Drosophila TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex

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The basal factor TFIIA has been shown to act early during initiation in both the mammalian and yeast transcription systems, but a TFIIA-like activity has not been identified in Drosophila. While characterizing the Drosophila TFIID complex, we discovered that a 30-kD protein that cofractionated with dTFIID was homologous to the previously identified, large subunit of yeast TFIIA. Here, we report the cloning and biochemical characterization of Drosophila TFIIA-L. Coimmunoprecipitation studies with anti-dTBP, anti-dTFIIA-L, and anti-TAF antibodies indicated a tight association of the endogenous dTFIIA and dTFIID. However, dTFIIA could be dissociated from dTFIID under conditions that did not elute the TAFs, and the eluted material had mobility shift and transcriptional activities associated with TFIIA. Peptide sequence and Western analysis with antibodies raised against the amino- and carboxy-terminal portions of recombinant dTFIIA-L revealed that a precursor 48-kD species was cleaved in vivo, giving rise to the 30- and 20-kD subunits of dTFIIA that remain associated with each other and with dTFIID. Protein–protein interaction assays identified dTBP and dTAFn110 as targets for binding TFIIA in the TFIID complex. These results suggest that TFIIA may form a specific complex with both TAFs and other components of the transcriptional machinery during formation of the initiation complex.

[Key Words: Drosophila; TFIIA; TFIID; TAFs; transcription]

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Accurate initiation of transcription from RNA polymerase II promoters requires the assembly of a complex containing various accessory factors, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (for review, see Zawel and Reinberg 1992). Recent studies revealed that several of these basal transcription components themselves contain multiple subunits. For example, TFIID consists of the TATA-binding protein (TBP) and seven or more associated factors called TAFs that range from 30 to 250 kD (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1992; Hisatake et al. 1993; Kokubo et al. 1993). Basal factors TFIIE and TFIIF each consist of two subunits (Burton et al. 1988; Inostroza et al. 1991), whereas TFIIH has been reported to contain five subunits (Fischer et al. 1992; Flores et al. 1992). It has been reported that TFIIA consists of two subunits in yeast (Ranish and Hahn 1991; Ranish et al. 1992), whereas three subunits of 35, 18, and 14 kD have been described for its mammalian counterpart (Cortes et al. 1992). Interestingly, TFIIA has not been identified in all fractionated transcription systems, including a fractionated Drosophila transcription system (Wampler et al. 1990). In an effort to reconstitute an active initiation complex in vitro from purified components, we have been systematically purifying subunits of the basal transcription machinery and isolating cDNAs that encode these essential transcription factors.

Our recent efforts have largely been directed at identifying, cloning, and characterizing all of the subunits associated with TFIID. Our interest in this particular factor stems from the finding that activation of transcription by site-specific enhancers, such as Sp1, CTF, NTF-1, and VP16, requires coactivators, which in some cases, turn out to be TAFs that are associated with TBP in the TFIID complex (Pugh and Tjian 1990, 1991; Dynlacht et al. 1991; Tanese et al. 1991). We now know that dTAFII110 and dTAFII40 can make direct contact with activation domains of Sp1 and VP16, respectively (Hoey et al. 1993; J. Goodrich, T. Hoey, C.J. Thut, A. Admon, and R. Tjian, in prep.). In addition to TAF/coactivator interactions, our studies also reveal numerous TAF/TBP and TAF/TAF interactions (Dynlacht et al. 1993; Ruppert et al. 1993; Weinzierl et al. 1993). Recently, we identified a specific interaction between a Drosophila TAF (dTAFII40) and a basal factor, TFIIIB (J. Goodrich, T. Hoey, C.J. Thut, A. Admon, and R. Tjian, in prep.). However, we anticipate that there will be other important and specific interactions between TAFs and basal factors, as TFIID appears to be the connection be-
tween activators and the basal transcriptional machinery.

We have thus far cloned, expressed, and partially characterized cDNAs encoding six of the dTFIID subunits, including TBP, dTAFII250, dTAFII110, dTAFII80, dTAFII60, and dTAFII40, as well as various human homologs [Hoey et al. 1990, 1993; Peterson et al. 1990; Dynlacht et al. 1993; Ruppert et al. 1993; Weinzierl et al. 1993; R. Weinzierl, S. Ruppert, B. Dynlacht, N. Tanese, and R. Tjian, both in prep.]. However, there are several additional subunits originally identified in the TFIID complex that remain to be purified and their cDNAs isolated. We therefore set out to isolate the remaining polypeptides associated with TFIID to begin a detailed biochemical analysis and in vitro reconstitution of this important transcription factor. We report here the purification, molecular cloning, expression, and partial characterization of a 30-kD polypeptide associated with dTFIID. Unexpectedly, DNA sequence analysis and biochemical characterization indicated that this 30 kD species is homologous to the largest subunit of yeast TFIIA (yTFIIA) [Ranish et al. 1992]. Moreover, antibody precipitation and Western analysis of dTFIID revealed a precursor product of 48 kD (TFIIA-L), which is proteolytically processed into 30- and 20-kD species, both of which are associated with dTFIID. Protein–protein interaction assays indicate that dTFIIA-L can bind dTBP as well as dTAFII110. These results suggest that at least a subpopulation of the cellular TFIIA is associated with TFIID, which may help resolve some anomalous behavior previously attributed to TFIIA.

Results

Purification and cloning of the dTFIIA large subunit

Antibody affinity chromatography of dTFIID was performed with partially purified nuclear extracts from 0- to 14-hr embryos [Dynlacht et al. 1989; Wampler et al. 1990]. Approximately 100 μg of purified dTFIID was subjected to preparative SDS-PAGE and transferred to PVDF membrane (Fig. 1). After staining, three polypeptides migrating with an apparent molecular mass between 30 and 27 kD were excised, treated with trypsin, and the resulting peptides were separated by reverse-phase HPLC. Partial amino acid sequences of individual peptides were determined by sequential Edman degradation. The amino acid sequences of the longest peptide derived from the protein indicated with the arrow in Figure 1 were used to generate two nonoverlapping, partially degenerate "guess-mers". In addition, shorter peptide sequences were used to design guess-mer oligonucleotide probes. The guess-mer probes were used to screen a Drosophila embryo cDNA library. These probes allowed us to isolate a cDNA of 1.6 kb that appears to be nearly full length as determined by Northern blot analysis, which detected a mRNA species of 1.8 kb (data not shown). The complete DNA sequence of this longest cDNA revealed an open reading frame encoding a protein of 366 amino acids with a predicted molecular mass of 39.2 kD and a calculated pl of 4.4 (Fig. 2A). In situ hybridization mapped this gene to position 97F5.6 on the X chromosome (data not shown).

Surprisingly, the deduced amino acid sequence of this TFIID-associated factor revealed a striking similarity to the largest subunit of yeast (286 amino acids) and human [376 amino acids] TFIIA. We therefore refer to this clone as dTFIIA-L. Both amino-terminal and carboxy-terminal regions of dTFIIA-L share ~40% sequence identity with the large subunit of yTFIIA and 60% sequence identity with the large subunit of human TFIIA [hTFIIA] (Fig. 2B), whereas the sequences in the middle of the proteins are less conserved. In addition, the yeast, human, and Drosophila proteins contain acidic rich stretches in the carboxy-terminal halves. We also noted that the recombinant Drosophila protein migrated on SDS–polyacrylamide gels with an apparent molecular mass of 48 kD, which is significantly larger than the 30-kD protein origi-

Figure 1. The dTFIID complex contains multiple proteins in the 30-kD size range. A portion (2%) of the dTFIID used for the preparative gel was resolved by SDS-PAGE and stained with silver (lane 2). Molecular mass markers (~100 ng per band) are shown in lane 1. The TAFs are indicated. Proteins in the 30-kD region are shown in higher resolution, and the band from which peptide sequence was obtained is indicated with an arrow.
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A

CATCGCATCCTGTAATCTTCTGCTGCAACGTTAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
81

CTCAAGGCTGGCGGAGAAAGTGCACAAATGGCGCTATGCCCCACCATTTATAATTCAGCAAATACGTCAATTAGCCCACCAGCGAACGGAAAAGC
162

GTCATCGGTCTGCTGTTGTCGCTGTAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
243

AACAAGGGGCTGCCATAGGCCCCGTGGCCTGGTGGCCTGGCTGGCTGGCCTGGCCTGGCCTGGCCTGGCCTGGC
324

MALCQTSVLKVKHAVIED
(18)

VITRNGVRAGFLGDVEQVLQEMKQKVNR
(45)

NKLLASROSIPDGSHPPIVAN
(72)

ATCCEAAGGGAAGCTGCATACCTGCTGTAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
405

NESSSSMSLSVGLKSLSSAGMAAGSGCRNG
(126)

CTGGTGGCCATCAGGGAAGAGTGCACATCTCAGAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
567

DAASSCCGSIPIVATLDFPNNIFVMV
(180)

ATACCGTGCTGCGGCCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
729

VEQVPSALQ
(257)

GAGAATTGCGCAATGGCGCTGCGGCCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
810

ENQLTQLTALHSSISMPLTTLSV
(234)

CCCGGACAGCCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
891

LIQQHVNALSQHKTLAABAQKLDGA
(261)

CCCGGACAGCCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
972

LDSSDEDESESSDONIDDDDLDKD
(288)

GATGCGAGGGATGGCGAGGCAATGGCGCTGCGGCCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
1053

HSMNAPSAEVMVSDDVESDVDDS
(315)

GGCGGAGGTAAGTGGCAGGGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
1134

AEMFDYKRSMKWF
(342)

CTCAAGGATGGCAATGGCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
1215

ELKDGIMNMRKDGKFGKRKF
(366)

GCATGGGCAGGCAGGGCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
1296

ACCGCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
1377

AGCAAGATGGCAATGGCGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
1458

CAACACATTGTAATAATGATGGCAGGGCTGCCAGGTCTGCAATCTGCAATCGCACACCCAGGGCAGCGAACGGAAAAGC
1539

AAAAA
1553

B

Figure 2.  (See facing page for legend.)

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Figure 3. The endogenous 30-kD TFIID-associated protein is antigenically related to the 48-kD recombinant dTFIIA-L. (A) Expression of recombinant proteins and specificity of antibodies. dTFIIA-L, dTFIIA-N, and dTFIIA-C were expressed in E. coli. In the cases of expressed dTFIIA-L and dTFIIA-N (lane 1,2), the insoluble E. coli protein was loaded on an SDS-polyacrylamide gel, whereas the E. coli sonicate supernatant was loaded in the case of dTFIIA-C (lane 3). The SDS-polyacrylamide gel was stained with Coomassie blue. Antibodies raised against the three E. coli-expressed proteins were used in Western analyses to determine their specificities for interacting with dTFIIA-L, dTFIIA-N, and dTFIIA-C. Baculovirus-expressed dTFIIA-L was included in the Western blots. A schematic of the recombinant proteins is shown. (B) Antibodies raised against dTFIIA-N can immunoprecipitate the dTFIID complex. Immunoprecipitated proteins were resolved by SDS-PAGE. A silver stain is shown with the components of dTFIID labeled. Western analysis with anti-dTAF_80 and anti-dTAF_40 confirmed the identity of these TAFs.

Antibodies directed against dTFIIA-L coimmunoprecipitate the dTFIID complex

To begin a more detailed biochemical characterization of dTFIIA-L, we have expressed full length and truncated versions in bacteria (Fig. 3A). These overproduced proteins, including dTFIIA-L [full length], dTFIIA-N [amino acids 1–222], and dTFIIA-C [amino acids 229–366] were then used to generate specific polyclonal antibodies. As shown in Figure 3A, anti-dTFIIA-L and anti-dTFIIA-N both cross-reacted with dTFIIA-L and dTFIIA-N and not with dTFIIA-C, whereas the anti-TFIIA-C cross-reacted with dTFIIA-L and dTFIIA-C. In addition, we also expressed both hemagglutinin (HA) tagged and untagged dTFIIA-L in SF9 cells using baculovirus. All three antibodies cross-reacted efficiently with a 48-kD species overproduced in baculovirus extracts expressing dTFIIA-L (Fig. 3A). As expected, α-dTFIIA-L also cross-reacted with a 30-kD species found in the endogenous antibody-affinity-purified dTFIID complex (Fig. 3B). Precipitation of the dTFIID complex with anti-dTAF_250 and anti-dTAF_40 antibodies also coprecipitated the 30-kD protein that was recognized by α-dTFIIA-L [data not shown]. In addition, antibodies raised against dTFIIA-L and dTFIIA-N were capable of immunoprecipitating the dTFIID complex from Drosophila nuclear extracts (Fig. 3B). These results confirmed that the cDNA we have isolated encodes a dTFIIA-like subunit that is found associated with affinity-purified dTFIID. However, it is evident that the full length recombinant protein is significantly larger than the species found copurifying with dTFIID. We shall return to this apparent anomaly later.
dTFIIA can be dissociated from dTFIID with 0.5 M salt

Because our data indicated that at least one TFIIA-like subunit is associated with purified dTFIID, it became important to determine how avid this interaction is and whether we can demonstrate TFIIA transcriptional activity associated with this protein. First, we tested the affinity of the dTFIIA interaction with dTFIID by washing the immunopurified dTFIID complex with increasing concentrations of salt. At 500 mM KCl, we observed that a 30-kD species was eluted (Fig. 4A), whereas the prototypical TAFs, such as dTAF$_{110}$, dTAF$_{80}$, and dTAF$_{40}$, remained bound to TBP on the complex. Western analysis with anti-dTFIIA-L confirmed that the 30-kD polypeptide present in the 0.5 M fraction was related to the protein encoded by our dTFIIA-L cDNA (Fig. 4B, lanes 2,3).

The mobility shift and transcriptional activities of both human and yTFIIA have been widely characterized (Reinberg et al. 1987; Ranish and Hahn 1991; Cortes et al. 1992; Ranish et al. 1992). Because our Western analysis indicated that the 0.5 M eluate contained the Drosophila homolog of the large subunit of TFIIA, we tested this fraction for TFIIA transcriptional and mobility shift activities. As shown in Figure 5, reconstituted in vitro transcription reactions from which the TFIIA fraction had been left out, directed a rather weak basal level of transcription from the adeno virus major late promoter. However, when the 0.5 M eluate fraction containing the putative dTFIIA was added back to this reaction, a significant stimulation of basal transcription was observed. These results are consistent with the notion that the 0.5 M eluate fraction contains an activity resembling TFIIA, which is frequently found to be stimulatory but not essential for basal transcription in reconstituted RNA polymerase II transcription systems (Cortes et al. 1992). As a control, we also performed reconstituted transcription reactions in which the TFIID fraction was omitted from the reaction, and under these conditions, addition of the 0.5 M eluate failed to complement or stimulate transcription, indicating that the stimulation of basal activity by the TFIIA-like fraction was not attributable to contamination of TBP or other TFIID subunits. We have also confirmed that the 0.5 M eluate contains dTFIIA by gel-shift analysis in the presence of recombinant dTBP. As expected, in the presence of both TBP and the 0.5 M eluate, a supershift characteristic of a TBP–TFIIA shift was observed (Fig. 5B). Significantly, the addition of affinity-purified anti-TFIIA-N antibodies to the reactions supershifted the DNA/TBP/IIA band (lanes 4,5) but not the DNA/TBP band (lane 2) thus, confirming the presence of dTFIIA in the shifted species. These findings, taken together, strongly suggest that dTFIIA subunits are associated with dTFIID under standard affinity purification conditions. However, dTFIIA subunits can be dislodged from the dTFIID complex with 0.5 M salt, whereas dTAFs remain bound and can only be eluted with urea or guanidine (Dynlacht et al. 1991; Tanese et al. 1991).

dTFIIA-L interacts with dTBP and dTAF$_{110}$

With which subunits of TFIID does TFIIA interact? To address this question, we carried out several protein–protein binding assays. By utilizing an HA-tagged version of full length dTFIIA-L, we were able to rapidly screen its potential interaction with individual dTAFs (40, 60, 80, and 110) and the basal transcription factor TFIIIB (Fig. 6A). These coprecipitation experiments revealed that dTAF$_{110}$ interacted efficiently with HA-tagged dTFIIA-L. To confirm this initial finding, we also performed the reciprocal experiment in which antibodies directed against HA-tagged dTAF$_{110}$ were used to coimmunoprecipitate full length dTFIIA-L (Fig. 6B). Additionally, we found that recombinant dTFIIA-L could be coprecipitated with recombinant dTBP using a dTBP-specific monoclonal antibody (Fig. 6B). These results establish that both HA-tagged dTAF$_{110}$ and dTBP interact with recombinant dTFIIA-L when either partner is immobilized on antibody beads. It was reported previously that dTAF$_{110}$ cannot interact directly with dTBP (Weinzierl et al. 1993). In Figure 6C we demonstrate that dTFIIA-L can tether dTAF$_{110}$ to immobilized dTBP, thus indicating that dTFIIA-L can interact with...
Figure 5. The 0.5 M eluate contains TFIIA activity. (A) The 0.5 M eluate can substitute for the hTFIIA fraction in a reconstituted in vitro transcription system. The complete RNA polymerase II transcription system (lane 1) consisted of five recombinant human basal transcription factors [TFIIB, TFIIE-34, TFIIE-56, RAP30, and RAP74] that were expressed in E. coli and purified to near homogeneity, highly purified HeLa RNA polymerase II, a hTFIID fraction (1 M wash from phosphocellulose), and a hTFIIA fraction (0.35 M wash from DE-52). The supercoiled template used contained the adenovirus major late promoter (-53 to +10) upstream from the G-less cassette (Sawadogo and Roeder 1985). The hTFIIA fraction was omitted from reactions 2–5. Increasing amounts of the Drosophila 0.5 M eluate were added to reactions 3, 4, and 5 (0.5, 1.5, and 4.5 μl, respectively). The hTFIID fraction was omitted from reactions 6–8. Reactions 7 and 8 contained 1 and 4.5 μl of the Drosophila 0.5 M eluate, respectively. The position of the full length G-less transcript is denoted by an arrow. (B) TFIIA in the 0.5 M eluate retards a TATA–dTBP complex in mobility shift assays. The 0.5 M eluate supershifts the dTBP–TATA oligonucleotide complex (lane 3), which was shifted further by the addition of affinity-purified anti-dTFIIA-N antibodies (lanes 4, 5, and 20 ng of antibody, respectively). The 0.5 M did not shift the TATA oligonucleotide in the absence of dTBP (lane 6), and the anti-TFIIA-N did not shift the TATA–dTBP complex in the absence of the 0.5 M eluate (lane 2).

dTAF_{110} and dTBP simultaneously. Finally, we also tested the ability of dTBP to interact with endogenous dTFIIA subunits present in the 0.5 M eluate [Fig. 6D]. Using an antibody against dTBP, we found that the dTFIIA-30 and a 20-kD species coprecipitate efficiently with dTBP. In some experiments we can also detect a 14-kD species that stains poorly with silver but can be seen in lane 2 of Figure 6D. These findings indicate that both recombinant 48-kD full length dTFIIA-L and the endogenous 30-kD dTFIIA can interact with TBP. In addition, recombinant dTFIIA-L can also interact selectively with dTAF_{110}. These TAF–basal factor interactions between subunits of TFIIID and TFIIA define a novel set of protein contacts that may explain the relatively tight association of TFIIA and the TBP/TAF complex.

The largest subunit of TFIIA is proteolytically processed into the 30- and 20-kD species in Drosophila cells

It was puzzling to consistently find that the endogenous dTFIIA large subunit is 30 kD, whereas the full-length recombinant protein migrates as a 48-kD species. To address this paradox, we raised antibodies against the amino- and carboxy-terminal regions of bacterially expressed dTFIIA-L [Fig. 3] and used these domain-specific antibodies to probe the structure of the endogenous dTFIIA subunits. To our surprise, when these two antibodies were used for Western blot analysis with the 0.5 M dTFIIA fraction that had been coprecipitated with dTBP, the amino-terminal-specific antibody cross-reacted exclusively with the 30-kD species while the carboxy-terminal specific antibody recognized the 20-kD polypeptide [Fig. 7A]. Furthermore, the 20-kD species, which is not antigenically related to the amino terminus of dTFIIA-L, was efficiently coprecipitated from both the 0.5 M eluate and a crude Drosophila nuclear extract with antibodies raised against the amino terminus of dTFIIA-L [Fig. 7B]. These unexpected findings suggested that the 20-kD subunit may actually be a proteolytic product of a precursor protein (the 48-kD species) that becomes processed into the 30- and 20-kD subunits, which remain associated. To confirm this notion, we determined the partial amino acid sequence of peptides derived from the Drosophila 20-kD species. Our results establish that peptides found in the 20-kD band correspond to sequences derived from the carboxy-terminal region of the full length dTFIIA-L [Fig. 2A, doubly underlined amino acids]. A similar result was obtained when the 18-kD human subunit of TFIIA was subjected to sequence analysis [A. Admon, D. Reinberg, and H. Handa, per. comm.]. Thus, it seems likely that in Drosophila and human, a precursor TFIIA-L is clipped by a protease generating the 30- and 20- (or 18-) kD subunits,
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Figure 6. Recombinant dTFIIA-L interacts with dTAFII110 and dTBP. (A) Co-precipitation of 35S-labeled TFIIB and dTAF with HA-tagged dTFIIA-L. 35S-labeled TFIIB, dTAFII40, dTAFII60, dTAFII80, and dTAFII110 were made in reticulocyte lysates. Equivalent portions of the labeled proteins were incubated with anti-HA and protein A beads in either the absence (even-numbered lanes) or presence (odd-numbered lanes) of recombinant HA-tagged dTFIIA-L. The percentage of the bound protein compared with the input in the dTAFII110 reaction was ~7.5%, whereas the amounts of the other proteins retained were <0.5%. (B) Coprecipitation of baculovirus-expressed dTFIIA-L with dTBP and HA-tagged TAFs. Protein A beads were preincubated with either anti-dTBP (lanes 2, 3) or anti-HA (lanes 4–7). Baculovirus-expressed dTFIIA-L was mixed with ~1 μg of the recombinant proteins as indicated above the lanes. After precipitation, dTFIIA-L was detected by Western blot. Lane 1 contains dTFIIA-L equal to 10% of the input. Lanes 3 and 7 are controls showing the dependence on the immunoprecipitation of dTFIIA-L on the recombinant TBP and TAFs. Lanes 5 and 6 are controls in which two other HA-tagged proteins (hTAFII48 and dTAFII30a) were tested. (C) Recombinant dTFIIA-L can tether dTAFII110 to immobilized dTBP. HA-tagged dTBP was immobilized on beads with anti-HA antibody. Half of the beads were incubated with dTFIIA-L before adding dTAFII110 (lane 2). After incubation with dTAFII110, samples were washed and analyzed by SDS-PAGE. The silver-stained gel revealed that dTAFII110 was coprecipitated with HA-tagged dTBP in the presence (lane 2) but not in the absence (lane 1) of dTFIIA-L. dTFIIA-L migrates with IgG heavy chain (IgH) and is not visible in this gel. (D) Proteins of 30, 20, and 14 kD coprecipitate from the 0.5 M eluate with recombinant dTBP. Recombinant dTBP was immobilized on protein A beads with a monoclonal antibody specific for dTBP. The beads were then incubated with the 0.5 M eluate fraction, and after precipitation the bound material was analyzed by SDS-PAGE. The silver-stained gel demonstrated that the 30-, 20-, and 14-kD proteins were coprecipitated with dTBP, but neither in the absence of dTBP (lane 1) nor in the absence of 0.5 M eluate (lane 3).

which appear to remain associated with one another and presumably also with the 14-kD subunit.

To determine whether this proteolytic activity is largely an in vitro artifact or a reaction that takes place in vivo, we have used antibodies to detect dTFIIA-L-related polypeptides in Drosophila cells directly lysed in SDS without further manipulation, thus eliminating the possibility of in vitro proteolysis. Western blot analysis with anti-dTFIIA-N revealed the presence of a 30-kD species but no detectable full length 48-kD species in whole-cell SDS lysates [Fig. 7C]. Using partially purified nuclear or polyvinyl alcohol (PVA) extracts we again observe that the only species clearly detectable is the 30-kD form, whereas none of the 48-kD precursor species can be detected [Fig. 7C]. These results strongly suggest that dTFIIA-L is clipped in vivo into smaller polypeptides of 30 and 20 kD, which are homologous to the largest subunits of hTFIIA [Cortes et al. 1992; D. Reinberg and H. Handa, pers. comm.].

Discussion

Ever since the discovery that TFIIID is a multisubunit complex consisting of TBP and TAFs that are required for activation of transcription [Pugh and Tjian 1990; Dynlacht et al. 1991; Tanese et al. 1991], the focus has been on defining the interactions between TAFs and activators. However, it has become increasingly obvious that TAFs must also interact in specific and functionally important ways with basal factors, especially because it is likely that TFIIID mediates interactions between activators and the basal transcriptional apparatus. In an effort to define the transcriptional components associated with TFIIID, we have purified a 30-kD protein associated with TFIIID and isolated a cDNA encoding this transcription factor. Analysis of the primary amino acid sequence deduced from the DNA sequence identified this gene product to be the large subunit of dTFIIA. Apparently, in Drosophila, the subunits of TFIIA copurify...
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Figure 7. Western blot analysis with amino- and carboxy-terminal–specific dTFIIA antibodies indicates that endogenous dTFIIA 30- and 20-kD subunits are both antigenically related to the 48-kD dTFIIA-L. (A) The 20-kD TFIIA subunit is recognized by anti-dTFIIA-C, whereas the 30-kD TFIIA subunit is recognized by anti-dTFIIA-N. Coimmunoprecipitation of the 0.5 m eluate with recombinant dTBP was performed as described for Fig. 6D. The position of the specific cross-reacting 20- and 30-kD species are indicated. Nonspecific crossreaction with two bands corresponding to protein G beads are also indicated. (B) The endogenous 20-kD subunit of dTFIIA coprecipitates with antibodies that specifically recognize the 30-kD TFIIA subunit. Nuclear extract (lane 1) and 0.5 M fraction (lane 2) were coprecipitated separately with anti-dTFIIA-N. The presence of the 20-kD dTFIIA subunit was analyzed by Western blot using anti-dTFIIA-C antibodies. (C) The endogenous 48-kD dTFIIA-L precursor is not detectable in Schneider cells. Schneider cells were harvested, boiled in SDS sample buffer, and loaded immediately onto an SDS–gel (lane 1). PVA-precipitated nuclear extract (lane 2) and a crude extract of SF9 cells expressing dTFIIA-L (lane 3) were loaded as controls. The presence of the 48-kD dTFIIA-L was analyzed by Western blot with anti-dTFIIA-N. The approximate ratio of nuclei between the Schneider cell sample and the nuclear extract was 1 : 1000.
present in yeast [Ranish et al. 1992]. Curiously, we have not been able to detect specific processing of recombinant dTFIIA-L overproduced in the host cells for the baculovirus expression system. It is intriguing that recent studies suggest that other nuclear transcription factors can also undergo a proteolytic processing event. For example, the host cell factor [HCF], which is a cofactor for the viral trans-activator VP16, is apparently processed from a precursor 300-kD protein into a multitude of shorter forms that remain associated and possess the appropriate functions necessary to form a VP16/HCF/Oct-1 complex [Wilson et al. 1993]. Similarly SREBP-1, a novel helix-loop-helix [HLH]-type transcription factor involved in regulation of the low-density-lipoprotein [LDL] receptor is apparently subject to cleavage by a protease [J. Goldstein and M. Brown, pers. comm.].

At present, it is not clear what the purpose of this processing event is and what consequences it may have on the function of TFIIA in these higher organisms. However, this unexpected finding does help to explain why in human extracts TFIIA has been found to consist of three subunits of 30, 18, and 14 kD [Cortes et al. 1992] while in yeast only two subunits have been identified [Ranish and Hahn 1991; Ranish et al. 1992]. Apparently both the 30- and 18- to 20-kD subunits in Droso-phila and human are derived from the product of a single gene, TFIIA-L, which is highly homologous to the yeast large subunit. Presently, we have not identified the gene encoding the smallest 14-kD subunit of TFIIA from Drosophila and man, but it is likely that once identified, it will be significantly similar in sequence to the yeast small subunit of TFIIA.

We have confirmed that recombinant and endogenous dTFIIA-L interacts specifically with dTBP as expected from earlier studies. However, we have also found that dTFIIA-L interacts selectively with at least one other component of TFIIID, dTAF8110. We reported previously that dTAF8110 is one of the targets of the activator Sp1 [Hoey et al. 1993] and that this coactivator assembles into the dTFIIID complex via interactions with dTAF8250 [Weinzierl et al. 1993]. Thus, dTAF8110 is able to make multiple contacts with several partners, including an activator, another TAF, and a basal factor. These findings lend strong support to the coactivator model [Pugh and Tjian 1990] in which TAFs serve as intermediaries, possibly transducing molecular signals between activators and the basal transcriptional machinery. As we continue to define more of these protein–protein and protein–DNA interactions that take place between the multisubunit components of the transcriptional complex, it may become possible to dissect the different pathways and mechanisms that govern the regulation of transcriptional initiation.

Materials and methods

Purification of TFIIID and peptide sequencing of the 30-kD protein

Nuclear extracts were prepared from 800 g of 0- to 14-hr em-bryos and precipitated with PVA as described previously [Dynlacht et al. 1989; Wampler et al. 1990]. TFIIID was immunoprecipitated either directly from PVA extracts or from a Q-Sepharose fraction [Wampler et al. 1990] using affinity-purified polyclonal antibodies against dTBP [Dynlacht et al. 1991]. Beads with bound TFIIID were washed extensively with HEMG buffer [25 mM HEPES at 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 HEMG containing 0.5 M KCl; finally, the TAFs were eluted in 1 M guanidine-HCl. The guanidine-eluted TAFs were precipitated in 100% TCA containing 4 mg/ml of deoxycholate. The TCA precipitate was resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), and stained with Ponceau S. Proteins in the 30-kD size range were excised from the membrane, fractionated by reverse-phase HPLC, and subjected to microsequencing.

Cloning of a cDNA for the 30-kD TFIIID-associated protein

The longest peptide sequence [KAVELPDSGDGHPFPIVAN-\textit{NPK}] was used to generate two nonoverlapping partially degenerate oligonucleotide primers [5'-AAGCCCGCT/CG/GACCCTGGGCGA/CT/TCCGCGCCAAGAT-3'; 5'-TCICACCGCICCAATGCGT/CG/GCCAACACCCCAAG-3']. A Drosophila cDNA library [Poole et al. 1985] was screened with the 32P-labeled guess-mers. After the tertiary screening, 10 positive clones were selected. Another peptide sequence [VYHAVIED-VITNV], which showed homology to the yTFIIA large subunit, was also used to generate a guess-mer [5'-GTITACCACGCCG\textit{NIT/TVAT/TGC/CG/GCCAACACCCCAAG-3'}]. A D. melanogaster cDNA library [Poole et al. 1985] was screened with the 32P-labeled guess-mers. After the secondary screening, we found that the positive clones from the first set cross-hybridized with the second set of positives, thus confirming that both peptides were contained in the same clones. Six of the clones were analyzed further. Four of them had inserts of ~1.6–1.7 kb, differing only in the 5' untranslated regions. The clones were sequenced, and each was found to contain a reading frame of 366 amino acids. Four peptide sequences were found within the amino-terminal 193 amino acids [Fig. 2A, singly underlined].

Expression of recombinant dTFIIA

EcoRI fragments from four clones were subcloned into Blue-script SK+ (Stratagene), and transcribed and translated in reticulocyte lysates. Translation products from the four independent clones exhibited identical SDS-PAGE mobilities consistent with a protein of 48 kD, which is substantially larger than the 30-kD protein from which the peptide sequences were obtained. To further investigate this discrepancy, we divided one of the clones into amino- and carboxy-terminal portions. An NdeI site was introduced into the 5' and 3' ends of the amino- and carboxy-terminal fragment by a PCR strategy. The NdeI fragments encoding the full length TFIIA-L, the amino-terminal 222 amino acids, and the carboxy-terminal 137 amino acids were cloned into pAR308 for Escherichia coli T7 expression [Rosenberg et al. 1987]. Proteins were expressed in E. coli [BL21(DE3)]. After 2 hr of induction with IPTG, cells were harvested, resuspended in HEMG(0.1) containing 0.1% NP-40, and sonicated. Because the recombinant TFIIA-C migrated at an anomalously large size in SDS gels [Fig. 3], we performed mass spectrometry on the recombinant protein. We found that the actual molecular mass of TFIIA-C was consistent with that predicted from the amino acid sequence.

Construnctions were also created for expression of dTFIIA-L and
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HA-tagged dTFIIA-L in the baculovirus system using SF9 cells by subcloning the eDNA into pVL1393 [Pharmingen] and pVL1392-HA [S. Ruppert, unpubl.]. In addition, PCR products were subcloned into Bluescript SK+ to ensure that the sequence of the clones were identical to the original eDNA clone.

Generation of polyclonal antibodies against dTFIIA

Polyclonal antisera against dTFIIA-L, dTFIIA-N, and dTFIIA-C were raised in rabbits. Crude extracts from E. coli expressing the three proteins were subjected to SDS–PAGE, and the corresponding fragments were excised from the gel after brief staining with 0.05% Coomassie blue in dH2O. The acrylamide gel slices containing ~300 μg of proteins were passed through 18-gauge needles and mixed with Freund’s complete or incomplete adjuvant. Injections were done subcutaneously. After the second injection, antisera were tested for reactivity in Western assays. The anti-dTFIIA-N was affinity purified using a column containing Affigel-10 (Bio-Rad) conjugated to E. coli-expressed dTFIIA-L in buffer containing 80 mM CaCl2 to compensate for the low pH of the protein. The eluted and dialyzed antibody had an average concentration of 200 ng/μl.

In vitro transcription

In vitro transcription assays were performed in a fractionated HeLa transcription system in which recombinant TFIIB, TFIIE, and TFIIF were used in place of the corresponding fractions. Recombinant TFIIB was a gift from Greg Peterson and had been expressed in E. coli and purified as described [Ha et al. 1991]. Recombinant TFIIE-34 and TFIIE-56 were gifts from Mary Maxon and had been expressed in E. coli and purified as described [Peterson et al. 1991]. Recombinant RAP30 and RAP74 were expressed in E. coli and purified as described [Aslo et al. 1992, Finkelstein et al. 1992]. The hTFIID fraction [phosphocellulose, 1.0 M eluate] and hTFIIF fraction [phosphocellulose, 0.1 M flowthrough: DE-52, 0.35 M wash] were isolated as described previously [Reinberg et al. 1987]. RNA polymerase IIO and IIA were purified from HeLa cell nuclear pellets through the DEAE-5PW step as described by Lu et al. (1991). Equal portions of the polymerase IIO and polymerase IIA pools were mixed and dialyzed into buffer containing 0.1 M ammonium sulfate. Transcription reactions were performed in a final volume of 25 μl in buffer containing 20 mM Tris–HCl (pH 7.9), 10% glycerol, 1 mM DTT, 4 mM MgCl2, 50 mM KC1, and 10 mM ammonium sulfate. Each reaction contained the following: 10 ng of TFIIB, 50 ng of RAP30, 10 ng of RAP74, 30 ng of TFIIE-34, 40 ng of TFIIE-56, 125 ng of RNA polymerase, 2 μg of TFIIID fraction, 3 μg of hTFIIA, and 200 ng of template DNA containing the adenovirus major late promoter (~33 to +10) upstream of the G-less cassette [Sawadogo and Roeder 1985]. The reactions did not contain 3’-O-methyl GTP or RNase T1. Transcription factors were preincubated with DNA for 30 min at 30°C. Nucleotides (2 μl of a 13× mixture) were added to give final concentrations of 500 μM ATP, 500 μM CTP, and 25 μM [α-32P]UTP (5 μCi). Transcription was allowed to proceed for 15 min at 37°C. Reactions were stopped by the addition of 100 μl of stop mix containing 3.1 mM ammonium chloride, 10 μg of carrier yeast RNA, and 15 μg of proteinase K. After ethanol precipitation the samples were resolved by denaturing PAGE. The polyacrylamide gels were dried and exposed to film.

Gel-shift assay

A synthetic oligonucleotide containing a TATA element was prepared as described previously [Peterson et al. 1990]. Approximately 500 counts per second (cps) of probe was used for one reaction. Baculovirus-expressed dTBP (~30 ng per reaction) was kindly provided by B. Dynlacht [Dynlacht et al. 1993]. A portion of the 0.5 M eluate (~20 ng of total proteins per reaction) was mixed with dTBP in HEMG buffer containing 0.05 M KCl, 0.005% NP-40, and 0.5 mM DTT in the presence of 32P-labeled probe. The mixture was incubated for 30 min at room temperature, then mixed further with different amounts of affinity-purified anti-dTFIIA-N and incubated for 30 min on ice. The samples were resolved on 5% acrylamide gel containing 0.5× Tris-glycine buffer, 0.02% NP-40, and 1 mM EDTA.

Protein–protein interaction assays

In communoprecipitation assays, the primary protein, e.g., HA-tagged dTFIIA-L, HA-tagged TAF110, or dTBP (1–2 μg), was incubated at 4°C with protein A-Sepharose beads (Pharmacia), which were preincubated with either anti-HA (Báfco) or anti-dTBP (kindly provided by R. Weinzierl, unpubl.). After 2 hr, the beads were washed with HEMG(0.1) containing 0.1% NP-40, 1 mM DTT, 0.2 mM AEBSF (Calbiochem), and subsequently incubated for 2.5 hr at 4°C with the second protein, e.g., in vitro-translated 35S-labeled TFIIA and TAFs (40, 60, 80, and 110), or a crude extract from SF9 cells infected with baculovirus-expressing dTFIIA-L (~1–2 μg). Beads were washed thoroughly, resuspended in 2× SDS sample buffer [10% glycerol, 0.7 M β-mercaptoethanol, 3% SDS, and 0.5× upper Tris buffer (pH 6.8)], and loaded on an SDS–polyacrylamide gel. In the case of baculovirus-expressed dTFIIA-L, the proteins were transferred to nitrocellulose and blotted with anti-dTFIIA. SDS–polyacrylamide gels containing 35S-labeled proteins were dried down and exposed to the film. These signals were also analyzed and quantitated with the use of a PhosphorImager (Molecular Dynamics).

Western blot of Schneider cell extracts

Drosophila Schneider cells were harvested, washed briefly with PBS, and directly resuspended in 2× SDS sample buffer. After heat denaturation, the protein was subjected to SDS–PAGE and analyzed by Western blot.

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