Molecular cloning and characterization of dTAF_{II}30 α and dTAF_{II}30 β : two small subunits of *Drosophila* TFIID

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The multisubunit transcription factor TFIID is an essential component of the RNA polymerase II initiation apparatus. Recent studies suggest that TFIID subunits, or TAFs associated with the TATA-binding protein (TBP), play a critical role in modulating transcriptional activation by sequence-specific DNA-binding factors. Thus far, six of the largest TAFs associated with *Drosophila* TFIID have been cloned and partially characterized. Here, we report the molecular cloning, expression, and subunit interaction specificities of two small molecular mass TAFs. Both dTAF_{II}30 α and dTAF_{II}30 β are associated with TFIID via interactions with other TAFs, including dTAF_{II}250, dTAF_{II}150, and dTAF_{II}110. In addition, dTAF_{II}30 α also contacts dTBP. The carboxy-terminal half of dTAF_{II}110 was found to contact a short 67-amino-acid region of dTAF_{II}30 α , which is predicted to form two potential α -helices, one of which is amphipathic. Interestingly, dTAF_{II}30 α also appears to multimerize through its carboxy-terminal region. Although neither dTAF_{II}30 α nor dTAF_{II}30 β have been found to interact with specific activators thus far, it is intriguing that both bind other TAFs such as dTAF_{II}110 and dTAF_{II}150, which are the targets of activation domains. Our studies suggest that both of the small subunits of TFIID play a role in the assembly of the complex and may contribute to the stability of multiple TAF–TAF interactions.

[Key Words: *Drosophila*; TFIID; RNA polymerase II; TAFs; dTAF_{II}30]

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Promoter-dependent transcription by RNA polymerase II requires a set of accessory factors, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (for review, see Zawel and Reinberg 1992). Among these basal transcription factors, TFIID has been of particular interest, because it is thought to be the first factor to enter an initiation complex by binding to the TATA box of template DNA (Buratowski et al. 1989). We now know that TFIID is a complex composed of TBP (TATA-binding protein) and multiple subunits, or TAFs (TBP-associated factors), which are required for mediating transcriptional activation by promoter- and enhancer-binding factors (Dynlacht et al. 1991; Pugh and Tjian 1991, 1992; Tanese et al. 1991; Zhou et al. 1992). To dissect the mechanism of transcriptional activation, we have endeavored to characterize the components and function of the TFIID complex. As a first step we have undertaken the molecular cloning and characterization of the *Drosophila* and human TAFs and have begun to study the protein–protein interactions that enable assembly of the TFIID complex.

Thus far, *Drosophila* TAF_{II}250, TAF_{II}150, TAF_{II}110, TAF_{II}80, TAF_{II}60, and TAF_{II}40 have been cloned and partially characterized (Dynlacht et al. 1993; Goodrich et al. 1993; Hoey et al. 1993; Kokubo et al. 1993; Weinzierl et

al. 1993a,b; P. Verrijzer, L. Attardi, K. Yokomori, J.-L. Chen, and R. Tjian, unpubl.). We have found that dTAF_{II}110 and dTAF_{II}40 interact with the transcriptional activation domains of sequence-specific DNA-binding factors Sp1 and GAL4–VP16, respectively (Goodrich et al. 1993; Hoey et al. 1993), indicating that at least some of the TAFs may serve as targets for specific activators to modulate transcription. Using recombinant proteins, we have also identified specific TAF–TAF interactions that occur during the assembly of the TFIID complex. However, before we can complete the picture, it is important to obtain all of the pieces of this molecular jigsaw puzzle. Our recent biochemical studies of the *Drosophila* TFIID complex revealed the presence of at least two smaller components of ~30 kD, which had remained elusive (Dynlacht et al. 1991; Tanese et al. 1991).

Here, we report the molecular cloning, expression, and partial characterization of the specific interactions involving these remaining small subunits, dTAF_{II}30 α and dTAF_{II}30 β . Our results suggest that each of these two TAFs joins the TFIID complex through interaction with one or more of the larger subunits and that only dTAF_{II}30 α may be in contact with TBP. Analysis of mu-

tant proteins reveals a short potentially α -helical domain in dTAF_{II}30 α that is necessary and sufficient for interacting with dTAF_{II}110, whereas dTAF_{II}30 β makes contact with dTAF_{II}150. In both of these cases, it is possible that the smaller subunits may be serving a function as stabilizers as well as transmitters of signals from activators to other components of the transcriptional machinery. Our studies suggest that there are multivalent networks of contacts between TAFs and TBP that help to form a stable complex that then acts as a modulator of transcriptional activation.

Results

Purification, cloning, and expression of dTAF_{II}30 α and dTAF_{II}30 β

Drosophila TFIID was purified from 0- to 14-hr embryos by anti-dTBP antibody affinity chromatography (Dylacht et al. 1989; Wampler et al. 1990). Nuclear extracts from 800 grams of embryos were used to isolate ~ 100 μ g of the TBP-TAF complex. The purified subunits were separated by SDS-PAGE and transferred to PVDF membrane. The region corresponding to polypeptides with an apparent molecular mass of 30 kD was excised from the membrane and digested with trypsin. After purification of individual peptides by reversed-phase HPLC, they were subjected to microsequencing. This 30-kD region of the gel contained at least three closely migrating polypeptides, which we assumed were distinct gene products (Fig. 1). Therefore, in designing hybridization probes, we used intrapeptide PCR or single oligonucleotide guessmers to screen a cDNA library derived from *Drosophila* embryo mRNA (Poole et al. 1985). Using a PCR product from peptide α (see Fig. 2A) as a probe, 10 related clones

Figure 1. The *Drosophila* TFIID complex contains multiple proteins in the 30-kD range. *Drosophila* TFIID complex was immunopurified from the embryo nuclear extracts with anti-dTBP antibody. TAFs were then eluted with guanidine and TCA precipitated. The polymerase II TAFs are indicated. (Lane 1) The IgG heavy-chain and dTBP remaining on the beads after elution; (lane 2) the TAF pattern in the complex. The multiple bands in the 30-kD range, from which the peptide sequences were obtained, are shown at higher resolution.

were isolated and the longest one consisting of 1.2 kb was chosen for further study. Northern blot analysis indicated that this clone ($\lambda\alpha$) is derived from a 1.4-kb mRNA species (data not shown). The DNA sequence of $\lambda\alpha$ revealed an open reading frame of 196 amino acids preceded by a stop codon in the same frame, confirming that we had most likely obtained the complete coding region (Fig. 2A). Three other peptide sequences obtained from the 30-kD region of the PVDF membrane were also found within this coding region (Fig. 2, underlined sequences), confirming that $\lambda\alpha$ encodes one of the 30-kD polypeptides. The amino acid sequence deduced from the DNA sequence predicts a protein with a calculated molecular mass of 21.6 kD and an estimated isoelectric point (pI) of 8.8.

A protein data base search did not uncover any genes with homology to our clone. To further characterize the product of $\lambda\alpha$, we have used both *Escherichia coli* and baculovirus expression systems to produce the recombinant protein. The bacterially expressed product was subsequently used to generate polyclonal antibodies in rabbits. Western blot analysis confirmed that antibodies against the $\lambda\alpha$ product specifically recognized a 30-kD protein in the dTFIID complex isolated by anti-dTBP affinity chromatography (Fig. 3A, lane 1). Furthermore, the baculovirus-expressed recombinant protein migrated with a mobility indistinguishable from that of the endogenous protein (Fig. 3A, lane 2). To ascertain that this clone encodes a bona fide TAF, we used antibodies against dTAF_{II}80 and dTAF_{II}250 to purify the dTFIID complex and confirmed the presence of a 30-kD protein in these complexes by Western blot analysis using $\lambda\alpha$ antisera (Fig. 3A, lanes 3–5). In addition, we used anti- $\lambda\alpha$ antibody to immunoprecipitate the TFIID complex and established the presence of other TAFs, such as dTAF_{II}40, by Western blot analysis (Fig. 3A, lane 6). These results, taken together, indicate that $\lambda\alpha$ encodes a 30-kD protein that is a subunit of TFIID; therefore, we have designated this clone as dTAF_{II}30 α . In situ hybridization mapped the dTAF_{II}30 α gene to position 86F1, 86F2 on the right arm of chromosome 3.

Because several of the peptide sequences that we had obtained were not included within the deduced amino acid sequence of dTAF_{II}30 α , we assumed that they must be derived from a separate gene product. We therefore designed a guess-mer oligonucleotide based on peptide β (see Fig. 2B) and rescreened the same λ gt10 library. We obtained one unique isolate ($\lambda\beta$) with a 750-bp insert. After DNA sequence analysis, peptide β and one other peptide were found within the 196-amino-acid open reading frame of $\lambda\beta$ (Fig. 2B, underlined sequences). The amino acid sequence deduced from the DNA sequence predicted a protein with a calculated molecular mass of 22.1 kD and an estimated pI of 4.7. There was no similarity to dTAF_{II}30 α , and no genes with homology to our clone were found in a data base search. A recombinant protein encoded by $\lambda\beta$ was expressed in *E. coli*, and it was used to generate polyclonal antibodies. To test whether the $\lambda\beta$ protein is part of the TFIID complex, we purified endogenous dTFIID by immunoprecipitation

A

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CCAAAAATCGCCCACTTACTGTACTTCCCAAACTTCAACCAACCGACCTACCAACCACTTGATTGACTCTGAA 81
AGAAACCCAAAAGCAATGTCGGATCTTTTACCCTTTCGATAGCAACGGCGTCGCGAGGCACCACTGCACCACAACCAC 162
      M S D L F T T F D S N G V A R H H L H N H (22)
AACTCCACATCGTCGCCACGGAGTGTCCACGACCCACCATGGCTCGCCCTCCAGCAGCTCCGATGACCAACAAAC 243
      N S T S S A S G L L H D P P M A S P S Q H S P M T N N (49)
AGCAACTCATCTCGCAGAACGGGACCGGTTTCCGTTTGGGTACGGAGCGGCCCATATCTGTTGGTAGCAAGTCA 324
      S N S S S Q N G G P V S G L G T G T G P I S G G S K S (76)
TCCAATCACACATCATCGCCCGCGGTTCGAGAACACTCCCATGCTTACCAACCGCGTCTCAGAGCTCGTCCGAGAG 405
S N H T S S A A G S E N T P M L T K P R L T E L V R E (103)
PEP  $\alpha$ 
GTGGATACCAACGACGAGTGGAGGAGTGTGAGGAGCTTCTGCTCAGATCATCGACGACTTGTGAGGGACACCGTC 486
      V D T T T T S A S G L L H D P P M A S P S Q H S P M T N N (130)
AAGTCGACGAGCGCTTCGCGAACGACGAAAGTCTAACAAGATCGAGGTGCGCGACGTGCAGCTTTGAGCGGAAG 567
      K S T S A F A K H R K S N K I E V R D V O L H F E R K (157)
TACAACATGTGGATACCGGCTTCGGTACGACGAAGTGGTCCCTACAAGCGGCGAGCTGTACGAGGCGCACAAACAG 648
Y N M W I P G F G T D E L R P Y K R A A V T E A H K O (184)
CGCCTTGCCCTCATACGGAACGATCAAGAACTACTAGAGGATGGATCTAATCGGGTCGAGGCTCTGTTTCGGTTGCC 729
R L A L T R K T I K K Y * (196)
GGATTTCGCGTATGTAAACGTGCACACGCCACAACTAATTTAAGCTCCAATTTAGATTAAATAACAAATTATCGTCGCT 810
CTATTGTAGATTATTGTAATAAAGTGCATATTGATTTCACATTCAAAAAATATATATATATATATATATATATATAT 863

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B

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CCCGATTTTTTTTAAATGGACGAATCTCTTCCACGCGAGCAAAAGAGCAACTCCCTAAGCGACGGGACGATGTCGAC 81
      M D E I L F P T Q Q K S N S L S D G D D V D (22)
CTGAAATTTCTCCAGTCGGCTCCGCGAGCGAAAGACAGCGACCTCGGATCCGGGAAACGATGCGGATCGTGATGCG 162
      L K F F Q S A S G E R K D S D T S D P G N D A D R D G (49)
AAAGATGCGGATGGGACACGACAAACAAGAACGAGCGAGATGTTGACTCTGGCGAGCGCGCACAAAAGCTCAAA 243
      K D A D G D N D N K N T D G D G D S G E P A H K K L K (76)
ACCAAGAAGGAAGTGGAGGAGGAGGCGCGAAGTGCAGGTTCTCGTTTCCAACTTTACTGAAGAAGCGTGGATGCG 324
      T K K E L E E E R E R M Q V L V S N F T E E Q L D R (103)
TACGAAATGTATCGTCGTCAGCTTTCCTCAAGCGCGGTCAAGCGTCTAATGAACTATCACCGGCTGTTCCGTGTC 405
      Y E M Y R R S A F P K A A V K R L M Q T I T G C S V S (130)
CAAAATGTTGTATAGCCATGTCGCGATTGCGAAGTCTCTCGTCGCGAGGTTGTGGAGGAACCCCTCGACGTGATGGAG 486
      Q N V V I A M S G I A K V F V G E V V E E A L D V M E (157)
PEP  $\beta$ 
GCCCAAGTGAATCCGGTGGCTGACGCCCAATTCATACGAGAGGCGAGTGCAGCAGTGGAGGACCAAGGATCGGATGCC 567
A O G T S G A L O P K F I R E A V R R L R T K D R M P (184)
ATAGGCAGATACGACGCGCTATTTCAGACTGAAGTACGAGTGCAGACATTAGAAATATAGTTTGAATCTGTTAGT 648
      I G R Y O O P Y F R L N * (196)
GAATATAAAATACATAAACAAGTAAAGTAAATAAATAAAGATTTTTCAGAAAAAATATATATATATATATATATAT 720

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Figure 2. DNA and amino acid sequences of dTAF_{II}30 α and dTAF_{II}30 β . Nucleotide and deduced amino acid sequences of dTAF_{II}30 α (A) and dTAF_{II}30 β (B) cDNA clones. Peptide sequences obtained from microsequencing of the purified polypeptides are underlined. Peptide α (A) and peptide β (B) are the peptide sequences used to generate probes for screening the *Drosophila* cDNA library.

with either anti-dTAF_{II}250 or anti-dTBP antibody. Western blot analysis with anti- $\lambda\beta$ antibody detected a protein in the purified TFIID complex that migrates in SDS-polyacrylamide gels with a mobility indistinguishable from that of the recombinant $\lambda\beta$ product (Fig. 3B, lanes 1–3), indicating that most likely we obtained a full-length clone. Furthermore, antibodies against $\lambda\beta$ were used to immunoprecipitate the TFIID complex, and the presence of other TAFs, such as dTAF_{II}40, was confirmed by Western blot analysis (Fig. 3B, lanes 4,5). These results established that $\lambda\beta$ is also a 30-kD TAF associated with the TFIID complex, and this clone was designated dTAF_{II}30 β .

dTAF_{II}30 α interacts with a carboxy-terminal domain of dTAF_{II}110

To investigate the relationship between dTAF_{II}30 α and other TAFs in the TFIID complex, the interaction of the recombinant protein with individual TAFs and TBP was

tested. A hemagglutinin antigen (HA)-tagged version of dTAF_{II}30 α expressed in *E. coli* was immobilized on protein A-agarose beads containing anti-HA antibody. This affinity resin was used to bind radiolabeled in vitro-translated dTAF_{II}30 β , dTAF_{II}60, and dTAF_{II}110 (Fig. 4A), as well as dTAF_{II}40, dTAF_{II}80, dTAF_{II}150, dTFIIA-L (Yokomori et al. 1993), and dTFIIB (data not shown). Among the TAFs and general transcription factors tested, dTAF_{II}110 showed a strong interaction with dTAF_{II}30 α , whereas none of the other components displayed significant binding (Fig. 4A, lanes 1–9). To confirm the interaction of dTAF_{II}30 α with dTAF_{II}110, HA-dTAF_{II}110 expressed in recombinant baculovirus-infected cells was immobilized on beads and mixed with radiolabeled dTAF_{II}30 α . As expected, dTAF_{II}30 α bound efficiently to HA-dTAF_{II}110 on the beads (Fig. 4A, lanes 11,14,15). We noted a 20-kD species present in the in vitro translation products of dTAF_{II}30 α that is similar in size to a polypeptide sometimes observed in preparations of dTFIID (Fig. 3A, lane 1). These results and antibody cross-reactivity suggest that this species may arise as a

Figure 3. Antibodies directed against recombinant dTAF_{II}30α and dTAF_{II}30β recognize proteins with the same mobility in the dTFIID complex. (A) Western blot analysis of the recombinant and endogenous dTAF_{II}30α in the TFIID complex. The endogenous TFIID complex (lane 1) and the recombinant protein expressed in insect cells (lane 2) were compared by Western blot analysis using anti-dTAF_{II}30α antibody. A cross-hybridizing smaller species of ~20 kD in the TFIID complex is indicated as Δ30α. Nuclear extract from *Drosophila* embryo was immunoprecipitated with a control antibody (lane 3), anti-dTAF_{II}80 antibody (lane 4), and anti-dTAF_{II}250 antibody (lane 5)—the latter two were shown to immunoprecipitate dTFIID complex (Dymlach et al. 1993)—and the presence of dTAF_{II}30α was detected by Western blot analysis using anti-dTAF_{II}30α antibody. The antibody directed against dTAF_{II}30α was used to immunoprecipitate the TFIID complex, and dTAF_{II}40 was detected using anti-dTAF_{II}40 antibody (lane 6). (B) Western blot analysis of the recombinant and endogenous dTAF_{II}30β in the TFIID complex. Recombinant dTAF_{II}30β expressed in *E. coli* (lane 1) was compared with the proteins present in the TFIID complex immunoprecipitated by anti-dTAF_{II}250 (lane 2) or anti-dTBP (lane 3) antibodies by Western blot analysis using anti-dTAF_{II}30β antibody. Nuclear extract from *Drosophila* embryo was immunoprecipitated either with preimmune rabbit sera (lane 4) or anti-dTAF_{II}30β antibody (lane 5), and the immunoprecipitated dTFIID was analyzed by Western blot using anti-dTAF_{II}40 antibody. Cross-reacting IgG heavy chain is indicated as IgH.

consequence of translation initiation from an internal ATG. An amino-terminal deletion mutant initiating from the second methionine [Δ30α (46–196)] comigrates with this small species (Fig. 4A, lanes 10,11). As illustrated in Figure 4A, this truncated product also appears to interact selectively with dTAF_{II}110.

We showed previously that dTAF_{II}110 acts as a coactivator for Sp1 through interactions with the amino-terminal region of dTAF_{II}110 (Hoey et al. 1993; Weinzierl et al. 1993a; G. Gill, E. Pascal, Z. Tseng, and R. Tjian, unpubl.). It was therefore of interest to map the domain in dTAF_{II}110 responsible for dTAF_{II}30α interaction to see whether the same or different regions of dTAF_{II}110 are involved in multiple contacts. As shown in Figure 4B, dTAF_{II}30α interacted with the carboxy-terminal half of dTAF_{II}110 but not with the amino-terminal region. Deletion of 69 amino acids from the carboxyl terminus in dTAF_{II}110 abolished this binding (Fig. 4B, lanes 3,9,10, see diagram), suggesting that this region is important for the interaction. These results indicate that the interface for binding dTAF_{II}30α lies between amino acid 572 and 921 of dTAF_{II}110, which is a region distinct from the activator contact domain for Sp1.

dTAF_{II}30α interacts with dTAF_{II}110 through a 67-amino-acid region with predicted α-helix potential

To further characterize the interaction of dTAF_{II}30α with dTAF_{II}110, a deletion analysis of dTAF_{II}30α was

performed. A series of carboxy-terminal deletion mutants was generated by in vitro runoff transcription and translation in the presence of [³⁵S]methionine in rabbit reticulocyte lysate. These mutants were then tested for their ability to interact with immobilized HA-dTAF_{II}110 (Fig. 5A). A deletion mutant (1–158), in which 38 amino acids were deleted from the carboxyl terminus, bound efficiently to HA-dTAF_{II}110 (Fig. 5A, lanes 1–6), whereas further deletions abolished the interaction (Fig. 5A, lanes 7–18). To narrow down the binding domain, an amino- and carboxy-terminal double-deletion mutant (91–158) containing 67 amino acids was tested (Fig. 5B). This mutant protein interacted with HA-dTAF_{II}110 as efficiently as the full-length product (Fig. 5B, lanes 1–3), whereas two other proteins tested, HA-dTAF_{II}30α and HA-dTAF_{II}30β, did not bind this fragment, demonstrating the specificity of the interaction. Interestingly, a Chou–Fasman and Robson–Garnier computer analysis predicted that this 67-amino-acid dTAF_{II}110-binding domain potentially forms two α-helices (Fig. 5C,D). We have initiated an investigation into the structure of this putative domain. Inspection of the sequence reveals that it is composed of ~30% hydrophobic residues and contains a potential five-turn amphipathic helix (Fig. 5D). Preliminary circular dichroism spectra of a synthetic 63-amino-acid peptide corresponding to dTAF_{II}30α amino acids 100–163 indicate >75% α-helix content. These results indicate that this short potentially α-helical region within the carboxy-terminal half of dTAF_{II}30α is sufficient to interact with dTAF_{II}110.

Figure 4. Recombinant dTAF_{II}30 α interacts with the carboxyl terminus of dTAF_{II}110. (A) dTAF_{II}30 α selectively interacts with dTAF_{II}110. HA-dTAF_{II}30 α expressed in *E. coli* was immobilized on anti-HA protein A beads and tested for binding to radiolabeled dTAF_{II}30 β , dTAF_{II}60, and dTAF_{II}110. (Lanes 1–3) The input proteins. Equal amounts of radiolabeled proteins were incubated with beads containing anti-HA antibody alone (lanes 5,7,9) or loaded with HA-dTAF_{II}30 α (lanes 4,6,8). HA-dTAF_{II}110 expressed in baculovirus was immobilized on beads with anti-HA, and binding of the radiolabeled full-length dTAF_{II}30 α or amino-terminal deletion mutant [Δ 30 α (49–196)], which initiates at the second methionine, was tested in the presence (lanes 12,14) or absence (lanes 13,15) of HA-dTAF_{II}110. The input materials are shown in lanes 10 and 11. (B) The carboxyl terminus of dTAF_{II}110 is necessary and sufficient to bind dTAF_{II}30 α . HA-dTAF_{II}30 α was immobilized on beads with anti-HA, and the binding of radiolabeled dTAF_{II}110 mutants was determined. The results of the deletion analysis are summarized. The structure of full-length dTAF_{II}110 was described previously (Hoey et al. 1993). The region on dTAF_{II}110 required for dTAF_{II}30 α binding is indicated.

dTAF_{II}30 α can interact with itself through a carboxy-terminal region distinct from the dTAF_{II}110 interaction domain

In the process of testing TAFs for interactions with dTAF_{II}30 α , we discovered that dTAF_{II}30 α can form homomultimers (Fig. 6A, lanes 1,3,4). We therefore also attempted to map the putative multimerization domain of dTAF_{II}30 α . The same set of deletion mutants used previously to study dTAF_{II}30 α –dTAF_{II}110 interactions was assayed for binding to HA-dTAF_{II}30 α . Figure 6A reveals that none of the carboxy-terminal deletion mutants bound to immobilized HA-dTAF_{II}30 α , including the mutant (1–158) that interacted efficiently with dTAF_{II}110 (Fig. 5A, lanes 2,5,6). In addition, the minimal dTAF_{II}110-binding domain, the double-deletion mutant (91–158), also did not interact with dTAF_{II}30 α (Fig. 5B). These results indicate that the dTAF_{II}30 α –dTAF_{II}30 α interaction requires the carboxy-terminal 38 amino acids, a highly basic region (Fig. 6B). Together, these results show that the binding domains in dTAF_{II}30 α for

dTAF_{II}110 and for dTAF_{II}30 α are different and that dTAF_{II}30 α dimerization is not required for the interaction of dTAF_{II}30 α with dTAF_{II}110.

Multiple interactions by dTAF_{II}30 α with TAF/TBP complex

We established previously that dTAF_{II}250 interacts with dTAF_{II}110 and that this largest subunit of TFIID provides surfaces for binding several other TAFs as well as TBP (Weinzierl et al. 1993a,b; P. Verrijzer, L. Attardi, K. Yokomori, J.-L. Chen, and R. Tjian, unpubl.). Because the dTAF_{II}110–dTAF_{II}30 α interaction was observed in vitro in the absence of other components of the TFIID complex, we were prompted to dissect this interaction in relation to the other subunits of the TAF/TBP complex, particularly dTBP and dTAF_{II}250, which appear to form a scaffold for the other components (Dynlacht et al. 1993; Weinzierl et al. 1993a). We therefore tested the interaction of dTAF_{II}30 α with *Drosophila* TBP, human

Figure 5. A 67-amino-acid region in dTAF_{II}30 α is sufficient for dTAF_{II}110 binding. (A) Radiolabeled carboxy-terminal deletion mutants of dTAF_{II}30 α were tested for their ability to bind HA-dTAF_{II}110 immobilized on beads. (Lanes 1,2,7–10) The input of dTAF_{II}30 α deletion mutants. The presence (+) or absence (–) of HA-dTAF_{II}110 on beads is indicated. (B) A double-deletion mutant of dTAF_{II}30 α (91–158) containing 67 amino acids was used to test the interaction with HA-dTAF_{II}110, HA-dTAF_{II}30 α , and HA-dTAF_{II}30 β on beads. (Lane 1) Input radiolabeled dTAF_{II}30 α mutant (91–158). The same amount of mutant protein was incubated with either anti-HA antibody alone (lane 2), HA-dTAF_{II}110 (lane 3), HA-dTAF_{II}30 α (lane 4), or HA-dTAF_{II}30 β (lane 5) bound to anti-HA protein A-agarose beads. (C) The results of the deletion analysis are summarized in a diagram; the minimal dTAF_{II}110-binding domain is indicated. Regions within dTAF_{II}30 α that are rich in certain amino acids (representing >25% of the residues within the region), including basic, acidic, serine-rich, and glycine-rich domains, are indicated. (D) A predicted secondary structure of a 67-amino-acid dTAF_{II}110-binding domain in dTAF_{II}30 α . Two potential α -helices based on the Chou–Fasman and Robson–Garnier computer analysis are indicated by stippled boxes, helix 1 and helix 2. Within helix 1, a helical wheel projection and a roll-out model show a potential amphipathic α -helix. Hydrophobic amino acids are indicated by solid boxes.

TAF_{II}250 (Ruppert et al. 1993), or *Drosophila* TAF_{II}250 by coimmunoprecipitation using dTBP or TAF_{II}250 immobilized on beads. dTAF_{II}30 α was found to interact with both dTBP and TAF_{II}250 from both human and *Drosophila* (Fig. 7A). To confirm these interactions, complexes formed in vivo by coinfection of recombinant baculoviruses were immunoprecipitated (Fig. 7B). Baculovirus expressing dTBP or a truncated dTAF_{II}250 was coinfecting into Sf9 cells with virus-expressing dTAF_{II}30 α . The resulting complexes were immunoprecipitated by either anti-dTBP or anti-dTAF_{II}250 antibody. Western blot analysis revealed that dTAF_{II}30 α formed complexes with both dTBP and dTAF_{II}250 in vivo. The extracts from cells infected with only dTAF_{II}30 α failed to be precipitated by these antibodies (Fig. 7B, lanes 1,3). The expression of dTAF_{II}30 α was comparable in all infections as confirmed by Western blot analysis (data not shown).

These results indicate that dTAF_{II}30 α is able to interact with dTBP and dTAF_{II}250 as well as human TAF_{II}250.

Our results indicate that dTAF_{II}110 does not interact with dTBP (Weinzierl et al. 1993a), whereas dTAF_{II}30 α interacts with both dTBP and dTAF_{II}110. Therefore, we also attempted a coinfection with viruses expressing all three proteins to test whether dTAF_{II}30 α could tether dTAF_{II}110 to dTBP and mediate the formation of a triple complex. Cell lysates were prepared from dTAF_{II}110 virus-infected cells, cells coinfecting with the dTAF_{II}110 and dTBP viruses, or triply infected cells with dTAF_{II}110, dTBP, and dTAF_{II}30 α viruses. Figure 7C shows that dTAF_{II}110 was coimmunoprecipitated efficiently by anti-dTBP only when dTAF_{II}30 α was coexpressed (Fig. 7C, lane 3). This result supports the notion that interactions among dTAF_{II}30 α , dTAF_{II}110, and dTBP are not mutually exclusive. Instead, these three

Figure 6. dTAF_{II}30 α can multimerize. (A) HA-dTAF_{II}30 α was immobilized on beads, and the same set of dTAF_{II}30 α deletion mutants used in Fig. 5 were tested for their ability to bind full-length dTAF_{II}30 α . (Lanes 1,2,7,8,9) The input radiolabeled proteins. (B) The results of the binding assays are summarized in the diagram. The region required for the 30 α –30 α association is indicated.

proteins appear to form a triple complex. Most of the 67-amino-acid minimal domain in dTAF_{II}30 α involved in dTAF_{II}110 binding could be removed (the resulting mutant contains amino acids 1–109) without affecting dTBP binding (data not shown), indicating that the binding domains within dTAF_{II}30 α for dTAF_{II}110 and for dTBP are distinct. Interestingly, deleting an additional 10 amino acids from the carboxyl terminus (1–99), which correspond to the extreme amino terminus of the minimal dTAF_{II}110-binding domain, abolished the dTBP interaction (data not shown). The deletion within this region disrupts a hypothetical α -helix, indicating the presence of a potentially unique structural feature within this region of dTAF_{II}30 α that may be involved in binding dTBP. We also attempted to build partial complexes on HA-hTAF_{II}250 immobilized on beads, by step wise addition of the crude extracts from recombinant baculovirus-infected cells expressing dTBP, dTAF_{II}110, and dTAF_{II}30 α . The silver-stained gel of this immunoprecipitated complex (Fig. 7D) indicates that dTBP, dTAF_{II}110, and dTAF_{II}30 α can be immunopurified by HA-TAF_{II}250 immobilized on beads by anti-HA antibody. These results indicate that the interactions between these TAFs and dTBP are highly specific and that dTAF_{II}30 α may participate in stabilizing TBP/TAF complexes by bridging multiple proteins.

dTAF_{II}30 β interacts with dTAF_{II}150 and dTAF_{II}250

Although dTAF_{II}30 β has not been studied as extensively as dTAF_{II}30 α , we found that a recently cloned large TAF, dTAF_{II}150, was able to interact selectively with dTAF_{II}30 β but not with dTAF_{II}30 α (data not shown). Figure 8 shows the interaction between dTAF_{II}150 and dTAF_{II}30 β . When either HA-dTAF_{II}30 β or dTAF_{II}150 immobilized on beads is incubated with the other, its interaction partner can be specifically retained on the beads and detected by Western analysis. Preliminary studies suggest that dTAF_{II}30 β may also interact with dTAF_{II}250 but not with dTBP. When an amino-terminal truncated version of dTAF_{II}250 or full-length dTBP is immobilized on beads, dTAF_{II}250 but not the dTBP resin

selectively binds dTAF_{II}30 β (Fig. 8, lanes 3–6). These results reveal that dTAF_{II}30 β displays TAF-binding properties quite distinct from that of dTAF_{II}30 α .

Discussion

The discovery that TAFs are required for transcriptional activation has instigated a detailed analysis of the TFIID complex and its associated subunits. One of our objectives is to reconstitute a fully functional TFIID complex with purified and well-defined recombinant proteins representing each TAF. During this past year we have cloned and partially characterized six *Drosophila* TAFs, including dTAF_{II}250, dTAF_{II}150, dTAF_{II}110, dTAF_{II}80, dTAF_{II}60, and dTAF_{II}40. As hypothesized previously, some of these subunits appear to mediate specific interactions between the transcriptional machinery and sequence-specific activators (Goodrich et al. 1993; Hoey et al. 1993; P. Verrijzer, L. Attardi, K. Yokomori, J.-L. Chen, and R. Tjian, unpubl.). In addition, interactions between TAFs and TBP have also been identified that are likely to enable assembly of the TFIID complex. During this process of analyzing TFIID components, we have noticed a cluster of at least three proteins of ~30 kD that copurify with the TFIID complex and that may be additional TAFs. Although we cannot exclude the possibility that other components of TFIID exist that we have failed to detect thus far, the 30-kD TAFs are some of the last TAFs that had been identified but remained to be cloned and characterized (Dynlacht et al. 1991; Tanese et al. 1991). Interestingly, we recently discovered that one of the three proteins in this molecular mass range is a proteolytically processed fragment of the large subunit of dTFIIA (Yokomori et al. 1993). In this paper we describe the sequence analysis and properties of the two remaining 30-kD proteins, dTAF_{II}30 α and dTAF_{II}30 β . These have been confirmed to be TAFs as defined by their tight association with the TFIID complex and by their ability to recognize and bind specifically to other TAFs or TBP. Moreover, antibodies directed against both dTAF_{II}30 α and dTAF_{II}30 β selectively immunoprecipitate the entire dTFIID complex.

Figure 7. dTAF_{II}30α tethers dTAF_{II}110 to dTBP and interacts with dTAF_{II}250. (A) TAF_{II}30α expressed by recombinant baculovirus was tested for its ability to bind either dTBP or TAF_{II}250 from human or *Drosophila*. Either anti-HA antibody alone (lane 2), HA-hTAF_{II}250 (lane 3), or HA-dTBP (lane 4) on anti-HA protein A beads were incubated with dTAF_{II}30α. In lanes 5 and 6, the protein A beads were incubated with anti-dTAF_{II}250 mAb and mixed with dTAF_{II}30α either in the presence (lane 5) or in the absence (lane 6) of the amino-terminal truncated dTAF_{II}250. In lanes 7 and 8, protein G beads were incubated with anti-dTBP mAb and incubated with dTAF_{II}30α in the presence (lane 7) or absence (lane 8) of dTBP. The presence of dTAF_{II}30α was detected by Western blot using anti-dTAF_{II}30α antibody. In lanes 7 and 8, a background cross-reaction of protein G, migrating slightly above dTAF_{II}30α, is indicated. (B) In vivo assembly of bipartite complexes containing dTAF_{II}30α and either dTBP or dTAF_{II}250. Sf9 cells were coinfecting with baculovirus-expressing dTAF_{II}30α and the second virus expressing either dTBP or dTAF_{II}250ΔN, and the cell lysates were immunoprecipitated with anti-dTBP antibody (lane 2) or anti-dTAF_{II}250 antibody (lane 4). The lysate was also prepared from cells infected with dTAF_{II}30α alone and immunoprecipitated with anti-dTBP antibody (lane 1) or anti-dTAF_{II}250 antibody (lane 3). The presence of dTAF_{II}30α was detected by Western blot analysis. (C) Triple complex formation was examined by coinfection of dTAF_{II}110-expressing baculovirus with either no virus (lane 1), dTBP-expressing virus (lane 2), or both dTBP- and dTAF_{II}30α-expressing viruses (lane 3). The lysates were immunoprecipitated by anti-dTBP antibody, and the presence of dTAF_{II}110 was detected by Western blot using anti-dTAF_{II}110 antibody. Cross-reaction of IgG heavy and light chain is indicated as IgH and IgL. (D) Silver staining of the in vitro-assembled partial TBP/TAF complex. HA-hTAF_{II}250 was immobilized on protein A-Sepharose beads (Pharmacia) containing anti-HA antibody. Beads were then incubated sequentially with Sf9 cell extracts infected with recombinant-expressing dTBP, dTAF_{II}110, and dTAF_{II}30α. Beads were washed thoroughly after each incubation. The bound proteins were eluted with the HA peptide (YPYDVPDYA), and the eluted complexes were resolved by SDS-PAGE followed by silver staining. The arrow indicates the upper band among two in the 30-kD region as dTAF_{II}30α. The lower band is a contaminant, the presence of which is not dependent on the addition of dTAF_{II}30α extract (data not shown).

So far, all the TAFs cloned were tested for their ability to incorporate into the partial complexes assembled in vitro, but the analysis of each interaction has not been carried out in detail. We believe, however, that to understand the function and assembly of the TFIID complex, it is important to determine the binding specificity of each component. Thus, in this paper we have analyzed dTAF_{II}30α interactions with other components of the TAF/TBP complex. We found that dTAF_{II}30α binds most efficiently to dTAF_{II}110 and can multimerize with itself. Deletion analysis mapped a small region in the carboxyl terminus of dTAF_{II}30α that is sufficient for dTAF_{II}110 interaction, whereas other regions distinct from this dTAF_{II}110-binding domain appear to be involved in dTAF_{II}30α–dTAF_{II}30α and dTAF_{II}30α–dTBP interactions. The 67-amino-acid minimal interface for dTAF_{II}110 binding is predicted to form α-helices, one of

which is amphipathic. As expected, dTAF_{II}30α interacts with a domain of dTAF_{II}110 distinct from its coactivator regions.

In addition to dTAF_{II}110, dTAF_{II}30α also appears to interact with dTAF_{II}250 and dTBP, whereas dTAF_{II}30β interacts with dTAF_{II}150 and dTAF_{II}250 but not with dTBP. It is intriguing that these relatively small subunits make multiple contacts. Our findings strengthen the notion that although individual TAF–TAF or TAF–TBP interactions may be relatively unstable, by simultaneously contacting multiple proteins, considerably more robust complexes may be formed, which are reminiscent of the highly stable TFIID complex.

Figure 9 summarizes all of the known TAF–TAF or TAF–TBP interactions identified so far. We note, however, that these figures do not imply that the order of assembly has been determined but rather that multiple

Figure 8. dTAF_{II}30 β interacts with dTAF_{II}150 and dTAF_{II}250. The binding of dTAF_{II}150 was examined on beads containing HA-dTAF_{II}30 β with anti-HA antibody (lane 1) or beads with anti-HA antibody alone (lane 2), and the presence of dTAF_{II}150 was detected by Western blot using polyclonal antibodies directed against dTAF_{II}150. The amino-terminal truncated dTAF_{II}250 (dTAF_{II}250 Δ N), dTBP, and dTAF_{II}150 were immobilized on beads by antibodies specific to each protein and incubated with dTAF_{II}30 β (lanes 3, 5, and 7, respectively). Beads containing each antibody alone were also incubated with dTAF_{II}30 β (lanes 4, 6, and 8, respectively). The bound protein was analyzed by Western blot using anti-dTAF_{II}30 β antibody.

TAF-TAF and TAF-TBP contacts have been identified. For convenience, incorporation of the larger TAFs is shown first, followed by the small subunits. Although the dTAF_{II}30 β -dTAF_{II}150 interaction has not been characterized extensively, we postulate that these smaller

TAFs may modulate or accentuate the coactivator function of dTAF_{II}110 and dTAF_{II}150. Alternatively, these smaller TAFs might serve as cross bridges to strengthen the ties between coactivator subunits such as dTAF_{II}110 and other subunits such as dTAF_{II}250. Finally, it is quite possible that both dTAF_{II}30 α and dTAF_{II}30 β could also serve as targets of specific activators that have yet to be identified.

Materials and methods

Purification of TFIID and peptide sequencing of the 30-kD proteins

Nuclear extracts from 800 grams of 0- to 14-hr embryos were precipitated with polyvinyl alcohol (PVA) as described previously (Dynlacht et al. 1989; Wampler et al. 1990). TFIID was affinity purified directly from PVA extracts or from a Q-Sepharose fraction (Wampler et al. 1990) using affinity-purified anti-dTBP antibody (Dynlacht et al. 1991). Beads with bound TFIID were washed with HEMG buffer [25 mM HEPES (pH 7.5), 12.5 mM MgCl₂, 0.1 mM EDTA, 10% (vol/vol) glycerol] containing 0.1 M KCl, 0.1% NP-40, and 1 mM DTT, then with the same buffer containing 0.5 M KCl, and eluted with 1 M guanidine-HCl. The guanidine-eluted TAFs were dialyzed against HEMG buffer containing 0.1 M KCl, TCA precipitated, and resolved by SDS-PAGE. The proteins were transferred to PVDF membrane and stained with Ponceau S. Proteins in the 30-kD size range were excised from the membrane and digested with trypsin. The eluted peptides were fractionated by reversed-phase HPLC and subjected to microsequencing.

Cloning of cDNAs for the 30-kD TFIID-associated proteins

For the cloning of dTAF_{II}30 α , the sequence of peptide α (KSS-NHTSSAAGSENTPLTK) was used to generate nonoverlapping degenerate oligonucleotides on complementary strands to

Figure 9. Schematic diagrams of TBP-TAF and TAF-TAF interactions. These diagrams represent specific TAF-TAF and TAF-TBP contacts that we have identified. The multiple interactions involved in forming TFIID are shown in three separate diagrams for convenience starting with the largest TAFs followed by the smaller TAFs. (A) The TBP-dTAF_{II}250 interaction, which suggests that this largest subunit may play a role as a core component; this helps to coordinate the entire TAF-TBP complex. (B) Other TAFs that interact with dTAF_{II}250 are shown, including dTAF_{II}150, which binds both TBP and dTAF_{II}250 (P. Verrijzer, L. Attardi, K. Yokomori, J.-L. Chen, and R. Tjian, unpubl.), whereas dTAF_{II}60 and dTAF_{II}110 interact with dTAF_{II}250 but not with TBP (Weinzierl et al. 1993a,b). dTAF_{II}80 was shown to be incorporated into a complex containing dTBP, dTAF_{II}250, dTAF_{II}110, and dTAF_{II}60 (Dynlacht et al. 1993). (C) illustrates the interaction of the smaller TAFs. dTAF_{II}40 primarily interacts with dTAF_{II}60 but not with the other TAFs or TBP (Weinzierl et al. 1993b). In contrast, dTAF_{II}30 α interacts with TBP, dTAF_{II}250, and dTAF_{II}110 and may also oligomerize with itself, whereas dTAF_{II}30 β interacts with dTAF_{II}250 and dTAF_{II}150 but not with TBP. These schematic diagrams represent the summary of known TAF-TAF and TAF-TBP interactions and are not meant to imply an assembly pathway for the TFIID complex in vivo or in vitro.

perform intrapeptide "touchdown" PCR (Don et al. 1991). To decrease the degeneracy of the oligonucleotides, four different combinations of degenerate oligonucleotides were synthesized corresponding to the same 5'-side region: 5'-1-1 (5'-AA/AG/TCITC/GACT/AA/CT/CA/CT/AC-3'), 5'-1-2 (5'-AA/GA/AGITC/GACT/AA/CT/CA/CT/AC-3'), 5'-2-1 (5'-AA/GA/AGIAG/CT/AA/CT/CA/CT/AC-3'), and 5'-2-2 (5'-AA/AG/TCIAG/CT/AA/CT/CA/CT/AC-3'). Four separate PCR reactions were carried out in the presence of the same 3'-side oligonucleotide (5'-A/AG/CAT/AGCT/GG/AGCT/GT/AG/TT/CT/TC-3'). DNA templates for the PCR reaction were prepared from a *Drosophila* embryo cDNA library, which was also used for obtaining the full-length cDNA clones (Poole et al. 1985). The PCR products were separated on 3% low-melt agarose gels (Sea Plaque, FMC Bioproduct), and a band of the expected size of 57 nucleotides was excised and cloned into pBluescript SK. The product encoding the correct amino acid sequences was identified after sequence analysis of multiple clones. This fragment was radiolabeled by random priming and used as a probe for the library screening. A cDNA clone containing a 1.2-kb insert was isolated and cloned into pBluescript SK. Sequence analysis revealed that the clone encodes the sequences of three other peptides obtained from peptide sequencing, thus confirming the authenticity of the cDNA clone.

For the cloning of dTAF_{II}30 β , a partially degenerate guess-mer oligonucleotide of 44 nucleotides was synthesized (5'-GTTTTCGTIGGIGAGGTGTGTGAGGAGGCCCTIGA/CT/GTIATGGA-3') based on the sequence of peptide β (KVVFGEVVEEALDVMEAQGESGALQPK). The oligonucleotide was end labeled by T4 polynucleotide kinase and used to screen the library. A cDNA clone containing a 750-bp insert was isolated and cloned into pBluescript SK.

Expression of recombinant dTAF_{II}30 α and dTAF_{II}30 β

NdeI sites were introduced at both ends of the open reading frames of dTAF_{II}30 α and dTAF_{II}30 β by PCR. NdeI fragments were cloned into pAR3038 with or without HA tag for expression in *E. coli* (BL21 : DE3) (Rosenberg et al. 1987). For the antigen preparation to immunize rabbits, protein expression was induced by 0.3 mM IPTG for 2 hr and the lysate was prepared by sonication in HEMG buffer containing 0.1 M KCl and 0.1% NP-40. Radiolabeled proteins were generated by in vitro transcription and translation of dTAF_{II}30 α full-length or the amino-terminal deletion mutants obtained by PCR in pAR3038 in TNT-coupled transcription translation system in rabbit reticulocyte lysate (Promega).

A construct was also created for expression of the full-length dTAF_{II}30 α in the baculovirus system using Sf9 cells by subcloning the cDNA into pVL1393 (Pharmingen).

Generation of polyclonal antibodies against dTAF_{II}30 α and dTAF_{II}30 β

Polyclonal antisera were raised in rabbits using bacterially expressed dTAF_{II}30 α and dTAF_{II}30 β in SDS-polyacrylamide gel mixed with Freund's complete or incomplete adjuvant as described previously (Yokomori et al. 1993). The anti-dTAF_{II}30 α antibody was affinity purified using a column containing Affigel-10 (Bio-Rad) conjugated to the recombinant protein expressed in *E. coli*.

Protein-protein interaction assays

In coimmunoprecipitation assays, the primary protein, for example, HA-dTAF_{II}110, HA-dTAF_{II}30 α , HA-dTAF_{II}30 β , HA-

hTF_{II}250, HA-dTBP, dTAF_{II}250 Δ N, dTBP, or dTAF_{II}150 (1–2 μ g), was incubated at 4°C with protein A-Sepharose beads (Pharmacia), which were preincubated with either anti-HA (Bafco), anti-dTBP, anti-dTAF_{II}250 monoclonal antibodies (kindly provided by R. Weinzierl, University of California, Berkeley) or polyclonal antibodies against dTAF_{II}150 (C.P. Verrijzer, unpubl.). After 2 hr, the beads were washed with HEMG containing 0.1 M KCl, 0.1% NP-40, 1 mM DTT, and 0.2 mM AEBSF (Calbiochem) and incubated for 2 hr at 4°C with the second protein, for example, in vitro-translated ³⁵S-labeled dTFIIA-L (Yokomori et al. 1993), dTFIIB, and dTAF_{II}40, dTAF_{II}60, dTAF_{II}80, dTAF_{II}110, and dTAF_{II}150), or a crude extract from *E. coli* expressing dTAF_{II}30 α or dTAF_{II}30 β , or a crude extract from Sf9 cells infected with virus expressing dTAF_{II}30 α or dTAF_{II}150 (1–2 μ g). The beads were then washed thoroughly with HEMG containing 0.1 M KCl and subsequently 0.15–0.2 M KCl, resuspended in 2 $\times$ SDS sample buffer (10% glycerol, 0.7 M β -mercaptoethanol, 3% SDS, and 62.5 mM Tris-Cl at pH 6.8). The bound proteins were resolved by SDS-PAGE. In the case of coimmunoprecipitation with nonradioactive proteins, the proteins were analyzed by Western blotting. For ³⁵S-labeled proteins, the proteins were visualized by autoradiograph.

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Molecular cloning and characterization of dTAFlI30 alpha and dTAFlI30 beta: two small subunits of Drosophila TFIID.

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