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The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18

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Nuclear receptors (NRs) bound to response elements mediate the effects of cognate ligands on gene expression. Their ligand-dependent activation function, AF-2, presumably acts on the basal transcription machinery through intermediary proteins/mediators. We have isolated a mouse nuclear protein, TIF1, which enhances RXR and RAR AF-2 in yeast and interacts in a ligand-dependent manner with several NRs in yeast and mammalian cells, as well as *in vitro*. Remarkably, these interactions require the amino acids constituting the AF-2 activating domain conserved in all active NRs. Moreover, the oestrogen receptor (ER) AF-2 antagonist hydroxytamoxifen cannot promote ER–TIF1 interaction. We propose that TIF1, which contains several conserved domains found in transcriptional regulatory proteins, is a mediator of ligand-dependent AF-2. Interestingly, the TIF1 N-terminal moiety is fused to B-raf in the mouse oncoprotein T18.

Key words: bromodomain/nuclear receptors/oncoprotein/ring finger/transcriptional intermediary factor

Introduction

There are two families of retinoic acid (RA) receptors, the RARs (α, β and γ and their isoforms), which bind and are activated by all-*trans* RA (T-RA) and 9-*cis* RA (9C-RA), and the RXRs (α, β and γ), which bind and are activated by 9C-RA only (reviewed in Leid *et al.*, 1992; Chambon, 1994; Kastner *et al.*, 1994; Mangelsdorf *et al.*, 1994). Both RARs and RXRs are members of the superfamily of steroid/thyroid hormone nuclear receptors, which act as ligand-dependent transcriptional regulatory proteins (Green and Chambon, 1988; Gronemeyer, 1991; Laudet *et al.*, 1992). Six regions of the nuclear receptors (A–F; Green and Chambon, 1988; Figure 1A) exhibit different degrees of evolutionary conservation. Region C encompasses the DNA binding domain (DBD), which recognizes cognate *cis*-acting response elements. C-Terminal to the DBD, region E contains both the ligand binding domain (LBD), a dimerization surface composed of conserved heptad repeats and a ligand-dependent transcriptional activation function (AF-2). The N-terminal A/B region contains an autonomous activation function (AF-1), which can activate transcription constitutively in the absence of the LBD (Green and Chambon, 1988; Tora *et al.*, 1989; Forman and Samuels, 1990; Gronemeyer, 1991; Chambon, 1994). An AF-2 activating domain (AF-2 AD) has recently been characterized in the C-terminal part of the E region and shown to correspond to an amphipathic α-helix motif (see Figure 5) whose main features are conserved between all known transcriptionally active members of the nuclear receptor superfamily (Danielan *et al.*, 1992; Barettino *et al.*, 1994; Durand *et al.*, 1994, and references therein).

The individual transactivating potential of AF-1 and AF-2 of the oestrogen receptor (ER), as well as that of RARs and RXRs, is dependent on the context of both the target promoter and the transfected cells (Tora *et al.*, 1989; Berry *et al.*, 1990; Nagpal *et al.*, 1992). AF-1 and AF-2 of ER, RARs and RXRs can also stimulate transcription in yeast, indicating that at least some of the transcriptional activation mechanisms are conserved throughout eukaryotes (Metzger *et al.*, 1988; Pierrat *et al.*, 1992; Heery *et al.*, 1993; our unpublished results). Interestingly, transcriptional interference (squelching) has been observed between the activation functions of the various steroid receptors (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tasset *et al.*, 1990). The activity of AF-2 of a given steroid hormone receptor can be squelched by over-expression of the AF-2-containing region E of the same receptor (auto-interference) or of a different steroid receptor (hetero-interference) in the presence of the cognate ligand (Meyer *et al.*, 1989; Tasset *et al.*, 1990). In this respect, the anti-oestrogen hydroxytamoxifen (OHT), whose antagonist activity is due to competitive inhibition of the oestrogen-dependent activity of AF-2 (Berry *et al.*, 1990), is less efficient than oestradiol (Meyer *et al.*, 1989). These transcriptional interferences, together with the promoter and cell context dependency of AF-1 and AF-2 activity, led us to propose that AF-1 and AF-2 activities are mediated by transcriptional mediators or intermediary factors (TIFs) interacting with the A/B and E regions of the receptor respectively (Tasset *et al.*, 1990). Furthermore, the interaction between the putative AF-2 mediator and region E is expected to be ligand-dependent.

The aim of the present study was to identify an intermediary factor(s) which mediate(s) the ligand-dependent activity of AF-2 with the transcription machinery in order to enhance initiation of transcription. Looking for mouse proteins which stimulate the transcriptional activity of RXRγ in yeast, we have isolated and characterized a protein (designated TIF1) which contains several conserved domains found in a number of nuclear regulatory proteins (e.g. the RING finger domain and the bromodomain) and may function as a mediator for the conserved AF-2 AD of nuclear receptors. Interestingly,

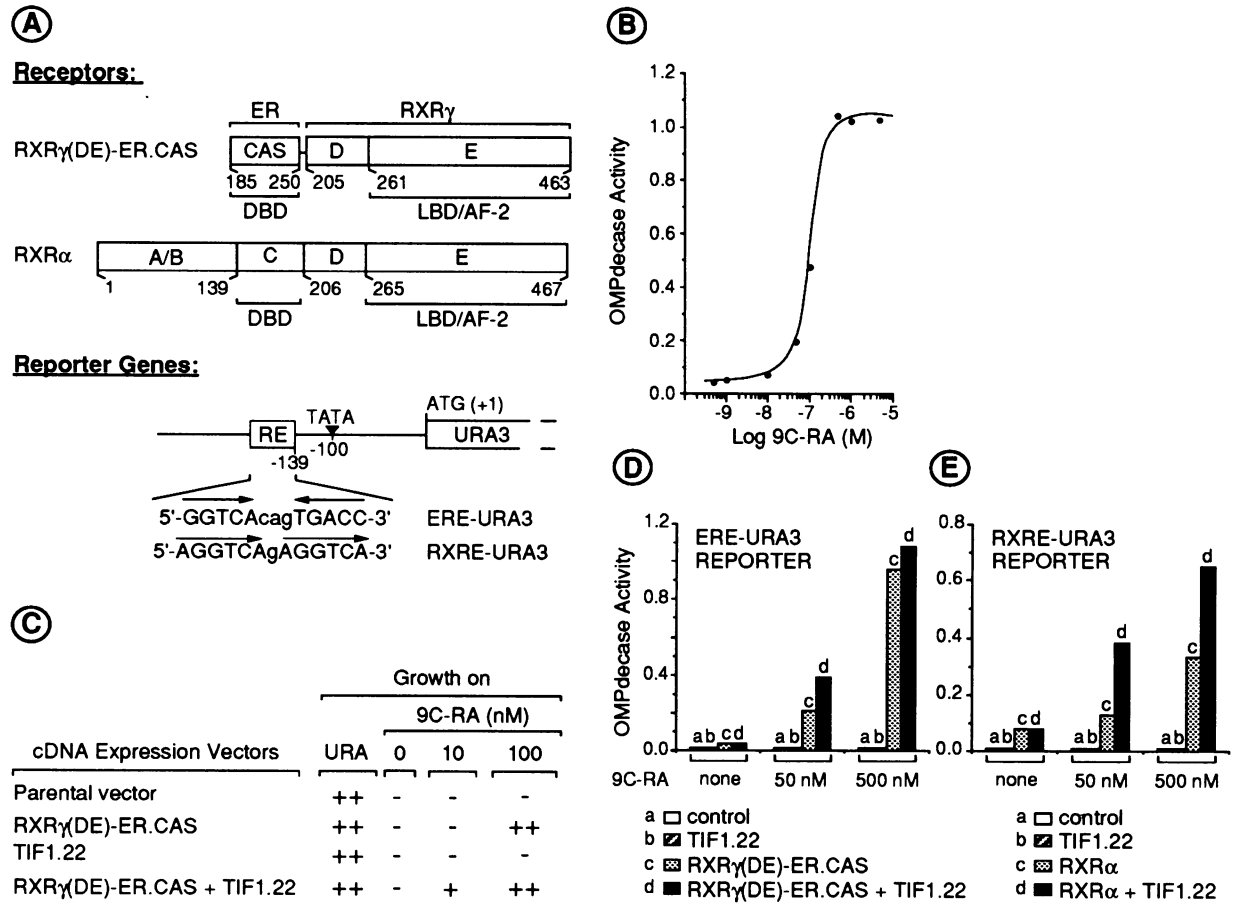


Fig. 1. Isolation and characterization of TIF1.22 cDNA. (**A**) A schematic representation of receptors (chimeric and wild-type) and reporter genes is shown. Numbers indicate the position of amino acid residues in wild-type receptors. The structure of the *URA3* reporter gene promoter region is schematized and the sequences of the response elements (RE) are given. (**B**) Dose–response curve of RXRγ(DE)–ER.CAS to 9C-RA. Yeast strain PL1 containing the integrated ERE–*URA3* reporter gene was transformed with a multicopy vector expressing RXRγ(DE)–ER.CAS. Cultures were grown in selective medium containing uracil and increasing amounts of 9C-RA. OMPdecase assays were performed on cell-free extracts. Enzyme activities are expressed in nmol substrate/min/mg protein. (**C**) The expression of TIF1.22 cDNA complements the growth defect of yeast strain PL1 expressing RXRγ(DE)–ER.CAS in the presence of 10 nM 9C-RA. PL1 was transformed with high copy number plasmids containing RXRγ(DE)–ER.CAS, TIF1.22 cDNA or no insert. Transformants were grown on selective medium containing uracil (URA) and spot tested on selective medium lacking uracil ± 9C-RA as indicated. Plates were incubated at 30°C for 3 days. ++, wild-type growth; −, no growth; +, weak growth. (**D**) RXRγ(DE)–ER.CAS is more efficiently activated by 9C-RA in yeast cells expressing TIF1.22. PL1 cells expressing RXRγ(DE)–ER.CAS and TIF1.22 either alone (b and c) or in combination (d) were grown in liquid medium containing uracil ± 9C-RA as indicated. Extracts were prepared and assayed for OMPdecase activity expressed as in (B). The values (±10%) are the mean of at least three independent experiments. Control (a) indicates the basal reporter activity (using 'empty' expression vectors). (**E**) TIF1.22 stimulates ligand-induced transactivation by RXRα in yeast. High copy number plasmids expressing RXRα (c), TIF1.22 (b) or both (d) were introduced into the yeast strain BP-G1 that contains an integrated *URA3* reporter gene driven by a RXRE (Figure 1A). Control (a) and transformant treatment were as in (D).

we also found that the N-terminal moiety of TIF1 is associated with the C-terminal moiety of B-raf in the mouse fusion oncoprotein T18 (Miki *et al.*, 1991).

Results

Isolation of a cDNA clone whose expression enhances the activity of AF-2 of RXR and RAR in yeast

A yeast genetic screen was designed to isolate mouse cDNAs encoding proteins that increase the activity of AF-2 of RXRγ. A chimeric receptor consisting of the DBD of ER (ER.CAS, ER amino acid residues 185–250) fused to the LBD of RXRγ [RXRγ(DE)–ER.CAS; Figure 1A] was expressed in the yeast strain PL1(α), which contains a *URA3* reporter gene under the control of an oestrogen response element (RE) (ERE–*URA3*; Figure 1A; Pierrat *et al.*, 1992). Addition of 9C-RA induced orotidine 5′-monophosphate decarboxylase (OMPdecase) activity in a dose-dependent manner (Figure 1B), indicating that RXRγ AF-2 is functional in yeast, as previously shown for RXRα AF-2 (Heery *et al.*, 1993). Half maximal response was achieved at ~100 nM, which was sufficient to induce yeast growth on a medium lacking uracil, while no growth was observed at 10 nM (Figure 1C). A P19 embryonal carcinoma (EC) cell oligo(dT)-primed cDNA expression library was constructed in the yeast multicopy expression vector pAS5, which contains the strong constitutive phosphoglycerate kinase (PGK) promoter, and introduced into PL1(α) expressing RXRγ(DE)–ER.CAS. Approximately 10^6 yeast transformants containing both plasmids were recovered and plated onto ura$^-$ plates containing 10 nM 9C-RA in order to isolate clones which can increase RXR AF-2 activity. Of the 112 clones which grew, only four required both the RXRγ(DE)–ER.CAS construct and the cDNA library plasmid to allow growth

at 10 nM 9C-RA. One 2.5 kb cDNA clone, designated TIF1.22, was further characterized.

The effects of TIF1.22 on activation by either RXRγ-(DE)−ER.CAS or RXRα were investigated using OMPdecase assays (Figure 1D and E). As expected, under a limiting 9C-RA concentration (50 nM), RXRγ(DE)−ER.CAS was a more efficient activator when co-expressed with TIF1.22 (Figure 1D). Moreover, TIF1.22 also enhanced the ligand-dependent activity of RXRα in the yeast strain BP-G1, which is identical to PL1(α) except that a retinoid XRE (RXRE) is substituted for the ERE in the reporter gene (Figure 1A and E). A similar ligand-dependent stimulatory effect was also observed in yeast at limiting concentrations of either T-RA or 9C-RA by co-expressing TIF1.22 and RARα(DEF)−ER.CAS, which contains RARα AF-2 (Heery *et al.*, 1993; data not shown). In contrast, TIF1.22 did not affect the constitutive activation function AF-1 of RARα when co-expressed with RARα1(A/B)−ER(C) (Heery *et al.*, 1993; data not shown). Thus, TIF1.22 appears to specifically enhance the activity of the ligand-dependent AF-2 of both RXR and RAR.

The 5′-moieties of full-length TIF1 and T18 fusion oncogene cDNAs are identical

Sequence analysis of TIF1.22 cDNA revealed a reading frame open at its 5′-end, suggesting that the 5′-portion of the cDNA was lacking. A randomly primed cDNA library made from mouse F9 EC cell poly(A)$^+$ RNA was screened to isolate additional TIF1 cDNAs. Successive screenings of this library with 5′ probes led to the identification of overlapping clones that allowed reconstitution of the sequence shown in Figure 2A. That 5′ and 3′ cDNA clones were derived from the same gene was confirmed by cloning and sequencing of the genomic DNA spanning the region −557 to +1728 of the cDNA (Figure 2A; data not shown). Furthermore, Northern blot analysis of poly(A)$^+$ RNA from P19 and F9 cells with DNA probes derived from either 5′- or 3′-portions of TIF1 cDNA revealed the same 4.2 kb mRNA species, which was also detected in all adult mouse tissues tested (heart, brain, spleen, lung, muscles, kidney and liver, with the highest expression level in the testis) and in several human cell lines (including HeLa, HepG2, HEK and MCF-7 cells; data not shown). A TIF1 cDNA isoform containing a 102 bp insertion (Figure 2B) between nucleotides 1728 and 1729 (filled triangle in Figures 2A and 3A) was also identified. This isoform is likely to correspond to an alternative splicing event, as consensus acceptor and donor sites were found in the genomic DNA at the ends of this insertion (data not shown). All subsequent studies were performed with the full-length cDNA isoform, which lacks the insertion and encodes a protein of 1017 amino acids with a predicted molecular weight of 112 kDa (Figure 2A).

A database search revealed that the sequence of the 5′-moiety of TIF1 cDNA was almost identical to that of the 5′-moiety of the mouse T18 oncogene cDNA, originally isolated from a furfural-induced hepatoma and shown to encode a chimeric protein of which the N-terminal region corresponds to a novel protein and the C-terminal region is identical to the 328 C-terminal residues of the murine homologue of the human B-raf proto-oncoprotein (Miki *et al.*, 1991). In fact, TIF1 cDNA between positions 397 and 1296 (332 amino acids) is identical to T18 cDNA between positions 144 and 1043 (Miki *et al.*, 1991). There are, however, a number of differences in the 5′-end of the two cDNAs, which certainly reflect sequencing difficulties related to the GC-richness of this region, as it was necessary to use dITP and formamide gels to unequivocally establish the sequence shown in Figure 2A, in which amino acid residues 3–32 of TIF1 (underlined in Figure 2A) replace the corresponding residues of the T18 sequence (GGCGEGGGGTGSGRSAAAARRAGRMR-PRA; see Miki *et al.* 1991). Moreover, we found the same 5′-TIF1 sequence by sequencing the corresponding genomic DNA (not shown). Thus, the N-terminal moiety of the T18 fusion protein appears to correspond to amino acids 1–332 of TIF1 (see Figures 2A and 3A).

The salient structural features of TIF1 are depicted in Figures 2A and 3. As noted by Kastner *et al.* (1992), the N-terminal portion of TIF1/T18 contains three Cys/His-rich clusters: an evolutionarily conserved zinc finger-like domain (the RING finger) precedes two zinc finger-like domains which have been designated as B boxes 1 and 2 (see Discussion). These three Cys/His-rich domains are followed by a putative coiled coil domain. Interestingly, these domains have been found in the N-terminal moiety of several nuclear proteins and in two other fusion oncoproteins, PML−RAR and RFP−ret (Figure 3A; see Kastner *et al.*, 1992, and Discussion). TIF1 also contains in its C-terminal region another highly conserved sequence, the bromodomain, which has been identified in a number of proteins, including several transcriptional regulatory proteins (see Discussion; Figures 2A and 3). The bromodomain is preceded in TIF1 by an additional Cys/His-rich cluster (Figures 2A and 3A). Other noteworthy features present in TIF1 include (Figures 2A and 3A): (i) short stretches of alanine (A) and proline (P) residues in the N-terminal region; (ii) a glutamine/proline-rich region (Q/P) in the central portion; (iii) several regions with high contents of serine (S) or acidic residues (E/D) in the C-terminal moiety (Figure 3A), which are common features of transcriptional activators (Tjian and Maniatis, 1994).

TIF1 and RXRα functionally interact in yeast

The yeast two-hybrid system (reviewed in Fields and Sternglanz, 1994) was used to investigate whether TIF1 and RXRα functionally interact. The hybrid protein corresponding to a fusion between the ER DBD (amino acids 176–282) and full-length TIF1 (DBD−TIF1, Figure 4A) and the hybrid protein resulting from a fusion between the acidic activating domain (AAD) of VP16 (amino acids 411–490) and RXRα (AAD−RXRα, Figure 4A) were expressed alone or in combination in the yeast strain PL3, which contains an integrated *URA3* reporter gene controlled by three tandem EREs (Figure 4A; Pierrat *et al.*, 1992). When expressed alone, none of these hybrid proteins transactivated the *URA3* reporter (Figure 4B and D and data not shown), indicating that full-length TIF1 could not activate transcription on its own when tethered to DNA through a heterologous DBD. In contrast, co-expression of the two hybrid proteins resulted in a 9C-RA-dependent ~25-fold increase in OMPdecase activity (Figure 4B and D), indicating that TIF1 and RXRα functionally interact in yeast cells. As expected, the

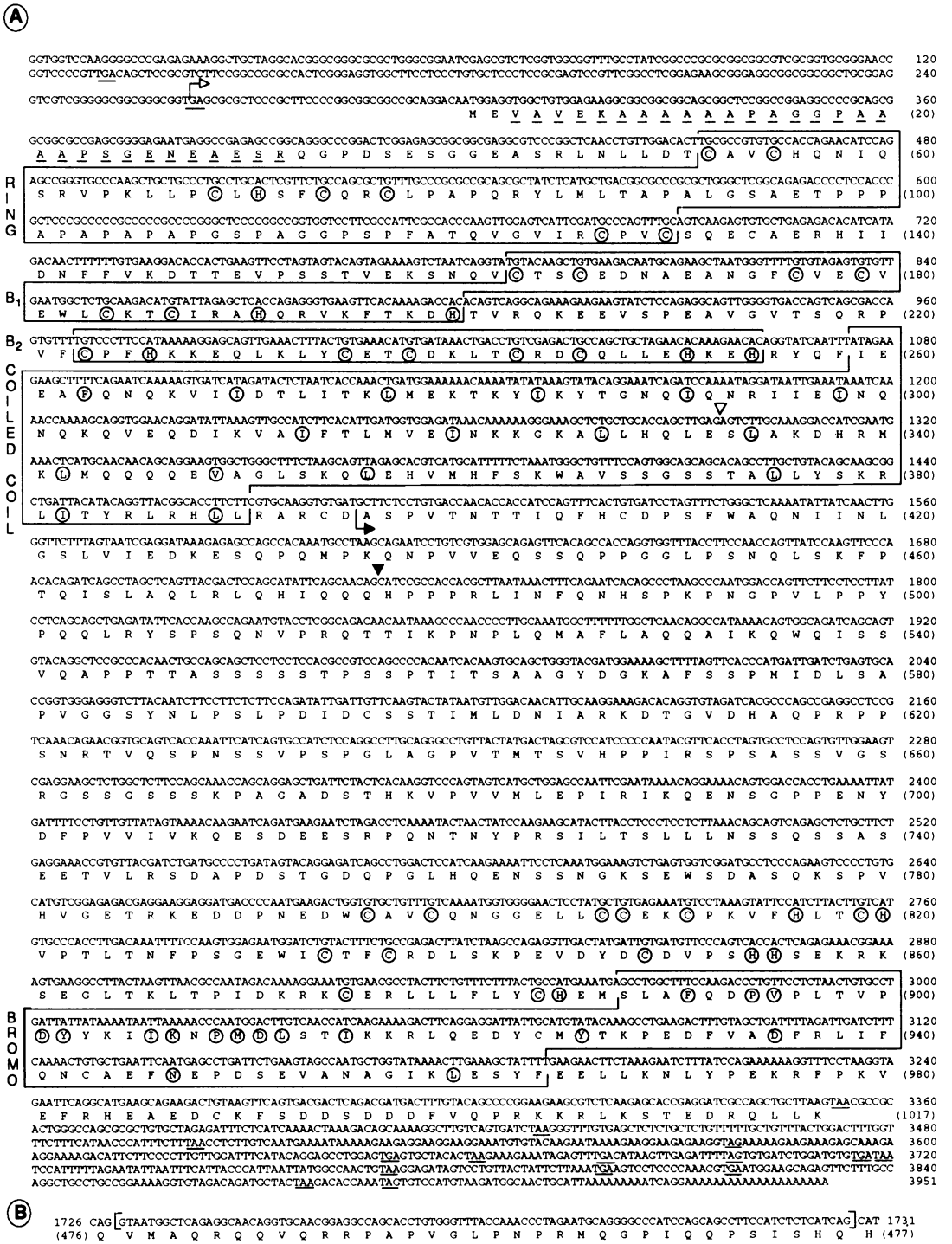
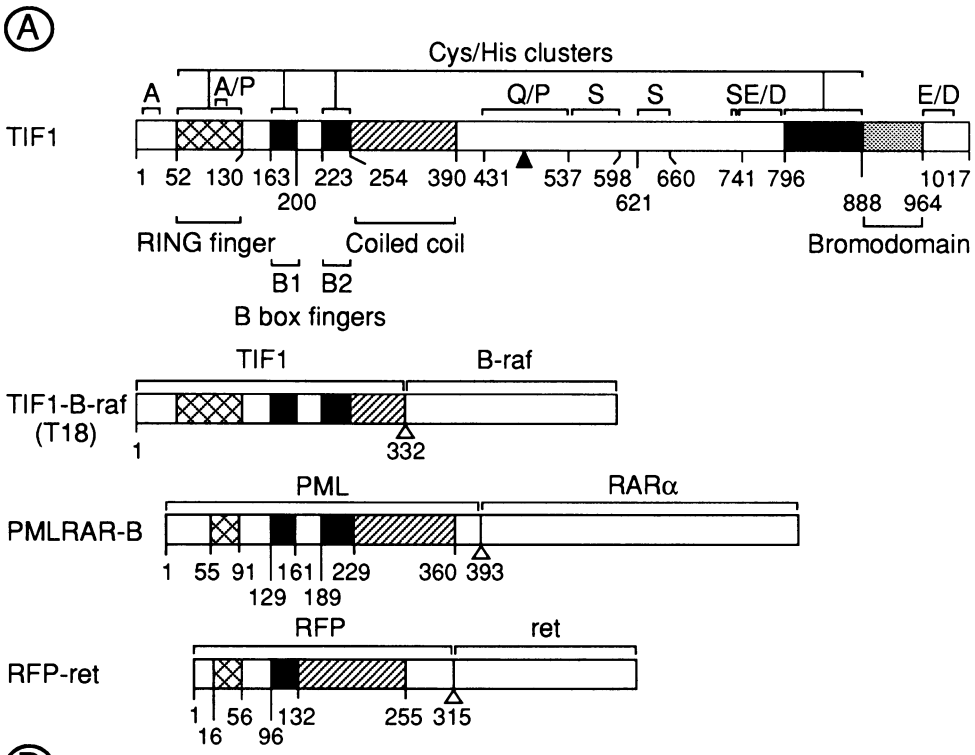


Fig. 2. Nucleotide and deduced amino acid sequences of TIF1. (**A**) The 3951 nucleotides of TIF1 cDNA, the 1017 amino acid open reading frame beginning at position 301 and flanking termination codons (underlined) are shown. The filled arrow indicates the 5′-end of TIF1.22 cDNA, which encodes residues 396–1017. The filled triangle denotes the location of a 102 bp insertion encoding 34 additional amino acids (see B). The open arrow (nt 263) indicates the 5′-end of T18 cDNA in Miki *et al.* (1991). The open triangle (amino acid residue 332) denotes the point of fusion between the TIF1 and B-raf sequences in T18. Differences between the TIF1 and T18 nucleotide sequences were found in several GC-rich regions (see text). The sequences GCG, AGGGT, ACGG, AGCGCCG, CGCGCCG and AGCGCAG, corresponding to positions 41–43, 55–59, 84–87, 99–105, 112–118 and 140–146 respectively in T18 (Miki *et al.*, 1991), are replaced by GCCG, AGGT, AGCG, AGGCCCCG, CGGCGCCG and AGCCGGCAG in TIF1 (positions 290–293, 305–308, 333–336, 348–355, 362–369 and 391–399 respectively). Amino acid residues of TIF1 differing from those of T18 are underlined (see text). Cysteine and histidine residues belonging to the Cys/His-rich clusters [RING finger, B1 and B2 boxes and the C-terminal Cys/His cluster (amino acids 796–885)] are highlighted with circles. The hydrophobic amino acids defining the heptad repeats of the putative coiled coil structure are also circled, as are conserved residues of the bromodomain (BROMO). Note that TIF1 contains a putative nuclear localization signal KRKSEGLTKLTPIDKRK in its C-terminal region (amino acids 858–874). (**B**) Sequence of the 102 bp internal insertion identified in several TIF1 cDNA clones from the F9 EC cDNA library.



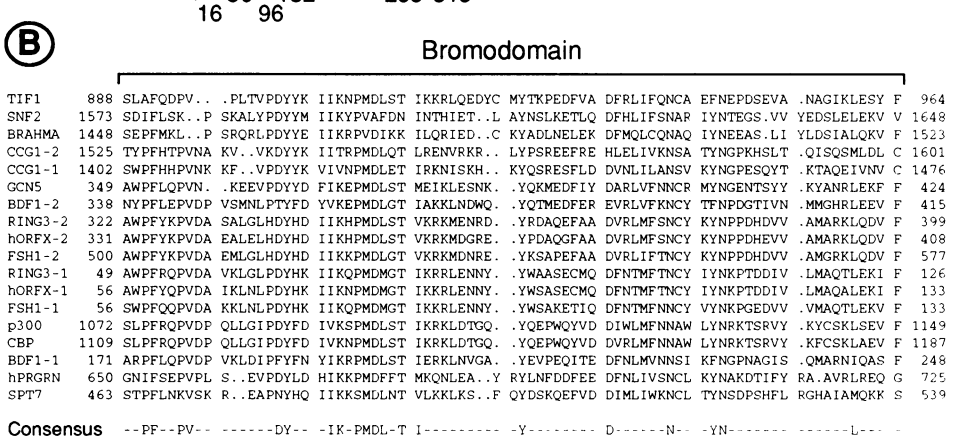


Fig. 3. Structural features of TIF1 and similarities with PML and RFP. (**A**) The salient structural motifs present in TIF1 are schematically represented. Regions rich in particular amino acids (abundance >20%) are indicated (see text). The corresponding N-terminal domains present in the fusion oncoproteins TIF1–B-raf (T18), PMLRAR-B and RFP–ret are similarly highlighted. (**B**) Comparative alignment of the TIF1 bromodomain with 17 other bromodomain sequences. Sequences homologous to the bromodomain of TIF1 were detected using BLAST (Altschul *et al.*, 1990). The alignment was generated using the PILEUP and PRETTY programs of the GCG sequence analysis software package (GCG, University of Wisconsin, Madison, WI). A consensus was derived by using a plurality occurence of 14 and is given at the bottom of the alignment. The coordinates of the first and last amino acids for each bromodomain are also given. Database accession numbers: RING3, P25440; GCN5, Q03330; FSH, P13709; hORFX, D26362; BDF1, Z18944; p300, U01877; SPT7, L22537; CCG1, P21675; SNF2, P22082; BRAHMA, P25439; hPRGRN, M91585; CBP, S39161. Several proteins contain more than one bromodomain, as indicated by the suffix -1 or -2.

LBD of RXRα (amino acids 263–467) was sufficient for functional interaction with TIF1 in the yeast assay [AAD–RXRα(263–467) in Figure 4B]. No activation of the reporter was detected with any of the other regions of RXRα (Figure 4B), demonstrating that the ligand-dependent functional interaction between TIF1 and RXRα is specific to region E.

Several deletion mutants of TIF1 were fused to the ER DBD and assayed for transactivation in the yeast strain PL3 (Figure 4D). In the presence of unfused AAD, none of the DBD–TIF1 fusions activated the reporter gene above the level of unfused DBD, except DBD–TIF1(209–433) and DBD–TIF1(539–750), which suggests that TIF1 may contain 'masked' activating domain(s). No significant increase in reporter activity was observed when fusion proteins bearing TIF1 residues 1–208, 209–433 and 792–1017, which include the RING finger, the coiled coil motif and the bromodomain respectively, were co-expressed with AAD–RXRα (Figure 4D). However, a 9C-RA-dependent ~60-fold enhancement was observed in the presence of DBD–TIF1(434–791) (Figure 4D). In all cases, the level of expression of the 'deficient' and of the 'active' deletion mutants were similar (see legend to Figure 4). The minimal interaction domain between RXRα and TIF1 appears to be located between TIF1 amino acid residues 539 and 750 [see DBD–TIF1(434–750) and

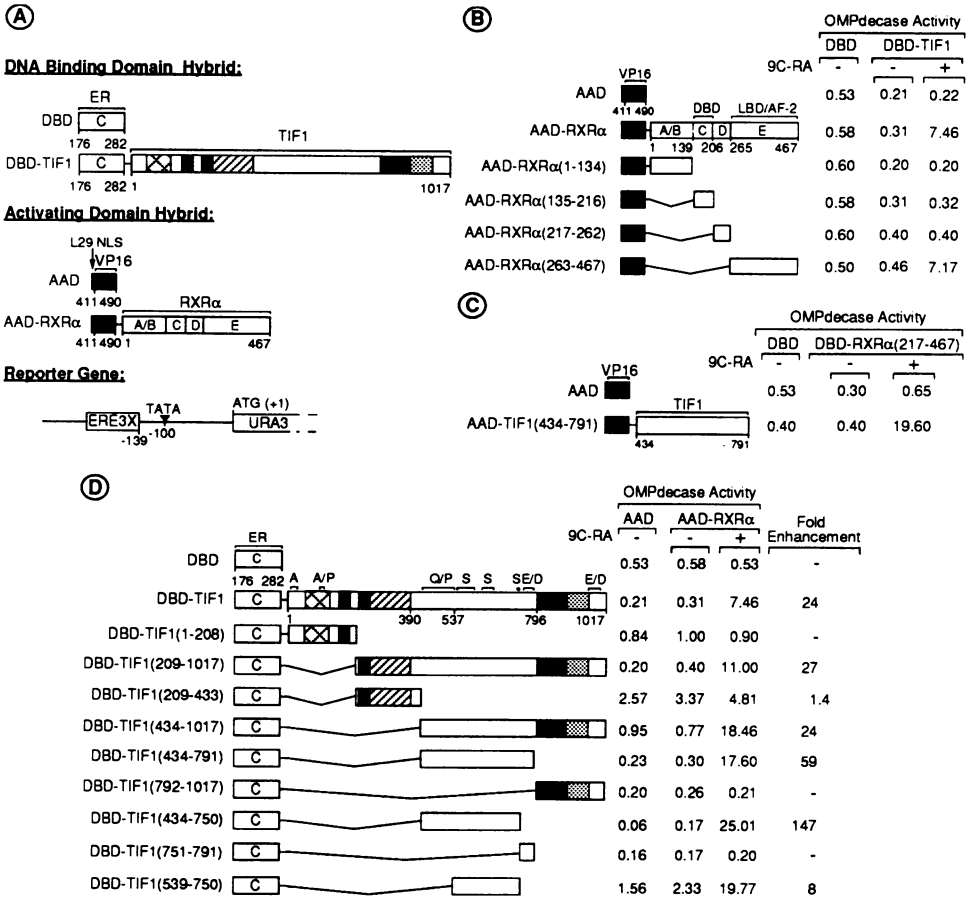


Fig. 4. TIF1 and RXRα functionally interact in yeast. (**A**) Schematic representation of the DBD of ER and of the VP16 AAD, unfused or fused to the complete coding sequences of TIF1 and RXRα respectively. Numbers refer to amino acid positions in the natural protein. AAD and AAD−RXRα also include codons specifying the NLS of the yeast ribosomal protein L29. The structure of the integrated *URA3* reporter gene, whose expression is regulated by three oestrogen response elements (ERE3×) in the yeast reporter strain PL3 is also shown. (**B**) The ligand binding domain of RXRα is sufficient for interaction with TIF1. Plasmids expressing RXRα or individual regions of RXRα (as indicated) fused to the VP16 AAD were co-transformed in PL3 with the ER DBD or DBD−TIF1 fusion constructs (A). Expression of these fusion constructs was confirmed in all cases by Western blot using the antibody 2GV-4 against VP16 (data not shown). Transformants were grown in liquid medium containing uracil in the presence or absence of 500 nM 9C-RA. Extracts were prepared and assayed for OMPdecase activity, which is expressed as in Figure 1B. (**C**) Co-expression of DBD−RXRα(217–467) with AAD−TIF1(434–791) stimulates the *URA3* reporter in a 9C-RA-dependent manner. PL3 transformants expressing the indicated proteins were treated as described in (B). (**D**) Residues 539–750 of TIF1 are sufficient for interaction with RXRα. Various regions of TIF1 were fused to the ER DBD and assayed for activation with either AAD or AAD−RXRα in PL3 grown in the presence or absence of 500 nM 9C-RA, as indicated. OMPdecase activities were expressed as in Figure 1B. The enhancement of OMPdecase activity resulting from the interaction between the DBD−TIF1 constructs and AAD−RXRα in the presence of 9C-RA is indicated. Similar OMPdecase activities were obtained when the DBD−TIF1 fusions were tested in the absence or in the presence of AAD (data not shown). Expression of the fusion proteins was confirmed in all cases by Western blot using the antibody F3 against the F region of ER (data not shown). In all panels the values (±10%) are the average of at least three independent experiments.

-(539–750) in Figure 4D]. Similar ligand-dependent activations were observed with the reciprocal constructions [see AAD−TIF1(434–791) and DBD−RXRα(217–467) in Figure 4C] and by substituting the ER DBD for the LexA DBD in another version of the two-hybrid system using *lexA* binding sites within the context of the *GAL1* promoter (Vojtek *et al.*, 1993) (data not shown).

TIF1 also functionally interacts with other members of the nuclear receptor superfamily

cDNAs encoding RARα1 and the LBD of either the vitamin D_3 receptor (VDR), progesterone receptor (PR) or ER were fused to VP16 AAD (Figure 5A). The fusion proteins were co-expressed in the yeast strain PL3 with either DBD or DBD−TIF1. In combination with DBD−TIF1 (but not DBD; data not shown), each of these VP16 AAD fusion proteins stimulated expression of the *URA3* reporter in the presence of the cognate ligand (Figure 5A and data not shown). Furthermore, no stimulation was detected when yeast cells co-expressing DBD−TIF1 and AAD−ER(DE) were grown in the presence of the anti-oestrogen OHT, which prevents ER AF-2 activity in mammalian and yeast cells (Berry *et al.*, 1990; Metzger *et al.*, 1992; Figure 5B). An excess of OHT inhibited oestradiol (E_2)-induced stimulation by ≥80% (Figure 5B), supporting the conclusion that the OHT-liganded protein AAD−ER(DE) cannot interact with TIF1.

Thus, TIF1 can functionally interact in yeast with several members of the nuclear receptor superfamily. In all cases tested, this interaction is ligand-dependent, and the ligand dependency appears to be specific for ligands that are able to induce AF-2 activity.

Conserved amino acid residues of the AF-2 activating domain (AF-2 AD) are required for functional interaction with TIF1

An amphipathic α-helical motif essential for AF-2 activity in both mammalian and yeast cells (the AF-2 activating domain, AF-2 AD; see Figure 5C) has been characterized in the C-terminal part of the RXRα region E (Durand *et al.*, 1994; Heery *et al.*, 1994). This AF-2 AD is conserved among transcriptionally active members of the nuclear receptor superfamily and deletion or point mutations in the conserved residues of this motif abrogate AF-2 activity of RXRα, RARα, TR, ER or GR (without drastically altering ligand or DNA binding), indicating that these residues are specifically involved in ligand-dependent transcriptional activation by nuclear receptors (Danielan *et al.*, 1992; Barettino *et al.*, 1994; Durand *et al.*, 1994; our unpublished results).

To determine whether AF-2 AD residues were involved in the interaction between TIF1 and RXRα, we introduced mutations into the AF-2 AD of RXRα. Western blots indicated that these mutants were expressed at similar levels as AAD−RXRαWT (see legend to Figure 5). In contrast to wild-type RXRα, the C-terminally truncated mutant RXRαΔ455–467, lacking AF-2 AD (but still binding 9C-RA; data not shown), as well as the point mutants F455A/L456A and M459A/L460A (in which the conserved hydrophobic residues phenylalanine, leucine and methionine were replaced with alanine; see Figure 5C), were unable to functionally interact with TIF1 in the yeast assay (Figure 5C) or to transactivate in mammalian cells (Figure 5D). Remarkably, a single point mutation in the glutamic acid residue 461 (E461Q) reduced the ability of RXRα to interact with TIF1 in yeast by 70%, while reducing by 52% its ability to transactivate in mammalian cells (Figure 5D). Similarly, an RARα mutant with the AF-2 AD deleted (Δ408–416) did not functionally interact with TIF1 (Figure 5C). This deletion mutant is known to act as a dominant negative mutant in mammalian cells while retaining T-RA and DNA binding functions (Durand *et al.*, 1994). Thus, ligand binding appears to be necessary, but not sufficient, for functional interaction with TIF1. Together, these results suggest that TIF1 interacts with the AF-2 AD of the nuclear receptors.

Ligand-dependent interaction between TIF1 and the LBD of nuclear receptors in vitro

To investigate whether the functional interaction of TIF1 with the liganded nuclear receptors in yeast corresponds

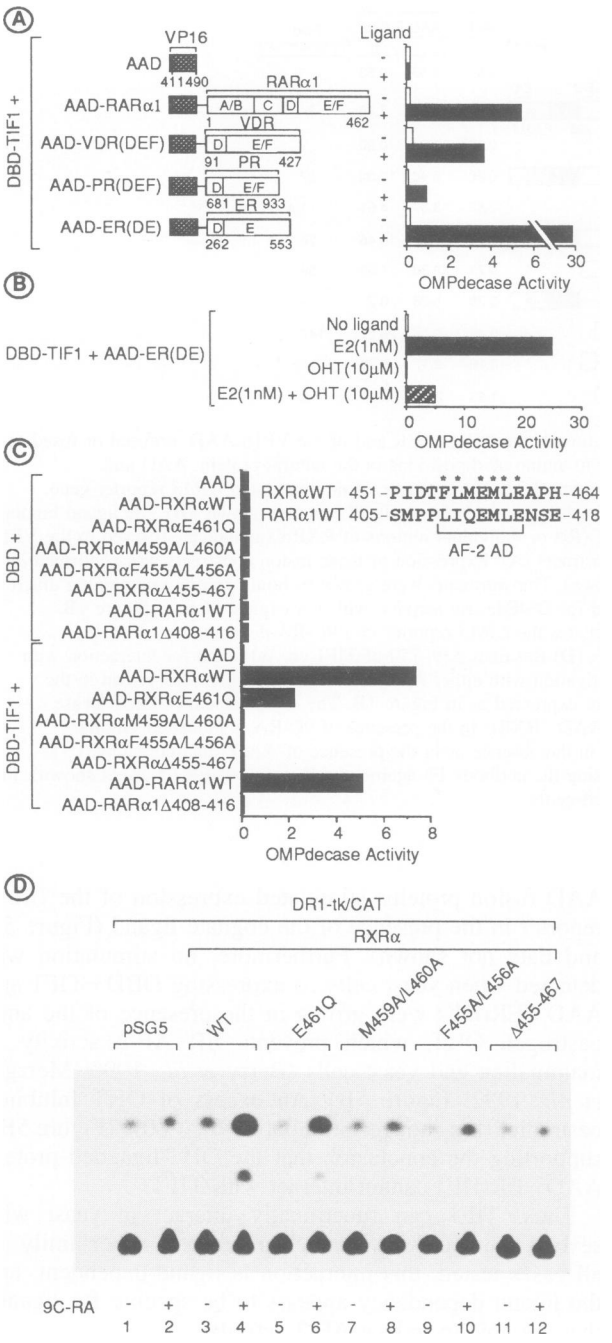


Fig. 5. Functional interaction between TIF1 and various nuclear receptors in yeast. (**A**) Schematic representations of the AAD fusion receptors are displayed on the left. Numbers indicate amino acid positions in human RARα1, human VDR, human PR form B and human ER. The indicated AAD fusion constructs were co-expressed with either the unfused ER DBD or the DBD−TIF1 construct in the yeast reporter strain PL3. Transformants were grown in the presence (+) or absence (−) of the cognate ligand (1 μM T-RA for RAR, 5 μM vitamin D3 for VDR, 10 μM R5020 for PR, 1 μM E_2 for ER). Extracts were prepared and assayed for OMPdecase activity, which was expressed as in Figure 1B. No increase in OMPdecase activity above background was observed when the AAD fusion proteins were co-expressed with the unfused ER DBD (data not shown). (**B**) No interaction is seen between TIF1 and the DE region of ER in the presence of the antagonist OHT. The PL3 reporter strain was co-transformed with plasmids expressing DBD−TIF1 and AAD−ER(DE) fusion constructs. Transformants were grown in the presence or absence of 1 nM E_2, 10 μM OHT or 1 nM E_2 plus 10 μM OHT as indicated and treated as described in (A). (**C**) The AF-2 AD is required for interaction with TIF1. The sequence of the AF-2 AD of mouse RXRα and mouse RARα1 is shown. The conserved hydrophobic and acidic amino acids are indicated by a star. RXRα and RARα1 and their mutants were fused to the AAD of VP16 and assayed for interaction with either ER DBD or DBD−TIF1 in the yeast reporter strain PL3 grown in the presence of 1 μM 9C-RA, as indicated. OMPdecase activities were expressed as in Figure 1B. Expression of the fusion proteins was confirmed in all cases by Western blot using the antibody 2GV-4 against VP16 (data not shown). (**D**) Transcriptional activation by wild-type and mutant RXRα receptors in mammalian cells. Cos-1 cells were transfected with the DR1T−tk/CAT reporter gene (2 μg), together with either 100 ng 'control' pSG5 or 100 ng receptor expression vector as indicated. Cells were treated with EtOH (−) or 100 nM 9C-RA (+) for 24 h and CAT activity was determined. All mutants were expressed at levels similar to that of wild-type RXRα, as determined by gel shift assays using the RXRα antibody 4RX.

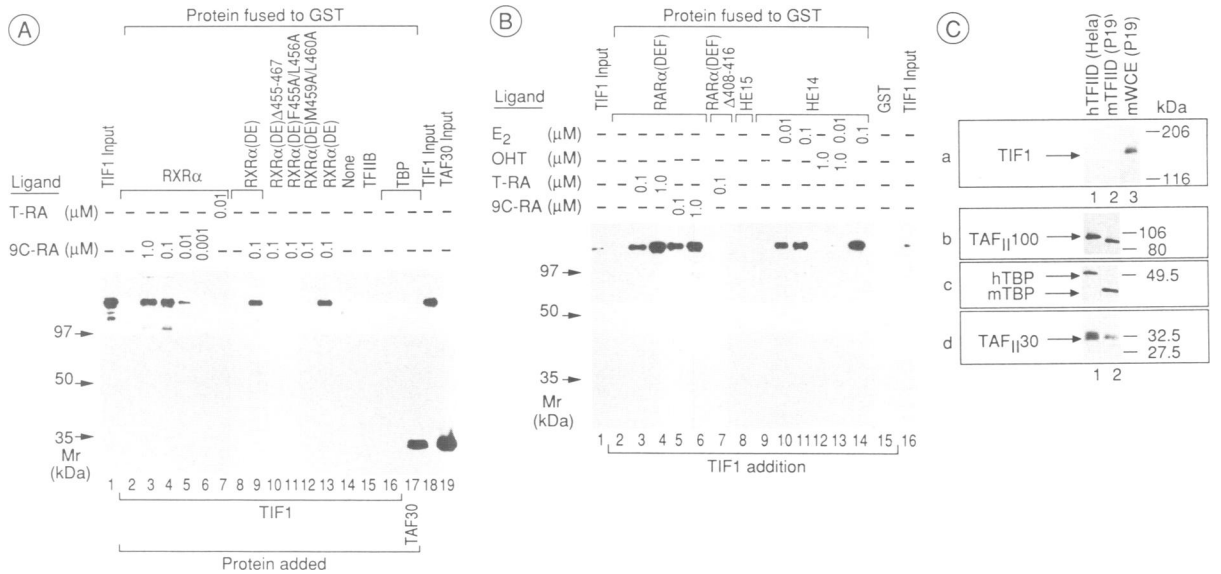


Fig. 6. TIF1 which interacts *in vitro* with the liganded LBD of nuclear receptors does not interact with TBP or TFIIB and is not present in TFIID complexes. (**A**) Purified His-TIF1 (lanes 2–16) was incubated in a batch assay with one of the GST−RXRα fusion proteins (lanes 2–13, as indicated), 'control' GST (lane 14), GST−TFIIB (lane 15) or GST−TBP (lane 16) bound to glutathione−Sepharose beads. As a positive control, His-$TAF_{II}30$ (TAF30) was also incubated with GST−TBP (lane 17). Lanes 1 and 18 correspond to the His-TIF1 input and lane 19 to the His-$TAF_{II}30$ input. Ligands (T-RA or 9C-RA) were added to the incubations as indicated. Bound proteins were identified by Western blotting. (**B**) Binding assays as in (A) reveal that interaction between TIF1 and RARα requires the AF-2 AD (compare lanes 3 and 7) and either T-RA or 9C-RA (compare lane 2 with lanes 3–6). E_2 (compare lane 9 with lanes 10, 11 and 14), but not the antagonist OHT (lanes 12 and 13), induces interaction between TIF1 and the ER LBD, while no interaction is seen with the N-terminal half (regions A, B and C) of ER (HE15, lane 8) or the GST 'control' (lane 15). (**C**) TIF1 is not present in TFIID. TBP-containing TFIID complexes from HeLa (lane 1) or P19 (lane 2) whole cell extracts were affinity-purified with an anti-TBP mAb (Brou *et al.*, 1993) and resolved by SDS−PAGE along with crude P19 whole cell extracts (lane 3). TIF1 (panel a), $TAF_{II}100$ (panel b), TBP (panel c) and $TAF_{II}30$ (panel d) were revealed by Western blotting with cognate mAbs.

to a direct interaction, *in vitro* binding assays were performed using purified TIF1 and nuclear receptors. TIF1 was tagged with six histidine residues at its N-terminal end (His-TIF1), expressed in SF9 cells using a baculovirus vector and purified. The complete open reading frame of RXRα was fused to glutathione S-transferase (GST) to generate GST−RXRα, which was expressed in *Escherichia coli*, bound to glutathione−Sepharose beads and incubated (±9C-RA) with purified His-TIF1. The matrix-associated TIF1 protein was revealed by Western blotting. His-TIF1 was retained on GST−RXRα beads in a 9C-RA-dependent manner (Figure 6A, lanes 2–6), but not on 'control' GST beads (lane 14). GST fusion proteins containing the LBD/AF-2 region of either RXRα [amino acids 205–467, GST−RXRα(DE)], RARα [amino acids 153–462, GST−RARα(DEF)] or ER (amino acids 282–595, GST−HE14) were produced, as well as a GST fusion protein containing the ABC regions of the ER which harbors the ER activation function 1 AF-1 (GST−HE15, amino acids 1–282; Kumar *et al.*, 1987). Only GST−LBD beads [GST−RXRα(DE), GST−RARα(DEF) and GST−HE14] specifically retained His-TIF1 in the presence of the cognate ligand (9C-RA, T-RA or 9C-RA and E_2 respectively; Figure 6A, compare lane 8 with lanes 9 and 13; Figure 6B, compare lane 2 with lanes 3–6 and lane 9 with lanes 10, 11 and 14). Moreover, GST−HE14 failed to retain His-TIF1 in the presence of the anti-oestrogen OHT (Figure 6B, lane 12) and OHT antagonized the E_2-dependent interaction between GST−HE14 and His-TIF1 (lane 13 and data not shown).

Several AF-2 AD-deficient RXRα and RARα mutants unable to functionally interact with TIF1 in yeast and to activate transcription in transfected mammalian cells (Figure 5D; Durand *et al.*, 1994) were also expressed as GST fusion proteins and tested for interaction with His-TIF1. No binding was observed with the C-terminally truncated RXRαΔ455–467 lacking AF-2 AD or with the RXRα mutants F455A/L456A and M459A/L460A (Figure 6A, lanes 10–12). Furthermore, the RARα mutant in which the AF-2 AD was deleted (Δ408–416) was also unable to bind His-TIF1 (Figure 6B, lane 7).

These results, which are in complete agreement with the functional interactions detected in yeast, demonstrate that the direct interactions observed *in vitro* between purified TIF1 and the liganded LBD of either RXRα, RARα or ER require the integrity of the AF-2 AD conserved motif.

TIF1 and ER interact in mammalian cells

Interaction between TIF1 and ER was tested by co-immunocytofluorescence analysis in monkey Cos-1 cells transiently transfected with the corresponding expression vectors. Interestingly, TIF1 was localized in the nucleus of the transfected cells, often displaying a punctate distribution pattern (Figure 7A and data not shown; endogenous Cos-1 TIF1 could not be detected with the present antibody; data not shown). This pattern was similar to, although not identical with, the speckled nuclear distribution of PML (Kastner *et al.*, 1992; our unpublished results).

To determine whether TIF1 and ER could interact in Cos-1 cells, we used an ER mutant ERΔNLS, which contains a deletion preventing its nuclear localization (see Ylikomi *et al.*, 1992, in which ERΔNLS was designated HE257G). ERΔNLS was present in the cytoplasm of most

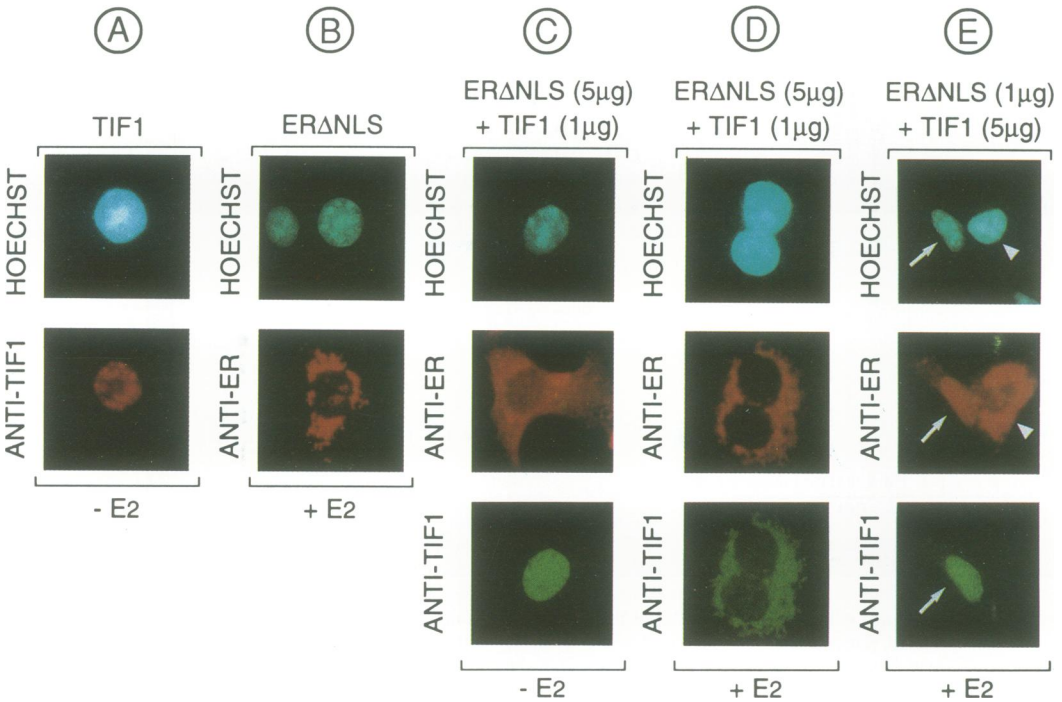


Fig. 7. Interaction between TIF1 and ER in mammalian cells. pSG5-based (Green *et al.*, 1988) expression vectors encoding either TIF1 (A and C–E) and/or the ER deletion mutant ERΔNLS (B–E) were transfected into Cos-1 cells and the corresponding proteins were revealed by immunocytofluorescence. In all cases the upper panels show the Hoechst DNA staining and the lower panels correspond to immunodetection with either anti-ER or anti-TIF1 mAbs, as indicated. (**A** and **B**) Cells were transfected with 1 μg TIF1 or ERΔNLS expression vector respectively. (**C–E**) Cells were co-transfected with both expression vectors in the absence or presence of 5×10^{-6} M E_2, as indicated.

of the transfected cells, irrespective of the presence of oestradiol (E_2) (Figure 7B and data not shown). ERΔNLS and TIF1 were then co-transfected in the presence or absence of E_2 and the intracellular localization of both proteins was determined by double labelling immunocytofluorescence. In the absence of E_2, ERΔNLS and TIF1 exhibited their characteristic cytoplasmic and nuclear localizations respectively (Figure 7C). In contrast, a co-localization of ERΔNLS and TIF1 was observed in E_2-treated cells. When the ERΔNLS expression vector was transfected in excess, TIF1 always adopted the localization of ERΔNLS (Figure 7D and data not shown). On the other hand, when the TIF1 expression vector was in excess, both proteins were nuclear in >90% of the transfected cells (Figure 7E, arrow, and data not shown). Interestingly, in the few co-transfected cells in which TIF1 was not over-expressed, ERΔNLS remained cytoplasmic (Figure 7E, arrowhead). Moreover, ERΔNLS also remained cytoplasmic when transfected together with a nuclear-localized TIF1 mutant deleted for the receptor interacting domain (TIF1Δ539–750; data not shown). Thus, TIF1 and ER interact in a ligand-dependent manner in mammalian cells, as observed in yeast cells and *in vitro*.

TIF1 which interferes with transactivation by nuclear receptors fails to interact with several transcription factors

The above results indicate that TIF1 could be a mediator transducing the effect of the ligand-dependent AF-2 of nuclear receptors to some component(s) of the transcription machinery. TIF1 and RXRα expression vectors were therefore transiently co-transfected into Cos-1 cells, together with the DR1–tk/CAT reporter, which contains

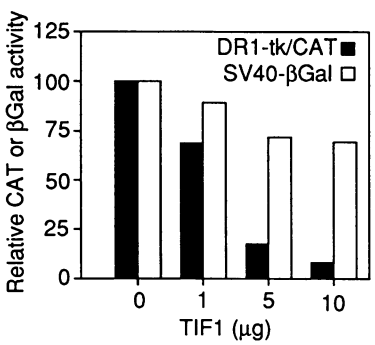


Fig. 8. TIF1 interferes with transactivation by RXRα. Cos-1 cells were transiently co-transfected with 4 μg DR1–tk/CAT reporter, 100 ng RXRα, 1 μg pCH110 (expressing β-galactosidase) and increasing amounts of pSG5-TIF1 expression vector, as indicated. In all cases, 9C-RA was added at a concentration of 1 μM, which resulted in a 6-fold increase of DR1–tk/CAT expression (in the absence of TIF1) when compared with background expression in the absence of ligand. Enzyme activities (average values ± 20% of at least three independent experiments) are expressed relative to those seen in the absence of TIF1 (taken as 100%).

a RXR response element (Mader *et al.*, 1993). Co-expression of TIF1 resulted in a marked decrease in transactivation by RXRα (Figure 8, CAT activity). Similar decreases were observed for ligand-dependent transactivation by RAR and ER, as well as by the GAL–RXRα(DE), GAL–RARα(DEF) and GAL–ER(EF) derivatives using a 17mer reporter gene (data not shown). However, under similar conditions, the activity of the SV40 early promoter/enhancer of the internal control vector pCH110 was not significantly affected (Figure 8, β-galactosidase activity). Note that the promoter of the RXRα and TIF1 pSG5-

based expression vectors is also the SV40 early promoter. Thus, TIF1 appears to interfere specifically with ligand-dependent AF-2-activated transcription. This interference has also been observed with a TIF1 mutant lacking the nuclear receptor interacting domain (TIF1Δ539–750; data not shown), which excludes the possibility that TIF1 could act as a ligand-dependent repressor. Therefore, the observed transcriptional interference, which is most easily interpreted as a sequestration by TIF1 of a limiting factor required to transduce AF-2 activity, supports the idea that TIF1 is an intermediary factor mediating AF-2 activity.

We thus investigated whether TIF1 could directly interact with TFIID complexes whose TBP-associated factors (TAFs) have been shown to interact with a number of transactivators (Jacq *et al.*, 1994, and references therein; Tjian and Maniatis, 1994). TBP-containing TFIID complexes were immunopurified from either mouse (P19 cells) or human (HeLa cells) whole cell extracts using an anti-TBP antibody (Brou *et al.*, 1993) and tested by Western blotting for the presence of TIF1. TIF1 could not be detected in TFIID under conditions where the TIF1 antibody mAb 4T readily reacted with P19 whole cell extract endogenous TIF1 (Figure 6C, panel a, lanes 1–3). In contrast, antibodies against $TAF_{II}100$, TBP and $TAF_{II}30$ (Jacq *et al.*, 1994) revealed the corresponding proteins in the immunoprecipitated TFIID complexes (Figure 6C, panels b–d, lanes 1 and 2). Thus, TIF1 does not appear to be tightly associated with TAFs or TBP of TFIID complexes.

A number of activators have been reported to directly interact *in vitro* with basal transcription factors, most notably TBP and TFIIB (Tjian and Maniatis, 1994, and references therein). Moreover, CBP, the putative mediator for the CREB activator, has recently been shown to directly interact with TFIIB (Kwok *et al.*, 1994). We therefore investigated whether His-TIF1 could interact *in vitro* with GST–TBP or –TFIIB fusion proteins (Figure 6A). GST–TBP beads were incubated with either His-TIF1 or His-$TAF_{II}30$ (as a positive control; see Jacq *et al.*, 1994). Western blotting (Figure 6A) revealed that, as expected, His-$TAF_{II}30$ interacted with GST–TBP (lane 17), whereas His-TIF1 was not bound (lane 16). Under similar conditions, GST–TFIIB beads were incubated with either His-TIF1 or TBP (as a positive control; see Ha *et al.*, 1993). His-TIF1 was not retained on GST–TFIIB beads (Figure 6A, lane 15), whereas TBP was bound as expected (data not shown). Thus, it appears that TIF1 does not interact stably *in vitro* with either TBP or TFIIB.

Discussion

TIF1 contains multiple evolutionarily conserved domains present in several families of regulatory proteins

The RING finger domain defines a family which comprises more than 50 viral to human proteins implicated in the control of development, cellular differentiation and/or cell growth and in processes such as transcription, DNA repair and site-specific recombination (reviewed in Freemont, 1993). In fact, TIF1 belongs to a nuclear protein subfamily whose RING finger is followed by one or two B box Cys/His-rich domains (B1 and B2; Reddy *et al.*, 1992; Bellini, 1993; Freemont, 1993) and a coiled coil domain with potential for dimerization (Kastner *et al.*, 1992). The RING finger, the B2 box and the coiled coil domains are present in all members of this subfamily, whereas both B boxes are present in only a subgroup, which to date includes TIF1, PML (Figure 3 and below) and the oestrogen-induced protein efp (Inoue *et al.*, 1993). Although the functions of the RING finger and of the B box fingers are unknown, it is currently thought that they may have a role in nucleic acid–protein and/or protein–protein interactions (Freemont, 1993; Boddy and Freemont, 1994). However, our present data indicate that the RING finger, B box and coiled coil domains are not indispensable for TIF1–RXR interaction.

The bromodomain (Haynes *et al.*, 1992; Tamkun *et al.*, 1992), located in the C-terminal part of TIF1, is conserved from yeast to humans in a number of transcriptional regulatory proteins. This domain has been found in the yeast SWI2/SNF2 protein, which belongs to the activating SWI/SNF multiprotein complex (reviewed in Carlson and Laurent, 1994). *Drosophila* and mammalian homologues of SWI2/SNF2 have been identified and the SWI/SNF complex appears to function as a molecular machine helping transcription factors to bind to chromatin nucleosomal DNA (Côté *et al.*, 1994; Imbalzano *et al.*, 1994; Kwon *et al.*, 1994; Richard-Foy, 1994, and references therein). Interestingly, in transfected cells the two human homologues of SNF2 (hbrm–hSNF2α and BRG1–hSNF2β) stimulate to various degrees transcriptional activation by either the glucocorticoid receptor (GR), RARα or ER (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993; Chiba *et al.*, 1994). Furthermore, the SWI/SNF proteins appear to be essential for GR function in yeast and a SWI3–GR physical interaction has been observed *in vitro* (Yoshinaga *et al.*, 1992). The bromodomain is also found in several proteins which, like TIF1, lack the DNA-dependent ATPase-like domain that is essential for the function of SWI2/SNF2 (Khavari *et al.*, 1993; Laurent *et al.*, 1993; Muchardt and Yaniv, 1993). These proteins include the yeast co-activator GCN5 (Georgakopoulos and Thireos, 1992), the human TFIID subunit $TAF_{II}250$ (identical to the cell cycle regulatory protein CCG1; Hisatake *et al.*, 1993; Ruppert *et al.*, 1993), the CREB binding protein CBP (Chrivia *et al.*, 1993) and the E1A-associated protein p300 (Eckner *et al.*, 1994). CBP and p300 also contain several functionally important regions of near identity, including several Cys/His-rich motifs (Arany *et al.*, 1994, and references therein). Moreover, CBP has recently been identified as a CREB and c-*jun* bridging co-activator/mediator which interacts with the basal transcription factor TFIIB (reviewed in Nordheim, 1994). p300 probably plays a similar role for a number of enhancer-bound transactivators (Eckner *et al.*, 1994). In both cases, Cys/His-rich regions appear to be important for protein–protein interactions and the bromodomain may also act as a surface for protein–protein interactions, although it is not required for CREB–CBP interaction (Eckner *et al.*, 1994; Kwok *et al.*, 1994). Interestingly, it is also dispensable for TIF1–RXR interaction.

In addition to these conserved domains, TIF1 contains several regions rich in either glutamine, proline, serine or acidic amino acid residues, which are also characteristic of a number of transcriptional activators (Tjian and Maniatis, 1994).

TIF1 is a putative mediator of the transcriptional activation function AF-2 of nuclear receptors

The above structural similarities with transcriptional regulatory proteins suggest that the nuclear TIF1 protein may function as a co-activator/intermediary factor mediating the nuclear receptor ligand-dependent activation function AF-2. Although other possibilities cannot be ruled out, our study provides functional evidence supporting this assumption. First, TIF1 was isolated in a yeast genetic screen designed to isolate factors which enhance the activity of RXRγ AF-2. Second, TIF1 functionally interacts in a ligand-dependent manner with the AF-2-containing E regions of several nuclear receptors in yeast. Most importantly, these ligand-dependent interactions are prevented by mutations which specifically affect amino acid residues of the conserved AF-2 AD. Third, TIF1 directly interacts with the liganded LBD of these nuclear receptors *in vitro* and this interaction is similarly prevented by AF-2 AD mutations which impair AF-2 activity. Moreover, the *in vitro* interaction appears to be specifically induced by ligands which activate AF-2, since no interaction with the ER was observed in the presence of the AF-2 antagonist OHT. Fourth, TIF1 and the ER interact in a ligand-dependent manner when co-expressed in transfected animal cells. These tight correlations between stimulation of transcription by AF-2 and interaction of TIF1 with the AF-2-containing region E strongly support a model in which TIF1 interacts with the conserved AF-2 AD, once the structure of the LBD has been modified following binding of the ligand (Durand *et al.*, 1994, and references therein; Keidel *et al.*, 1994; Leid, 1994). Thus, the interference/squelching phenomenon which has been observed between nuclear receptor AF-2s (Meyer *et al.*, 1989; Tasset *et al.*, 1990) and has recently been shown to involve the AF-2 AD (Barettino *et al.*, 1994) may reflect the sequestration of TIF1 and/or of a mediator(s) further 'downstream' present in limiting intracellular concentrations (see below).

Further support for TIF1 playing a key role in mediating ligand-induced AF-2 activity is provided by the experiments in which TIF1 or a TIF1 mutant lacking the nuclear receptor interacting domain was expressed in transfected animal cells together with either RXR, RAR or ER (Figure 8 and our unpublished results). In all three cases, a strong decrease in transactivation by the nuclear receptor was observed, whereas the activity of the SV40 early promoter/enhancer was not affected. These drops in specific transactivation probably reflect the squelching of a limiting factor(s) which interacts 'downstream' of TIF1 to further transduce the activity of liganded AF-2. A cell line lacking TIF1 may be required to see whether over-expressed TIF1 enhances transactivation by co-transfected nuclear receptors, as was observed for over-expressed human SWI2 (hbrm), which also could not stimulate transcription in a SWI2-containing cell line (Muchardt and Yaniv, 1993). In this respect, note that at least two regions of TIF1 stimulated expression of the *URA3* reporter gene in yeast when fused to the ER DBD (see Figure 4D).

In addition to the region which has been shown here to be sufficient for functional interaction with RXRα (residues 539–750), TIF1 contains several conserved regions which may provide surfaces for further protein–protein interactions (see above). However, we did not detect physical interactions between TIF1 and components of the transcription machinery (e.g. TBP, TFIIB or TAFs) which are known to interact with activators or co-activators (reviewed in Tjian and Maniatis, 1994). Such interactions may be too weak to be detected *in vitro* and/or TIF1 may interact with some of the other components which are involved in control of initiation of transcription, e.g. the SWI/SNF complex (see above).

We have recently characterized a new TBP-associated factor ($TAF_{II}30$), which, unlike TIF1, specifically interacts *in vitro* in a ligand-independent manner with the N-terminal part of the AF-2-containing LBD of the ER (Jacq *et al.*, 1994). Thus, at least in the case of the ER, the activation function AF-2 may correspond to two activating domains acting synergistically on different components of the transcription machinery. Moreover, Halachmi *et al.* (1994) and Cavaillès *et al.* (1994) have recently found several as yet uncharacterized proteins exhibiting properties of oestradiol-dependent binding to the ER which are very similar to those reported here for TIF1, in that the integrity of the AF-2 AD was required for interaction and anti-oestrogens did not promote their binding. These similarities raise the interesting possibility that, as for CBP and p300 (see above), TIF1 may belong to a multigene family of co-activators/mediators.

Oncogenicity of proteins containing the RING finger, B box and coiled coil domains

It is remarkable that three out of the eight presently known members of the RING finger–B box–coiled coil (RBCC) protein subfamily (PML, TIF1 and RFP) have been identified in the context of chimeric oncoproteins. In all three cases, only the RBCC domains are retained as the N-terminal moiety of the fusion proteins [PMLRAR, TIF1–B-raf (T18) and RFP–ret respectively, see Figure 3A]. This strongly suggests that these domains contribute to the transforming activity of the oncoproteins. Recent studies on PMLRAR give some clues as to how this could be achieved.

PMLRAR, which is associated with acute promyelocytic leukemia (APL), corresponds to a fusion between the RBCC domains of PML and the B–F regions of RARα, which on its own is not known to be oncogenic (reviewed in Warrell *et al.*, 1993; Figure 3A). PML is a nuclear protein (Kastner *et al.*, 1992) located within a discrete compartment corresponding to matrix-associated nuclear bodies (NB), which are disrupted into multiple smaller nuclear clusters in APL cells and are currently assumed to be the oncogenic target of PMLRAR (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). Since the integrity of the RING finger is required for proper nuclear localization of PML (Kastner *et al.*, 1992), it is likely that the dominant negative effect of PMLRAR on NB assembly reflects the formation of inactive and inappropriately located PML/PMLRAR heterodimers resulting from coiled coil domain interactions. In addition, PMLRAR may have a dominant negative effect on the function of the nuclear receptors which require RXR as an heterodimer partner, due to formation of PMLRAR/RXR heterodimers (Perez *et al.*, 1993; Weis *et al.*, 1994). Thus, the oncogenicity of PMLRAR appears to involve the RBCC domains and may result from an abnormal subnuclear organization diverting subsets of proteins from their natural function.

In contrast to the RAR component of PMLRAR, the N-terminally truncated B-raf component of TIF1−B-raf (T18) encodes a serine/threonine protein kinase domain which is likely to be oncogenic on its own, due to the removal of N-terminal negative autoregulatory domains (Miki *et al.*, 1991; Magnuson *et al.*, 1994, and references therein). By analogy with PMLRAR, the RBCC domains of TIF1 may potentiate the oncogenicity of N-terminally truncated B-raf through two mechanisms which are not mutually exclusive. First, the formation of TIF1/TIF1−B-raf heterodimers involving the coiled coil domain may generate dominant negative effects through the sequestration of TIF1 and/or TIF1-associated proteins in an inappropriate location. Second, when associated with the TIF1 RBCC domains, N-terminally truncated B-raf may acquire a different intracellular localization, thereby increasing its oncogenic potential. Note in this respect that TIF1 is a nuclear protein and that the localization of TIF1−B-raf is presently unknown. Interestingly, TIF1, which is not firmly attached to the nuclear matrix, displays a subnuclear distribution different from that of PML (our unpublished observations), even though the RBCC domains of the two proteins are closely related. Finally, similar mechanisms may also be involved with RFP−ret, in which the RBCC domains of the nuclear matrix protein RFP are fused to a tyrosine kinase domain of the ret protein (Takahashi *et al.*, 1988; Isomura *et al.*, 1992). In conclusion, it is tempting to speculate that the oncogenic potential of the RBCC domains reflects their natural function(s), which might include the targetting of proteins to different subnuclear compartments. For PMLRAR these RBCC domains are indispensable for the generation of an oncogenic protein, whereas for TIF1−B-raf and RFP−ret they would potentiate the oncogenicity of N-terminally truncated B-raf and ret proteins.

Materials and methods

All recombinant DNA work was performed according to standard procedures (Ausubel *et al.*, 1992); details concerning the plasmid constructions, which were all verified by sequencing (see figures), are available upon request.

Plasmids

Receptor cDNAs used in this study correspond to the mouse RXRs (α and γ) and RARα1 and human ER, VDR and PR, unless indicated otherwise. RXRγ(DE)−ER.CAS and RXRα were expressed from the yeast HIS3 multicopy vector YEp90 described by Pierrat *et al.* (1992). DBD and AAD fusion proteins were expressed from the yeast multicopy plasmids pBL1 and pASV3 respectively, as will be described in detail elsewhere (Le Douarin *et al.*, 1995). Briefly, pBL1 contains the *HIS3* marker and directs the synthesis of epitope (F region of human ER)-tagged ER DBD fusion proteins. pASV3 contains the *LEU2* marker and a cassette expressing a nuclear VP16 acidic activating domain (AAD), preceding a polylinker with cloning sites for the cDNA and stop codons in all reading frames. All inserts cloned into pBL1 and pASV3 were obtained by PCR. For transfection studies in mammalian cells, RXRα cDNA and the mutant derivatives shown in Figure 5 were cloned into pSG5 (Green *et al.*, 1988). DR1−tk/CAT has been described (Mader *et al.*, 1993). For *in vitro* binding assays, the indicated cDNAs were fused to GST in the pGEX-2T plasmid (Jacq *et al.*, 1994). Protein overexpression (in XL1-Blue) and preparation of extracts were as described by Zechel *et al.* (1994). His-$TAF_{II}30$ was a gift from X.Jacq. The TIF1 coding region was cloned into the pAcSG HIS NT-A (Pharmingen)-based transfer vector and His-TIF1 baculovirus was obtained by complementation of defective virus. Infection, expression and purification by Ni^{2+} chelating chromatography have been described (Chen *et al.*, 1994).

cDNA library screening

Construction of the P19 embryonal carcinoma cell cDNA library will be described in detail elsewhere. In brief, cDNAs expressed from the multicopy *LEU2* plasmid pAS5 were introduced by electroporation into the *Saccharomyces cerevisiae* PL1 reporter strain (MATα *ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::1ERE-URA3*) expressing RXRγ(DE)−ER.CAS from the His3 YEp90 vector. Yeast transformants (10^6) were selected on 40 15 cm his^-leu^- plates and replated at a multiplicity of eight onto $ura^-his^-leu^-$ plates containing 10 nM 9C-RA. After 6 days, 112 clones were isolated. Library plasmids were rescued in *E.coli* EC350 (*leuB*$^-$), re-introduced into PL1 containing either YEp90-RXRγ(DE)-ER.CAS or YEp90 and the phenotypes were re-tested.

To isolate clones containing the 5′-end of the TIF1 cDNA, a randomly primed mouse F9 embryonal carcinoma cell cDNA library constructed in λZAPII was screened with a 0.5 kb *Sfi*I−*Hin*dIII fragment corresponding to the 5′-end of the original clone, TIF1.22. The 5′-end of the longest isolate (nucleotides 739–2201; see Figure 2A) was used to re-screen the F9 cDNA library, which resulted in the isolation of λ27 (nucleotides 97–1775), among others. P19 poly(A)$^+$ RNA was reverse transcribed with a λ27-specific primer (nucleotides 129–154) and RACE PCR was performed as described (Frohman, 1991). The +1 position of the reconstituted TIF1 sequence shown in Figure 2A corresponds to the 5′-end of the longest RACE product (nucleotides 1–152).

Transactivation assays

Yeast transformants were grown exponentially for about five generations in selective medium containing uracil in the presence or absence of ligand. Yeast extracts were prepared and assayed for OMPdecase activity as described (Pierrat *et al.*, 1992). Transient transfections of Cos-1 cells and CAT and β-galactosidase assays were performed as described (Bocquel *et al.*, 1989).

Antibodies and immunocytofluorescence

Monoclonal (mAbs) anti-TIF1 antibodies were raised against synthetic peptides ('3T', amino acids 487–503, and '6T', amino acids 33–52) coupled to ovalbumin and the purified proteins TIF1.22.2 ('4T', amino acids 682–1017) and TIF1.22.1 ('5T', amino acids 396–682). mAB 2GV-4 is directed against VP16 (White *et al.*, 1992). mAbs B10 and F3 are directed against the B and F regions of human ER respectively (Ali *et al.*, 1993); 9α and 4RX are mAbs directed against the F region of RARα (Gaub *et al.*, 1992) and the DE regions of RXRα (Rochette-Egly *et al.*, 1994) respectively. Immunocytofluorescence experiments were performed as described (Kastner *et al.*, 1992).

In vitro binding assays

Glutathione−Sepharose was equilibrated with binding buffer (BB; 50 mM Tris−HCl, pH 8.0, 100 mM NaCl, 0.3 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 0.1 % NP40), loaded with GST fusion proteins and washed as described by Jacq *et al.* (1994). A single binding reaction comprised 8 μg fusion protein [14 μg in the case of GST−RXRα(DE) and the mutants derived therefrom], 2 μg His-TIF1 (or 1 μg His-$TAF_{II}30$) plus ligand or carrier in a final volume of 100 μl. Incubation was for 2 h at 4°C with gentle agitation. Variation of the NaCl concentration in BB revealed that the TIF1−RXR interaction is stable between 40 and 500 mM.

Immunoprecipitation of the TBP-containing TFIID complexes and immunoblotting with antibodies against TIF1, $TAF_{II}100$, hTBP or $TAF_{II}30$ were performed as described by Jacq *et al.* (1994).

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