to mutagenize the entire open reading frame of TAF11 (Fig 3.3). The resulting mutant library contained sufficient homologous sequences for in vivo recombination to a plasmid encoding the Gal4 activation domain (AD-taf11). Mutant libraries were transformed into the yeast strain MaV103 expressing toa2^{I27K} fused to the Gal4 DNA binding domain (DB- toa2^{I27K}), and compensatory interactions between toa2^{I27K} and taf11 derivatives were identified using the yeast two-hybrid assay. The transformed yeast strains contain the *HIS3* gene under the control *GAL1* promoter with four Gal4 binding sites and two-hybrid interactions were detected by activation of the *HIS3* gene. Compensatory mutants were selected by their ability to grow on 3-aminotriazole, (AT), a competitive inhibitor of the *HIS3* gene product. Strains in which *HIS3* is highly expressed due to interactions between DB- toa2^{I27K} and AD-taf11 are able to grow on AT (AT^{R}). Approximately 20,000 transformants were screened for AT^{R}, and a total of 75 independent compensatory alleles were isolated.

3.4c taf11^{E182G} can confer compensatory interaction with toa2^{I27K}.

The number of compensatory alleles isolated suggested that diverse substitutions in TAF11 could confer a compensatory interaction with toa2^{I27K}. To identify mutations that allow interaction with toa2^{I27K}, 30 of the 75 isolated alleles were sequenced. Each compensatory allele contained multiple amino acid

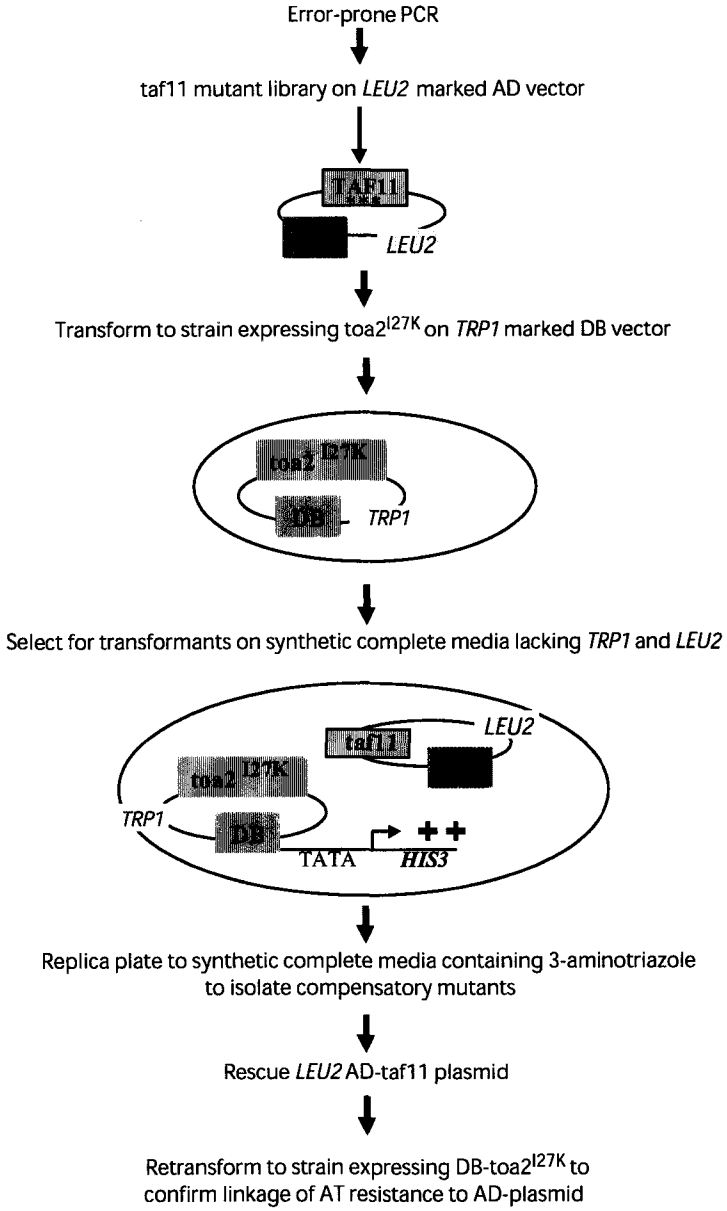


Figure 3.3 Scheme used for genetic selection for TAF11 compensatory mutants Error-prone PCR was used to create a TAF11 mutant library fused to the *LEU2* marked Gal4 activation domain vector (AD). TAF11 mutant libraries were transformed to a strain expressing toa2^{I27K} fused to the *TRP1* marked Gal 4 DNA binding domain vector, DB. Transformants were selected on synthetic complete media lacking tryptophan and leucine and then replica plated to synthetic complete media containing 3-aminotriazole (AT). Compensatory mutants were identified by their ability to grow on media containing AT (AT^{R}). The *LEU2* marked AD-TAF11 plasmids were rescued from AT^{R} strains and retransformed to strain expressing DB- toa2^{I27K} to confirm linkage of AT^{R} to the AD plasmid.

substitutions randomly distributed throughout the protein (Table 3.1). Despite this complexity, a few mutations were common to a number of alleles in the collection. Approximately 60% of the sequenced alleles contained a mutation in glutamic acid at position 182 (E182). Other amino acids frequently mutated were lysine 46 (K46), phenylalanine 171 (F171), and isoleucine 178 (I178). To determine if substitutions at these specific residues contributed to the compensatory interaction with toa2^{I27K}, single point mutations at each of the common residues were constructed and independently assayed for interaction with toa2^{I27K} using the yeast two-hybrid assay. Mutation of E182 to alanine or glycine conferred a strong compensatory interaction with toa2^{I27K} (Fig 3.4). Residue I178 when mutated to methionine could also interact with toa2^{I27K}, however to a much weaker extent. Neither K46R nor F171V could confer compensatory interaction alone; thus alleles containing these substitutions require additional mutations to interact with toa2^{I27K}.

3.4d Compensatory mutations map to a solvent exposed surface of TAF11.

Since TAF11 is highly conserved throughout evolution, we used the three-dimensional structure of the human TAF13/TAF11 heterodimer (9) as a template to model the location of residues involved in the compensatory interaction. The residues E182 and I178 map to a solvent exposed surface within the α-2 helix of the histone fold domain (HFD), a surface not involved in dimerization with TAF13 (Fig 3.5). We also tested whether the point mutant E185A, a residue whose location flanks that of E182, could confer compensatory interaction with toa2^{I27K}

TABLE 3.1

Amino Acid Substitutions in TAF11 Compensatory Alleles

Allele	Mutations[a]
1	D9G F139S E182G Y322C
2	I19T K29R K46R N59S K66R I90V I95T V141A F171S I178K N262D Y322C
3	T2A E182G S236T L264C
4	F26L I82T T145A E182G K212R F222L
5	Q77H N83S K153R N161D V175A E182V
6	K68E N192H
7	K66R I90V K133E N227D
8	N15S M31T Y67H N83S Q123L S157G Q278L S307C E317G N331S
9	Q135R E182G D327G
10	K46R K63R I178V T257A K268R K281E Q294R
11	K84R E182G
12	Q34H K71R K125R N130D E182K K287R F345L
13	Y64C G181A E182G L290R L392S
14	V35A F142L V170A F171V E182K Q229R K255R D279G
15	Q40L I178M
16	K84I E182G S223P N227S Y230F
17	E122G F142L V170A F171V E182V Q232R E252K E339G F345L
18	E38G K68E F124Y K133N S165G D250G
19	I74V I82T Q97R L155Q F171S N227S D233G S238G D249G Q266R K281R N307D
20	I74V L118P F171S Y179H E185V N192D K297E N307D T328A
21	K46R I57N E87D F91L E122G V128A N130I L172R F222S
22	K46R N109S E112G N161D N167D G176R K212E Q294L Y322C
23	S56P E140G V152A E182G K205R N306D
24	T28A I57T N62S E182V N227D Q337R
25	Y64H S81P I95V F171L E182G L329C
26	N148D F171L E182V Q337R
27	I82T F171L E182G S223P D242G S273P
28	F91A K125E V152D I224T
29	F91S F142S T150A K177E E182G Q228R
30	N88D Q135R V152G N192D L284P
31	I19M K46R K71R L131S F142L S146P E182G I184T V239A E257G N307D N331T

[a] Substitutions are listed in the single-letter amino acid code. Wild-type amino acid residue numbers are followed by the mutant amino acid. Common amino acid substitutions are highlighted in yellow, blue, green and red.

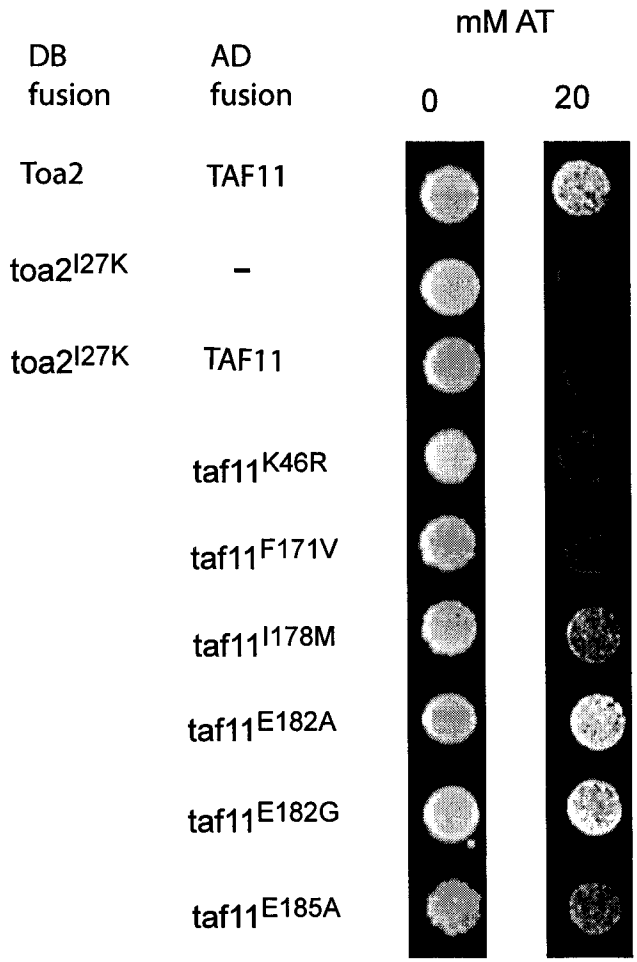
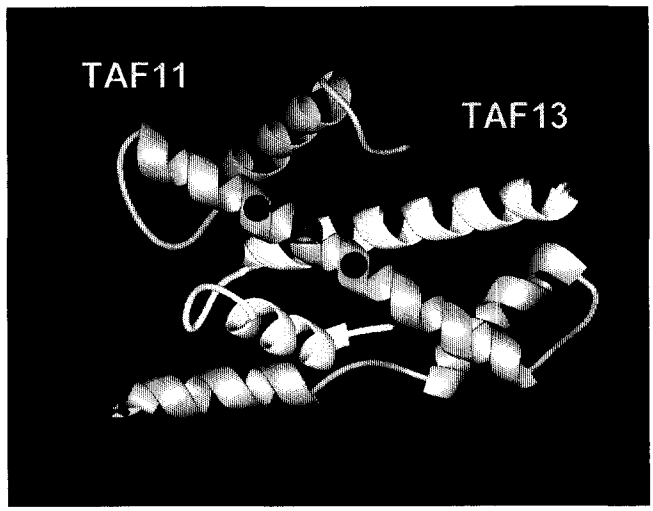


Figure 3.4 TAF11 derivatives E182G, I178M and E185A confer compensatory interaction with toa2^{I27K}. Two-hybrid assay was used to determine if substitutions at specific TAF11 amino acids could interact with toa2^{I27K}. Approximately 10^4 cells were spotted on synthetic complete plates containing either 0 or 20 mM AT. Growth on AT is indicative of an interaction between the two hybrid proteins.

A



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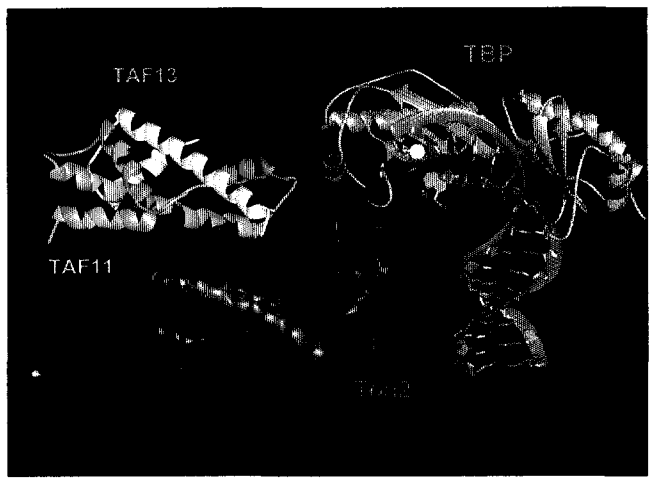

Figure 3.5 Crystal structure of the human TAF11/TAF13 heterodimer alone (A) and with the yeast TFIIA-TBP-DNA complex (B). (A) TAF11 is shown in blue ribbon and TAF13 in yellow ribbon. The location of residues homologous to yeast amino acids E182 in red and I178M and E185 are in blue. (B) Crystal structure of theTAF11/TAF13 heterodimer combined with the crystal structure of the TFIIA-TBP-DNA complex. DNA is shown in gray ribbon, TBP in orange ribbon, and the two subunits of TFIIA are shown with Toa2 in blue ribbon and Toa1 in green ribbon. Shown in red is the location of I27 of Toa2 within the four-helix domain. The TAF11/TAF13 heterodimer as described above is rotated 90^0 forward placing the location of the surface containing the compensatory residues in proximity of I27.

(Fig 3.4). As predicted E185A, like I178M, could interact weakly with toa2^{I27K}. Therefore, mutations on this surface can accommodate interaction with toa2^{I27K}. Based on these results, we conclude that the region encompassing these residues within the α-2 helix of the HFD defines a distinct region of TAF11 important for interaction with Toa2.

3.4e TAF11 mutants exhibit different abilities to maintain wild type TAF11 interactions.

To determine if compensatory mutations affect TAF11 interaction with TAF13 or wild type TFIIA, we assayed our panel of site-directed compensatory point mutants for interaction with wild type Toa2 and TAF13 using the yeast two-hybrid assay (Table 3.2). All of the point mutants conferring compensatory interaction were competent for interaction with TAF13. Consistent with the predicted location of these amino acids, TAF11 appears to interact with TFIIA and TAF13 simultaneously. In addition, a non-compensatory substitution located on the same surface, F171V, does not affect interaction with TAF13. This same mutation however, results in loss of interaction with wild type Toa2 in this assay, indicating that specific mutations on this surface can also disrupt TAF11-TFIIA interaction. Further analysis of site directed mutants identified an additional mutation, K46R, which results in loss of interaction with Toa2. This N-terminal residue is at a distinct distance from the HFD, suggesting that a second region of TAF11 may be required for interaction with TFIIA.

TABLE 3.2

Interaction and Viability Assessment Of TAF11 Derivatives

Mutant	I27K[a]	Toa2[a]	TAF13[a]	Viability[b]
WT	-	+	+	+
Q40L	-	-	+	+
K46R	-	-	+	+
F171V	-	-	+	+
I178M	+	+	+	+
E182G	++	+	+	+
E185A	+	+	+	+
taf11-14	+	-	+	slg 38°C
taf11-15	+	-	+	+
taf11-16	+	-	+	slg 38°C

[a] The indicated strains expressing Gal4 DNA binding domain fusions were tested for interaction with site directed TAF11 mutants fused to Gal4 activation domain fusions using the two hybrid assay. A "+" indicates a positive two hybrid interaction and " – " indicates no two hybrid interaction was detected.

[b] Viability denotes the growth (+) or no growth (-) phenotype of cells when the indicated. TAF11derivatives were tested for the ability to cover a genomic deletion of *TAF11* gene in a plasmid shuffle experiment. Strains supporting cell viability but exhibiting slow growth phenotypes at 38°C are indicated by "slg 38°C".

We extended the analysis of functional TAF11 interactions to the entire collection of compensatory alleles. TAF11 compensatory alleles were grouped into three classes based on their ability to interact with Toa2 and TAF13 (Table 3.3). The majority of isolated compensatory alleles (Class I) are competent for interaction with both Toa2 and TAF13 even though these alleles contain multiple mutations. This interaction profile is in keeping with our observations for single substitutions conferring compensatory interaction, E182G, I178M and E185A. significant number of isolated compensatory alleles (Class III) maintain interactions with Toa2 but are defective for interaction with TAF13. Since the compensatory alleles contain multiple mutations, certain substitutions allow interaction with toa2^{I27K} but disrupt interaction with TAF13. Further examination of mutations from Class III might reveal substitutions that specifically disrupt interaction with TAF13. The smallest class of compensatory alleles (Class II) is defective for interaction with Toa2. Since there were only three alleles in this class, examination of the substitutions in these alleles allowed us to distinguish between mutations that confer compensatory interaction with toa2^{I27K} versus mutations that disrupt interaction with Toa2. One allele in particular, taf11- 15 contained two substitutions, Q40L and I178M (Table 3.1). We previously demonstrated that mutation I178M could confer compensatory interaction with toa2^{I27K} without affecting interaction with wild type Toa2. We predicted then, that Q40L would be defective for interaction with Toa2. As expected, Q40L is not competent for interaction with Toa2 but maintains interaction with TAF13 (Table3.2).

TABLE 3.3

TAF11 Compensatory Mutant Classes

	Interactions			
Class	toa2^{I27K} [a]	Toa2 [b]	TAF13 [c]	Total
WT	-	+	+	
I	+	+	+	54
II	+	-	+	3
III	+	+	-	18
				75

[a] Indicates interactions with strains expressing Gal4 DNA binding domain fusion to toa2^{I27K}.

[b] Indicates interactions with strains expressing Gal4 DNA binding domain fusion to Toa2.

[c] Indicates interactions with strains expressing Gal4 DNA binding domain fusion to TAF13.

Taken together, our interaction studies identified compensatory mutations whose locations implicate the HFD as a region important for TFIIA interaction. The interaction profiles of TAF11 compensatory alleles also demonstrate that TAF11 uses the HFD to interact with TFIIA and TAF13 simultaneously.

3.4f TAF11 mutants support cell viability and exhibit conditional phenotypes.

To further assess the functional significance of the TAF11-TFIIA interaction in vivo, we tested our panel of site-directed TAF11 mutants for the ability to support cell viability in a TAF11 deletion strain. We included in this analysis the three alleles from Class II, which were defined by our two-hybrid analysis as defective for interaction with wild type Toa2. All strains expressing TAF11 alleles supported cell viability in a strain in which the chromosomal copy of TAF11 had been deleted (Table 3.2). In addition, TAF11 strains were assayed for conditional phenotypes (Table 3.4). These included growth on alternative carbon sources, galactose, raffinose, glycerol and ethanol, media lacking inositol, and temperature sensitive growth at 15 and 38^0 C. Strains were assayed for stress related phenotypes by growth on hydrogen peroxide, diamide, cadmium sulfate and benomyl. Strains expressing site directed TAF11 derivatives did not exhibit conditional phenotypes; however, two of the three compensatory alleles, taf11-14 and taf11-16, exhibited slow growth phenotypes at 38^0C (Table 3.4).

TABLE 3.4

Phenotypic Characterization[a] of TAF11 Mutant Strains

Mutant	Galactose	Raffinose	Glycerol	Ethanol	Inositol	Benomyl	Diamide	Hydrogen Peroxide	Cadmium Sulfate	15	38
WT	+	+	+	+	+	+	+	+	+	+	+
Q40L	+	+	+	+	+	+	+	+	+	+	+
K46R	+	+	+	+	+	+	+	+	+	+	+
F171V	+	+	+	+	+	+	+	+	+	+	+
I178M	+	+	+	+	+	+	+	+	+	+	+
E182G	+	+	+	+	+	+	+	+	+	+	+
E185A	+	+	+	+	+	+	+	+	+	+	+
Δ N	+	+	+	+	+	+	+	+	+	+	-
taf11-14	+	+	+	+	+	+	+	+	+	+	slg[b]
taf11-15	+	+	+	+	+	+	+	+	+	+	+
taf11-16	+	+	+	+	+	+	+	+	+	+	slg[b]

[a] Growth on media containing the indicated compounds. A "+" indicates growth and "-" no growth
[b] "slg" indicates slow growth at 38°C

3.4g The N terminus of TAF11 is important for interaction with Toa2.

Our two-hybrid analysis identified two mutations, Q40L and K46R, which reside within the N-terminal region of TAF11, can disrupt interaction with TFIIA. We were interested in examining further whether more than one region of TAF11 is required for interaction with TFIIA. To this end, we generated a series of deletion derivatives encompassing TAF11 domains. Using the yeast two-hybrid assay, TAF11 deletion derivatives were tested for interaction with TFIIA and TAF13 (Fig 3.6B). All deletion derivatives were expressed at levels similar to full length TAF11 (Fig 3.12). As predicted, disrupting the HFD by deletion of the α-3 helix (ΔC) is detrimental for TAF11 interaction with TAF13. This deletion derivative is also defective for interaction with TFIIA, confirming our findings that the HFD provides an interaction surface for TFIIA. On the other hand, the TAF11 derivative (ΔN), which includes the HFD but is missing the first 73 N-terminal amino acids can interact with TAF13 but is not sufficient for interaction with TFIIA. This indicates that loss of 73 N-terminal residues is detrimental for TFIIA association and suggests that this region is important for mediating interaction with TFIIA. Moreover, neither the HFD nor the N terminus alone is sufficient for TFIIA interaction since both ΔN and ΔHFD derivatives fail to interact with TFIIA. We conclude that the HFD is necessary and sufficient for interaction with TAF13; however TAF11 requires both the N terminus and the HFD for interaction with TFIIA.

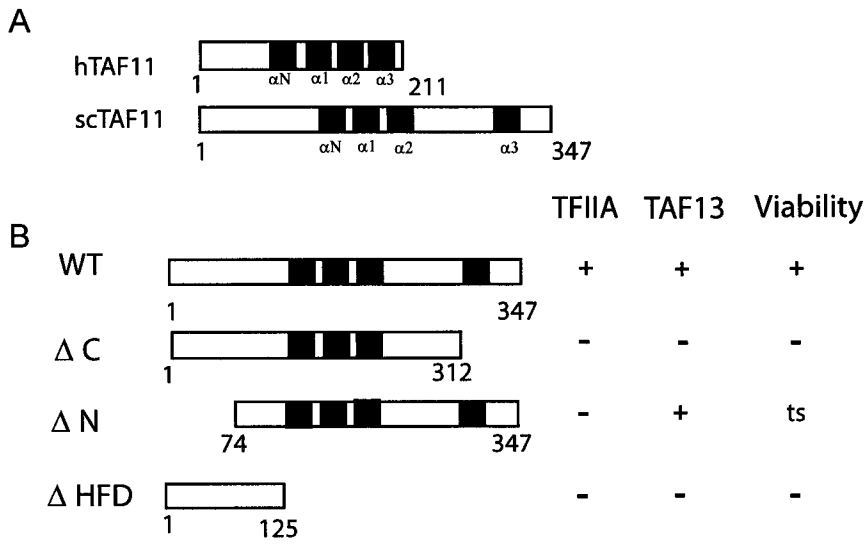

Figure 3.6 Interaction with TFIIA requires both the N terminus and the HFD of TAF11 (A) Sequence alignment of human and yeast TAF11. The conserved regions, which comprise the helices the histone fold domain are indicated by black boxes. (B) The N and C terminal residues are labeled for each deletion construct. The ability of deletion constructs to interact with TFIIA and TAF13 are denoted with a "+" or "-". Viability denotes the growth (+) or no growth (-) phenotype of cells when the indicated derivative is tested for its ability to cover a genomic deletion of TAF11 gene in a plasmid shuffle experiment.

In order to determine how loss of specific interactions affects TAF11 function in vivo, we next tested deletion derivatives for their ability to support cell viability.TAF11 deletion derivatives were expressed (under the control of the TAF11 promoter and terminator) in a strain in which the chromosomal copy of TAF11 had been deleted. There was a strong correlation between cell viability and the ability of TAF11 to interact withTAF13 and TFIIA (Fig 3.6B). That is, TAF11 deletions that disrupt interaction with both TAF13 and TFIIA were unable to support cell viability, whereas loss of TFIIA interaction results in a conditional growth phenotype.

3.4h The N terminus of TAF11 is not required for TFIID integrity.

Of particular interest to us was the TAF11 derivative, ΔN, which supported cell viability yet exhibited a temperature sensitive phenotype. Because this derivative was specifically defective for interaction with TFIIA, the inability to grow at the elevated temperature could result from loss of this interaction. Although ΔN can interact with TAF13, it is possible that the N terminus is necessary for functional contacts within TFIID and the observed conditional phenotype is due to loss of TFIID function. Co-immunoprecipitation assays were performed to examine TFIID integrity in yeast strains expressing the wild type or the ΔN TAF11 derivatives at both the permissive and restrictive temperatures. We used TAF1 as a marker for TFIID, and TAF deletion strains expressing TAF11 derivatives were modified by integrating a HA tag at the C terminus of TAF1 at the chromosomal locus. Whole cell extracts were prepared from TAF11 strains

expressing HA-tagged TAF1 and myc-tagged TAF11 derivatives. Polyclonal antibodies directed at TBP were used for immunoprecipitation, and the precipitated complexes were analyzed by immunoblot, using monoclonal antibodies against the myc or HA epitopes. Immunoprecipitation by antibodies directed to TBP indicates that wild type TAF11 associates with both TBP and TAF1. In addition, immunoprecipitates from the TAF11 strain expressing the ΔN derivative also show association with TBP and TAF1 at both 30^0C and 38^0C, indicating that TFIID is intact (Fig 3.7). These results indicate that deletion of the N terminus of TAF11 does not affect TFIID integrity even at the non-permissive temperature. Therefore, the temperature sensitive phenotype exhibited by strains expressing this TAF11 derivative could be due to loss of TFIIA interaction.

3.4i The TAF11 derivative ΔN exhibits transcriptional defects a number of Pol II transcribed genes.

We tested TAF11 derivative strains for molecular defects that impair transcription at Pol II transcribed genes using S1 nuclease assays. RNA was isolated from strains expressing wild type and TAF11 derivatives at both the permissive and restrictive temperatures. TAF11 strains expressing the ΔN derivative exhibited transcription defects at all the genes tested (Fig 3.8). The most significant reductions in transcription were observed for the TAF-dependent genes *RPS4* and *HTA2* with 3 and 5 fold decreases respectively. Similar reductions in transcription were observed for this strain at 38^0C (Fig 3.9). The TAF11 associated transcription defects for these genes are consistent with

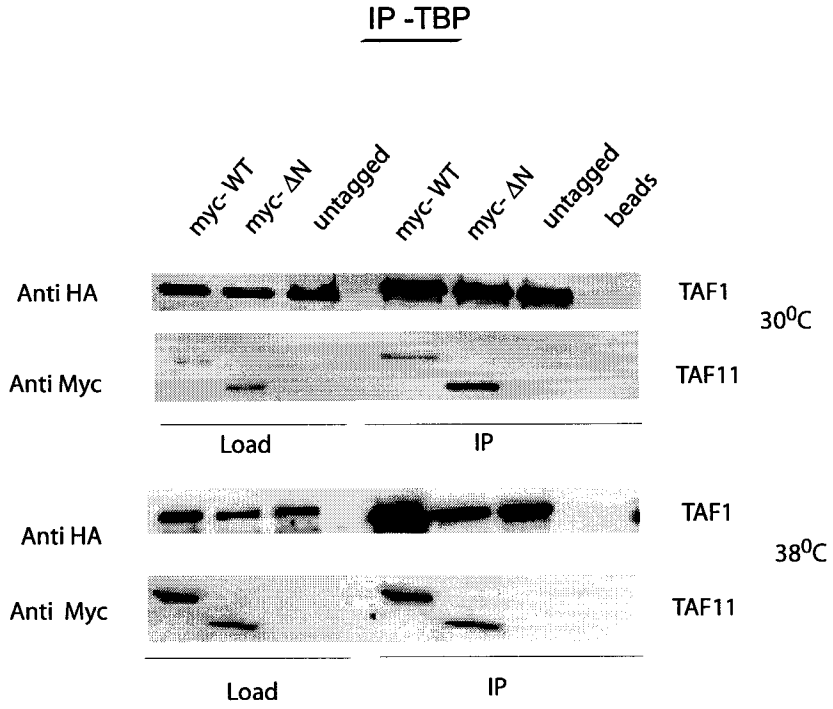

Figure 3.7 TAF11 derivative ΔN is stably associated with TFIID in vivo. Co-immunoprecipitation was performed on cell extracts from strains expressing myc-tagged TAF11 derivatives or untagged vector alone at 30^0C and 38^0C. Complexes were immunopreciptitated (IP) by polyclonal antibodies to TBP. Immunoprecipitated complexes were resolved by SDS-PAGE and probed by immunoblot analysis using monoclonal antibodies to myc or HA. Input from each extract (Load) and sample of antibody conjugated Protein-A beads (beads) are indicated.

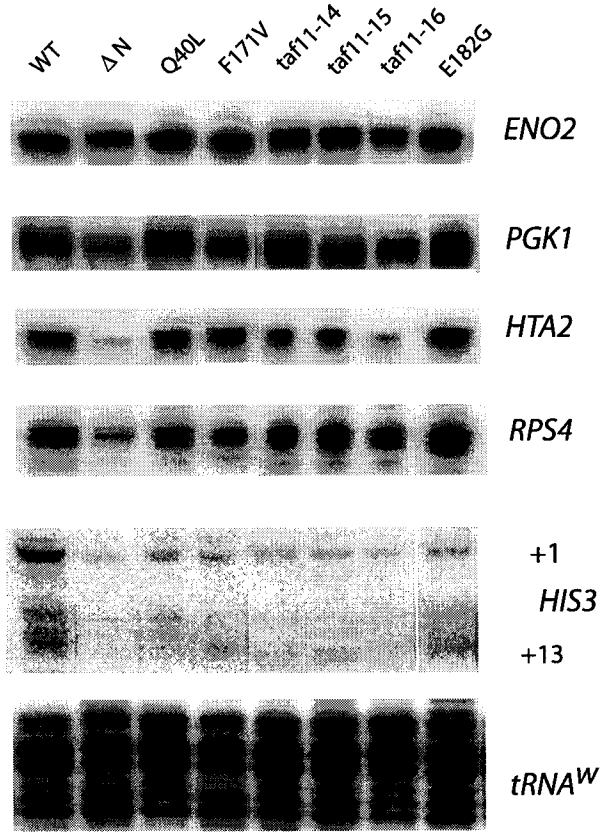

Figure 3.8 Transcription analysis of TAF11 derivatives for a collection of Pol II- transcribed genes at 30^{0}C. Wild-type (WT) and the indicated TAF11 derivatives were grown to log phase. Twenty-five to 30 μg of total RNA was hybridized with 100-fold excess of the indicated probe and treated with S1 nuclease. The *HIS3* +1 and +13 sites are indicated. The Pol III transcribed gene tRNA^W served as a loading control.

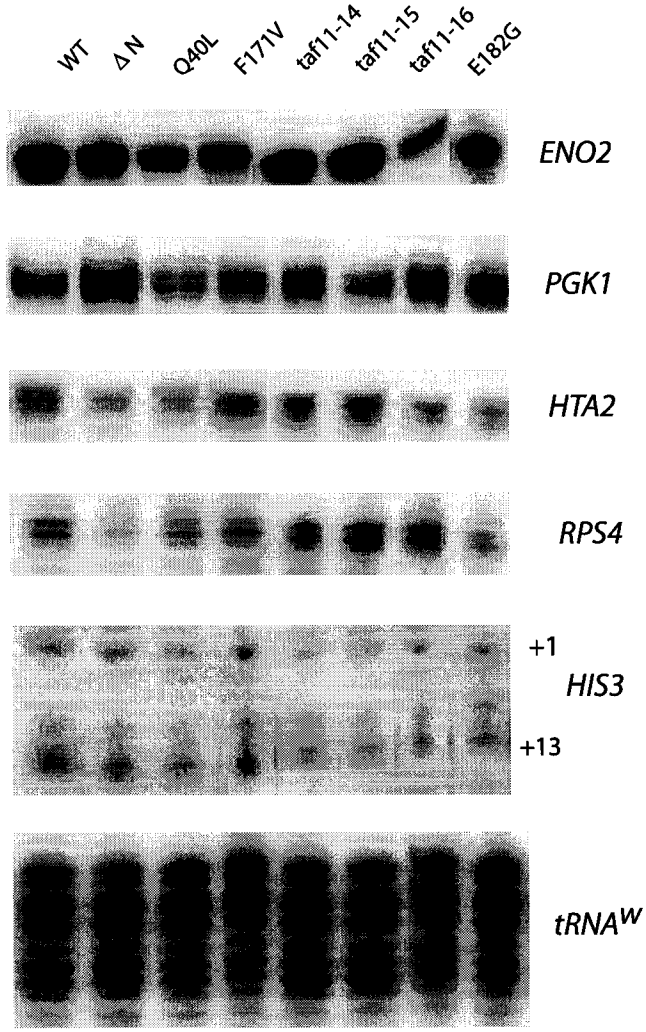

Figure 3.9 Transcription analysis of TAF11 derivatives at 38^{0}C for a collection of Pol II- transcribed genes. Wild-type (WT) and the indicated TAF11 derivatives were grown to early log phase at 30^{0}C. Cells were made heat tolerant by incubation at 38^{0}C for 15 min, then returned to 30^{0}C for 1 hour. Cells were then shifted to 38^{0}C for I hour prior to harvest. Twenty-five to 30 μg of total RNA was hybridized with 100-fold excess of the indicated probe and treated with S1 nuclease. The *HIS3* +1 and +13 sites are indicated. The Pol III transcribed gene *tRNA^W* served as a loading control.

previous studies (59, 60). In addition to the ΔN strain, the TAF11 strain expressing the compensatory allele taf11-16 also exhibited a 3-fold decrease in *HTA2* mRNA levels. The ΔN and compensatory strains share similar conditional phenotypes and contain more complex types of mutations, all of which may contribute to transcription defects observed. Transcription levels for *RPS4, HTA2, ENO2,* and *PGK1* in the remaining strains expressing TAF11 derivatives were comparable to the wild type TAF11. It is interesting to note that transcription levels for theTAF11 compensatory mutant E182G strain did not differ from mutant strains expressing non-compensatory mutations, suggesting that this single compensatory mutation does not drastically affect TAF11 function in vivo.

3.4j TAF11 derivatives exhibit defects for transcription derived from a weak TATA element.

In contrast to these genes, when we tested TAF11 strains for *HIS3* gene expression, all mutant strains exhibited a significant reduction in constitutive transcription when compared to wild type cells (Fig 3.8). Transcription was diminished for both the +1 and +13 transcripts of *HIS3.* Interestingly, quantitation of sample replicates revealed that mRNA levels for the +1 transcript were 2-fold more affected than the +13 transcript. The +1 transcript is generated from a non-canonical promoter element, while the +13 transcript is derived from a conventional TATA element. This suggests that TAF11 interaction with TFIIA is important for transcription at both types of promoters, but those with non-

canonical or weak promoters are more sensitive to loss of interaction between TFIIA and TAF11.

3.4k TAF11 derivatives are competent for Gcn4 activated transcription.

We next assessed TAF11 mutant strains for activator mediated transcription by examining Gcn4 induced *HIS3* gene expression. Cells were cultured to early log phase, AT, a competitive inhibitor of the *HIS3* gene product, was added and the cells were incubated for an additional hour before harvesting. Activation of *HIS3* transcription in all TAF11 mutant strains was indistinguishable from wild type (Fig 3.10). The lack of defect in induced *HIS3* transcription despite the diminished constitutive levels is consistent with previous observations that demonstrate TAF depletion has little effect on transcriptional response to activators and provides evidence that TAF11 is not required for activated transcription (87).

3.4l TFIIA-TBP-DNA complex formation is enhanced and stabilized by TAF11 under non-optimal conditions.

The preferential decrease in *HIS3* +1 transcripts derived from a non-consensus element observed in our TAF11 derivative strains is consistent with the transcription profiles of strains in which TAF11 or TAF13 has been conditionally depleted (70, 86, 87). Our previous studies indicate that sequence variations at the TATA element compromise TBP and TFIIA complex formation

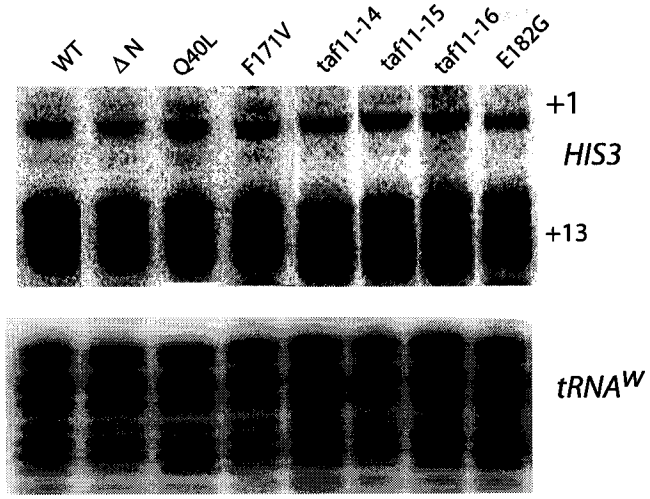

Figure 3.10 Analysis of Gcn4 dependent activation of the HIS3 transcription at 30^{0}C in TAF11 derivatives. Gcn4 activated transcription was measured by treating cells with 20mM AT for 1hour prior to harvest and examining levels of *HIS3* (+1 and +13 start sites indicated) and tRNA^W expression by S1 analysis. Total RNA (25 to 50 μg) was hybridized with excess *HIS3* and *tRNA^W* probes and treated with S1 nuclease. The Pol III transcribed gene *tRNA^W* served as a loading control.

and result in diminished levels of transcription in vivo (117). It is possible that TAF11 performs a specific function at weak promoters where TBP-TFIIA-DNA complex formation is compromised. To this end, we used electrophoretic mobility assays to compare TAF11-induced TFIIA-TBP- DNA complex enhancement on promoter fragments containing either the consensus sequence TATAAA or the sequence, CATAAA. The CATAAA sequence functionally replaces the non-consensus TATA element at the *HIS3* promoter, but does not support high levels of transcription (51). As such, the CATAAA promoter provides us a means to examine TAF11 induced complex enhancement at a weak promoter where TFIIA-TBP complex formation is not optimal. Recombinant TFIIA and TAF11/TAF13 were incubated with radiolabeled promoter fragments, and complexes were resolved on 5% acrylamide gels (Fig 3.11A). TBP and TFIIA form a complex on both the TATAAA and CATAAA probes. At limiting concentrations of TFIIA, there is slightly less TBP-TFIIA-DNA complex formed on CATAAA than TATAAA; however addition of TAF11 significantly enhanced TBP-TFIIA-DNA complex formed. Quantitation of complexes reveals a four-fold stimulation on the CATAAA promoter compared to a two-fold for the TATAAA promoter. Therefore, TAF11 has a greater effect at promoters that are compromised for TFIIA-TBP complex formation.

Our previous studies determined that TBP-TFIIA complexes formed on promoters containing the CATAAA sequence are significantly less stable than those formed on TATAAA containing promoters (117). It is possible that the observed augmentation in complex enhancement is due to the ability of TAF11 to

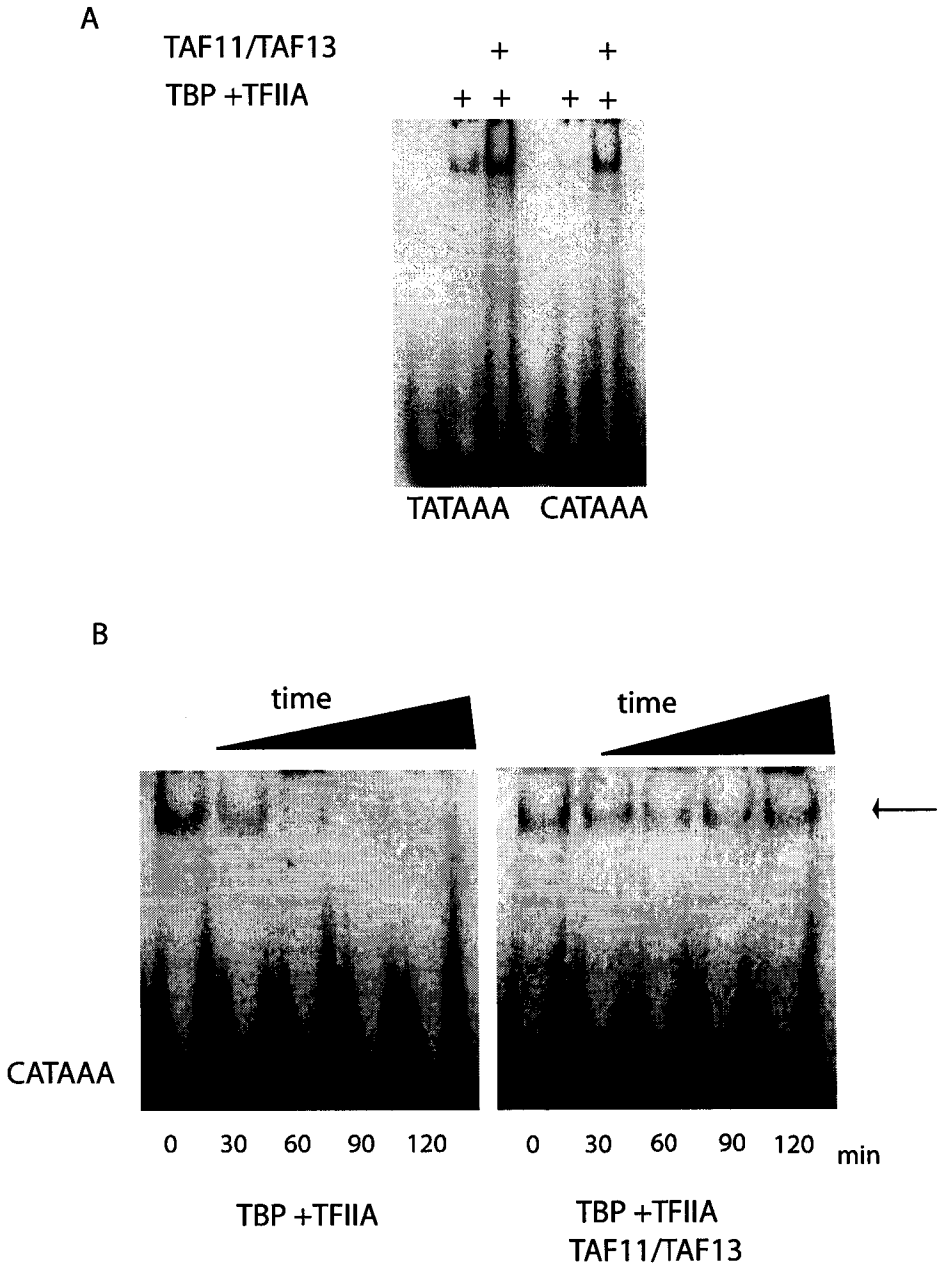

Figure 3.11 TAF11 enhances and stabilizes TFIIA-TBP complexes on the non-consensus CATAAA promoter (A) For all reactions, 0.1 pmole of radiolabeled CATAAA or TATAAA probe was used. The DNA probes were incubated the TBP, TFIIA and TAF11/TAF13 where indicated for 30 minutes then resolved on 5% acrylamide gels. All reactions contain (30nM) TBP, (5 nM) TFIIA and where indicated (18.6 nM) of TAF11/TAF13 heterodimer. (B) Reactions are as described in panel A, except that following 30 minute incubation, complexes were challenged with poly [dAdT] at 30 minute intervals with increasing time was added to each reaction.

stabilize TFIIA and TBP interactions on the CATAAA promoter. To test this hypothesis, we again used the CATAAA promoter as a model for weak promoters. TFIIA-TBP complexes were formed in the presence and absence of TAF11 and then challenged with a molar excess of specific competitor, (poly [dAdT]), for various time periods. Consistent with previous studies, the TFIIA-TBP complex on the CATAAA promoter is rapidly lost in the presence of competitor. In contrast, the TAF11-TFIIA-TBP complex remains stable for the entire two-hour time course, indicating that TAF11 stabilizes TFIIA-TBP complexes formed on a non-consensus promoter (Fig 3.11B). Thus, through interaction with TFIIA, TAF11 enhances protein interactions leading to stable association of TBP and TFIIA at the promoter, a property that is particularly important for complexes formed on weak TATA elements.

3.5 Discussion

3.5a TAF11 interacts with TFIIA via two distinct regions.

Isolation of TAF11 compensatory mutants confirms that toa2^{I27K} is specifically defective for interaction with TAF11 and effectively maps the TAF11 interaction surface to the four-helix bundle domain of TFIIA, a surface not involved in interaction with TBP or DNA. TAF11 compensatory mutants identified regions of TAF11 important for TFIIA interaction. Through the analysis of over-represented substitutions, we were able to identify a single substitution that could restore interaction with toa2^{I27K}. Moreover, mapping the location of these

compensatory mutations to the surface of the structurally defined α-2 helix of the HFD implicates the involvement of this region for interaction with TFIIA.

The predominance of HFD in TAFs and the identification of TAF-TAF dimers led to the proposal that, like histones, the HFD of TAFs dictate dimerization specificity. Recently, we and others demonstrate that this domain provides an accessible surface for other TAF-TAF interactions (33, 138). In this study we show that the HFD of TAF11 can mediate interactions with both TAF13 and TFIIA. Compensatory mutants map to an exposed surface opposite to that involved with TAF13 interaction. In addition mutations that either confer compensatory interactions or disrupt interactions with TFIIA have no effect on TAF13 interactions. Because these interactions require separate surfaces, TAF11 interactions with TFIIA and TAF13 need not be mutually exclusive. Furthermore, this data suggests that the accessibility of HFDs in TFIID may extend to additional protein interactions required for efficient initiation complex assembly. These findings are further supported by studies of human TAF11, which demonstrate that mutations in residues on the solvent exposed surface of the α-2 helix abolish synergistic transcription activation by nuclear receptors (66). Interestingly, the residues implicated in this activity correspond to the same residues we identified as important for interaction with TFIIA (E182 and E185), indicating the functional importance of this interaction surface in the human system.

In addition to the HFD, TAF11 mutational analysis indicated that the N-terminus also provides an important surface for TFIIA interaction. Mutations at

specific N-terminal residues and deletion of the first 73 N-terminal residues result in loss of interaction with TFIIA. Cells expressing the TAF11 derivative ΔN, (lacking the 73 N-terminal residues), are temperature sensitive and exhibit transcription defects at a number of Pol II transcribed genes. We confirmed that deletion of these N-terminal residues does not alter TAF11 interactions within TFIID, since cells expressing this TAF11 derivative can associate with TAF1 and TBP. In addition, two-hybrid analysis determined that interaction with TAF13 was not affected by this mutation. Although we can not rule out that other TAF11 interactions might be affected by the N-terminal deletion, loss of interaction with TFIIA could contribute to the conditional growth defects and impaired transcription observed. Similar defects were observed when TAF11-TFIIA interaction was disrupted by mutations in Toa2 (60).

3.5b The TAF11-TFIIA interaction is important for transcription.

All strains expressing TAF11 derivatives defective for interaction with TFIIA supported cell viability, and most strains exhibited only minor defects in transcription. It is likely that disruption of the TAF11-TFIIA interaction through single mutations can be compensated by redundant interactions that contribute to the formation of the TAF11-TFIIA-TBP complex (138). In addition, the TAF11 derivatives characterized in this study were isolated in a genetic screen that selected for gain of TAF11 function. As such, the inherent bias of the genetic selection may have identified substitutions that are not sufficient to result in complete loss of interaction with TFIIA. However, TAF11 inactivation by deletion

of N-terminal residues (taf11-ΔN) or through multiple mutations (taf11-14 and taf11-16) results in temperature sensitive phenotypes and selective defects in transcription. This is consistent with other studies, which demonstrate that multiple mutations are necessary to confer a temperature sensitive phenotype (59, 70). Moreover, the transcription defects observed for taf11-ΔN and taf11-16 are similar to those reported for conditional TAF11 alleles carrying multiple mutations (59).

Activated transcription in response to the Gcn4 activator was also not affected in strains expressing TAF11 derivatives, suggesting that theTAF11-TFIIA interaction is not a requirement for this activator response. This data is in keeping with other studies that suggest TAFs are not required for transcription activation (86, 95).

In contrast, all of the TAF11 derivatives exhibited greater defects for transcription derived from the non-consensus TATA element of the *HIS3* promoter. Similar defects were observed upon inactivation of TAF11 and TAF13 (70, 86, 87) suggesting that the TAF11-TFIIA interaction is critical for transcription at promoters lacking consensus TATA elements.

3.5c TAF11 enhances TBP-TFIIA complex formation without direct interaction with the DNA.

While the binding of TBP to the TATA box is critical for the assembly of the transcription apparatus, other DNA-protein interactions affect preinitiation complex formation. The TFIIA-TBP interaction is an example of one such

interaction and the importance of this association for transcription has been extensively examined (52, 116, 117). Several lines of evidence indicate that TAFs orient and stabilize the transcription machinery on the promoter, and DNA binding studies have revealed position dependent contacts between certain TAFs and DNA (13, 16, 63, 94,126). In previous studies, we show that TAF11 specifically enhances TFIIA-TBP-DNA complex formation (60). Using protein-DNA crosslinking assays, we demonstrate that TAF11/TAF13 affects TBP and TFIIA promoter interactions without observable DNA interactions. Instead, these changes to TBP and TFIIA promoter contacts are mediated through protein-protein interactions and lead to an overall increase in TFIIA-TBP-DNA complex formation. Although TAF11/TAF13 modestly alters TBP and TFIIA-DNA contacts, TAF11-induced complex enhancement is TFIIA dependent. Therefore, we conclude that TAF11 interaction with TFIIA predominantly influences enhanced complex formation. The TFIIA dependency of TAF11 mediated complex enhancement is in accord with other studies which show that TFIID association with yeast promoters is facilitated by TFIIA (109) and that TFIIA interaction with TFIID induces changes to specific TAF-DNA contacts (94). Clearly, this study demonstrates that the interplay between TAF11, TFIIA and TBP is beneficial for complex formation at promoters. This evidence, in conjunction with the previous observation that TFIIA mutants defective for interaction with TBP and TAF 11 do no support cell viability (60), indicate that the interaction between these proteins is essential for transcription.

3.5d The TAF11-TFIIA interaction has critical core promoter functions.

In this study we show that TAF11-mediated enhancement of TFIIA-TBP interactions at a promoter with a non-consensus TATA sequence leads to stabilization of the protein-DNA complex. Cells expressing TAF11 mutations that affect interaction with TFIIA further demonstrate the importance of TAF11-induced stabilization. These strains exhibit defects in transcription when a non-consensus TATA element is utilized. Preferential defects in transcription derived from promoters with non-consensus TATA elements have also been observed upon conditional depletion of TAF11 and TAF13 (70, 86, 87).

The ability of TAF11 to affect promoter associations at weak TATA elements suggests that the TAF11-TFIIA interaction is particularly important when conditions for TBP-TFIIA-DNA complex formation are not optimal. In addition, TFIIA-TBP complex enhancement by TAF11/TAF13 is only observed on a consensus TATA element when concentrations of TBP and or TFIIA are limiting. This suggests that by interaction with TFIIA, TAF11 can increase the effective concentration of TFIIA at the promoter. TAF11 has no effect on TFIIA-TBP-DNA complex formation when concentrations of TFIIA and TBP are in excess. This could account for the absence of dramatic changes to the pattern of TBP and TFIIA interactions with the promoter in photocrosslinking and DNAse I footprinting experiments, in which DNA -protein interactions can only be observed under conditions in which the protein template binding is saturated. Thus, in accord with the model that TAF promoter contacts affect the affinity and specificity of TFIID for certain promoters, TAF11 imposed effects on TFIIA and

TBP promoter interactions provide a similar function in yeast. Moreover, this promoter function is critical when protein concentrations are limiting.

The characterization of the TAF11-TFIIA interaction presented here has clearly defined the regions within both proteins important for this association. Combined with structural information presented in a number of studies, this new data provides the foundation for understanding the molecular architecture and organization of TFIIA-TFIID-promoter complexes. We have demonstrated that TAF11 interaction with TFIIA has a mechanistic role during complex formation, and loss of interaction results in molecular defects that affect cell growth and transcription in vivo. Taken together, our data provide further evidence that TAF11 can serve as a functional link between TFIIA and TFIID.

3.6 Acknowledgments

We thank Song Tan for recombinant TAF11/TAF13 heterodimer and Karolin Luger for creating Figures 3.5A and 3.5B.

Supplemental Figures for Chapter 3

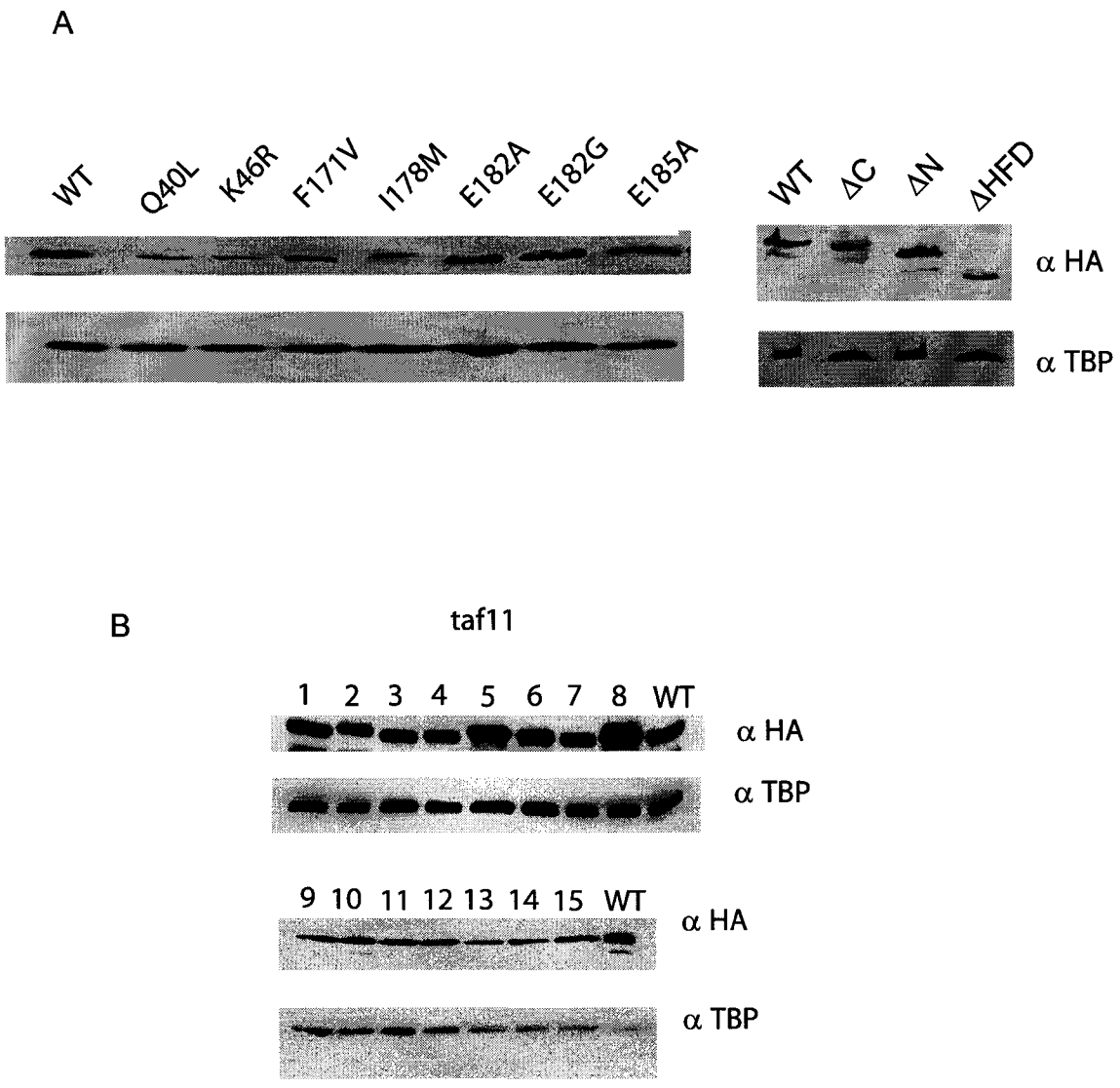

Fig. 3.12 TAF11 protein expression in yeast two hybrid strains expression AD taf11 derivatives (A) or AD- compensatory alleles (B) are comparable to wild type strains. Cells were grown to log phase and whole cell extracts were prepared. 25μg of total protein was separated by 10% SDS-PAGE and transferred to nitrocellulose. Membrane blots were probed with antibodies to HA to detect AD-taf11 fusion protein or with antibodies to TBP to serve as a load control.

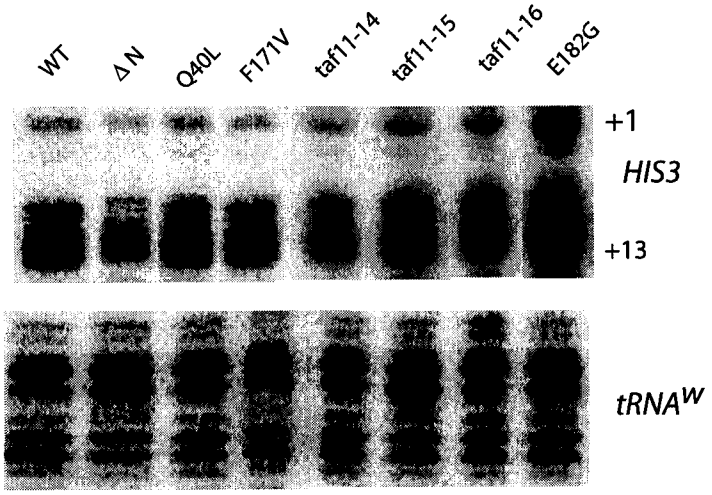

Figure 3.13 Analysis of Gcn4 dependent activation of the *HIS3* transcription in TAF11 derivatives at 38^0C. Wild-type (WT) and the indicated TAF11 derivatives were grown to early log phase at 30^0C. Cells were made heat tolerant by incubation at 38^0C for 15 min, then returned to 30^0C for 1 hour. Cells were then shifted to 38^0C. Gcn4 activated transcription was measured by treating cells with 20mM AT for 1hour prior to harvest and examining levels of *HIS3* (+1 and +13 start sites indicated) and *tRNA*^{W} expression by S1 analysis. Total RNA (25 to 50 μg) was hybridized with excess *HIS3* and *tRNA*^{W} probes and treated with S1 nuclease. The Pol III transcribed gene *tRNA*^{W} served as a loading control.

CHAPTER 4

PERSPECTIVES AND FUTURE DIRECTIONS

The work presented in this dissertation is focused on understanding the mechanistic requirements for TFIIA and TFIID interactions in RNA polymerase II transcription regulation. I have utilized biochemical and genetic analyses to investigate the interaction between TFIIA and TAF11. Through protein-protein interactions TAF11 alters TFIIA-DNA and TBP-DNA contacts at the promoter. TAF11-induced changes lead to an overall increase in TFIIA-TBP-DNA complex formation and stability. It appears this function is particularly important when TFIIA-TBP complex is compromised due to sequence variations at the promoter or when protein concentrations are limiting.

I have also refined our understanding of the intramolecular contacts between TFIIA and TAF11. Isolation of compensatory mutants unambiguously defines the hydrophobic surface of the four-helix bundle domain of TFIIA, as a surface required for interaction with TAF11. In addition, TAF11 compensatory mutations implicate the histone fold domain (HFD) of TAF11 as one surface important for interaction with TFIIA. Although the HFD of TAF11 is used for dimerization with TAF13, the interaction profiles of TAF11 compensatory mutants reveal that the TAF11 interaction with TFIIA and TAF13 need not be mutually exclusive. Analysis of TAF11 compensatory mutants also identified N- terminal substitutions that disrupt interaction with TFIIA. In addition, we found that

deletion of the first N-terminal 73 amino acids of TAF11 (taf11-ΔN) abolishes interaction with TFIIA, but had no effect on the ability of TAF11 to interact with TAF13. As such, our results indicate that in addition to the HFD, the N-terminal region of TAF11 is important for TFIIA interaction as well. Phenotypic and transcription analysis of the taf11-ΔN allele revealed conditional phenotypes and molecular defects at the transcriptional level. The taf11-ΔN derivative is fully functional for TFIID integrity, as indicated by co-immunoprecipitation experiments. This suggests that the conditional phenotypes and transcription defects observed for taf11-ΔN could be attributed to loss of interaction with TFIIA, further indicating the functional importance of this association in vivo.

4.1 TAF11 stabilization of the TFIIA-TBP complex correlates with the ability to support activated transcription.

The work presented in this study shows that the interplay between TFIIA and TAF11 is important for productive association of TFIIA-TBP complexes at promoters. TAF11 induced complex enhancement and stabilization appears to have a greater impact when the conditions for TFIIA-TBP-DNA complex formation are compromised. Our in vitro experiments using the CATAAA promoter fragment allowed us to examine TAF11 stabilization. Determining whether TAF11 promoter functions directly correlate with the ability to support transcription could extend this analysis. These experiments can be performed using a β-galactosidase reporter system previously employed to measure the functional activity of the variant TATAAA promoters. The ability to support

activated transcription driven from the CATAAA promoter could be assayed in yeast strains expressing either wild type TAF11 or the TAF11 derivative, taf11-ΔN. Since the taf11-ΔN is defective for interaction with TFIIA we might expect that this strain would exhibit an impaired response for transcriptional activation when compared with wild type strains.

4.2 Identify novel TAF11 interactions by isolating suppressors of the temperature sensitive phenotype of taf11- ΔN.

The TAF11 derivative taf11-ΔN identified in this study provides a reagent for further study TAF11 functions. In a broader context, since TAF11 appears to be a TFIID specific component, TFIID functions can be explored with this allele. The tight temperature sensitive phenotype exhibited by taf11-ΔN is ideal for use in a genetic screen to select for suppressors. Suppressors would be identified by their ability to grow at the elevated temperature (38^0C). RNA analyses could be used to determine if the suppressing phenotype is manifested at the transcriptional level. Analysis of suppressor strains would identify factors that are important for TAF11 functions in transcription such as chromatin remodeling factors, activators, repressors, or components of the holoenzyme. Further characterization of novel interactions would extend our understanding of the complex regulatory circuitry of RNA polymerase II and define mechanistic requirements for specific interactions with TFIID.

4.3 Investigate the importance of the TAF11-TFIIA in response to oxidative stress response.

Microarray analysis was used to examine the transcription profile of Toa2 allele toa2^{I27K} and this revealed altered expression of genes regulated by the activator Yap1. Yap1 is a bZIP transcription factor involved in the expression of genes required for cellular response to oxidative stress. Since cells expressing toa2^{I27K} are defective for interaction with TAF11 it is possible that the TAF11-TFIIA interaction is required for transcription mediated by Yap1. This is consistent with a coactivator role in which TAFs are required to mediate activator signals to the general transcription machinery. A number of TAF- activator interactions have been reported (reviewed in 14). To investigate this potential connection, strains expressing TAF11 derivatives defective for interaction with TFIIA were screened for oxidative stress phenotypes. Strains were streaked to media containing oxidative stress related compounds, benomyl, hydrogen peroxide, diamide, and cadmium sulfate; however, no growth defects were observed. In this assay, conditional phenotypes reflect global defects in oxidative stress response. It is possible that loss of interaction with TFIIA in TAF11 derivatives causes defects at the molecular level. This could be tested by examining transcription of Yap1 regulated genes in strains expressing taf11-ΔN. David Goldstrohm is investigating the role of TFIIA in Yap1 mediated transcription. The taf11-ΔN derivative strain can be used to determine if the TAF11-TFIIA interaction can be linked to oxidative stress response.

4.4 Reveal novel TFIID functions through characterization of TAF11 mutants defective for interaction with TAF13.

Characterization of the compensatory alleles identified in the genetic screen for compensatory mutants identified 18 alleles that are defective for interaction with TAF13. Additional conditional alleles identified from this collection could be used to study TFIID functions as described for the taf11-ΔN allele.

An interaction between TAF13 and the transcriptional repressor NOT (negative on transcription) complex was recently reported (70). NOT proteins along with other repressors, Mot1, and Spt3 are associated in a complex that serves to regulate TBP function (22). In addition, mutations in NOT proteins cause an increase in transcription at genes with promoters containing non-consensus TATA elements. This evidence in conjunction with identification of an interaction between NOT protein and TAF13 suggest these associations might regulate TBP or TFIID associations with weak or non-consensus promoters. Furthermore, this study and others provide evidence that TAF11 and TAF13 confer promoter functions that are required for transcription at weak promoters (70, 86, 87). The TAF13 and TAF11 alleles identified in this study could be used to investigate the functional relationship between TAFs and the NOT complex proteins and how the interplay between these proteins contribute to the mechanisms required for transcription of genes lacking consensus TATA elements.

4.5 Summary

The work presented in this dissertation provides a solid foundation for advancing our understanding of RNA polymerase II regulation, with a particular emphasis on the interaction between TFIIA and TAF11. The experiments proposed above will extend this analysis and hopefully provide further insights into the functional requirement for this interaction in vivo.

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