Human TAF_{II}250 interacts with RAP74: implications for RNA polymerase II initiation

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Accurate and regulated transcription by RNA polymerase II requires the assembly of an initiation complex involving multiple protein–DNA and protein–protein interactions. A key event is binding of TFIID, a complex consisting of TBP and associated factors (TAFs) to the template DNA. The TAF subunits of TFIID carry out diverse functions critical for transcription, including specific contact with enhancer proteins and binding to core promoter DNA. However, the role of TAFs in RNA polymerase II-mediated transcription initiation and cross talk with other basal factors remains poorly characterized. Here, we report the specific interaction of TAF_{II}250 with RAP74, an essential subunit of the basal transcription factor IIF. Using various *in vitro* binding assays we have mapped recognition interfaces between TAF_{II}250 and RAP74. *In vivo* complementation of a temperature-sensitive TAF_{II}250 cell line reveals that the RAP74 interaction is critical for cell viability. Because TFIIF is thought to be responsible for binding and recruiting RNA polymerase II, the ability of TAF_{II}250 to interact selectively with RAP74 is likely to contribute a critical contact for the assembly of an active transcription complex.

[Key Words: RNA Pol II; TAF; RAP74; transcription]

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It is well established that transcriptional regulation in eukaryotic cells requires the assembly of an elaborate molecular apparatus at the site of transcriptional initiation. For protein-coding genes, binding of site specific activators to distal enhancer and/or proximal promoter DNA elements is thought to help recruit, stabilize, or otherwise influence the formation of an activated initiation complex consisting of RNA polymerase II (Pol II) and basal accessory factors. An early and key event during the process of transcriptional activation is the binding of the basal factor, TFIID, to the core promoter. Subsequently, TFIIA, TFIIB, TFIIF/RNA Pol II, TFIIIE, and TFIIF are assembled at the site of transcriptional initiation (Buratowski et al. 1989; for review, see Zawel and Reinberg 1993).

Biochemical purification and molecular analysis have established that TFIID is a multisubunit complex comprised of the TATA-binding protein (TBP) and at least eight stably associated factors called TAFs (TBP-associated factors; Dynlacht et al. 1991; Tanese et al. 1991). Recent studies suggest that different subunits of the TFIID complex perform different functions that are not only critical for promoter recognition but also for gene specific activation (Chen et al. 1994; Verrijzer et al.

1995). It was found, for example, that in addition to TBP, which binds to the TATA box, at least two different TAFs in the TFIID complex participate in DNA recognition and promoter selectivity: TAF_{II}150 and TAF_{II}250 recognize and interact with initiator and downstream core promoter elements (Verrijzer et al. 1994, 1995; S. Hansen, pers. comm.). Several of the TAFs have also been shown to serve as coactivators that bind directly to activation domains of enhancer proteins. These include TAF_{II}110, which interacts with certain glutamine-rich activators (Hoey et al. 1991), and TAF_{II}40/60, which target the acidic class of activators (Goodrich et al. 1993; Thut et al. 1995). Both mutational and biochemical studies suggest that these TAF/activator interactions are essential for mediating enhancer dependent transcriptional activation (Chen et al. 1994; Goodrich and Tjian 1994a). However, the mechanism by which TAFs in the TFIID complex transduce or relay signals from activators to the basal machinery remains unknown.

The simplest hypothesis would be that some TAFs in the TFIID complex directly contact other basal factors, thereby recruiting and stabilizing the assembly of an active initiation complex. Previous studies have documented the interaction of TAF_{II}40 with the basal factor TFIIB, which is essential for transcription (Goodrich et al. 1993). However, it is unlikely that interactions between TFIID and TFIIB alone are sufficient to account for

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all the critical contacts necessary to direct transcriptional initiation by RNA Pol II. It is more likely that multivalent interactions between components of TFIID and other basal factors contribute to the signaling pathways connecting activators to the transcriptional apparatus. For example, it is unclear how RNA Pol II is recruited to the template. Early studies showed that the basal factor TFIIF plays a crucial role in RNA Pol II binding. The two subunits of TFIIF, called RAP74 and RAP30, were initially identified as RNA Pol II-associated proteins (Sopta et al. 1985; Burton et al. 1986) and subsequently found to be basal transcription factors essential for transcriptional initiation (Burton et al. 1988). Thus, a good candidate for bridging TFIID and RNA Pol II would be TFIIF.

Here, we report our attempts to identify components of the initiation complex that interact specifically with TFIID. Using a variety of protein-protein binding assays we have assessed the potential interaction of TAF_{II}250, the core subunit of TFIID, with different basal factors. In addition, we have mapped regions of TAF_{II}250 responsible for the recognition of specific targets in the initiation complex and vice versa. Finally, we have used the temperature sensitive cell line ts13 which is defective for TAF_{II}250 function to assess the *in vivo* importance of TAF_{II}250/basal factor interactions. Our results suggest that TAF_{II}250 recognizes and binds to a unique domain of RAP74 and that this interaction may contribute to transcription initiation by RNA Pol II.

Results

Human TAF_{II}250 binds to RAP74

Preinitiation complex formation at RNA Pol II promoters has been reported previously to occur in an ordered assembly of basal factors, beginning with TFIID binding to DNA followed by recruitment of TFIIA and TFIIB (Buratowski et al. 1989; Zawel and Reinberg 1993). The resulting TFIID/TFIIA/TFIIB complex is competent to interact with TFIIF and RNA Pol II. A critical step in the recruitment of RNA Pol II to the initiation complex appears to involve TFIIF. Because both subunits of this essential basal factor, RAP74 and RAP30, bind to RNA Pol II (Burton et al. 1988), it was of particular interest to test the potential interaction of TFIIF and components of TFIID. We began our study by assessing the ability of TFIID to bind the largest subunit of TFIIF, RAP74. Immunopurified TFIID was isolated from HeLa nuclear extracts (Fig. 1a, lane 2) and the resulting complex was allowed to bind *in vitro* translated human RAP74 (Finkelstein et al. 1992; Fig. 1a, lane 4). Our results indicate that RAP74 binds efficiently to a resin containing TFIID but not to a control resin (Fig. 1a, lanes 1,3). This result suggests that one or more components of TFIID is able to bind RAP74.

Thus far, no direct interaction between RAP74 and TBP has been documented. Therefore, the binding of RAP74 to immunopurified TFIID was likely to be medi-

Figure 1. Human TAF_{II}250 binds directly to RAP74. (a) RAP74 binds to TFIID. Human TFIID was immunopurified from HeLa nuclear extract by binding to a monoclonal anti-hTAF_{II}150 antibody (lanes 2,4). Lanes 1 and 3 are controls without TFIID. Duplicate samples as shown on the silver-stained gel (lanes 1,2) were incubated with radiolabeled human RAP74 (lanes 3,4). The bound protein was visualized by autoradiography. The arrow points to RAP74. Molecular masses of protein size markers are given in kD in all of the figure legends. (b) *In vitro*-translated RAP74 binds to hTAF_{II}250. Human TAF_{II}250 (hTAF_{II}250) was expressed as a HA-tagged protein in the baculovirus expression system, purified via binding to αHA antibody, immobilized to protein A beads, and analyzed by SDS-PAGE followed by Coomassie blue staining (lane 1, HA-hTAF_{II}250). As a control untransfected SF9 cells were processed identically (lane 2, SF9 control). The asterisk indicates the IgG. RAP74 was *in vitro* transcribed/translated and used in coimmunoprecipitation assays with either purified HA-hTAF_{II}250 (lanes 4,5) or SF9 control extract (lanes 6,7). The autoradiograph shows 10% of the input RAP74 (lane 3), the supernatant of the final washes (W; lanes 4,6) and RAP74 bound (B; lane 5,7) to HA-hTAF_{II}250 beads and SF9 control beads, respectively. (c) hTAF_{II}250 binds selectively and directly to RAP74. The indicated proteins were analyzed by Far Western blot analysis for their direct binding to ³⁵S-labeled hTAF_{II}250. The autoradiograph shows a band corresponding to RAP74 (lane 3).

ated via TAFs. Several different protein-binding assays were used to identify the TAFs that bind to RAP74, including coimmunoprecipitation and Far Western blot analysis. First, full-length human TAF_{II}250 (hTAF_{II}250; Ruppert et al. 1993) containing a hemagglutinin (HA) epitope tag was immunopurified from SF9 cells infected with a recombinant baculovirus encoding TAF_{II}250 (Fig. 1b, lane 1) and used to bind RAP74. Figure 1b, lane 5,

shows that the purified recombinant hTAF_{II}250 recognizes and binds efficiently to radiolabeled *in vitro* synthesized RAP74. Control resins containing HA antibody-immunopurified proteins from uninfected SF9 cells (Fig. 1b, lane 2) retained only a low background level of RAP74 (Fig. 1b, lane 7). We also tested each of the other TAFs that were available as cloned gene products but did not find any additional components of TFIID that interacted specifically and directly with RAP74 (data not shown).

As an additional test of specificity and to demonstrate direct interaction between hTAF_{II}250 and RAP74, we performed a Far Western analysis (Kaelin et al. 1992; Ruppert et al. 1993). Recombinant RAP74, transferred to a nitrocellulose membrane after SDS-PAGE, denatured, and subsequently renatured, was found to bind selectively to ³⁵S-labeled TAF_{II}250 (Fig. 1c, lane 3). The identity of the signal obtained was verified by reprobing the same filter with a polyclonal anti RAP74 antibody (data not shown). We also tested several other human basal factors including TFIIE(p56) (Peterson et al. 1991), TFIIE(p34) (Peterson et al. 1991), RAP30 (Sopta et al. 1989), TFIIB (Ha et al. 1991) and the subunits of RNA Pol II by Far Western analysis and found that none of these components of the initiation complex bound to hTAF_{II}250 (Fig. 1c, lanes 1,2,4–7). Taken together, these results suggest that hTAF_{II}250 is able to interact selectively and directly with RAP74.

An amino-terminal domain of RAP74 interacts with TAF_{II}250

Having established an interaction between the largest subunit of TFIIF and the largest subunit of TFIID, we next attempted to map the region of RAP74 responsible for binding hTAF_{II}250. We began by generating a series of carboxy-terminal deletions of RAP74 and expressing them as radiolabeled *in vitro*-translated proteins. Next, we tested the binding of these truncated proteins to full-length HA-hTAF_{II}250 tethered to a resin via the HA epitope tag. Whereas several larger truncated molecules also bound efficiently to hTAF_{II}250 (data not shown), remarkably, molecules containing only the amino-terminal 183 or 139 amino acid residues of RAP74 were as efficient in binding to hTAF_{II}250 as the full-length protein (Fig. 2, a, lanes 1–9, and b). Removal of an additional 19 amino acids from the carboxyl terminus to generate mutant N120 resulted in a truncated product that was ~50% as active in binding hTAF_{II}250 (Fig. 2, a, lanes 10–12, and b). Deletion of sequences between residues 120 and 100 rendered the resulting mutant protein severely impaired for binding TAF_{II}250 (N100; Fig. 2, a, lanes 13–15, and b). Further removal of sequences to residue 83 produced an inactive protein (N83; Fig. 2, a, lanes 16–18, and b). These results define a region between residue 1 and 120 that appears necessary and sufficient to provide an interface for binding to hTAF_{II}250.

Figure 2. Human TAF_{II}250 binds to the amino-terminal globular domain of RAP74. (a) Binding of carboxy-terminal RAP74 truncated proteins to hTAF_{II}250. HA-hTAF_{II}250 bound to protein A beads via the αHA antibody was incubated with ³⁵S-labeled full-length RAP74 (lanes 1–3) or with carboxy-terminal truncated proteins thereof (lanes 4–18). Each set of data includes 10% of the protein input (I), the final wash of the beads (W), and the bound proteins (B). The carboxy-terminal amino acid included in the truncated proteins is indicated by the number given above each set. Asterisks indicate the presence of full-length RAP74 that is generated because of incomplete restriction digestion of the template. The proteins indicated were analyzed on 8%–15% SDS-PAGE, depending on their molecular weight, and visualized by autoradiography. (b) Schematic representation of full-length RAP74, carboxy-terminal RAP74 truncated proteins, and quantitation of their binding to hTAF_{II}250. Based on its primary sequence, an amino-terminal globular domain (amino acid residues 1–178), a cluster of charged amino acid residues (179–356) and a carboxy-terminal globular domain (amino acid residues 357–517) have been described for RAP74 (Finkelstein et al. 1992). The truncated RAP74 proteins used in the coimmunoprecipitation assay (above) are shown schematically with respect to their position within full-length RAP74. The amount of each individual protein binding to hTAF_{II}250 is given as the percentage of binding of full-length RAP74, which was set at 100%.

A domain of 150 amino acids within hTAF_{II}250 is required for binding RAP74

Given the large size of human TAF_{II}250 (1893 amino acids; Ruppert et al. 1993) it has been difficult to carry out a detailed mutational analysis of its functional domains. Thus far, only the region of the *Drosophila* homolog (p230/250; Kokubo et al. 1993; Weinzierl et al. 1993) involved in binding to TBP has been studied (Kokubo et al. 1994). As a first step toward defining re-

Figure 3. Carboxy- and amino-terminal truncations of hTAF_{II}250 define the domain interacting with RAP74. (a) Far Western analysis of carboxy-terminal hTAF_{II}250 deletion proteins. The silver-stained gel (lane 1) shows the RAP74 preparation used for the Far Western analysis. The autoradiographs show the binding of full-length hTAF_{II}250 (lane 2) and carboxy-terminal truncated proteins thereof (lanes 3–11) to RAP74. The carboxy-terminal amino acid residue retained in the respective truncated proteins are indicated above each autoradiograph. All filters were exposed for the same time. The arrow points to RAP74. (b) Binding of RAP74 to GST–hTAF_{II}250ΔN proteins. Amino-terminal truncations of hTAF_{II}250 were expressed as GST–fusion proteins (GST–hTAF_{II}250ΔN), purified from baculovirus-infected SF9 cells by binding to GST beads, and used for binding to RAP74 (lanes 2–10). These beads, beads bound with GST encoded by the plasmid pGEX2TK (Pharmacia; GST, lane 11), and SF9 control proteins (SF9, lane 12) were incubated with ³⁵S-labeled RAP74. After binding, the complexes were analyzed on an 8% SDS–polyacrylamide gel, and bound RAP74 (indicated by an arrow) was visualized by autoradiography. (Lane 1) 10% of RAP74 used in the binding assays. The arrow points to the largest polypeptide, RAP74. (c) Schematic representation of the carboxy- and amino-terminal truncated hTAF_{II}250 proteins and quantitation of their interaction with RAP74. hTAF_{II}250 including some of its features is shown schematically. Shaded boxes indicate both carboxy- and amino-terminal acidic domains, bars indicate proline-rich sequences, arrows indicate two direct 120 amino acid residue direct repeats, each containing a bromo-domain (B), and SWI4 indicates sequence homology to the yeast protein SWI4 [Sekiguchi et al. 1991; Hisatake et al. 1993]. The names of the individual carboxy- and amino-terminal hTAF_{II}250 truncations (N and ΔN, respectively) are given on the left and the extent of their truncation is indicated by the lines. The signals obtained in Fig. 3, A and B, were quantified using a PhosphorImager and are presented as percent binding to filter-bound RAP74 (carboxy-terminal truncated hTAF_{II}250 proteins) and percent binding of radiolabeled RAP74 (amino-terminal truncated hTAF_{II}250 proteins), setting the binding to the full-length protein hTAF_{II}250 to 100%.

gions of TAF_{II}250 responsible for interacting with RAP74, we have carried out a systematic analysis of amino- and carboxy-terminal deletions. To assay the RAP74-binding properties of carboxy-terminal deletion mutants of hTAF_{II}250, we employed the Far Western assay in which radiolabeled in vitro-synthesized mutant versions of hTAF_{II}250 were allowed to bind full-length RAP74 (Fig. 3a, lane 1) tethered to membrane filters (Figs. 3a,c). Deletions that removed the carboxy-terminal 500 amino acids resulted in truncated molecules that

were as active in binding RAP74 as full-length hTAF_{II}250 (Fig. 3, a, lanes 2–5, and c). However, removal of an additional 214 residues from the carboxy-terminal region of hTAF_{II}250 produced a protein (N1184) that binds RAP74 at least fivefold less efficiently as determined by PhosphorImager quantitation (Fig. 3, a, lane 6, and c). Further removal of sequences to residue 1054, as well as more extensive deletions of hTAF_{II}250 resulted in a series of truncated products unable to bind RAP74 (Fig. 3, a, lanes 7–11, and c). These results suggest that

the region between amino acid residues 1184 and 1398 contains sequences important for recognition and binding to RAP74.

To map the amino-terminal boundary of the putative RAP74 interaction domain we generated a series of amino-terminal deletions as glutathion *S*-transferase (GST)-fusion proteins and tested their ability to bind radiolabeled RAP74 (Fig. 3b,c). Our results indicate that deletions of the first 700 amino acids from the amino terminus of hTAF_{II}250 have no significant effect on the binding of the resulting truncated proteins to RAP74 (Fig. 3, b, lanes 1–5, and c). However, removal of additional sequences to residue 1162 gave rise to a deletion mutant that was ~30% as active in binding RAP74 as the full-length molecule (Δ N1162; Fig. 3, b, lane 6, and c). Additional amino-terminal deletions to residue 1206 further reduced binding efficiency to ~15% of wild type (Δ N1206; Fig. 3, b, lane 7, and c). Removal of the next 67 residues (Δ N1273) as well as further amino-terminal deletions essentially abolished binding to RAP74 (Fig. 3, b, lanes 8–10, and c). In addition to defining this amino-terminal boundary using hTAF_{II}250 truncated proteins fused to GST, we performed Far Western blot analysis and obtained similar results (data not shown). These results taken together with the carboxy-terminal deletion studies identify a region of ~150 amino acids between residues 1120 and 1270 that likely contains an important interface for binding to the amino-terminal domain of RAP74.

The RAP74 binding region of hTAF_{II}250 is required to rescue a temperature-sensitive mutant

Thus far, we have documented the specific interaction between an essential subunit of TFIID, TAF_{II}250, and one component of the basal factor TFIIF, RAP74. How-

ever, these in vitro protein-protein-binding assays do not address the functional relevance of this interaction. When dealing with mammalian transcription factors it is very difficult, if not impossible, to assess the contribution of specific interactions to the transcription process in the context of the intact cell. Given the difficulties of assessing the function of individual components in the context of a multisubunit complex, we have attempted to gain some insights concerning the importance of the hTAF_{II}250/RAP74 interaction by in vivo complementation. The existence of the temperature-sensitive cell line ts13 (Talavera and Basilico 1977) that bears a conditional lethal TAF_{II}250 mutation leading to a block in cell cycle progression at the nonpermissive temperature (39.5°C) allows us to perform complementation studies (Sekiguchi et al. 1991; Wang and Tjian 1994). Thus, it should be possible to test various hTAF_{II}250 mutants for their ability to complement the ts13 phenotype and rescue the growth of these cells at the high temperature.

To perform such complementation assays, we have generated eukaryotic expression vectors containing either full-length wild-type hTAF_{II}250 or carboxy-terminal deletions that span the RAP74 interaction domain (Fig. 4a; Table 1). As expected, ts13 cells transfected with vectors expressing wild-type hTAF_{II}250 and subsequently shifted to the nonpermissive temperature were able to overcome the cell cycle block and grow at 39.5°C (Table 1; Fig. 4b). Similarly, ts13 cells expressing carboxy-terminal truncated versions of hTAF_{II}250 (N1784, N1681, and N1398; Table 1; Fig. 4a) that retain the ability to bind efficiently to RAP74 (Fig. 3) also allowed growth at the nonpermissive temperature (Fig. 4b). In contrast, expression of TAF_{II}250 mutants N1184 and N1054, which failed to bind RAP74 efficiently in vitro (see Fig. 3), failed to rescue the temperature-sensitive phenotype and no colonies were observed at the nonpermissive temperature (Table 1). In addition, a TAF_{II}250 mutant (N1225), which lacks part of the RAP74 interaction domain, also failed to rescue the ts13 phenotype (Fig. 4). Expression of the full-length proteins N1398, N1184, and N1054 in ts13 cells kept at permissive temperature was comparable, whereas expression of N1225 was slightly higher (data not shown). These results were consistent with the hypothesis that the region of hTAF_{II}250 defined as the RAP74 interaction domain is necessary to provide some essential function required to assemble a TFIID complex capable of mediating activated gene transcription (Fig. 5). However, we cannot rule out the possibility that this deleted region also contains amino acids important for other functions of hTAF_{II}250 that have not been defined.

Discussion

Eukaryotic transcription initiation requires the coordinate assembly of multiple regulatory factors at the promoter culminating with the recruitment of RNA Pol II (Buratowski et al. 1989; Zawel and Reinberg 1993). An early event in the formation of preinitiation complexes is the binding of TFIID to the promoter (Buratowski et al. 1989). Recent evidence suggests that the TBP, as well as TAF subunits of TFIID, participate in DNA binding and

Table 1. Summary of ts13 in vivo complementation experiments

Constructs	Percent binding to RAP74	Rescue of ts13
N1893	100	+
N1784	91	+
N1681	137	+
N1398	110	+
N1225	10	–
N1184	18	–
N1054	2	–

hTAF_{II} 250 mutants that fail to bind efficiently to RAP74 also fail to rescue the ts13 mutant phenotype. In addition to the constructs shown in Fig. 4A, ts13 cells were transfected with carboxy-terminal deletion constructs pCMV-HA-hTAF_{II}250-N1784, N1681, N1184, and N1054. N1893 encodes full-length hTAF_{II}250. The percent binding of RAP74 to the indicated hTAF_{II}250 mutants was derived from Fig. 3 except for mutant N1225 for which the percent binding to RAP74 was derived from a separate experiment using GST-fusion proteins (GST-hTAF_{II}250 Δ N1054/N1398 and GST-hTAF_{II}250 Δ N1054/N1225).

Figure 4. The RAP74 interaction domain is required for cell viability. (a) Constructs used for the *in vivo* complementation assay. The extent of the coding region of the carboxy-terminal hTAF_{II}250 deletion clones used to rescue the ts13 phenotype is shown schematically. The RAP74 interaction domain is completely included in the full-length construct pCMV-HA-hTAF_{II}250 and carboxy-terminal deletion construct pCMV-HA-hTAF_{II}250N1398 and is partly deleted in pCMV-HA-hTAF_{II}250N1225. Additional constructs used in the ts13 rescue experiment are listed in Table 1. (b) The construct pCMV-HA-hTAF_{II}250N1225 fails to rescue the ts13 phenotype. The constructs shown in Fig. 4A and Table 1 were used to transfect ts13 cells. After shifting to the nonpermissive temperature cell viability was monitored after 10–14 days in culture.

promoter recognition (Verrijzer et al. 1995). After binding of TFIID to the template, the basal factors TFIIA and TFIIB are recruited to the promoter and the resulting DAB complex is subsequently recognized by RNA Pol II in association with TFIIF (Buratowski et al. 1989). Abortive initiation studies suggested that this DAB-PolF complex is sufficient to initiate transcription, whereas additional factors, TFIIE and TFIIF, appear to direct later steps such as promoter clearance, elongation, and transcription coupled DNA repair (Goodrich and Tjian 1994b). Direct interactions between TAFs in the TFIID complex and activators bound to enhancer elements are responsible for transcriptional activation by unknown mechanisms (Dynlacht et al. 1991; Tanese et al. 1991; Goodrich et al. 1993; Chen et al. 1994). Assuming that this order of events is relevant for assembly of transcription complexes *in vivo*, the recruitment of the RNA Pol II/TFIIF complex represents an essential step in active initiation complex formation.

Facilitated preinitiation complex formation is likely to be the consequence of multiple stabilizing protein–protein interactions. For example, previous studies suggested that binding of RNA Pol II/TFIIF requires the presence of TFIID and TFIIB at the promoter (Buratowski et al. 1989). These earlier studies also showed that TBP could replace TFIID without significant consequences for preinitiation complex formation indicating that a TBP–TFIIA–TFIIB complex was sufficient to recruit RNA Pol II/TFIIF *in vitro*. However, recent studies indicate that *in vivo*, TBP exists as a stable complex with TAFs to form holo-TFIID (Dynlacht et al. 1991; Tanese et al. 1991; Goodrich and Tjian 1994a). In addition, *in vitro* binding studies suggested that whereas both TBP and TFIID could support recruitment of TFIIB, it required TFIID to support activator-mediated recruitment

of RNA Pol II/TFIIF to form a functional preinitiation complex (Choy and Green 1993). However, the individual interactions that must occur between TFIID subunits and other components of the basal transcription machinery that ultimately lead to RNA Pol II recruitment and transcription initiation remained poorly defined. Thus, it became of particular interest to determine whether specific contacts between TAFs and basal factors exist that would contribute to the transcription initiation by RNA Pol II. Here, we demonstrate a specific and direct interaction between TAF_{II}250, the core subunit of TFIID, and RAP74, the largest subunit of TFIIF.

Binding of RAP74 was observed both in isolation and in the context of holo-TFIID suggesting that the required interaction surfaces are accessible in these complexes. Deletion mapping studies identified an amino-terminal domain of ~120 amino acids in RAP74 that is sufficient to bind TAF_{II}250. Interestingly, a larger amino-terminal domain of 171 amino acids had been reported previously to bind RAP30, whereas deletion mutants truncated to position 128 failed to bind RAP30 (Yonaha et al. 1993).

Figure 5. A direct contact between TAF_{II}250, the core subunit of TFIID, and RAP74, the largest subunit of TFIIF, contributes to transcription initiation mediated by RNA Pol II. TAF_{II}250 contacts directly the TBP (Ruppert et al. 1993) and RAP74, which in addition makes contacts to RAP30 and RNA Pol II. Other TAFs of the TFIID complex are indicated by shading.

Thus, it seems likely that the binding of RAP30 and TAF_{II}250 to RAP74 require distinct interfaces and that these interactions can occur concomitantly. A comparison of sequences in the human RAP74 domain (Aso et al. 1992; Finkelstein et al. 1992) involved in binding TAF_{II}250 with the corresponding region from *Xenopus* (Gong et al. 1992) and *Drosophila* (Gong et al. 1993; Kephart et al. 1993) revealed a highly conserved domain. Consistent with this high degree of sequence similarity, we have observed that human TAF_{II}250 can also bind to the *Drosophila* homolog of RAP74. The region of TAF_{II}250 that is involved in binding of RAP74 (amino acid residues 1120–1270; Ruppert et al. 1993) is also conserved between human and *Drosophila* (Kokubo et al. 1993; Weinzierl et al. 1993). The more amino-terminal conserved region overlaps a domain with some homology to SWI4 (Hisatake et al. 1993) whereas the carboxy-terminal conserved region includes a portion of the so called “HMG box” domain of TAF_{II}250 (Sekiguchi et al. 1991; Hisatake et al. 1993; Landsmann 1993; Weinzierl et al. 1993).

In addition to establishing direct binding between TAF_{II}250 and RAP74, we have also obtained some evidence that this interaction is functionally important in the context of intact cells. In particular, complementation of a mutant cell line (ts13; Talavera and Basilico 1977), temperature sensitive for TAF_{II}250 function (Sekiguchi et al. 1991) reveals a strong correlation between TAF_{II}250 mutants that fail to bind RAP74 in vitro and those that are unable to rescue the temperature-sensitive phenotype at the nonpermissive temperature. Recently, it has been shown that the failure to progress from G₁ to S phase is attributable to a point mutation in TAF_{II}250 (Hayashida et al. 1994) that results in an inability to respond to certain activators at the nonpermissive temperature (Wang and Tjian 1994). In line with these observations our findings suggest that the TAF_{II}250 mutant N1225 fails to rescue ts13 cells because it is unable to relay signals from upstream bound activators to the transcriptional machinery. We hypothesize that this mutant is unable to bind efficiently to RAP74 in vivo and thus may fail to recruit the RNA Pol II/TFIIF complex to the promoter. Although it is equally possible that interactions between TAF_{II}250 and RAP74 plays some role in later steps of the transcription cycle, such as promoter clearance. Thus, our results suggest that a direct interaction between TAF_{II}250 and RAP74 may be essential for the assembly of an active transcription initiation complex (Fig. 5) and that the contact between RAP74 and TAF_{II}250 plays some role in either the recruitment of RNA Pol II to the initiation complex or in triggering transcription initiation after formation of the preinitiation complex.

Material and methods

Construction of vectors and plasmids

pVL1392GST and *pVL1393GST* The HA epitopes of pVL1392HAX and pVL1393HAX (Ruppert et al. 1993) were replaced by a PCR fragment encoding GST flanked by unique

NcoI and *NdeI* cloning sites. GST was amplified using standard conditions, the vector pGEX2TK (Pharmacia), and the following oligonucleotides: 5'-GTATCCATGCCCCCTATACTAGGT-TATTG-3', containing a *NcoI* site, and 5'-TCCCGGCATATGAACAGATGCACGACGAG-3', containing a *NdeI* site. The resulting PCR product was digested with *NcoI* and *NdeI* and cloned into pVL1392HAX and pVL1393HAX (Ruppert et al. 1993) to yield pVL1392GST and pVL1393GST, respectively. Both vectors were sequenced across the inserted fragment and found to have the correct sequence. The vectors have the following features/restriction sites: pVL1392GST: polyhedrin promoter–*NcoI*–GST coding region–thrombin cleavage site–kinase domain–*NdeI*–*SpeI*–*XbaI*–*NotI*–*EcoRI*–*XbaI*–*Asp718*–*SmaI*–*BamHI*. pVL1393GST: polyhedrin promoter–*NcoI*–GST coding region–thrombin cleavage site–kinase domain–*NdeI*–*SmaI*–*PstI*–*EcoRI*–*NotI*–*PstI*–*BglIII*.

pT β HAX-hTAF_{II}250 was constructed by inserting the 6-kb *NdeI*/*SpeI* fragment from pHAF_{II}250 (Ruppert et al. 1993) into *NdeI*–*XbaI*-cut pT β HAX (S. Ruppert, unpubl.), a derivative of pT β -STOP (Jantzen et al. 1992). The carboxy-terminal deletion series of hTAF_{II}250 was constructed by isolating the respective cDNA fragments from pvhTAF_{II}250 (Ruppert et al. 1993) with *NdeI* and a second restriction enzyme as indicated below and subsequent cloning into *NdeI*–*StuI*-cut pETHAX: pETHAX-hTAF_{II}250N1781 (*NdeI* and *SphI*, position cDNA 5368); pETHAX-hTAF_{II}250N1681 (*NdeI* and *XhoI*, position cDNA 5052); pETHAX-hTAF_{II}250N1398 (*NdeI* and *NarI*, position cDNA 4204); pETHAX-hTAF_{II}250N1184 (*NdeI* and *Tth1111*, position cDNA 3564); pETHAX-hTAF_{II}250N1054 (*NdeI* and *HincII*, position cDNA 3174); pETHAX-hTAF_{II}250N885 (*NdeI* and *AflIII*, position cDNA 2666); pETHAX-hTAF_{II}250N757 (*NdeI* and *MscI*, position cDNA 2286); pETHAX-hTAF_{II}250N643 (*NdeI* and *ScaI*, position cDNA 1943); pETHAX-hTAF_{II}250N434 (*NdeI* and *EcoRV*, position cDNA 1315).

The amino-terminal deletion series of hTAF_{II}250 was constructed as follows. Amino-terminal fragments were generated by digestion of the plasmid pHAX-hTAF_{II}250 (Ruppert et al. 1993) followed by exonuclease III/S1 nuclease digestion, Klenow fill-in and *SpeI* digestion (cutting in the 3'- untranslated region of the cDNA). The resulting fragments were separated on a 0.6% agarose gel, and fragments were isolated using Qiaex (Qiagen) and cloned into *HincII*–*XbaI*-cut pT β STOP (Jantzen et al. 1992). This leaves a unique *NdeI* site, providing the ATG translational start codon immediately 5' to the insertion site. The resulting clones (pT β hTAF_{II}250 Δ N) were sequenced to confirm in-frame deletions. The *NdeI*–*BamHI* inserts of confirmed deletion clones were subcloned into pVL1392GST (see above) to yield the pBGSThTAF_{II}250 Δ N baculovirus transfer vectors.

pCMV-HA-hTAF_{II}250 was generated by replacing the glucocorticoid receptor coding region within pCMVGR (Wang and Tjian 1994) with the 6-kb *BamHI* fragment derived from pvHAX-hTAF_{II}250 (Ruppert et al. 1993) containing the complete coding information of hTAF_{II}250 and an HA epitope tag. The carboxy-terminal deletion constructs pCMV-HA-hTAF_{II}250N1784, N1681, N1398, N1184, and N1054 have been generated by replacing the coding region of the full-length construct pCMV-HA-hTAF_{II}250 with the *EcoRV* (position 1315 in cDNA)–*NotI* fragment derived from pETHAX-hTAF_{II}250N1784, N1681, N1398, N1184, and N1054, respectively (see above). The resulting constructs were characterized and subjected to partial DNA sequence analysis. One construct had lost the *EcoRI* site at position 3686. Upon sequencing this construct completely, a 1-bp deletion resulting in a frameshift after amino acid position 1225 and a translational stop after incorporating 25 additional amino acids was found. This clone was named pCMV-HA-hTAF_{II}250N1225.

Expression of recombinant proteins in the baculovirus expression system and purification of HA- and GST-tagged proteins

Recombinant baculovirus was generated by cotransfecting baculovirus transfer vectors and BaculoGold virus DNA (Pharmin-gen) into SF9 cells (ATCC no. CRL-1711) using lipofectin (GIBCO-BRL). After 4–5 days of incubation the virus released into the medium was used for amplification and protein expression as described (O'Reilley et al. 1994). Extracts from recombinant virus-infected SF9 cells and control SF9 cells were prepared as described (Ruppert et al. 1993). HA epitope-tagged hTAF_{II}250 was immunopurified from overexpressing SF9 cells. Briefly, monoclonal α HA antibody (12C5, BABCO) was coupled for 1 hr at 4°C to protein A beads (Pharmacia) in 0.1 M KCl–HEMG [25 mM HEPES, 12.5 mM MgCl₂, 0.1 mM EDTA, 20% glycerol, containing 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF (phenylmethylsulfonyl fluoride) and 0.1 mM sodium metabisulfite]. After washing, crude extract overexpressing HA-tagged hTAF_{II}250 or control SF9 cell extract (both made in 0.4 M KCl–HEMG) were added to the beads and nutated at 4°C for 1.5 hr. After washing off the nonbound proteins, the quality of the recovered proteins was analyzed by SDS-PAGE. GST-fusion proteins were purified similarly by binding to GST beads (Pharmacia).

In vitro binding assays

Binding of RAP74 to TFIID Human TFIID was immunopurified from HeLa nuclear extracts using the monoclonal antibody 1A5 (anti-hTAF_{II}150) as described (Ruppert et al. 1993). ³⁵S-Labeled RAP74 (see below) was added to this TBP/TAF complex and incubated in 0.1 M KCl–HEMG. After washing and SDS-PAGE, binding was visualized by autoradiography.

Coimmunoprecipitation assays Binding of RAP74 and carboxy-terminal truncated proteins thereof to HA–hTAF_{II}250 bound to protein A beads via the α HA antibody was performed as follows. ³⁵S-Labeled full-length RAP74 (derived from the construct pET–RAP74; Finkelstein et al. 1992) and radiolabeled carboxy-terminal truncated RAP74 proteins were generated using the coupled transcription/translation system (Promega). The templates for runoff transcription/translation were generated from pET–RAP74 by restriction enzyme digestion: N183 (Sau3A), N139 (ApaI), N120 (AflIII), N100 (NciI), and N83 (RsaI). Binding of proteins and subsequent washings were done in 0.1 M KCl–HEMG.

Binding of RAP74 to GST-hTAF_{II}250ΔN fusion proteins Radiolabeled RAP74 was incubated with beads containing ~1–2 μ g of bound GST fusion proteins, GST alone or the equivalent of a SF9 control extract. After washing bound RAP74 was visualized by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics).

Far Western analysis ³⁵S-Labeled proteins were generated from the carboxy-terminal deletion construct series pETHAX–hTAF_{II}250N (see above) using the coupled transcription/translation system (Promega) and incubated with filter-bound proteins as described (Kaelin et al. 1992; Ruppert et al. 1993). Recombinant proteins for RAP74, TFIIE(p56), TFIIE(p34), TFIIB, and RAP30 were purified from *Escherichia coli*, and RNA Pol II was purified from HeLa nuclear extracts. The amount and integrity of the proteins used were analyzed by SDS-PAGE followed by silver staining (data not shown). Similar amounts of the respective proteins were used in subsequent experiments. The signals obtained were quantitated (PhosphorImager; Molecular Dynamics).

Transfections

ts13 cells were transfected at ~30%–50% confluency with the full-length construct pCMV–HA–hTAF_{II}250 and the truncated constructs pCMV–HA–hTAF_{II}250N1784, N1681, N1398, N1225, N1184, and N1054 using the calcium phosphate method as described (Wang and Tjian 1994). Four plates were used for each transfection. Cells were incubated for 1 day at the permissive temperature (33.5°C). Thereafter, cells were washed twice with PBS, fresh medium was added, and incubation was continued for several days at the nonpermissive temperature (39.5°C; two plates) and at the permissive temperature (33.5°C; two plates). Cell viability was monitored daily by light microscopy. After 2 days, whole-cell extracts were prepared from the cells kept at the permissive temperature and analyzed for expression of the respective TAF_{II}250 proteins by Western blotting using the monoclonal antibody 6B3 (Ruppert et al. 1993). When ts13 cells were cotransfected with the full-length construct pCMV–HA–hTAF_{II}250 and each individual truncated construct (see above), viable colonies were observed at the nonpermissive temperature.

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