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Identification of TATA-binding Protein-free TAF_{II}-containing Complex Subunits Suggests a Role in Nucleosome Acetylation and Signal Transduction*

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Recently we identified a novel human (h) multiprotein complex, called TATA-binding protein (TBP)-free TAF_{II}-containing complex (TFTC), which is able to nucleate RNA polymerase II transcription and can mediate transcriptional activation. Here we demonstrate that TFTC, similar to other TBP-free TAF_{II} complexes (yeast SAGA, hSTAGA, and hPCAF) contains the acetyltransferase hGCN5 and is able to acetylate histones in both a free and a nucleosomal context. The recently described TRRAP cofactor for oncogenic transcription factor pathways was also characterized as a TFTC subunit. Furthermore, we identified four other previously uncharacterized subunits of TFTC: hADA3, $hTAF_{II}150$, hSPT3, and hPAF65β. Thus, the polypeptide composition of TFTC suggests that TFTC is recruited to chromatin templates by activators to acetylate histones and thus may potentiate initiation and activation of transcription.

Initiation of transcription of protein-encoding genes by RNA polymerase II requires transcription factor TFIID that is comprised of the TATA-binding protein (TBP)[1] and series of TBP-associated factors (TAF_{II}s) (1). TFIID directs preinitiation complex assembly on both TATA-containing and TATA-less promoters. Previously, we have shown that functionally distinct TFIID complexes composed of both common and specific TAF_{II}s exist in human HeLa cells (for review, see Ref. 2).

We have isolated and partially characterized a novel human (h) multiprotein complex, which contains neither TBP nor TBP-like factor but is composed of several TAF_{II}s and a number of uncharacterized polypeptides (3). This novel complex, called TBP-free TAF_{II}-containing complex (TFTC) is able to direct preinitiation complex formation and initiation of transcription on both TATA-containing and TATA-less promoters in *in vitro* transcription assays and can mediate transcriptional activation by GAL-VP16 (3).

Following the discovery of the TFTC complex, TAF_{II}s have also been described in different histone acetyltransferase (HAT) complexes: the yeast SPT-ADA-GCN5 acetyltransferase (SAGA) complex and the human PCAF-GCN5 and the human STAGA complexes (4–6). Histone acetylation and deacetylation have been strongly linked to the regulation of transcription (7). Yeast (y) Gcn5 has HAT activity and is a transcriptional coactivator required for correct expression of various genes (8, 9). Transcriptional activators, such as VP16 or GCN4, interact directly with the SAGA complex and direct nucleosomal acetylation to potentiate transcriptional activation (10). The yeast SAGA complex consists of yGcn5 and various Ada (Ada1, Ada2, and Ada3) and Spt (Spt3, Spt7, Spt8, and Spt20) proteins (11). In addition to these proteins the SAGA complex also contains a distinct set of $yTAF_{II}$s ($TAF_{II}90$, $TAF_{II}68$, $TAF_{II}60$, $TAF_{II}25$, and $TAF_{II}17/20$) (4). To date two human homologues of the yGcn5 have been identified. The first human homologue of yGcn5 is hGCN5 (called hGCN5-L), which is highly homologous to yeast GCN5 but contains an extended amino-terminal domain (12, 13). Furthermore, in human cells an incompletely spliced transcript may exist that would encode a shorter GCN5 protein (called hGCN5-S) with a similar size to yeast Gcn5 (12–14). The second human yGcn5 homologue is hPCAF, which was isolated as a p300/CBP-associated factor (15). PCAF is highly similar to hGCN5 throughout the entire length of the protein (15). Overexpression of either hPCAF or hGCN5-S in HeLa cells allowed the isolation of large multiprotein complex(es) containing previously identified TAF_{II}s ($TAF_{II}31$, $TAF_{II}30$, and $TAF_{II}20/15$) (5). In addition to the TAF_{II}s, the PCAF-GCN5 complexes also contain the human homologues of the yeast Ada (hADA2 and hADA3) and Spt (hSpt3) proteins and two other proteins that resemble previously identified TAF_{II}s (PAF65α and PAF65β) (5). A third TAF_{II}-HAT complex was recently described and termed hSTAGA complex (6). In this multiprotein complex the hGCN5 is associated with $hTAF_{II}31$ and hSPT3 (6).

When the polypeptide composition of TFTC was compared with that of the other TAF_{II}-HAT-containing complexes, it became clear that these complexes share a number of common features (2). Neither TFTC nor the other TAF_{II}-HAT complexes contain TBP, $hTAF_{II}250/yTAF_{II}145$ (the TFIID HAT (16)), $hTAF_{II}28/yTAF_{II}40$, and $hTAF_{II}18/yTAF_{II}19$. To further study the similarities between TFTC and the TAF_{II}-HAT complexes, we investigated whether TFTC also contains a HAT activity and identified six previously unidentified components of the TFTC complex. Our results indicate that TFTC is very similar in its polypeptide composition and its HAT specificity to the previously described SAGA, PCAF, and STAGA complexes.

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[1] The abbreviations used are: TBP, TATA-binding protein; TAF, TBP-associated factor; TFTC, TBP-free TAF_{II}-containing complex; HAT, histone acetyltransferase; h, human; y, yeast; r, recombinant; PAGE, polyacrylamide gel electrophoresis; NR, nuclear receptor; HFMC, histone fold motif-containing; pol II, polymerase II.

EXPERIMENTAL PROCEDURES

Mononucleosome Preparation—Mouse P19 cells were washed and resuspended in buffer N (15 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and protease inhibitors). Cells were lysed in buffer N containing 0.6% Nonidet P-40, and nuclei were recovered by centrifugation at 1500 rpm. Nuclei were then incubated with micrococcal nuclease at 37 °C for 30 min in buffer N and centrifuged at 6000 rpm (17). The pellet was resuspended in 2 mM EDTA and centrifuged at 12,000 rpm. The supernatant contained the mononucleosomes. To verify the mononucleosomes, they were either deproteinated with proteinase K, and the length of the DNA was analyzed on a 1% agarose gel or the histone composition was analyzed by SDS-PAGE.

HAT Assays—To overexpress PCAF the *Eco*RI/*Kpn*I fragment (containing the PCAF cDNA together with a FLAG tag) from the pCX-FLAG-PCAF vector (15) was inserted into the corresponding sites of the baculovirus expression vector pAcSG-His NT-C (PharMingen). To overexpress GCN5, the hGCN5-S-containing cDNA was PCR-amplified from the pcDNA3-hsGCN5 plasmid (14) and inserted in the *Xho*I-*Bam*HI sites of the pET15bEpB10 expression vector (18). Double-tagged hPCAF was expressed using baculovirus in Sf9 cells and purified in the presence of 1 μM acetyl-CoA first on a Ni^{2+} column, then by an anti-FLAG immunoprecipitation (Sigma) and peptide elution. His-tagged rGCN5-S was expressed in *Escherichia coli* and purified on a Ni^{2+} column (Qiagen). TFTC and TFIIDβ complexes were purified as described previously (3). TFTC, TFIIDβ, rPCAF, or rGCN5-S was incubated with 1.6 nmol of [^{14}C]acetyl-CoA (ICN) and 1 μg of core histones, histone H1 (Roche Molecular Biochemicals) or mononucleosomes in a buffer containing 50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 0.1 mM EDTA, 50 mM KCl, 20 mM sodium butyrate, 1 mM dithiothreitol for 1 h at 30 °C. The samples were separated on a 4–20% gradient (core histones and mononucleosomes) or a 10% (histone H1) SDS-PAGE gel. Gels were stained with Coomassie Brilliant Blue, enhanced with diphenyloxazol, dried, and exposed to x-ray films for 5 days.

The synthetic peptides and the HAT assay using these peptides were described previously (19), except that 1 μg of each peptide was used.

Western Blot Analysis—Western blots were carried out by standard procedures and treated with either polyclonal rabbit or monoclonal mouse antibodies followed by incubation with peroxidase-conjugated goat-anti rabbit or goat-anti mouse secondary antibodies (Jackson ImmunoResearch). Chemiluminescence detection was performed according to manufacturer's instructions (Amersham Pharmacia Biotech). Polyclonal antibodies against hGCN5 and hPCAF (12, 15), TRRAP (20), SPT3 (6), and the monoclonal antibodies against $TAF_{II}30$, $TAF_{II}100$, and $TAF_{II}135$ (21, 22) were previously characterized and described. The polyclonal antibody raised against $hTAF_{II}150$ was a kind gift from S. Smale. The polyclonal antibodies against hADA2, hADA3, and hPAF65β were raised against bacterially produced recombinant proteins.[2]

Microsequencing of TFTC Subunits—TFTC was prepared as described in Ref. 3, and approximately 100 μg of TFTC was separated on a preparative 8% SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue, excised, and digested with trypsin. The eluted peptides were fractionated by reverse-phase high performance liquid chromatography and microsequenced as described in Ref. 23.

RESULTS AND DISCUSSION

Histone Acetylase Activity in the TFTC Complex—The similarity between TFTC and the TAF_{II}-HAT-containing complexes (2) prompted us to test whether TFTC contains a subunit with HAT activity. The previously characterized TAF_{II}-HAT complexes mediate HAT activity through either a GCN5 or a PCAF subunit (4–6). We first tested the presence of hGCN5 in the TFTC complex using Western blot analysis. The previously characterized anti-hGCN5 antibodies (12) reacted specifically with a ~98-kDa protein species in the TFTC complex and recognized 50 ng of recombinant hGCN5-S (Fig. 1*A*), indicating that the TFTC complex contains the hGCN5 (hGCN5-L) protein (12, 13). Note that the anti-hGCN5 antisera used in these Western blots does not cross-react with hPCAF (12). Next, the presence of hPCAF was tested by Western blot analysis using anti-hPCAF antibodies specific for hPCAF (12). The anti-hPCAF antibodies recognized 50 ng of recombinant (r) PCAF and hPCAF present in the PCAF complex (5), but did not cross-react with any protein species in the TFTC complex (Fig. 1*B*), in spite the fact that about five times more TFTC was loaded on the gel than PCAF complex (as judged from their respective $TAF_{II}30$ composition; Fig. 1*B*, *lanes 2* and *3*). These results indicate that TFTC contains hGCN5, but no or only trace amounts of hPCAF. Interestingly, the previously described PCAF complex also contains substantial amounts of hGCN5 as detected by Western blot analysis (Fig. 2).

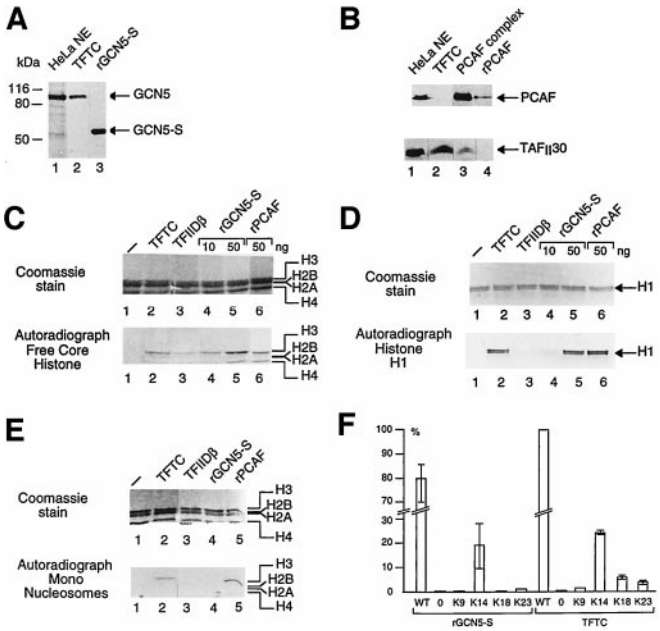

FIG. 1. **GCN5 histone acetyltransferase activity in the TFTC complex.** *A*, an anti-GCN5 serum recognizes 50 ng of rGCN5-S and endogenous hGCN5 in 50 μg of HeLa cell nuclear extract (*NE*) and in the TFTC complex when tested by Western blot analysis. *B*, anti-PCAF antibodies recognize 10 ng of rPCAF, endogenous hPCAF in 50 μg of HeLa cell NE, in the PCAF complex (5), but does not detect any PCAF in TFTC. The amount of $TAF_{II}30$ in each fraction was also tested by Western blot analysis. Histone acetylase activity was measured using either free core histones (*C*), histone H1 (*D*), or mononucleosomes (*E*) as substrates. Acetylase assays were performed with insulin (*lanes 1*), TFTC (*lanes 2*), TFIIDβ (*lanes 3*), rGCN5-S (*lanes 4* and *5*), and rPCAF (*lanes 6*; *lane 5* in *E*). The amount of TFTC used in each panel is equivalent to 10 ng of rGCN5-S (normalized by Western blot). Histones were separated by SDS-PAGE, stained with Coomassie Brilliant Blue (*top panels*), then acetylated histones were visualized by autoradiography (*bottom panels*). The positions of each of the core histones and that of H1 are indicated. *F*, the TFTC complex directly acetylates lysines other than lysine 14 on histone H3 peptides. rGCN5-S or purified TFTC were incubated together with wild type peptide (*WT*, without any acetylated lysines), peptide with all lysines acetylated (*0*), or peptides with only a single nonacetylated lysine at any given position (*K9*, *K14*, *K18*, or *K23*). Each *bar* represents the average of four independent experiments. Data are expressed relative to the activity obtained by TFTC on the wild type (*WT*) (nonacetylated) peptide. 100% corresponds to 2000 cpm. The *error bars* represent the minimal and maximal values obtained.

We next tested the HAT activity of the TFTC complex using either free core histones (Fig. 1*C*), H1 linker histone (Fig. 1*D*), or mononucleosomes (Fig. 1*E*). Assays were performed using either TFTC, TFIIDβ (3), rGCN5-S, or rPCAF proteins. We estimated that 10 μl of TFTC complex (the amount used in the following experiments) contains about 10 ng of GCN5 using Western blot analysis (data not shown). When free histones were used as substrate, the TFTC complex (Fig. 1*C*, *lane 2*), rGCN5-S and rPCAF (*lanes 4–6*) all strongly acetylated histone H3 and weakly modified histone H4. The acetylation of free histones by TFTC was comparable with that obtained with 10 ng of rGCN5-S (Fig. 1*C*, *lanes 2* and *4*). In contrast, TFIIDβ

[2] Y. Nakatani, manuscript in preparation.

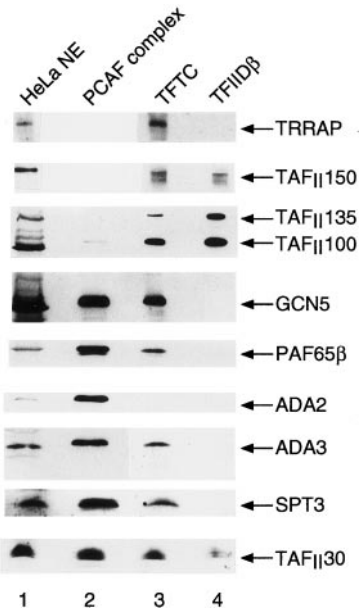

FIG. 2. **Newly identified components of the TFTC complex.** 50 μg of HeLa cell nuclear extract (*NE*; *lane 1*), purified PCAF (*lane 2*), TFTC (*lane 3*), and TFIIDβ (*lane 4*) complexes were separated by SDS-PAGE, blotted on a nitrocellulose filter, and examined for the presence of the indicated proteins by using the corresponding antibodies.

acetylated histones H3 and H4 with the same intensity (Fig. 1C, *lane 3*; Ref.16). Moreover, when histone H1 was used as a substrate, the TFTC complex acetylated histone H1 about five times better than the same amount of rGCN5-S (Fig. 1*D*, *lanes 2* and *4*), whereas TFIIDβ did not acetylate histone H1 (*lane 3*). When mononucleosomes were used as substrates, the level of acetylation by the TFTC complex was easily detectable and about two times weaker than the acetylation obtained by 50 ng of rPCAF (Fig. 1*E*, *lanes 2* and *5*), whereas rGCN5-S did not acetylate nucleosomes (*lane 4*). These results together indicate that the TFTC complex contains the hGCN5 HAT activity and is capable of efficiently acetylating histones in both a free and a nucleosomal context. Thus, acetylation specificity of the hTFTC complex is very similar to that of the ySAGA and the hPCAF-GCN5 complexes (4, 5), suggesting that these complexes may have a similar function in the cells. The TFTC complex acetylates histone H1 (an artificial but potential target) much better than the rGCN5, suggesting that additional proteins may increase the efficiency of acetylation in the TFTC complex and that ySAGA, hPCAF-GCN5, and hTFTC complexes may also acetylate substrates other than nucleosomes.

As expanded lysine acetylation specificity has been reported for the SAGA complex on histone H3 compared with recombinant yGcn5, which predominantly acetylates lysine 14 (19, 24), we investigated the lysine acetylation specificity of TFTC on histone H3. To investigate the acetylation specificity of the TFTC complex, we used a set of peptides that spanned the histone H3 amino terminus (19). The wild type peptide carries nonacetylated lysines at all four positions (Lys-9, Lys-14, Lys-18, and Lys-23), while derivatives of this peptide were either acetylated at all four lysines or retained only single nonacetylated lysines at positions Lys-9, Lys-14, Lys-18, or Lys-23 (19). These peptides were used in HAT assays to determine the lysine specificity of the TFTC complex in comparison with rGCN5-S. Consistent with previous studies carried out with either yGcn5 (19, 24) or mouse GCN5-L (13), rGCN5-S was able to efficiently acetylate only the wild type peptide or the peptide with nonacetylated Lys-14 (Fig. 1*F*). However, TFTC was able to acetylate Lys-18 and Lys-23 in addition to Lys-14, indicating an expanded lysine specificity over rGCN5-S (Fig. 1*F*). Thus, TFTC and SAGA show a similar expanded pattern of H3 lysine acetylation, suggesting that the expanded histone acetylation specificity of GCN5 in both complexes may be influenced by the same additional factors.

A Newly Identified Subunit of TFTC, TRRAP, May Link TFTC Function to Signal Transduction—To characterize unidentified polypeptides of TFTC, certain proteins of TFTC were microsequenced. From a ~300-kDa protein species we obtained four peptide sequences (GTQASHQVLR, LHNLAQFEGGESK, TIPNVIISHR, and VVAVSPQMR). Data base searches indicated that these peptides were contained in the sequence of the hTRRAP protein. The TRRAP protein was recently identified as an essential cofactor for oncogenic transcription factor pathways and shown to interact specifically with c-Myc and E2F-1 transactivation domains (20). The presence of TRRAP in the TFTC complex was also confirmed by Western blot analysis using an anti-hTRRAP-specific antisera (Fig. 2) (20). These results indicate that TRRAP is a component of the TFTC complex. Thus, it is possible that TFTC is able to play an adaptor role between Myc/Max and/or E2F/DP and the basic transcriptional machinery. Recently, hTRRAP (also called PAF400) and its yeast homologue, Tra1, were shown to be components of the hPCAF-GCN5 and the yeast SAGA complexes, respectively (25–27). The fact that TRRAP is a TFTC subunit further strengthens the link between TFTC and the other TAF_{II}-HAT complexes. The isolation of multiple transcriptional adaptor-HAT complexes containing TRRAP (or yTra1) suggests that the function of TRRAP/Tra1 is to directly interact with transcriptional activators and to recruit the HAT and the transcription initiation activities to promoters. Targeting these activities to activator bound promoter regions would then efficiently promote localized histone acetylation and stimulation of specific initiation of transcription.

*ADA3, SPT3, TAF_{II}*150, and PAF65β Are Subunits of the TFTC Complex—Since hTFTC is similar to ySAGA and hPCAF-GCN5 complexes, we tested whether these complexes also share other common subunits. Antibodies specifically recognizing hADA2 or hADA3[2] demonstrated that hADA3, but not hADA2, is present in the TFTC complex (Fig. 2), while these antibodies recognized both hADA2 and hADA3 in the hPCAF complex (Fig. 2). Our finding is not unexpected, since it has been demonstrated that hGCN5 exists in stable macromolecular complexes lacking hADA2 (28). Recently it was shown that ligand-dependent transactivation of different nuclear receptors (NRs) was mediated by the yeast ADA complex in which ADA3 directly bound to the ligand binding domain of some NRs (18). This observation further suggests that TFTC may interact with several activators to mediate transcriptional stimulation.

We next investigated whether, as in the case of the other TAF_{II}-HAT complexes (4–6), hSPT3 would be a component of TFTC. An antibody raised against hSPT3 (6) specifically recognized a protein species both in the TFTC and PCAF complexes (Fig. 2), indicating that hSPT3 is a component of the TFTC complex. Thus, as TFTC contains neither $hTAF_{II}28$ nor $hTAF_{II}18$, hSPT3, which contains two intramolecular histone fold motifs very similar to those found in $hTAF_{II}28$ or $hTAF_{II}18$ (29), may replace these two TAF_{II}s in TFTC. Thus, out of the five histone fold motif-containing (HFMC) TAF_{II}s (29–31), the TAF_{II}-HAT-containing complexes contain only three HFMC TAF_{II}s ($hTAF_{II}80$, $hTAF_{II}31$, and $hTAF_{II}20$ or their yeast homologues), while SPT3 may replace the two HFMC TAF_{II}s ($hTAF_{II}28$/$yTAF_{II}40$ and $hTAF_{II}18$/$yTAF_{II}19$; Table I).

Next we tested whether TFTC contains $hTAF_{II}150$, a TAF_{II} that was not always found associated with the different TFIID preparations (32, 33), but which was shown to bind sequences

TABLE I

The composition of TAF_{II}-containing complexes

TFIID complexes and TBP-free TAF_{II}-HAT complexes are shown. TAF_{II}s are represented with their apparent molecular weights. Structural or functional properties of certain factors are given at the left. Yeast (y) and human (h) proteins within the same horizontal row show homology to each other. ND, nondefined.

	TAF_{II}-HAT complexes					
	Containing TBP		Lacking TBP			
	y TFIID	hTFIID	y SAGA	hTFTC	h PCAF/GCN5-S	hSTAGA
TAF_{II}s						
HAT and bromo domain	145 (130)	250				ND
Initiator/DNA binding	TSM1	150		150	ND	ND
		135		135		
WD 40 repeats	90	100	90	100	100[a]	
Histone H4-like	60	80 (70)	60	80		ND
					(PAF65α)	
	67	55		55*	ND	ND
Histone H3-like	17 (20)	31 (32)	17 (20)	31 (32)	31 (32)	31 (32)
	25	30	25	30	30	ND
Histone-like	40	28			ND	ND
Histone H2B-like	68 (61)	20 (15)	68 (61)	20 (15)	20 (15)	ND
Histone H4-like	19 (FUN81)	18			ND	ND
TATA box binding	yTBP	hTBP				
SPTs, ADAs, GCN5, and other components						
HAT and bromo domain			yGcn5	hGCN5	hPCAF/hGCN5-S	hGCN5
			yADA1	ND	ND	ND
			yADA2		hADA2	ND
			yADA3	hADA3	hADA3	ND
			ySPT3	hSPT3	hSPT3	hSPT3
			ySPT8	ND	ND	ND
			ySPT20 (yADA5)	ND	ND	ND
WD 40 repeats			ND	hPAF65β	hPAF65β	ND
			yTRA1	hTRRAP	hTRRAP	ND
			Other uncharacterized proteins			

[a] The factor is a substoichiometric component of the respective complex.

overlapping the start site of transcription on pol II promoters (34). Antibodies raised against $hTAF_{II}150$ specifically recognized a polypeptide migrating around 140 kDa in both the TFTC and TFIIDβ complexes (Fig. 2). This indicates that both $TAF_{II}30$-containing complexes, TFTC and TFIIDβ, contain $hTAF_{II}150$ (Table I). While our results are consistent with $hTAF_{II}150$ (CIF150) being a component of hTFIID complex (33), the presence of $hTAF_{II}150$ in the TFTC complex is surprising since $hTAF_{II}150$ and its yeast homologue, TSM1, have not been identified from the hPCAF and the ySAGA complexes. Interestingly, in contrast to the hPCAF and the ySAGA complexes which are not able to nucleate pol II transcription initiation, TFTC contains a certain number of additional TAF_{II}s (Table I). The presence of $hTAF_{II}150$, together with the additional TAF_{II}s, in TFTC may account for the promoter specific sequence binding capability and the transcription initiation activity of TFTC (3) and explain the functional differences observed between TFTC and the other TAF_{II}-HAT complexes.

The PCAF complex contains hPAF65β, a WD40 repeat-containing factor having similarity to $hTAF_{II}100$ (5). Antibodies raised against hPAF65β revealed a band around 65 kDa in both the PCAF and the TFTC complexes (Fig. 2), indicating that hPAF65β is a subunit of TFTC. Note, that a monoclonal antibody raised against $hTAF_{II}100$ recognized $hTAF_{II}100$ not only in TFTC, but detected also a weak band in the PCAF complex (Fig. 2), indicating that the presence of several WD40 repeat containing proteins in these complexes is not mutually exclusive.

Taken together the above results indicate that hTFTC, hPCAF-GCN5, and ySAGA complexes share very similar, but not identical, polypeptide composition (Table I). This suggests that different TAF_{II}-HAT complexes may exist to carry out distinct but related functions.

RNA polymerase II subunits, general transcription factors, and components of the Srb-mediator complex are highly conserved among eukaryotes. Moreover, many SAGA components have human homologues in the TFTC and PCAF-GCN5 complexes. Thus, the gene regulatory mechanisms regulated by the TAF_{II}-GCN5-HAT complexes seem to be conserved in all eukaryotes. The differences observed in the polypeptide composition of the distinct TBP-free TAF_{II}-HAT complexes suggest that different subpopulations of TAF_{II}-GCN5-HAT complexes may exist in the cells (Table I). Thus, these different TAF_{II}-HAT complexes may permit a broad range of regulatory capabilities in pol II transcription. It is likely that several components of the TAF_{II}-HAT complexes provide interaction surfaces for distinct activators (*i.e.* TRRAP for c-Myc or E2F-1 or ADA3 for NRs). This complex network of interactions may then lead to the recruitment of the TAF_{II}-GCN5-HAT complexes, to specific acetylation of the nucleosomes surrounding the promoters, and subsequently to efficient initiation and activation of transcription.

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