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Identification of human TFIID components and direct interaction between a 250-kDa polypeptide and the TATA box-binding protein (TFIIDτ)

(RNA polymerase II/transcriptional activation/transcription initiation/subunit structure)

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ABSTRACT **Previous studies have indicated that human transcription initiation factor TFIID is a large complex that contains a TATA-binding polypeptide (TFIIDτ or TBP) and other components that qualitatively alter promoter interactions and are uniquely required for activator-dependent (versus basal) transcription. TFIIDτ-specific antibody columns have been employed to identify a number of human TFIID polypeptides that are tightly associated with TFIIDτ. These differ in size from polypeptides in known general initiation factors, including the initiator-binding factor (TFII-I) which shares some promoter binding characteristics with TFIID. The largest component (p250) identified in TFIID was shown to interact directly and tightly with TFIIDτ, suggesting that it may play a major role in the assembly of the TFIID complex.**

In eukaryotes transcription initiation on protein-coding genes involves the ordered assembly of RNA polymerase II and a number of general initiation factors (TFIIA, -B, -D, -E, -F, -G/J, -H, and -I) into a functional preinitiation complex on core promoter elements (TATA and initiator elements) (reviewed in refs. 1 and 2). The assembly and function of these complexes are further regulated by distal control elements and cognate gene-specific DNA-binding factors, which, in the case of activators, may contain various chemically distinct (acidic, glutamine-rich, proline-rich) activation domains (reviewed in refs. 3 and 4).

TFIID was identified initially as one of several chromatographically distinct initiation factors (5) and shown to initiate the assembly of a functional preinitiation complex by primary interactions with the TATA box and secondary interactions extending over and downstream of (to +35) the initiator region (6–10). Consistent with its key role in preinitiation complex assembly, early demonstrations of physical and functional interactions between TFIID and several activators (7, 11–14) further implicated TFIID as a target for regulatory factors. Although substantial progress in the purification of TFIID by conventional (8) and DNA affinity (M.H., unpublished observations) chromatography indicated the presence of multiple polypeptides, problems of stability and recovery precluded purification to homogeneity of sufficient amounts by these methods.

The subsequent identification of a yeast TATA-binding protein that could substitute for human TFIID in basal (core promoter) transcription (15, 16) led to its purification (17–19) and to the cloning of cDNAs encoding the yeast polypeptide (18–22) and corresponding highly conserved TATA-binding polypeptides from other organisms, including human (23–25), *Drosophila* (26, 27), plant (28), and *Schizosaccharomyces pombe* (29, 30). These polypeptides were active in basal transcription but, in contrast to native human and *Drosophila* TFIID species (6, 7), showed promoter interactions restricted to the TATA element and, importantly, failed to effect activator-dependent transcription (23, 24, 26). Along with the vast differences in molecular masses between the TATA-binding polypeptides (22–40 kDa) and the corresponding TFIID species (up to 750 kDa, refs. 31–33), these results cumulatively indicated that native TFIID from higher organisms contains, in addition to the TATA-binding subunit (designated TFIIDτ or TBP), polypeptides that are required specifically for activator function.

To further investigate TFIID structure–function relationships we have employed an antibody affinity method to identify the polypeptides associated with TFIIDτ in a fully functional human TFIID fraction. Similar results concerning the complex molecular structure of human TFIID were published during the course of this work (34, 35). Here we show, in addition, that a high molecular mass component (p250) present in TFIID can bind directly to TFIIDτ and may serve as major anchor for the other polypeptides in TFIID.

MATERIALS AND METHODS

Antibody-Affinity Column Chromatography. Rabbit anti-TFIIDτ sera were prepared by injection of TFIIDτ N-terminal peptides (Fig. 1*A*) coupled to bovine serum albumin. Anti-TFIIDτ sera were incubated with peptide-conjugated resin at 4°C, and anti-TFIIDτ specific antibodies were eluted with EGTG buffer [0.2 M glycine hydrochloride, pH 2.5 at 25°C, containing 50% (vol/vol) ethylene glycol and 10% (vol/vol) Tween 20]. Anti-TFIIDτ antibodies were immobilized on cyanogen bromide-activated Sepharose 4B (Pharmacia) (1.6 mg/ml) after further purification with protein A-agarose resin.

For the purification of TFIIDτ-associated polypeptides, a P11 0.85 M KCl fraction of TFIID from HeLa cell nuclear extract (8) was incubated with anti-TFIIDτ antibody-conjugated resin or antibody-free resin at 4°C in the following buffer: 20 mM Tris·HCl, pH 7.9 at 4°C, 0.75 M KCl, 15% (vol/vol) ethylene glycol, 10% (vol/vol) glycerol, 0.5% (vol/vol) Tween 20, 0.2 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The adsorbed resins were washed three times and eluted with EGTG buffer. The eluted fraction was adjusted to pH 8 with 2 M Tris.

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; NTA, nitrilotriacetic acid.

Immunoblot Analysis. Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to a nitrocellulose membrane. After blocking with 5% skim milk in Tris-buffered saline, the membrane was incubated for 1 hr at 37°C in 500-fold-diluted rabbit anti-TFIIDτ serum that had been preincubated in 2% bovine serum albumin solution. The membrane was stained using the Promega Protoblot Western blot AP system (rabbit).

Labeled and Unlabeled TFIIDτ Expressed in Bacteria. TFIIDτ was expressed from the 6 HisT-pET11d vector in BL21(DE3) plysS bacteria in the presence of 0.1 mCi of [^{35}S]methionine per ml (Amersham SJ1515; 1 Ci = 37 GBq) and 100 μg of rifampicin per ml. Bacterial extract was mixed batchwise with Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen), and labeled proteins bound to Ni^{2+}-NTA agarose were eluted with Tris buffer containing 0.25 M KCl, 20% (vol/vol) glycerol, and 200 mM imidazole hydrochloride, pH 7.9 at 4°C (36). The specific activity of purified labeled TFIIDτ was 3 × 10^6 cpm/μg. The 6 HisT-pET11d vector with no insert was subjected to the same expression/affinity purification protocol, and labeled proteins eluted from the Ni^{2+}-NTA agarose were used as a negative control probe in the experiment of Fig. 4.

The procedure for preparation of unlabeled TFIIDτ was the same as that for labeled TFIIDτ except that expression was done in the absence of [^{35}S]methionine. After purification of 6 HisT TFIIDτ using Ni^{2+}-NTA agarose, TFIIDτ was further purified using S-Sepharose resin (Pharmacia) in batch. The 6 HisT-pET11d vector was subjected to the same procedure, and derived proteins were used as a negative control in the competition analysis of Fig. 5.

Far Western Blot Analysis. After electrotransfer of the proteins from an SDS/polyacrylamide gel to the nitrocellulose membrane, proteins were denatured with 6 M guanidine hydrochloride in buffer A [20 mM Tris·HCl, pH 7.9 at 4°C, 0.25 M KCl, 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM PMSF, and 10% (vol/vol) glycerol] for 30 min, followed by successive 10-min treatments with 3.0, 1.5, 0.75, and 0.375 M guanidine hydrochloride in buffer A at 4°C. The membrane was washed with buffer A twice and treated with buffer A containing 1% skim milk for >3 hr. The membrane was then soaked in buffer A containing 1% skim milk and 0.02 μg of ^{35}S-labeled TFIIDτ per ml in the presence or absence of a 100-fold molar excess of unlabeled competitor TFIIDτ for 12 hr at room temperature. The membrane was washed with buffer A prior to autoradiographic analysis.

RESULTS

Anti-TFIIDτ Antibodies. To analyze TFIIDτ-associated polypeptides by an affinity method we prepared antibodies against an N-terminal region of TFIIDτ (Fig. 1*A*). This region was selected since previous results suggested that the nonconserved N-terminal region of yeast TFIIDτ was nonessential for cell growth (37–41) and more antigenic than the conserved C-terminal core (M.H., unpublished results). Immunoblot analysis (Fig. 1*C*) showed reactivity of immune serum but not preimmune serum with purified TFIIDτ (lane 4 versus lane 5) and with TFIIDτ in partially purified (phosphocellulose fraction) TFIID (lane 1 versus lane 2), although reactivity with other polypeptides was also observed in the latter case. However, affinity purification on an antigen-coupled column generated a purified antibody which was specific for TFIIDτ in partially purified TFIID (lane 3 versus lane 6). The apparent difference in mobility between recombinant TFIIDτ and endogenous TFIIDτ is shown more clearly in Fig. 1*D* and may reflect either HeLa-specific post-translational modification or a four-residue difference in the

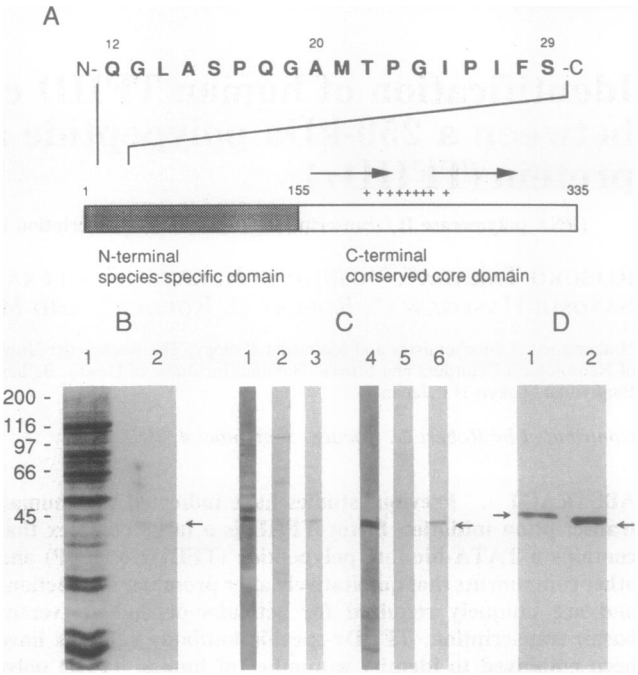

FIG. 1. Specificity of the antibody against TFIIDτ. (*A*) Amino acid sequence of the peptide used as antigen to obtain antibody against TFIIDτ. (*B*) SDS/PAGE and Coomassie brilliant blue staining of protein fractions used for immunoblot analysis. Lane 1, phosphocellulose TFIID fraction; lane 2, bacterially expressed TFIIDτ. (*C*) SDS/PAGE and immunoblot analysis of different TFIIDτ-containing fractions. Phosphocellulose fraction TFIID (lanes 1–3) and bacterially expressed TFIIDτ (lanes 4–6) blots were probed with crude immune serum (lanes 1 and 4), crude preimmune serum (lanes 2 and 5), or affinity-purified TFIIDτ specific antibody (lanes 3 and 6). (*D*) SDS/PAGE and immunoblot analysis of natural HeLa versus recombinant TFIIDτ. Phosphocellulose fraction TFIID (lane 1) and bacterially expressed TFIIDτ (lane 2) were analyzed. Arrows indicate the position of TFIIDτ (lower panels); positions of standard molecular mass markers are indicated on the left (in kDa).

number of glutamines between natural HeLa and Namalwa cell cDNA-derived TFIIDτ species (23–25).

Polypeptides Tightly Associated with TFIIDτ. Affinity-purified antibody specific for TFIIDτ was coupled to Sepharose, and partially purified TFIID (phosphocellulose fraction) was loaded onto antibody-coupled and control (noncoupled) Sepharose columns under high salt conditions (*Materials and Methods*). After extensive washing antibody-bound TFIIDτ and associated polypeptides were eluted under low pH conditions and analyzed by SDS/polyacrylamide gel electrophoresis and silver staining (Fig. 2*A*). In addition to TFIIDτ (lower arrow) this analysis detected several polypeptides [approximate molecular masses of 250, 150, 120, 100, 80, 75, 65, and 60 kDa (upper arrows); see also figure legend] that appeared specific for the TFIIDτ antibody-coupled column (lane 1) versus the control column (lane 2). The major contaminants include a background band (upper asterisk) also present in the control column eluate and IgH polypeptides (lower asterisk). That the 38-kDa polypeptide represents TFIIDτ was confirmed by Western blot analysis (Fig. 2*B*). The polypeptides specifically retained by the antibody column (see also Fig. 3*A*) are candidate TFIID subunits although it is not clear at this stage how they are all bound (directly or indirectly) to TFIIDτ. Except for our failure to clearly resolve specific low molecular weight bands, these results are consistent with the results of other analyses (34, 35).

Possible Relationship of TFII-I to TFIIDτ-Associated Polypeptides. TFII-I is a 120-kDa initiator-binding transcription

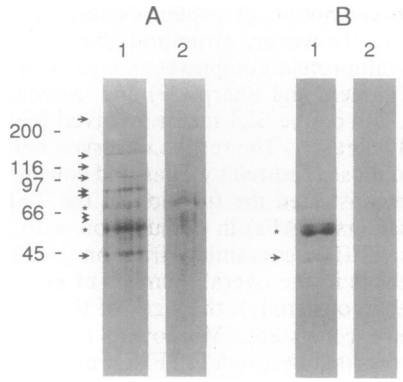

FIG. 2. Analysis of polypeptides associated with TFIIDτ. Antibody-containing and control resins were incubated independently with a phosphocellulose fraction of TFIID and after washing bound polypeptides were eluted at low pH and separated by SDS/PAGE. (*A*) Silver staining of bound polypeptides from antibody (lane 1) or control (lane 2) resins. The lower arrow indicates TFIIDτ; upper arrows indicate other polypeptides specifically and reproducibly retained on the antibody resin. The arrowheads indicate minor (65 and 60 kDa) specifically bound polypeptides that were more apparent in other analyses. The stoichiometries of the specifically bound polypeptides indicated here were more equivalent when monitored by sequence analysis following elution from preparative gels. Asterisks indicate immunoglobulin heavy chain (lower) and nonspecific (upper) polypeptides. Molecular masses are indicated in kDa. (*B*) Immunoblot analysis of TFIIDτ in bound polypeptides from antibody (lane 1) and control (lane 2) resins.

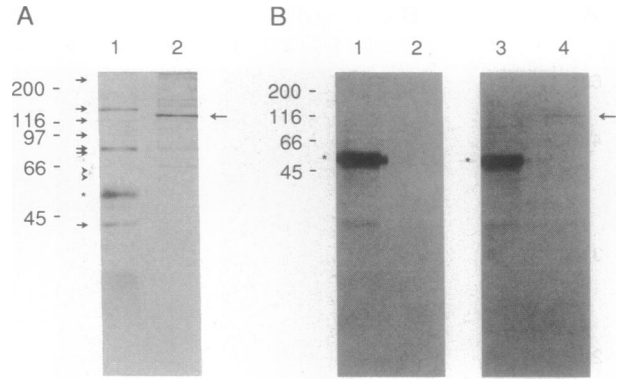

FIG. 3. Absence of TFII-I in the TFIIDτ-associated polypeptides. A preparation of TFIIDτ-associated polypeptides eluted from an antibody column and a partially purified preparation of TFII-I were subjected to SDS/PAGE and analyzed. (*A*) Silver staining of TFIIDτ-associated polypeptides (lane 1) or TFII-I (lane 2) fractions. (*B*) Immunoblot analysis of TFIIDτ-associated polypeptides (lanes 1 and 3) or TFII-I (lanes 2 and 4) using preimmune serum (lanes 1 and 2) or an immune serum against USF (lanes 3 and 4) that reacts with TFII-I (42). The right arrows indicate TFII-I. The other marks are the same as described in the legend to Fig. 2.

initiation factor that has been shown to interact cooperatively with TFIIDτ on the adenovirus major late promoter, generating complexes that showed broad DNase footprints similar to those exhibited by native TFIID preparations under certain conditions (ref. 42; A. L. Roy and R.G.R., unpublished observations). Although TFII-I is readily purified as a component separable from TFIID (42), these observations raised the possibility that the 120-kDa TFII-I polypeptide might be an integral component of native TFIID. However, a comparative analysis of TFIIDτ antibody affinity column-purified polypeptides with a TFII-I preparation showed clear differences in the apparent sizes of TFII-I and the 120-kDa component of TFIID (Fig. 3*A*). Moreover, immunoblot analysis with a USF antibody that also recognizes TFII-I (42) failed to reveal a corresponding immunoreactive component in the TFIIDτ-associated polypeptides (Fig. 3*B*). Additionally, a comparative sequence analysis of cDNAs encoding the large TFIIDτ-associated polypeptides (K. Hisatake, R.T., S.H., S. Hashimoto, Y.N., M.H., and R.G.R., unpublished results) revealed no sequence similarity with that of TFII-I (A. L. Roy, P. Gregor, E. Martinez, and R.G.R., unpublished observations). These results suggest that although TFII-I can interact with TFIIDτ on the promoter, it does not interact as strongly as the other TFIIDτ-associated polypeptides and is not likely to be an integral subunit of TFIID.

Direct Interaction of TFIIDτ with p250. The antibody affinity analysis indicated that several polypeptides interact directly or indirectly with TFIIDτ. The stability of these interactions to high salt conditions along with size estimates of around 700 kDa for native TFIID (31–35) further indicate that these polypeptides comprise a native TFIID complex(es). To determine which polypeptides make direct contacts with TFIIDτ, various preparations of TFIID were subjected to SDS/PAGE and transferred to nitrocellulose membranes. After protein renaturation filters were probed with purified ^{35}S-labeled TFIIDτ (^{35}S-TFIIDτ) and with control ^{35}S-labeled proteins from bacteria lacking TFIIDτ coding sequences in the expression vector (Fig. 4*A*). As shown in Fig. 4*B*, under the conditions employed ^{35}S-TFIIDτ was observed to interact exclusively with a 250-kDa polypeptide in the affinity-purified associated polypeptides (lane 1), in partially purified TFIID (lane 2), and in crude HeLa nuclear extracts (lane 3), whereas no interactions were observed with control ^{35}S-labeled bacterial polypeptides (lanes 4–6). These observations suggest a strong and highly specific interaction between p250 and TFIIDτ.

To further demonstrate the specificity of the observed interaction, especially in light of the presence of contaminating proteins in the purified ^{35}S-TFIIDτ preparation, the interaction studies were repeated in the presence of a homogeneous preparation of unlabeled TFIIDτ or a corresponding mock preparation from bacteria containing a vector with no TFIIDτ coding sequences (Fig. 5*A*). As shown in Fig. 5*B*, unlabeled TFIIDτ completely blocked p250 interactions with the ^{35}S-TFIIDτ preparation (lanes 1–3), whereas the mock preparation had no effect (lanes 4–6). This result confirms the

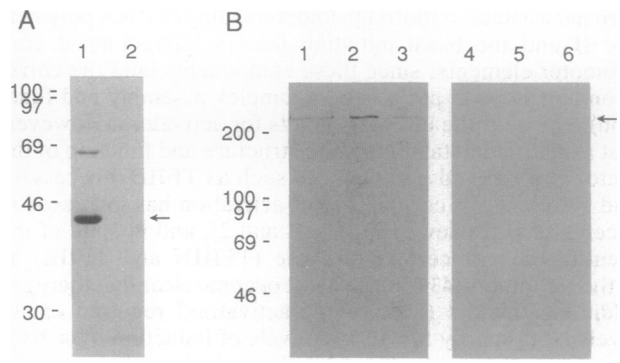

FIG. 4. Direct binding of TFIIDτ to p250 in the TFIIDτ-associated polypeptides. (*A*) Analysis of purified ^{35}S-labeled probes by SDS/PAGE and autoradiography. Lane 1, purified labeled TFIIDτ; lane 2, labeled control polypeptides from bacteria with the no-insert 6 HisT-pET11d vector. The arrow indicates the position of labeled TFIIDτ; the left column shows the positions of labeled standard molecular mass markers (in kDa) (Amersham). (*B*) Analysis of direct binding between TFIIDτ and TFIIDτ-associated polypeptides. TFIIDτ-associated polypeptides (lanes 1 and 4), phosphocellulose fraction TFIID (lanes 2 and 5), and HeLa cell nuclear extract (lanes 3 and 6) were subjected to SDS/PAGE and analyzed by far Western blot analysis using purified labeled TFIIDτ probe (lanes 1–3) or purified control probes (lanes 4–6). The arrow indicates the position of p250.

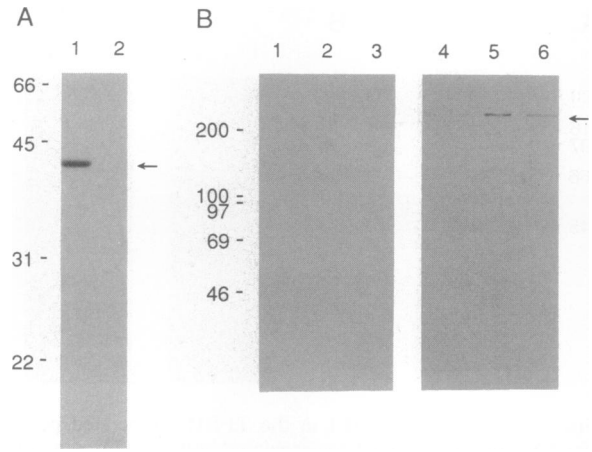

FIG. 5. Direct binding of TFIIDτ to p250 in the TFIIDτ-associated polypeptides assessed by competition assay. (*A*) Analysis of purified unlabeled competitors. Purified TFIIDτ (lane 1) and control proteins from bacteria containing the no-insert 6 HisT-pET11d vector (lane 2) were separated and stained with Coomassie brilliant blue. The arrow indicates the purified 6 HisT-TFIIDτ. There were no detectable proteins in the higher molecular mass zone (data not shown). (*B*) Inhibition of direct interaction between labeled TFIIDτ and p250 by addition of homogeneous unlabeled TFIIDτ. TFIIDτ-associated polypeptides (lanes 1 and 4), phosphocellulose TFIID fraction (lanes 2 and 5), and HeLa cell nuclear extract (lanes 3 and 6) were separated by SDS/PAGE and analyzed by far Western blot analysis using purified labeled TFIIDτ probe (as in Fig. 4) in the presence of 100-fold molar excesses of purified unlabeled TFIIDτ competitor (lanes 1–3) or control proteins (lanes 4–6). The arrow indicates the position of p250. Molecular masses are indicated in kDa.

specificity of the p250–TFIIDτ interactions and further argues that p250 is an integral component of TFIID.

DISCUSSION

As information on the diversity, structure, and regulation of various gene-specific factors has accumulated, there has been increasing interest in the actual mechanisms by which various types of activators activate target promoters. This in turn necessitates a thorough understanding of RNA polymerase II and the basal initiation factors interacting at core promoter elements, since these components (and the corresponding steps in preinitiation complex assembly and function) represent the ultimate targets for activators. However, just as our understanding of the structure and function of the factors (or derived polypeptides such as TFIIDτ) necessary and sufficient for core promoter activation has increased in recent years (reviewed in refs. 1 and 2), and in spite of the identification of certain of these (TFIIDτ and TFIIB) as activator targets (43–46), so has it become clear that there are additional factors (cofactors/coactivators) required exclusively or primarily for normal levels of induction by activators (reviewed in refs. 1 and 47). These include those present (with TFIIDτ) in native TFIID as well as more readily separable factors (48, 49).

In the present analysis we have identified, through the use of specific antibodies, a complex array of polypeptides that are associated with TFIIDτ in functional TFIID preparations. That these polypeptides may represent integral subunits of TFIID is suggested by their tight association (stable to detergents and 0.75 M KCl) with TFIIDτ and by the native molecular mass estimates for TFIID. The lack of reliable information on the stoichiometry of these components (legend to Fig. 2) precludes conclusions about the presence of a homogeneous versus heterogeneous species in the conventional TFIID fraction that is active in basal and activator-dependent transcription of protein-coding genes by RNA polymerase II. However, structural diversity in TFIIDτ-containing multiprotein complexes is clear from the recent studies of Timmers and Sharp (33) and especially those of Comai *et al.* (50) on the SL1 factor involved in transcription by RNA polymerase I. The results observed here for TFIID are similar to those reported by Tjian and colleagues (34, 35), who also demonstrated the function of the TFIIDτ (TBP)-associated factors (TAFs) in conjunction with a separated endogenous TFIIDτ-containing fraction. Although there were differences in the overall number of polypeptides detected (fewer in our study), the sizes of the observed polypeptides were consistent. Moreover, recently published work (51) shows that *Drosophila* TFIIDτ associates primarily with six different polypeptides with sizes relatively similar to these reported here. However, a *Drosophila* counterpart to the human p250 and human analogues to two low molecular weight *Drosophila* polypeptides were not observed. Whether these differences reflect true species differences or variable analytical methods is not clear, although our own analyses of *Drosophila* nuclear extracts have indicated a somewhat larger array of polypeptides (T.K., R.T., S. Yamashita, D.-W. Gong, R.G.R., M.H., and Y.N., unpublished results).

At present the TFIIDτ-associated polypeptides that have been identified as distinct entities on SDS gels are implicated in activator-dependent transcription only by circumstantial evidence. However, assuming that the complex array of polypeptides are integral components of TFIIDτ, the question arises as to their individual functions. Perhaps the most obvious possibility, also suggested by Tjian and colleagues (34, 35, 50), is that they might have selective functions for different classes of activators. Possible roles as adaptors or transducers of signals from activators to basal factors or as stabilizing factors that facilitate direct interactions between activators and basal factors can be envisioned along with other possibilities (discussed further in ref. 1). It is interesting in this regard that although direct interactions of various activators with TFIIB and TFIIDτ have been documented (43–46) and are likely relevant to the activation mechanisms, components specific to native TFIID are still essential for the function of these activators. Thus, our earlier demonstrations (11, 12) of qualitative effects of activators on native TFIID interactions with target promoters remain relevant and are indicative of additional interactions and conformational changes within TFIID that are important for the full manifestation of activator function (1). Questions about the specific roles of individual TFIID components await their further characterization, including cloning of corresponding cDNAs and development of suitable probes and functional assays. However, one general function of the p250 component is apparent from the present study demonstrating strong interactions with TFIIDτ. Thus, though interactions of other components with TFIIDτ cannot yet be excluded, this result suggests that the large 250-kDa polypeptide may function at least in part to tether other components to TFIIDτ.

The restriction of direct human TFIIDτ interactions to one (or a few) of the tightly associated polypeptides would thus explain how the relatively small TFIIDτ can associate stably with so many different polypeptides and still exhibit more readily reversible interactions with other factors (and with DNA). Factors in the latter category include the basal factors TFIIA (52–54) and TFIIB (52, 53), the initiator factor TFII-I (ref. 1; A. L. Roy and R.G.R., unpublished observations), and a group of negative cofactors (48, 49, 55). These factors, as well as other positive cofactors whose sites of interaction are not clear (48), appear distinct from the factors tightly associated with TFIIDτ in native TFIID. This is apparent on the basis of different chromatographic properties and on the basis of direct comparisons, as shown here for TFII-I. Nonetheless, these latter factors might be variably associated

with TFIIDτ in the cell to generate complexes with distinct biochemical characteristics and biological functions.

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