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Human $TAF_{II}28$ promotes transcriptional stimulation by activation function 2 of the retinoid X receptors

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Transcriptional activation *in vitro* involves direct interactions of transactivators with the TATA binding protein (TBP) and the TBP-associated factors (TAF_{II}s) which constitute the TFIID complex. However, the role of TAF_{II}s in transcriptional regulation in mammalian cells has not been addressed. We show that activation function 2 of the retinoid X receptors (RXR AF-2) does not activate transcription from a minimal promoter in Cos cells. However, coexpression of human (h) $TAF_{II}28$ promotes a strong ligand-dependent activity of the RXR AF-2 on a minimal promoter and potentiates the ability of the RXRα AF-2 to activate transcription from a complex promoter. The expression of $hTAF_{II}28$ also potentiated transactivation by several nuclear receptors, notably the oestrogen and vitamin D3 receptors (ER and VDR), whereas other classes of activator were not affected. The effect of $hTAF_{II}28$ on RXR AF-2 activities did not appear to require direct RXR–$TAF_{II}28$ interactions, but correlated with the ability of $hTAF_{II}28$ to interact with TBP. In contrast to Cos cells, the RXR AF-2s had differential abilities to activate transcription from a minimal promoter in HeLa cells, and a lesser increase in their activity was observed upon $hTAF_{II}28$ coexpression. Moreover, coexpression of $hTAF_{II}28$ did not increase but rather repressed activation by the ER and VDR AF-2s in HeLa cells. In agreement with these data, showing that $TAF_{II}28$ is limiting in the AF-2 activation pathway in Cos cells, $TAF_{II}28$ is selectively depleted in Cos cell TFIID.

Keywords: oestrogen receptor/TFIID/transcriptional intermediary factors/vitamin D3 receptor

Introduction

The transcription of protein coding genes in eukaryotes involves a multiprotein complex containing the RNA polymerase II (pol II) core enzyme and a series of auxiliary factors, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (for reviews see Buratowski and Sharp, 1993; Conaway and Conaway, 1993; Buratowski, 1994; Zawel and Reinberg, 1995). Although these factors can be assembled in an ordered fashion *in vitro* to form a preinitiation complex, in yeast and also in mammalian cells many of these factors are associated with the RNA pol II core enzyme in a holoenzyme complex (Kim *et al.*, 1994; Koleske and Young, 1994; Ossipow *et al.*, 1995).

One critical pol II transcription factor is TFIID, itself a multiprotein complex comprising the TATA binding protein (TBP) and TBP-associated factors (TAF_{II}s; Dynlacht *et al.*, 1991; Pugh and Tjian, 1991; Tanese *et al.*, 1991; Timmers *et al.*, 1992; Zhou *et al.*, 1992; Brou *et al.*, 1993a; Chiang *et al.*, 1993; for a review see Hernandez, 1993). In *Drosophila* (d) embryos, TFIID has been reported to exist as a homogenous complex comprising TBP and eight $dTAF_{II}$s (Chen *et al.*, 1994, and references therein). In contrast, we have shown that HeLa cell human (h) TFIID exists in several chromatographically separable and functionally distinct forms (Brou *et al.*, 1993a,b). Purification of hTFIID by chromatography and/or sequential immunoprecipitation with antibodies against hTBP and $hTAF_{II}30$ identified two hTFIID populations, hTFIIDα and hTFIIDβ, which lack or contain $hTAF_{II}30$, respectively (Jacq *et al.*, 1994). An analysis of the $hTAF_{II}$ composition of the hTFIIDα and hTFIIDβ complexes led us to propose the existence of core $hTAF_{II}$s, exemplified by $hTAF_{II}250$, $hTAF_{II}135$, $hTAF_{II}100$ and $hTAF_{II}28$, present in all hTFIID complexes, and specific $hTAF_{II}$s, exemplified by $hTAF_{II}30$, $hTAF_{II}20$ and $hTAF_{II}18$, present in only the hTFIIDβ complexes (Jacq *et al.*, 1994; Mengus *et al.*, 1995).

The cDNAs encoding many *Drosophila* and human TAF_{II}s have been isolated (Hoey *et al.*, 1993; Yokomori *et al.*, 1993; Jacq *et al.*, 1994; Kokubo *et al.*, 1994; Chiang and Roeder, 1995; Klemm *et al.*, 1995, and references therein; Lu and Levine, 1995; Mengus *et al.*, 1995). More recently, yeast homologues of the metazoan TAF_{II}s have been identified and an analysis of their cDNA sequence shows that TAF_{II}s have been highly conserved during evolution (Reese *et al.*, 1994; Poon *et al.*, 1995). Nevertheless, $hTAF_{II}30$, $hTAF_{II}18$ and $hTAF_{II}55$ have no known *Drosophila* counterparts, while no human counterpart for $dTAF_{II}150$ (Verrijzer *et al.*, 1994) has as yet been described. These results suggest that either these $dTAF_{II}$s have not yet been isolated or that they are not expressed in *Drosophila* embryos but only in differentiated adult tissues.

Based on the observation that transactivation *in vitro* can be supported by TFIID, but not TBP (Hoey *et al.*, 1990; Pugh and Tjian, 1990; Zhou *et al.*, 1992; Brou *et al.*, 1993a), it was proposed that TAF_{II}s may function as coactivators required for activated, but not basal, transcription (for reviews see Tjian and Maniatis, 1994; Zawel and Reinberg, 1995). Indeed, many of the TAF_{II}s have been shown to act as coactivators *in vitro* by interacting selectively and directly with transcriptional activators. For example, Sp1 interacts with $dTAF_{II}110$, while the acidic activation domain of VP16 interacts with $dTAF_{II}40$ (Goodrich *et al.*, 1993; Hoey *et al.*, 1993; Gill *et al.*, 1994). We have also shown that ligand-independent transactivation *in vitro* by the DE region of the oestrogen

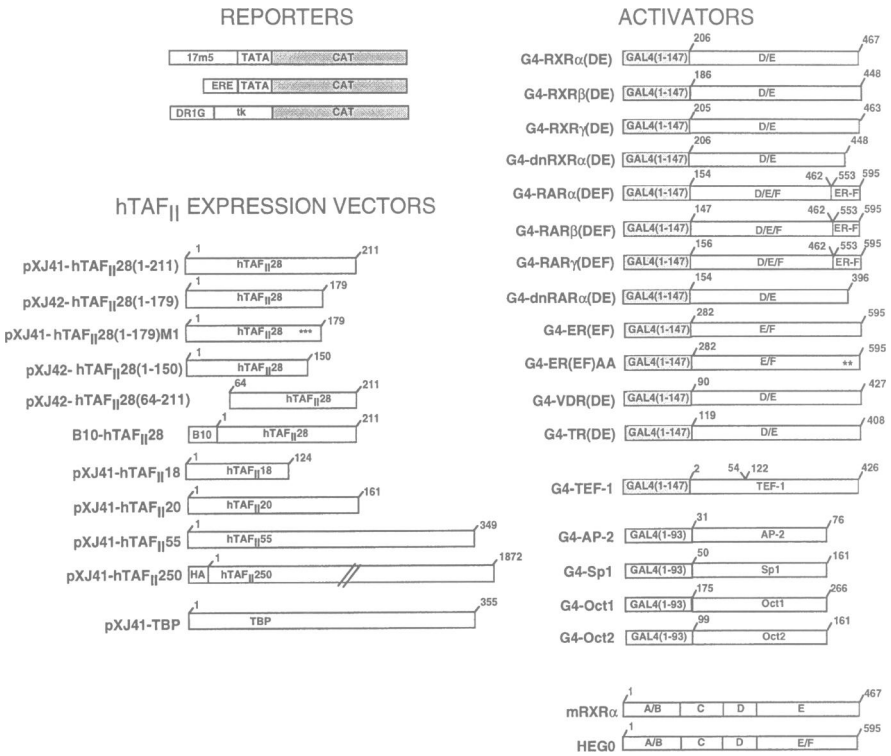

Fig. 1. Schematic representation of the reporter gene and expression vectors. Reporters: 17m5–TATA–CAT contains five GAL4 binding sites inserted 38 nucleotides upstream of the adenovirus major late promoter and the CAT gene. ERE–TATA–CAT and DR1G–tk–CAT containing the oestrogen and 9-*cis*-retinoic acid (RA) REs are as described previously (Tora *et al.*, 1989; Nagpal *et al.*, 1992). hTAF$_{II}$ expression vectors: the vectors expressing wild-type, tagged and mutant derivatives of hTAF$_{II}$28, and wild-type hTAF$_{II}$18, hTAF$_{II}$20, hTAF$_{II}$55, hTAF$_{II}$250 and TBP are schematized. The asterisks in hTAF$_{II}$28(1–179)M1 indicate the positions of the E164P, E167P and E168R amino acid substitutions. The numbers are the amino acid coordinates in each case. HA is the haemagglutinin epitope for monoclonal antibody 12CA5, and B10 is the ER epitope for monoclonal antibody B10. Activators: the vectors expressing all mouse RAR, RXR derivatives, HEG0 and G4–TEF-1(2–426)Δ55–121 are schematized. GAL4 is abbreviated to G4. G4–ER(EF)AA contains amino acid substitutions M543A and L544A (indicated by asterisks) in G4–ER(EF). G4–AP-2, G4–Sp1, G4–Oct1 and G4–Oct2, containing proline- or glutamine-rich ADs from the respective activators, are also depicted. G4–VDR(DE) and G4–TR(DE) contain the DE regions of the human vitamin D3 and chicken thyroid hormone (α) receptors, respectively, cloned in vector pXJ440. In all cases, the numbers indicate the amino acid coordinates in the native proteins.

receptor (ER) involves direct interactions with hTAF$_{II}$30 (Jacq *et al.*, 1994). In addition to acting as coactivators, hTAF$_{II}$s also participate directly in promoter recognition and selectivity (Verrijzer *et al.*, 1995).

The mechanism by which transactivator proteins act through coactivators, such as the TAF$_{II}$s, to stimulate transcription is the subject of intense study. One class of activators whose activating domains, referred to also as activation functions (AFs), have been studied genetically, biochemically and biophysically is the nuclear receptor superfamily comprising the receptors for steroid/thyroid hormones, retinoic acid and vitamin D3. The ability of these factors to activate transcription is regulated by the binding of their cognate ligands (reviewed in Parker, 1993; Chambon, 1994; Giguère, 1994; Glass, 1994; Mangelsdorf *et al.*, 1994; Tsai and O'Malley, 1994). The nuclear receptors generally comprise two AFs: AF-1, located in the N-terminal A/B region, and AF-2, located in the ligand binding domain (LBD) in the C-terminal E region. The activity of the AF-2 is ligand inducible and requires a conserved amphipathic α-helix at the carboxyl end of the LBD, designated the AF-2 activating domain core (AF-2 AD core; Danielan *et al.*, 1992; Barettino *et al.*, 1994; Durand *et al.*, 1994). Comparison of the crystal structures of the unliganded retinoid X receptor (RXR) with the liganded retinoic acid (RAR) and thyroid hormone (TR) receptors suggests that the binding of the ligand induces a conformational change bringing the AF-2 AD core into contact with α-helix H4 of the LBD (Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995; Wurtz *et al.*, 1996). This conformational change generates an altered interaction surface, which allows the receptors to interact with several putative transcriptional intermediary factors (TIFs) required for AF-2 activity (Cavaillès *et al.*, 1995; LeDourain *et al.*, 1995; Lee *et al.*, 1995; Swaffield *et al.*, 1995; Vom Bauer *et al.*, 1995).

Although TAF$_{II}$s have been shown to function as transcriptional coactivators *in vitro*, their function in living mammalian cells has not yet been addressed directly. Here we show that the expression of hTAF$_{II}$28 promotes the transactivation of a minimal promoter by the AF-2s of the RXRs in Cos cells, where they are otherwise inactive. Transactivation by the AF-2s of several other nuclear receptors, in particular the ER and vitamin D3 receptor (VDR), was also stimulated by the coexpression of hTAF$_{II}$28, whereas no significant effect was seen with activators belonging to other families. The coactivator activity of hTAF$_{II}$28 did not appear to involve direct interactions with the receptor AF-2s, but did correlate with the ability of hTAF$_{II}$28 to interact with the TBP. In contrast to that observed in Cos cells, the RXR AF-2s activate transcription to varying degrees from a minimal

promoter in HeLa cells, and their activity is affected to a lesser extent by the coexpression of hTAF$_{II}$28. Furthermore, the expression of hTAF$_{II}$28 in HeLa cells did not increase but rather repressed transactivation by the ER and VDR AF-2s. In agreement with these results, showing that TAF$_{II}$28 is a limiting factor for AF-2 activity in Cos cells, the simian homologue of hTAF$_{II}$28 was selectively depleted in immunopurified Cos cell TFIID. These results show that TAF$_{II}$28 can act as a specific coactivator in mammalian cells.

Results

hTAF$_{II}$28 promotes transactivation by members of the nuclear receptor superfamily in transfected Cos cells

It has been shown previously that a chimera comprising the RXRβ DE region (containing the ligand-inducible AF-2) fused to the DNA binding domain of the yeast activator GAL4 [G4–RXRβ(DE)] does not activate transcription from a minimal promoter containing two GAL4 binding sites upstream of a TATA element in transfected Cos cells (Nagpal *et al.*, 1993). Similarly, this chimera does not activate transcription from a minimal promoter containing five GAL4 binding sites in either the presence or absence of ligand when between 0.25 and 1.00 μg of expression vector were transfected (Figure 2A, lanes 1, 2 and 9, and data not shown; for reporter and activator plasmids, see Figure 1). Strikingly, when cotransfected with G4–RXRβ(DE), hTAF$_{II}$28 promoted a strong ligand-dependent transcriptional activation (Figures 2A, lanes 13–15, and 3A and B). Coexpression of hTAF$_{II}$28 also promoted activation by the RXRα and RXRγ AF-2s (Figure 3A and B). Maximal transactivation was observed using 1.0 μg RXR and 2.0 μg hTAF$_{II}$28 expression vectors (Figure 2A, lane 15). A much weaker, yet significant, effect on the activity of the RXRβ AF-2 was observed with another TFIID subunit, hTAF$_{II}$20 (Figure 2A, lanes 10–12).

The ability of hTAF$_{II}$28 to potentiate transactivation was not limited to the RXR AF-2s. Coexpression of hTAF$_{II}$28 increased transcriptional activation by 5- to 7-fold for the AF-2s of the ER [G4–ER(EF), Figures 2B, lanes 4 and 8–10, and 3A and B] and VDR [G4–VDR(DE), Figure 3A and B], by 4-fold for the AF-2 of the RARγ [G4–RARγ(DEF)], but by only 2- to 3-fold for the AF-2s of the RARα, RARβ and thyroid hormone receptor [G4–TR(DE)]. Similar results were observed even when lower amounts of the RAR (α and β forms) and the TR expression vectors were transfected, showing that the modest effect of hTAF$_{II}$28 was not caused by saturating levels of activation (data not shown). For each AF-2, the ability of hTAF$_{II}$28 to potentiate activation was strictly dependent on the presence of the cognate ligands, with the exception of a modest (between 5 and 8% of that seen in the presence of ligand) ligand-independent activation of transcription observed with RARα and RARβ (Figure 2A and B, and data not shown).

In contrast to the above results, the expression of hTAF$_{II}$28 had no significant effect on transactivation by five chimeric activators which do not belong to the nuclear receptor superfamily but have diverse classes of AFs (G4–Sp1 in Figure 2B, lanes 11 and 14–15; G4–TEF-1, G4–AP-2, G4–Oct1 and G4–Oct2 in Figure 3A and B]. Therefore, although hTAF$_{II}$28 may potentiate transactivation by activators other than those tested, hTAF$_{II}$28 is clearly an activator-specific coactivator.

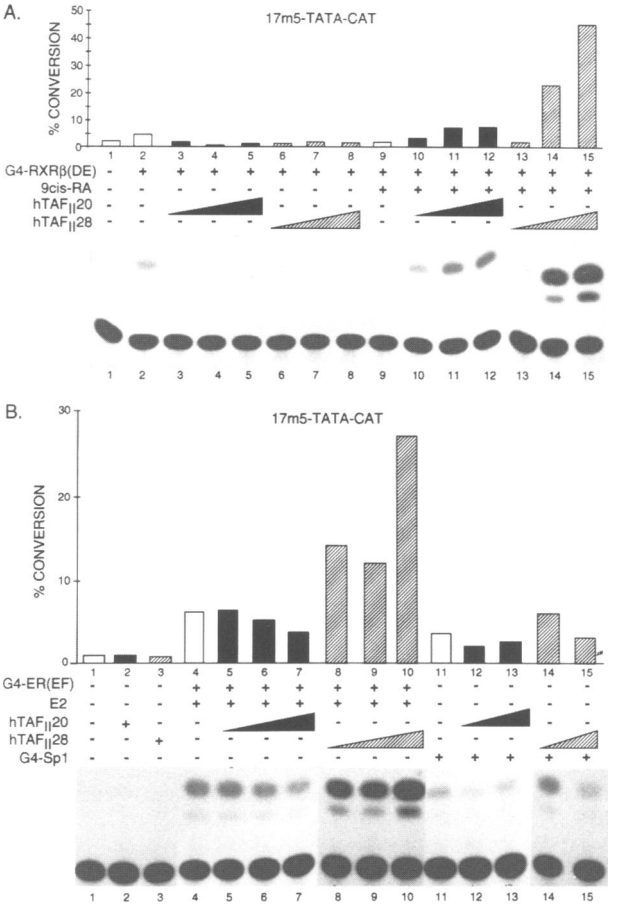

Fig. 2. (**A**) Expression of hTAF$_{II}$28 in Cos cells promotes transactivation by the RXRβ AF-2. The lower panel shows the autoradiography of CAT assays performed with extracts from cells transfected with the expression vectors shown above each lane in the presence (+) or absence (–) of 100 nM 9-*cis*-RA. Transfections contained 1.0 μg of the 17m5–TATA–CAT reporter and G4–RXRβ(DE) expression plasmids with 0.0, 0.5, 1.0 or 2.0 μg of the hTAF$_{II}$20 or hTAF$_{II}$28 expression vectors. The upper panel shows the quantitative phosphorimager analysis of the CAT assays represented in the lower panel. Values are expressed as percentages of the total chloramphenicol which was acetylated. (**B**) Expression of hTAF$_{II}$28 potentiates transactivation by G4–ER(EF) but not by G4–Sp1. The lower panel shows the autoradiography of CAT assays performed with extracts from cells transfected with the expression vectors indicated above each lane in the presence or absence of 15 nM oestradiol (E2). Transfections contained 1.0 μg of the 17m5–TATA–CAT reporter plasmid, 250 ng of the G4–ER(EF) or G4–Sp1 expression vectors, and 0.0, 0.5, 1.0 and 2.0 μg of the hTAF$_{II}$28 or hTAF$_{II}$20 expression vectors. In lanes 2 and 3, 2.0 μg of the hTAF$_{II}$ expression vectors were transfected. The upper panel shows the quantitative phosphorimager analysis, as in (A).

Next we tested the ability of hTAF$_{II}$28 to promote transactivation by wild-type receptors bound to their cognate response elements. The transfection of wild-type RXRα results in a ligand-dependent activation of transcription from a reporter comprising the thymidine kinase (tk) promoter and a DR1G RXR response element (RE) (Nagpal *et al.*, 1992; see Figure 4A, columns 1 and 2). Cotransfection of hTAF$_{II}$28 and wild-type RXRα resulted in a 5-fold ligand-dependent increase in transcriptional

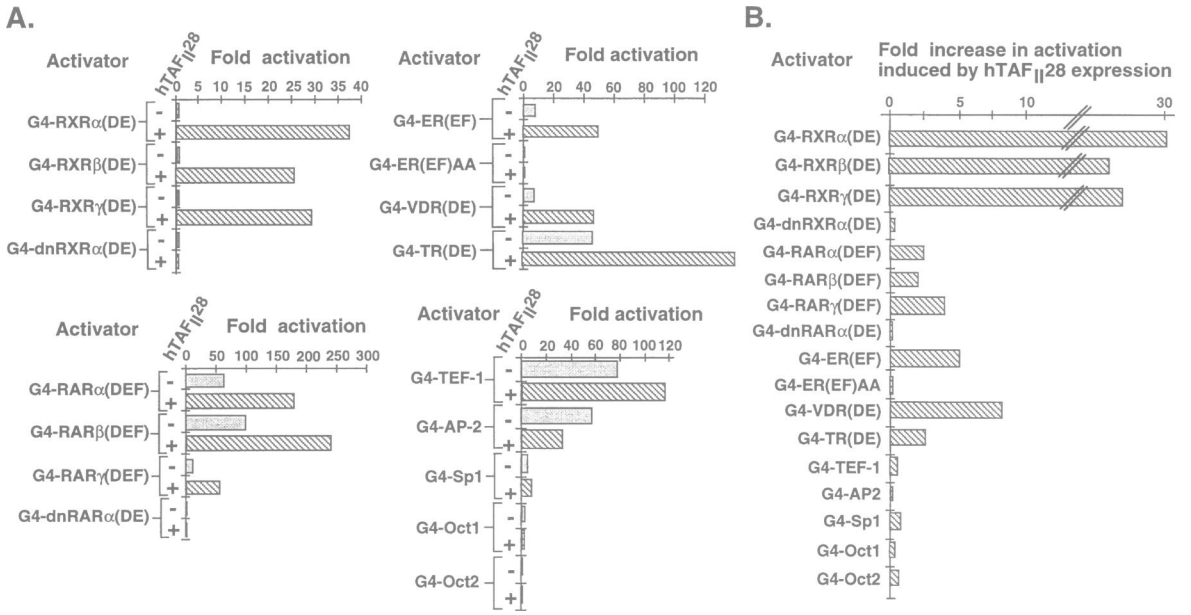

Fig. 3. (**A**) Transactivation by chimeric activators in the absence or presence of coexpressed hTAF$_{II}$28. The ability of each activator to transactivate the 17m5–TATA–CAT reporter in transient transfections in Cos cells was determined by a quantitative phosphorimager analysis of the CAT assays. The transfected activator is shown to the left of the panels. All values (±20%) represent the average of at least three transfections. The fold activation, relative to basal transcription, in the absence or presence of hTAF$_{II}$28 is shown by the filled or hatched bars respectively. All values were determined in the presence of the cognate ligands. 9-*cis*-RA and *all trans*-RA were added to a final concentration of 100 nM, oestradiol to 15 nM, 1,25-dihydroxy-vitamin D3 to 100 nM and thyroid hormone (3,5,3′-triiodo-L-thyronine) to 1 μM. In addition to pRSV–Luc as the internal standard and 0.0 or 2.0 μg of hTAF$_{II}$28, transfections contained 1.0 μg of the 17m5–TATA–CAT reporter and RXR chimeras; 100 ng of the RAR, VDR, TR and TEF-1 chimeras; and 250 ng of the ER, AP-2, Sp1, Oct1 and Oct2 chimeras. The expression of G4–Oct1 and G4–Oct2, for which no activation was seen in the presence or absence of hTAF$_{II}$28, was verified by a Western blot analysis using the anti-GAL4 antibodies 2GV3 and 3GV2. (**B**) Potentiation of transactivation by hTAF$_{II}$28 expression. The effect of hTAF$_{II}$28 shown in (A) is summarized. The value 1 represents no increase relative to activation in the absence of hTAF$_{II}$28.

activation (Figure 4A, columns 2, 3 and 6). Similarly, cotransfection of hTAF$_{II}$28 led to a 7-fold increase in activation by the wild-type ER from a minimal promoter with an upstream oestrogen response element (ERE) (Figure 4B, compare columns 2–3 with 5–6). These experiments show that hTAF$_{II}$28 can also potentiate activation by wild-type receptors bound to their cognate REs, excluding the possibility that the effect of hTAF$_{II}$28 requires a cryptic AF present in GAL4(1–147).

As described above, the activity of the nuclear receptor AF-2s requires a conserved amphipathic α-helical motif at the C-terminus of the LBD, designated the AF-2 AD core. Deletion of the AF-2 AD core abolishes transcriptional activation by the RARs and RXRs in both the absence (see also Durand *et al.*, 1994) and presence of hTAF$_{II}$28 [G4–dnRARα(DE) and G4-dnRXRα(DE) in Figure 3A and B]. Analogous results were obtained with a double amino acid substitution within the ER AF-2 AD core [M543A; L544A; G4–ER(EF)AA; Figure 3A and B]. These results indicate that hTAF$_{II}$28 does not induce the activity of a novel AD functioning independently of the AF-2 AD core.

The ability of two further TFIID subunits, hTAF$_{II}$55 and hTAF$_{II}$250, to potentiate transactivation by the receptor AF-2s was also tested. While in the same experiment in which hTAF$_{II}$28 potentiated activation by the RXR and ER AF-2s, no equivalent effect was seen with hTAF$_{II}$250 or hTAF$_{II}$55 (Figure 5A and B). Similarly (with the exception of the RXRs and hTAF$_{II}$20; Figure 2A), the expression of hTAF$_{II}$250, hTAF$_{II}$55, hTAF$_{II}$20 and hTAF$_{II}$18 did not result in increased transcriptional activation using any of the activators tested (see Figure 2B, and data not shown). These results indicate that the ability to potentiate activation by these activators in Cos cells was not a general property of all hTAF$_{II}$s but was specific to hTAF$_{II}$28.

The coactivator function of hTAF$_{II}$28 correlates with its ability to interact with TBP and involves a putative amphipathic α-helical region

Transcriptional activation *in vitro* has been reported to require direct activator–hTAF$_{II}$ interactions (see above). Therefore we investigated whether hTAF$_{II}$28 would interact with the AF-2 of the RXR. To analyse this interaction under conditions which most closely resemble those in which a functional effect is observed, vectors expressing wild-type hTAF$_{II}$28(1–211) and G4–RXRβ(DE) or, as a control, TBP were transfected into Cos cells in either the presence or absence of ligand. The transfected cell extracts were then immunoprecipitated with monoclonal antibodies (mAbs) against TBP (mAb 3G3), hTAF$_{II}$28 (mAb 15TA) or the GAL4 DBD (mAb 2GV3). The precipitated proteins were analysed on Western blots. Under conditions where cotransfected hTAF$_{II}$28 and TBP form a stable immunoprecipitable complex (Figure 6A, lanes 4–6; see also Mengus *et al.*, 1995 and below), no coimmunoprecipitation of hTAF$_{II}$28 and G4–RXRβ(DE) was observed in either the presence or absence of ligand (Figure 6B). Similarly, no significant hTAF$_{II}$28–RXR interactions could be detected *in vitro* using GST–RXR and purified recombinant hTAF$_{II}$28 or in the yeast two-hybrid system; nor were ligand-dependent interactions detected between hTAF$_{II}$28

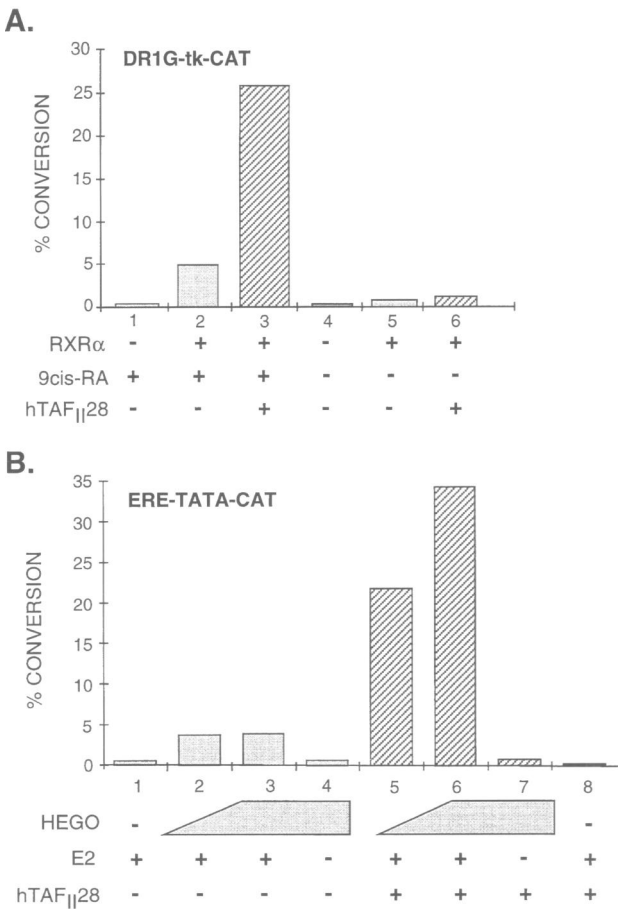

Fig. 4. (**A**) hTAF$_{II}$28 potentiates the transactivation of DR1G–tk–CAT by wild-type RXRα. A quantitative phosphorimager analysis of the CAT assays. Transfections contained 1.0 μg of the DR1G–tk–CAT reporter and RXRα expression plasmids and 0.0 or 2.0 μg of the hTAF$_{II}$28 expression plasmids, as indicated. (**B**) hTAF$_{II}$28 potentiates transactivation by the wild-type ER. Transfections contained 1.0 μg of the ERE–TATA–CAT reporter, 1.0 or 2.0 μg of the HEG0 expression vector and 0.0 or 2.0 μg of the hTAF$_{II}$28 expression vector, as indicated.

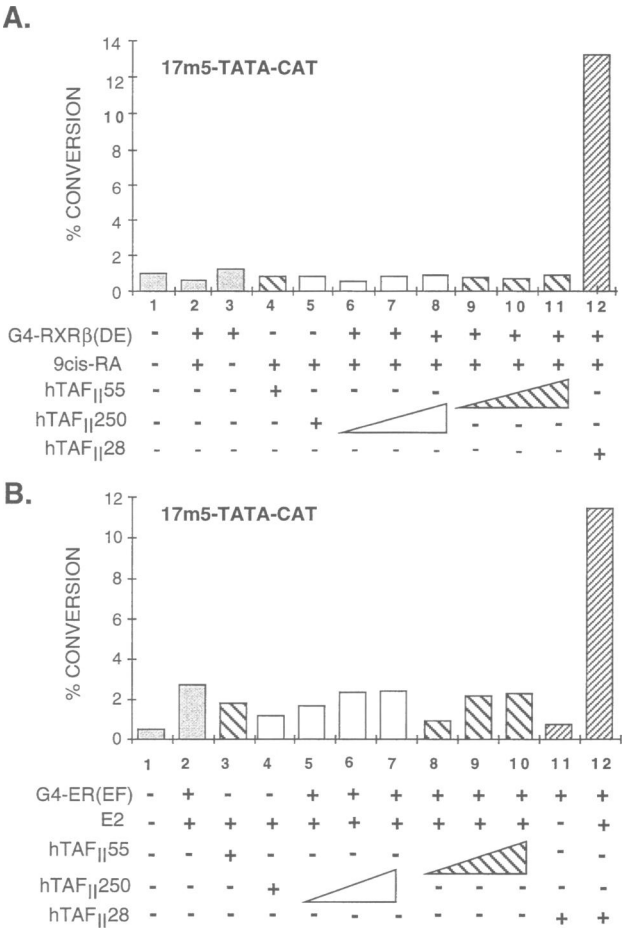

Fig. 5. (**A**) Coexpression of hTAF$_{II}$55 and hTAF$_{II}$250 does not potentiate transactivation by the RXRβ AF-2. Transfections contained 1.0 μg of the 17m5–TATA–CAT reporter and RXRβ AF-2 expression plasmids, 0.5, 1.0 and 2.0 μg of the hTAF$_{II}$55 expression plasmid, 1.0, 2.5 and 5.0 μg of the hTAF$_{II}$250 expression plasmid, and 2.0 μg of the hTAF$_{II}$28 expression plasmid, as indicated. The transfections shown in columns 4 and 5 contained 2.0 and 5.0 μg of the corresponding hTAF$_{II}$ expression vectors. (**B**) Cotransfection of hTAF$_{II}$55 or hTAF$_{II}$250 does not potentiate transactivation by the ER AF-2. Transfections contained the same amounts of each plasmid, except that the RXRβ expression vector was replaced by 250 ng of the ER AF-2 expression vector. The transfections in columns 3 and 4 contained 2.0 and 5.0 μg of the hTAF$_{II}$ expression vectors.

and the AF-2s of the VDR or the TR (data not shown). Thus, the ability of hTAF$_{II}$28 to induce the activity of the RXR AF-2s does not appear to require direct activator–hTAF$_{II}$ interaction.

We have shown previously that hTAF$_{II}$28 interacts with TBP both *in vitro* and in transfected Cos cells (see above and Mengus *et al.*, 1995). Next we asked whether the ability of hTAF$_{II}$28 to function as a coactivator required its ability to interact with TBP. A previous deletion analysis had shown that while wild-type hTAF$_{II}$28(1–211) interacted with TBP, no interaction was observed with the hTAF$_{II}$28 deletion mutant (1–150). To define further the region required for hTAF$_{II}$28–TBP interaction, a novel mutant, hTAF$_{II}$28(1–179), was made, and its ability to interact with TBP was determined. Following cotransfection, the hTAF$_{II}$28 deletion mutant (1–179) could be coimmunoprecipitated with TBP (Figure 6A, lanes 9–11). Together, these results indicate that hTAF$_{II}$28 amino acids between 150 and 179 are critical for interaction with TBP. A computer analysis indicated that amino acids 161–179 have the potential to form an amphipathic α-helix which can align the six acidic amino acids present in this region on one face of the helix (see Materials and methods). Therefore we mutated three of the glutamic acid residues (E164P, E167P and E168R) to generate hTAF$_{II}$28(1–179)M1, disrupting the α-helix and changing the charge. This triple amino acid substitution reduced the interaction between cotransfected hTAF$_{II}$28 and TBP (Figure 6A, lanes 12–14). Surprisingly, however, deletion of the N-terminus of hTAF$_{II}$28, hTAF$_{II}$28(64–211), also reduced the interaction with TBP in transfected Cos cells, showing that determinants for interaction with TBP are present not only between amino acids 150 and 179 but also in the N-terminal 63 amino acids (Figure 6A, lanes 15–17).

In cotransfections with G4–RXRβ(DE), wild-type hTAF$_{II}$28(1–211) and the (1–179) deletion mutant promoted AF-2 activity, whereas the (1–150), (64–211) and (1–179)M1 mutants had no significant effect (Figure 6C). These results show that the induction of RXRβ AF-2 activity correlates with the ability of hTAF$_{II}$28 to bind efficiently to TBP.

The above results suggest that hTAF$_{II}$28 may act as a

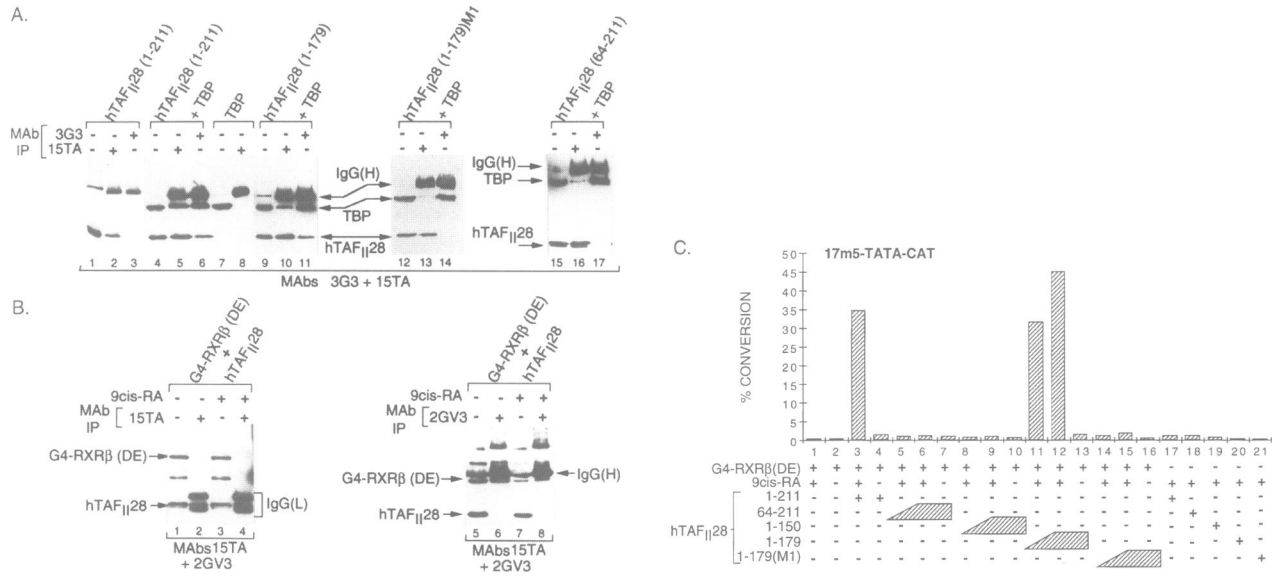

Fig. 6. (**A**) Coimmunoprecipitation of TBP with wild-type and mutated $hTAF_{II}28$. Extracts of Cos cells transfected with the expression vectors shown above the panel were precipitated with the antibodies shown above each lane. Lanes 1, 4, 7, 9, 12 and 15 show aliquots of the unprecipitated starting extracts. The precipitated proteins were revealed on Western blots using the mAbs shown below the panel. mAb 3G3 is directed against TBP and mAb 15TA is directed against $hTAF_{II}28$. The positions of $hTAF_{II}28$ and TBP are indicated, along with that of the heavy chain [IgG(H)] of the antibody used in the immunoprecipitations (IP). (**B**) The RXRβ DE region does not coimmunoprecipitate with $hTAF_{II}28$. Extracts from Cos cells transfected with the expression vectors shown above the panel were immunoprecipitated with the antibodies indicated above each lane. Lanes 1, 3, 5 and 7 show the unprecipitated cell extracts. The precipitated proteins were detected with the antibodies shown below the panel. The positions of the G4–RXRβ(DE) fusion and $hTAF_{II}28$ are indicated. The presence or absence of 50 nM 9-*cis*-RA during the transfection and the immunoprecipitation is also indicated above the lanes. As the G4–RXRβ(DE) fusion migrates close to the heavy chain of the antibodies used in the immunoprecipitation, lanes 1–4 were revealed with a peroxidase-conjugated antibody directed against the light chain [IgG(L)], which detects two species (whose positions are indicated) migrating immediately above and below $hTAF_{II}28$. Note that no coimmunoprecipitation of $hTAF_{II}28$ and G4–RXRβ(DE) was detected, even when cell extracts and immunoprecipitations were performed under less stringent salt conditions (0.25 M KCl). (**C**) Effect of $hTAF_{II}28$ mutants on RXRβ AF-2 activity. Cells were transfected with 1.0 μg of the 17m5–TATA–CAT reporter and RXRβ(DE) expression plasmids and 1.0 or 2.0 μg of the $hTAF_{II}28$ expression vectors, as indicated. The transfections represented in columns 3, 4 and 17–21 contained 2.0 μg of the indicated $hTAF_{II}28$ expression vectors.

bridging factor between TBP and the receptor AF-2s, if not directly, at least indirectly via one of the TIFs required for AF-2 function (see Discussion). If this were the case, the loss of function of the mutants described above may be ascribed to their inability to either interact efficiently with TBP and/or interact with another cofactor required for AF-2 function. Consequently, if $hTAF_{II}28$ acts as a bridging factor and these mutations only affect interactions with TBP but not other factors required for RXR AF-2 activity, the mutants should act as dominant-negative repressors of $hTAF_{II}28$-induced AF-2 activity. Alternatively, if the mutant $hTAF_{II}28$ proteins no longer interact with any cellular factors required for AF-2 activity, they should have no effect. To distinguish between these possibilities, the $hTAF_{II}28$(1–150) and (64–211) deletion mutants were cotransfected along with $hTAF_{II}28$(1–179) in the presence of G4–RXRα(DE) and ligand. The co-expression of $hTAF_{II}28$(1–179) and G4–RXRα(DE) resulted in transactivation (Figure 7A, column 3), while no effect was seen with either of the other two deletion mutants (Figure 7A, columns 7 and 10). Strikingly, co-expression of $hTAF_{II}28$(64–211) or (1–150) repressed the $hTAF_{II}28$(1–179)-induced activation (Figure 7A, columns 4–6 and 8–9). Similar results were obtained with the ER AF-2, where the increase in activation seen upon coexpression of $hTAF_{II}28$(1–179) was strongly diminished in the presence of $hTAF_{II}28$(1–150) or (64–211) (Figure 7B). In contrast, the expression of wild-type $hTAF_{II}28$ or the deletion mutants had no significant effect on transactivation by G4–TEF-1 (Figure 7C). These results strongly suggest that, although these mutants no longer interact efficiently with TBP, they can interact with and titrate other factors specifically required for receptor AF-2 activity.

Expression of $hTAF_{II}28$ has distinct effects in Cos and HeLa cells on the activities of the RXR, VDR and ER AF-2s

Next the ability of $hTAF_{II}28$ to act as a coactivator in HeLa cells was investigated. In transient transfection, $hTAF_{II}28$ was coexpressed with the RXR, ER and VDR AF-2s because these are the activators for which the most dramatic effects were observed in Cos cells. In contrast to Cos cells, where all the RXR AF-2s were inactive on a minimal promoter, the RXR AF-2s had differential abilities to activate transcription in HeLa cells. The strongest activation was seen with the RXRα AF-2, where a 10-fold ligand-dependent activation was observed with 1.0 μg of expression vector (Figure 8A, columns 1–4). A lower activation (2.5-fold) was seen with the RXRβ AF-2 (Figure 8A, columns 7–9). In contrast, only a low 2-fold activation was seen with 1.0 μg of the RXRγ AF-2 expression vector (Figure 8A, columns 12–14). Coexpression of $hTAF_{II}28$ with 0.25 μg of the RXRα or RXRβ AF-2 expression vector increased activation by a further 8.5- and 6.0-fold, respectively (Figure 8A, columns 3, 5, 8 and 10), whereas only a 1.5-fold stimulation was seen with 1.0 μg of the vector (Figure 8A, columns 4, 6, 9 and

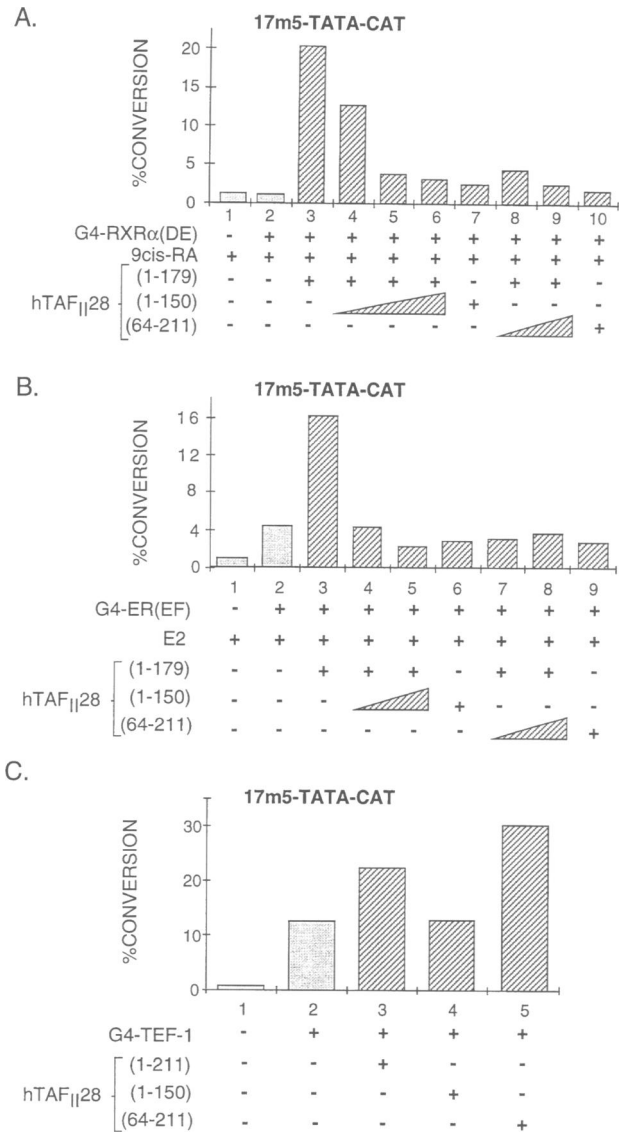

Fig. 7. (**A**) Dominant negative phenotype of hTAF$_{II}$28 deletion mutants. A quantitative phosphorimager analysis of the CAT assays from cells transfected with 1.0 μg G4–RXRα(DE), 2.0 μg hTAF$_{II}$28(1–179) and 2.0, 5.0 or 10.0 μg hTAF$_{II}$28(1–150) or 2.0 and 5.0 μg hTAF$_{II}$28(64–211), as indicated below each column. 2.0 μg of the hTAF$_{II}$28 mutant expression vectors were transfected in columns 7 and 10. (**B**) Transfections were performed as in (A), except that 250 ng G4–ER(EF) expression vector replaced the RXR expression vector. (**C**) Transfections contained 100 ng of the G4–TEF-1 expression vector and 2.0 μg of the indicated hTAF$_{II}$28 expression vectors.

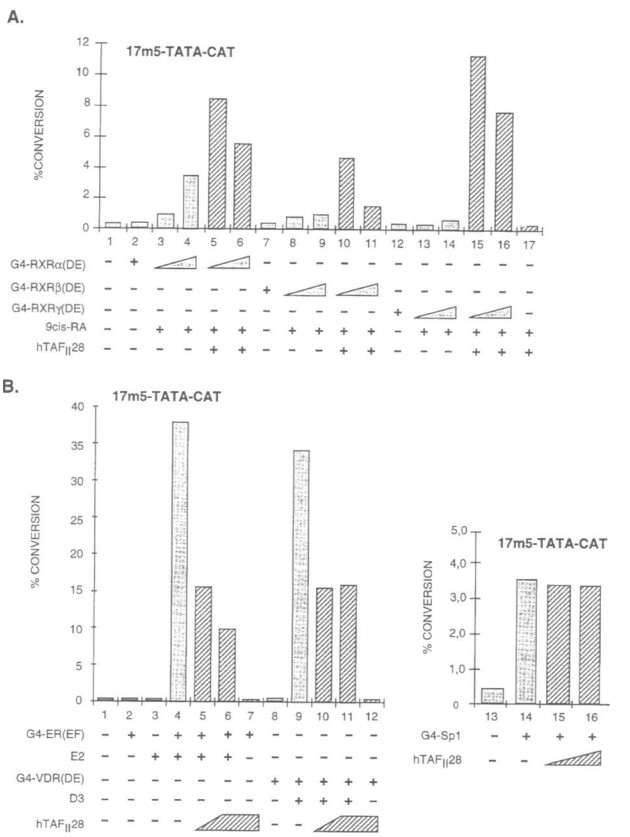

Fig. 8. (**A**) The effect of hTAF$_{II}$28 coexpression on the activity of the RXR AF-2s in HeLa cells. A quantitative phosphorimager analysis of the CAT assays from a representative experiment. In addition to 1.0 μg of the RSV–Luc internal standard, transfections contained 0.25 or 1.0 μg of the G4–RXR, and 0.0 or 2.0 μg of the hTAF$_{II}$28 expression vectors, as indicated below the graph. The presence or absence of 100 nM 9-*cis*-RA is also indicated. (**B**) Dominant-negative effect of hTAF$_{II}$28 on ER and VDR AF-2 activity in HeLa cells. Transfections contained 100 ng of the G4–ER(EF), 250 ng of the G4–VDR(DE) and 2.0 or 5.0 μg of the hTAF$_{II}$28 expression vectors, as indicated below the graph. Transfections contained 50 nM oestradiol (E2) or 100 nM vitamin D3, as indicated. Similar results for all transfections were obtained in two other independent experiments. The transfections in columns 13–16 contained 250 ng of the G4–Sp1 and 0.0, 2.0 or 5.0 μg of the hTAF$_{II}$28 expression vectors.

11). In contrast, a strong increase in activation by the RXRγ AF-2 was seen at both concentrations of expression vector in the presence of hTAF$_{II}$28 (31- and 11-fold; see Figure 8A, columns 13–16). These results show that the RXR AF-2s have differential abilities to activate transcription from a minimal promoter in HeLa cells, but that, with the exception of the RXRγ AF-2, the effect of hTAF$_{II}$28 coexpression is less pronounced than in Cos cells.

A strong ligand-dependent stimulation of transcription was seen in HeLa cells with the ER and VDR AF-2s (Figure 8B, columns 3 and 4, and 8 and 9) in the absence of coexpressed hTAF$_{II}$28. In striking contrast to the increase in activation seen in Cos cells, the coexpression of hTAF$_{II}$28 in HeLa cells in fact repressed activation by the VDR and ER AF-2s by 2- to 3-fold (Figure 8B, columns 4–6 and 9–11). However, as observed in Cos cells, coexpression of hTAF$_{II}$28 had no significant positive or negative effect on the 6-fold transactivation by G4–Sp1 in HeLa cells (Figure 8B, columns 13–16). Together, the above results show that hTAF$_{II}$28 has different effects on activation by the RXR, ER and VDR AF-2s in Cos and HeLa cells.

TAF$_{II}$28 is depleted in Cos cell TFIID

The above observations suggest that, while TAF$_{II}$28 is limiting for the activities of the RXR, VDR and ER AF-2s in Cos cells, it is less limiting in HeLa cells because the RXR AF-2s are active and, with the exception of the RXRγ AF-2, overexpression of hTAF$_{II}$28 has a lesser effect in HeLa than in Cos cells. This prompted us to ask whether HeLa and Cos cell TFIIDs contained equivalent amounts of TAF$_{II}$28. Total TFIID was immunopurified

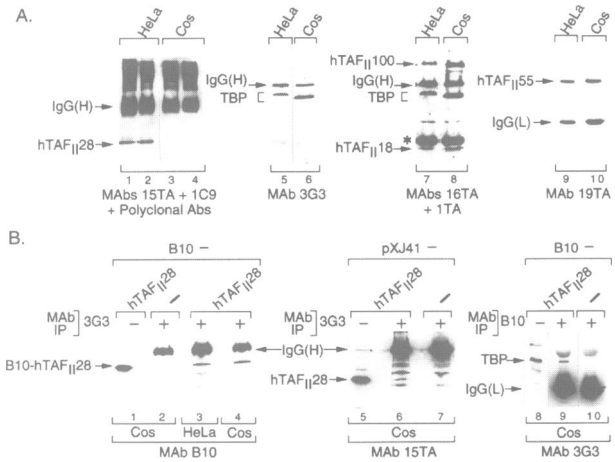

Fig. 9. (**A**) Cos cell TFIID is specifically depleted in hTAF$_{II}$28. Western blots of immunopurified HeLa and Cos cell TFIIDs were probed with the antibodies shown below each panel. As hTAF$_{II}$55 comigrates with the IgG(H) chain, it was detected using a secondary antibody directed against the IgG(L). The positions of hTAF$_{II}$28, TBP, hTAF$_{II}$18, hTAF$_{II}$55 and hTAF$_{II}$100 are indicated, along with the IgG(H) and IgG(L). The asterisk indicates an artifactual signal caused by the presence of the 3G3 epitope peptide used to elute the immunopurified TFIID. Lanes 1, 3 and 5–10 contain the first eluted fraction, and lanes 2 and 4 contain the wash fraction (see Materials and methods). (**B**) Transfected hTAF$_{II}$28 stably associates with endogenous Cos cell TBP/TFIID. In all, 15 dishes of Cos or HeLa cells were transfected with 3.0 μg of the expression vectors indicated above each lane (the dash indicating the empty expression vector). The transfected cell extracts were pooled and immunoprecipitated with the mAbs indicated for each panel. For mAb 3G3, the immunoprecipitated material was eluted using the corresponding epitope peptide, while for mAb B10 the beads were resuspended directly and boiled in loading buffer. Lanes 1, 5 and 8 show aliquots of the transfected cell extracts to indicate the positions of the precipitated proteins. The heavy and light chains of the mAbs used in the immunoprecipitations are indicated.

from HeLa or Cos cell extracts using the anti-TBP monoclonal antibody 3G3 (mAb 3G3) and eluted with the corresponding epitope peptide (Brou *et al.*, 1993a; see Materials and methods). Aliquots of the immunopurified TFIIDs were subjected to SDS–PAGE and transferred to nitrocellulose filters. The filters were then probed with mAb 3G3 or a mixture of two mAbs, 15TA and 1C9, and two rabbit polyclonal antisera, all of which recognize distinct epitopes in hTAF$_{II}$28 (see Materials and methods). Although more immunopurified Cos cell TFIID was present on the filter, as proved by the amounts of TBP (Figure 9A, lanes 5 and 6), no TAF$_{II}$28 could be detected in the Cos cell TFIID, whereas hTAF$_{II}$28 was clearly detected in the HeLa cell TFIID (Figure 9A, compare lanes 1 and 2 with 3 and 4, containing two eluted TFIID fractions). The Cos and HeLa cell TFIIDs were probed further with antibodies against hTAF$_{II}$18, hTAF$_{II}$20, hTAF$_{II}$30, hTAF$_{II}$55, hTAF$_{II}$100 and hTAF$_{II}$135. Approximately equivalent amounts of each of these TAF$_{II}$s were observed in HeLa and Cos cell TFIIDs (Figure 9A, lanes 7–10, and data not shown). Therefore, although TBP and six other TAF$_{II}$s could readily be detected in Cos cell TFIID, no simian homologue of hTAF$_{II}$28 was detected. These results show that this TAF$_{II}$ is either absent or dramatically depleted in Cos cell TFIID.

Next we verified that, as described previously for hTAF$_{II}$100, hTAF$_{II}$70 and hTAF$_{II}$30 (Weinzierl *et al.*, 1993; Jacq *et al.*, 1994; Dubrowskaya *et al.*, 1996), transfected hTAF$_{II}$28 associates with endogenous TBP/TFIID. As the anti-hTAF$_{II}$28 monoclonal antibodies do not efficiently immunoprecipitate the TFIID complex because of masking of the epitopes, a vector expressing a derivative of hTAF$_{II}$28 tagged at its N-terminus with the B10 epitope of the ER (Mengus *et al.*, 1995) was transfected into Cos cells. The transfected extracts were immunoprecipitated with mAb 3G3 against TBP or mAb B10 directed against the tag. Immunoprecipitation of the transfected cell extracts (10–15 times more extract than used in Figure 6A) with mAb 3G3 resulted in the coimmunoprecipitation of B10–hTAF$_{II}$28 (Figure 9B, lane 4). Similar results were obtained in extracts from HeLa cells transfected with B10–hTAF$_{II}$28 (Figure 9B, lane 3). In the converse experiment, TBP was coprecipitated by mAb B10 from Cos cells transfected with B10–hTAF$_{II}$28 but not from cells transfected with the empty B10 expression vector (Figure 9B, lanes 9 and 10). In analogous experiments, untagged hTAF$_{II}$28 was coprecipitated by mAb 3G3 from extracts of Cos cells transfected with the pXJ41–hTAF$_{II}$28 expression vector, whereas no TAF$_{II}$28 was detected after the precipitation of extracts from cells transfected with the empty vector (Figure 9B, lanes 6 and 7). Thus, although no endogenous TAF$_{II}$28 was detected stably associated with the Cos cell TFIID, the transfected hTAF$_{II}$28 does stably associate with the endogenous TBP/TFIID.

Discussion

TAF$_{II}$28 is an essential cofactor for the activity of the RXR AF-2s in vivo

We have shown previously (Mengus *et al.*, 1995) that hTAF$_{II}$28 is the homologue of dTAF$_{II}$30β (Yokomori *et al.*, 1993). Although these *Drosophila* and human TAF$_{II}$s show 50% identity, indicating a high evolutionary conservation, several differences in their interactions with other TFIID subunits were observed, notably in their reported abilities to interact with TBP (Mengus *et al.*, 1995). Thus, despite the fact that TAF–TAF interactions involving dTAF$_{II}$30β and hTAF$_{II}$28 had been studied, no function had been ascribed to either the *Drosophila* or human proteins.

The results presented here show that hTAF$_{II}$28 can function as a specific coactivator for several nuclear receptors in Cos cells. The most dramatic effect is observed with the RXR AF-2s, which do not activate transcription from a minimal promoter in the absence of coexpressed hTAF$_{II}$28, whereas a strong stimulation is seen in the presence of hTAF$_{II}$28. The ER and VDR AF-2s do activate transcription from a minimal promoter in Cos cells in the absence of hTAF$_{II}$28, but activation by these AF-2s is stimulated further by the expression of hTAF$_{II}$28. A weaker, yet significant, effect was observed with the AF-2s of the TR and RARs, which strongly stimulated transcription in the absence of hTAF$_{II}$28. The expression of hTAF$_{II}$28 also increased activation by wild-type RXRα or ER bound to their cognate REs. In addition, the results obtained with the DR1G–tk–chloramphenical acetyl transferase (CAT) reporter show that the coactivator activity of hTAF$_{II}$28 is not limited to a minimal promoter, but that it can also be observed when the RXRα AF-2 cooperates with its own AF-1 and/or the AFs of upstream factors on

a more complex promoter. The coactivator effect of hTAF$_{II}$28 in Cos cells requires the integrity of the AF-2 AD core as deletions or mutations of this sequence abolish activation in both the absence and presence of hTAF$_{II}$28. This result shows that hTAF$_{II}$28 does not promote the activity of a novel AD functioning independently of the AF-2, although we do not exclude the possibility that hTAF$_{II}$28 mediates the activity of an AD located within the receptor DE region which would function only in cooperation with the AF-2. In comparison with these observations of nuclear receptors, the expression of hTAF$_{II}$28 had no effect on activation by a series of activators whose activating domains are characterized by high proline or glutamine contents, irrespective of whether they function as strong or weak activators.

In contrast to Cos cells, in which the RXR AF-2s were inactive on a minimal promoter, RXR AF-2s had differential abilities to activate transcription from this promoter in HeLa cells. The strongest activation was observed with the RXRα AF-2, while only weak activation was observed with the RXRγ AF-2. In Cos cells, the coexpression of hTAF$_{II}$28 promoted a strong increase in the activities of all of the RXR AF-2s, whereas in HeLa cells a comparable strong increase was seen only with the RXRγ AF-2. A more dramatic difference was observed when comparing the effect of hTAF$_{II}$28 expression on activation by the ER and VDR AF-2s in Cos and HeLa cells. In Cos cells, the expression of hTAF$_{II}$28 increased activation, whereas in HeLa cells activation was reduced. Thus, the ectopic expression of hTAF$_{II}$28 has distinct effects in Cos and HeLa cells on the activities of these AF-2s.

One interpretation of the above results is that TAF$_{II}$28 is limiting for AF-2 activity in Cos cells but less so in HeLa cells, although its concentration is clearly suboptimal for the RXRγ AF-2. For the ER and VDR AF-2s, the concentration of endogenous hTAF$_{II}$28 in HeLa cells may be close to the optimum because overexpression of hTAF$_{II}$28 may actually begin to titrate other factors required for the activity of these AF-2s, resulting in decreased activation. This interpretation is supported further by the fact that we did not detect TAF$_{II}$28 in immunopurified Cos cell TFIID. This shows that, relative to HeLa cell TFIID, Cos cell TFIID contains significantly lower amounts of this TAF$_{II}$. It is unlikely that our inability to detect Cos cell TAF$_{II}$28 may be explained by the absence of all four human epitopes in the simian protein because hTAF$_{II}$28 shows a high evolutionary conservation (note that 11 of the 15 amino acids of the mAb 1C9 epitope are conserved, even in dTAF$_{II}$30β; Mengus *et al.*, 1995). In accordance with this observation, the anti-hTAF$_{II}$28 antibodies recognize mouse TAF$_{II}$28 in TFIID immunopurified from F9 embryonal carcinoma cells (our unpublished data). Furthermore, the simian homologues of six other hTAF$_{II}$s were detected in Cos cell TFIID. Nevertheless, we could not detect TAF$_{II}$28 in Cos or HeLa cell nuclear extracts with the mAbs used here because of its low abundance (our unpublished data). Thus, we cannot rule out the possibility that Cos cells contain hTAF$_{II}$28 but that it is not stably and functionally associated with the TFIID as in HeLa cells. Importantly, however, the transfected hTAF$_{II}$28 does associate stably with Cos cell TBP/TFIID, thereby increasing significantly the level of TAF$_{II}$28-containing TFIID; this correlates with the potentiation of transactivation by the receptor AF-2s.

Our experiments show that the RXR AF-2s can activate a minimal promoter in HeLa cells where the TFIID contains endogenous hTAF$_{II}$28, but are inactive in Cos cells where the TFIID is depleted in hTAF$_{II}$28. The ectopic expression of hTAF$_{II}$28 increases the levels of Cos cell TFIID containing TAF$_{II}$28, allowing activation by the RXR AF-2s. These observations imply that the activation of a minimal promoter by the RXR AF-2s absolutely requires the presence of TAF$_{II}$28 in the TFIID complex. On the other hand, TAF$_{II}$28 is not absolutely required for cooperation between the RXRα AF-2 and other AFs on complex promoters because full-length RXRα activated transcription from the DR1G–tk–CAT reporter in Cos cells. Similarly, the ER and VDR AF-2s activate transcription from a minimal promoter in Cos cells in the absence of TAF$_{II}$28, showing that, in contrast to the RXR AF-2s, they can function, albeit at reduced levels, via a TAF$_{II}$28-independent pathway perhaps involving other TFIID subunits. However, for the ER and VDR AF-2s, as well as for the RXRα AF-2 on a complex promoter, the ectopic expression of hTAF$_{II}$28 increases activation further, possibly by providing an additional pathway. In contrast to the RXRs, the RAR (at least the α and β forms) and the TR AF-2s strongly activate transcription in Cos cells, showing that these AF-2s work efficiently in the absence of TFIID-associated TAF$_{II}$28 and that their activity is only mildly stimulated by hTAF$_{II}$28 coexpression. These results show that the receptor AF-2s have differential abilities to activate transcription in the absence of TFIID-associated TAF$_{II}$28, and suggest that they can act by distinct molecular pathways, some of which are TAF$_{II}$28 dependent. The possible existence of alternative pathways for AF-2 activity has also been proposed to explain the E1A-dependent and -independent activation by the RAR (Berkenstam *et al.*, 1992; Keaveney *et al.*, 1993). It is also worth noting that although E1A and TBP cooperate to mediate activation by the RAR AF-2, like hTAF$_{II}$28, no direct E1A–RAR interactions were detected.

The coactivator function of hTAF$_{II}$28 requires interactions with TBP and other cofactors essential for the activities of the RXR AF-2s

Several different, but not mutually exclusive, molecular mechanisms may be invoked to explain the coactivator activity described here. It is possible that overexpressed hTAF$_{II}$28 acts by sequestering or inactivating a negatively acting factor(s) which would repress the receptor AF-2s to different degrees. Indeed, a negatively acting factor has been described recently which binds to the unliganded RAR and TRs (Chen and Evans, 1995; Hörlein *et al.*, 1995) and can in some conditions repress AF-2 activity (Kurokawa *et al.*, 1995). Although all our results cannot be explained by titration or inactivation of this factor, the existence of other related factors cannot be excluded at present.

Alternatively, as TAF$_{II}$28 is a TFIID subunit, the simplest interpretation of our results would be that it functions as a bridging factor between the receptor AF-2s and the basal transcription apparatus via TBP. However, we did not detect direct ligand-dependent receptor–hTAF$_{II}$28 interactions. This raises the possibility that hTAF$_{II}$28 exerts

its effect by interacting with a TIF(s) (see Introduction for references), which itself interacts with the receptors and is required for AF-2 function. This is supported further by the observation that deletions or mutations of the AF-2 AD core which affect the ability of the receptors to interact with putative TIFs, such as mSUG-1 or TIF1 (LeDourain *et al.*, 1995; Vom Bauer *et al.*, 1995), abolish activation in the presence of hTAF$_{II}$28. At present, we have not detected significant interactions between hTAF$_{II}$28 and TIF1 or mSUG1, but several other putative TIFs, such as RIP140 (Cavaillès *et al.*, 1995), ERAP160 (Halachmi *et al.*, 1994; Kurokawa *et al.*, 1995) and SRC-1 (Onate *et al.*, 1995), have been identified which interact with the receptors in a ligand- and/or AF-2 AD core-dependent manner. Further experiments will be required to determine whether these factors interact with hTAF$_{II}$28. Alternatively, hTAF$_{II}$28 may act via interactions with the SWI–SNF complex, some of whose components have been shown to influence receptor activity in yeast and mammalian cells (Yoshinaga *et al.*, 1992; Muchardt and Yaniv, 1993; Chiba *et al.*, 1994).

Further indication that hTAF$_{II}$28 may act as a bridging factor between the nuclear receptors and their associated TIFs and the basal transcription machinery comes from the observation that the ability of hTAF$_{II}$28 to act as a coactivator correlates with its ability to interact with TBP. We have shown previously that the carboxyl 61 amino acids of hTAF$_{II}$28 were required for interaction with TBP in Cos cells (Mengus *et al.*, 1995). The results presented here delineate this region to amino acids 150–179. Previously we were unable to determine the effect of deletions in the N-terminal region of hTAF$_{II}$28 on this interaction because of the low expression of the B10 epitope-tagged deletion mutants. Here we show that the untagged hTAF$_{II}$28(64–211) mutant is expressed efficiently in transfected Cos cells but interacts only weakly with TBP. This shows that determinants for the TBP–hTAF$_{II}$28 interaction are present in both the N- and C-terminal regions of hTAF$_{II}$28. The deletion of either of these determinants abolishes the ability of hTAF$_{II}$28 to promote RXR AF-2 activity. Furthermore, the amino acid substitutions within the putative amphipathic α-helical region between amino acids 161 and 179 reduce hTAF$_{II}$28–TBP interactions and abolish transactivation.

Previously the RXR and ER have been reported to interact directly with TBP *in vitro* and/or in yeast two-hybrid assays (Sadovsky *et al.*, 1995; Schulman *et al.*, 1995). Hence, it is possible that hTAF$_{II}$28 interacts with these receptors not via a TIF(s) but via a TBP. However, RXR and TBP could not be coimmunoprecipitated from extracts of cotransfected Cos cells, and the overexpression of TBP alone in Cos cells did not potentiate transactivation by RXR (our unpublished data). Moreover, the cotransfection of hTAF$_{II}$28 deletion mutants (1–150) and (64–211), which do not interact with TBP, represses hTAF$_{II}$28-mediated activation by the RXR and ER AF-2s but does not affect activation by the AF of TEF-1. Thus, although interactions with TBP are required to promote receptor AF-2 activity, hTAF$_{II}$28 also interacts with other factors specifically required for the activity of the receptor AF-2s. Therefore it is possible that hTAF$_{II}$28 acts as a bridging factor between the receptor and its associated TIF(s) and TBP, or that the receptor interacts with the basal transcription complex directly via TBP, with hTAF$_{II}$28 acting on a downstream target. Irrespective of the molecular mechanisms involved, our results clearly show that changes in the intracellular concentration of hTAF$_{II}$28 modulate AF-2 activity, indicating that hTAF$_{II}$28 can act as a novel regulator of nuclear receptors.

Materials and methods

Construction of recombinant plasmids

The hTAF$_{II}$28 expression vectors were generated by PCR amplification using appropriately positioned primers containing *Bam*HI and *Eco*RI restriction sites. The resulting fragments were cloned in the corresponding sites in the pXJ41 or pXJ42 vectors (Xiao *et al.*, 1991). Computer predictions using the Chou and Fasman algorithm in the GCG (Genetics Computer Group, University of Wisconsin, WI) software package and the PHD programme (EMBL) indicated that amino acids 161–179 (*FVGEVVEEALDVCEKWGEM*) of hTAF$_{II}$28 had the potential to form an amphipathic α-helix with a highly hydrophobic face (shown in italic) and a hydrophilic face where six out of the seven amino acids (with the exception of K175) are acidic (shown in bold). Site-directed mutagenesis of (1–179) single-strand DNA was performed to mutate E164, E167 and E168 to P, P and R, respectively, changing charge and disrupting the putative α-helix to generate hTAF$_{II}$28(1–179)M1. hTAF$_{II}$55 was cloned by screening a HeLa cell cDNA library with degenerate oligonucleotides derived from tryptic peptide sequences of hTAF$_{II}$55 immunopurified using anti-TBP antibodies (our unpublished data). pXJ41–hTAF$_{II}$55 was constructed by PCR amplification of a clone containing the complete hTAF$_{II}$55 open reading frame, followed by cloning of the resulting fragment between the *Bam*HI and *Xho*I sites of pXJ41. pXJ41–hTAF$_{II}$250 was constructed by inserting a *Spe*I fragment comprising the HA-tagged hTAF$_{II}$250 open reading frame into the *Sma*I site in pXJ41 after filling in the *Spe*I extremities. The vectors expressing the other hTAF$_{II}$s have been described previously (Mengus *et al.*, 1995). G4–VDR(DE) was constructed by PCR amplification of the human VDR DE region and cloning of the resulting fragment between the *Xho*I and *Bam*HI sites in plasmid pXJ40–GAL4(1–147) (Xiao *et al.*, 1991). Similarly, G4–TR(DE) was constructed by PCR amplification of the chicken TRα with the appropriate oligonucleotide primers and cloning between the Asp718 and *Bam*HI sites of pXJ40–GAL4(1–147). All constructions were verified using an Applied Biosystems automated DNA sequencer.

The vectors expressing all mouse RAR, RXR derivatives, HEG0 and G4–TEF-1(2–426)Δ55–121 have been described previously (Tora *et al.*, 1989; Nagpal *et al.*, 1992, 1993; Hwang *et al.*, 1993; Durand *et al.*, 1994). G4–AP-2, G4–Sp1, G4–Oct1 and G4–Oct2 containing proline- or glutamine-rich ADs from the respective activators are as described previously (Seipel *et al.*, 1992). All the reporter plasmids are as described previously (Tora *et al.*, 1989; Nagpal *et al.*, 1992).

Transfections, CAT assays and immunoprecipitations

Cos and HeLa cells were transfected by the $Ca_3(PO_4)_2$ precipitate technique, as described previously (Mengus *et al.*, 1995). In addition to the expression vectors or reporters described in each figure, all transfections contained 0.5 μg (1.0 μg in HeLa) of the luciferase reporter pRSV–Luc as an internal standard and pBSK$^-$ DNA as a carrier. Cells were harvested 48 h after transfection, and luciferase and CAT assays were performed by standard procedures. Transfections were performed in dextran charcoal-treated medium, and ligands were added at the indicated concentrations at the same time as the DNA–$Ca_3(PO_4)_2$ coprecipitate. A quantitative phosphorimager analysis was performed on a Fujix BAS 2000 apparatus. Immunoprecipitations were performed essentially as described previously (Mengus *et al.*, 1995). Cells were transfected with 2.0 μg of the TBP expression vector and 5.0 μg of the hTAF$_{II}$28 or G4–RXRβ(DE) expression vector as indicated. Cell extracts were prepared by three cycles of freeze–thaw in 100 μl buffer A (50 mM Tris–HCl, pH 7.9, 20% glycerol, 1 mM dithiothreitol, 0.1% NP-40) containing 0.5 M KCl and 2.5 μg/ml leupeptin, pepstatin, aprotinin, antipain and chymostatin. Extracts were mixed with ~1.0 μg of the monoclonal antibodies and 50 μl protein G–Sepharose, and incubated at 4°C for 2 h with rotation. The precipitated proteins were washed four times with 1 ml buffer A containing 1.0 M KCl and once with buffer A containing 0.1 M KCl. The proteins were then detected on Western blots using an Amersham enhanced chemiluminescence kit.

Total TFIID was prepared from extracts of untransfected cells using

antibody 3G3, essentially as described previously (Brou *et al.*, 1993a; Chaudhary *et al.*, 1994). Briefly, total Cos or HeLa cell TFIID was immunoprecipitated with mAb 3G3 and eluted by the addition of an excess of the corresponding epitope peptide (Lescure *et al.*, 1994) in buffer A containing 0.1 M KCl. The resin was washed one more time with buffer A containing 0.1 M KCl.

Antibody preparation

The monoclonal antibodies against TBP (3G3), hTAF$_{II}$18 (16TA), hTAF$_{II}$100 (1TA), hTAF$_{II}$30 (4G2), the B10 and HA tags, and GAL4(1–147) (2GV3, 3GV2) have been described previously (White *et al.*, 1992; Brou *et al.*, 1993a; Eberhardt *et al.*, 1993; Jacq *et al.*, 1994; Lescure *et al.*, 1994; Mengus *et al.*, 1995). mAb 1C9 was raised against a synthetic peptide corresponding to amino acids 106–120 (MQILVSSFSEEQLNR) of hTAF$_{II}$28, as described previously (Mengus *et al.*, 1995). mAb 15TA was raised against purified GST–hTAF$_{II}$28, and the epitope was mapped first by immunofluorescence using hTAF$_{II}$28 deletion mutants. Subsequent fine mapping was performed by an enzyme-linked immunosorbent assay using a series of overlapping peptides. mAb 15TA recognizes the sequence between amino acids 71 and 86 (REDSSLLNPAAKKLKI) of hTAF$_{II}$28. The rabbit polyclonal antisera were generated by immunizing rabbits with two hTAF$_{II}$28 peptides corresponding to amino acids 56–75 (GELESQDVSDLTTVEREDSS) and 185–204 (KHMREAVRRLKSKGQIPNSK) coupled to ovalbumin. After three injections, the rabbit antisera were tested on Western blots for their ability to recognize recombinant hTAF$_{II}$28 and hTAF$_{II}$28 present in immunopurified HeLa cell TFIID. Monoclonal antibodies against hTAF$_{II}$20 (22TA), hTAF$_{II}$55 (19TA) and hTAF$_{II}$135 (20TA) were generated by immunization with the appropriate purified GST–hTAF$_{II}$ fusion proteins, as described previously (Mengus *et al.*, 1995).

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