The EMBO Journal vol.15 no.14 pp.3667–3675, 1996

TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors

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Nuclear receptors (NRs) act as ligand-inducible transcription factors which regulate the expression of target genes upon binding to cognate response elements. The ligand-dependent activity of the NR activation function AF-2 is believed to be mediated to the transcription machinery through transcriptional mediators/intermediary factors (TIFs). We report here the cloning of the 160 kDa human nuclear protein TIF2, which exhibits all properties expected for a mediator of AF-2: (i) it interacts *in vivo* with NRs in an agonist-dependent manner; (ii) it binds directly to the ligand-binding domains (LBDs) of NRs in an agonist- and AF-2-integrity-dependent manner *in vitro*; (iii) it harbours an autonomous transcriptional activation function; (iv) it relieves nuclear receptor autosquelching; and (v) it enhances the activity of some nuclear receptor AF-2s when overexpressed in mammalian cells. TIF2 exhibits partial sequence homology with the recently isolated steroid receptor coactivator SRC-1, indicating the existence of a novel gene family of nuclear receptor transcriptional mediators.

Keywords: ligand binding domain/retinoid receptors/steroid receptors/transcriptional coactivator/transcriptional squelching

Introduction

Nuclear receptors (NRs) represent a large family of ligand-inducible transcription regulators which trigger complex events during development, growth and homeostasis by controlling gene expression upon binding of small hydrophobic ligands, such as steroid and thyroid hormones, vitamin D and retinoids. All NRs display a modular structure, with five to six distinct regions (termed A–F) exhibiting different degrees of evolutionary conservation. The N-terminal A/B region contains an autonomous activation function (AF-1), the highly conserved region C encompasses the DNA-binding domain, and region E contains the ligand-binding domain (LBD), a dimerization surface and the ligand-dependent transcriptional activation function AF-2 (Beato *et al.*, 1995; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995; reviewed in Mangelsdorf *et al.*, 1995; Thummel, 1995; Gronemeyer and Laudet, 1995).

Transcriptional interference/squelching, observed between the AFs of steroid receptors, provided initial evidence for the existence of transcriptional mediators or intermediary factors (TIFs)/coactivators which mediate AF activity to the transcription machinery (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tasset *et al.*, 1990). This concept was further supported by the observation that both AF-1 and AF-2 of the oestrogen receptor (ER) (Tora *et al.*, 1989), as well as those of the retinoic acid (RA) receptors (RARs and RXRs) (Nagpal *et al.*, 1992, 1993) activate transcription in a promoter- and cell-specific fashion. Several putative TIFs for the NR AF-2 have been characterized, including a 160 kDa protein (Cavaillès *et al.*, 1994; Halachmi *et al.*, 1994; Kurokawa *et al.*, 1995), and some have been cloned (Jacq *et al.*, 1994; Cavaillès *et al.*, 1995; Le Douarin *et al.*, 1995; Lee *et al.*, 1995a,b; Onate *et al.*, 1995; vom Baur *et al.*, 1996). However, with the exception of the recently isolated steroid receptor coactivator SRC-1 (Onate *et al.*, 1995), none of these putative mediators has been shown unequivocally to stimulate AF-2 activity.

We report here the cloning of the 160 kDa nuclear protein TIF2, which exhibits all properties expected for a TIF/mediator of AF-2: it interacts directly with the LBDs of several NRs in an agonist- and AF-2-integrity-dependent manner *in vitro* and *in vivo*, harbours an autonomous activation function, relieves NR autosquelching and enhances the activity of NR AF-2s when overexpressed in mammalian cells. Furthermore, sequence similarities between TIF2 and SRC-1 indicate the existence of a novel gene family of NR transcriptional mediators.

Results

Identification and cDNA cloning of a 160 kDa nuclear receptor-interacting protein

In agreement with previous reports (Cavaillès *et al.*, 1994; Halachmi *et al.*, 1994; Kurokawa *et al.*, 1995), we observed agonist-dependent binding *in vitro* of a 160 kDa protein from ^{35}S-labelled whole-cell extracts (HeLa, Cos-1, P19.6, MCF-7) to the glutathione *S*-transferase (GST)-tagged LBDs of retinoic acid (RAR) and oestrogen (ER) receptors (Figure 1a, and data not shown). Note that the proteins with masses of 50–55 kDa interacting with the NR affinity matrices bound equally well to the GST matrix (see Figure 1a, lane 1), and therefore do not interact with the nuclear receptor LBDs. The same protein species were detected by Far-Western blotting with agonist-bound ^{32}P-labelled RAR and ER LBD probes (data not shown). To isolate the coding sequence of the 160 kDa NR-interacting protein, a randomly primed human placenta cDNA expression library was screened with the oestradiol (E2)-bound ^{32}P-labelled ER LBD, since this probe produced a better signal-to-noise ratio than the RAR LBD probe. Only one of five distinct cDNA clones identified by screening 340 000 clones encoded a protein fragment (termed

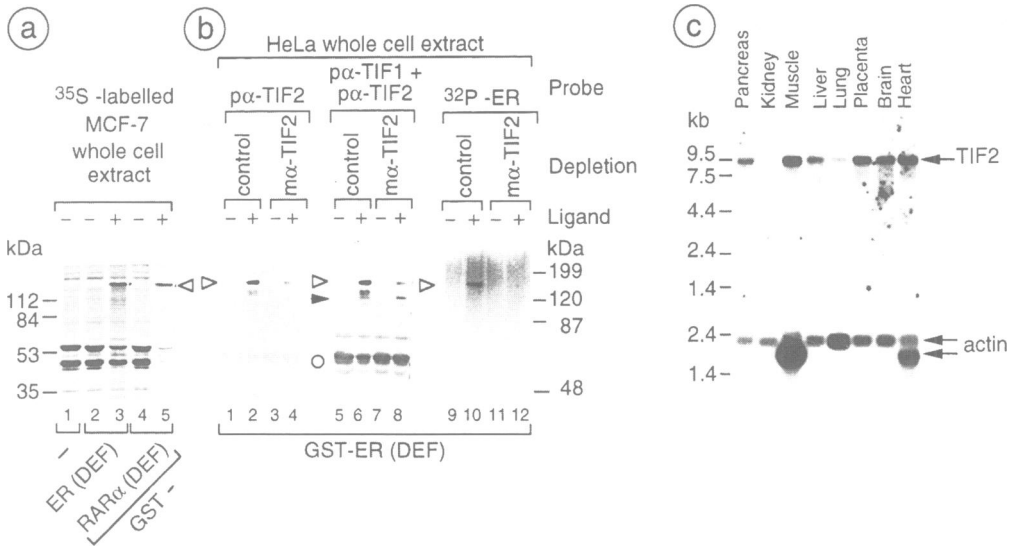


Fig. 1. TIF2 is the 160 kDa nuclear receptor-interacting factor. (**a**) GST pull-down experiments identify a 160 kDa protein that interacts with liganded ER and RARα LBDs [ER(DEF) and RARα(DEF), respectively]. Note that less material was loaded in lane 5 than in lanes 1–4, resulting in a less prominent appearance of the GST-binding 50–55 kDa proteins in lane 5. (**b**) Immunodepletion followed by Far-Western detection demonstrates the identity of TIF2 with the biochemically identified 160 kDa protein. Open triangle, TIF2; arrowhead, TIF1; circle, antibody cross-reaction to GST–ER(DEF). The pα-TIF2-immunodetected species smaller than TIF2 (lanes 2 and 6) most probably is a degradation product of TIF2, as it is removed by immunodepletion with mα-TIF2 (lanes 4 and 8). Note: Far-Western detection with ^{32}P-ER is less sensitive than TIF1/TIF2 immunodetection on Western blots. The residual TIF2 (lane 12) and low TIF1 (lanes 10 and 12) amounts retained on the affinity column are therefore not sufficient for Far-Western detection. (**c**) Northern blotting reveals an ~9 kb TIF2 transcript in various human tissues.

TIF2.1) that interacted on Far-Western blots with three different ^{32}P-labelled NR LBD probes (ER, RAR and RXR) in an agonist-dependent manner (data not shown), and could therefore correspond to the 160 kDa protein. The full-length TIF2 coding sequence (Figure 2a), preceded by in-frame stop codons 5′ of the initiator AUG, was obtained upon rescreening the same library with a ^{32}P-labelled TIF2.1 cDNA probe.

Human TIF2 cDNA encodes a protein with a predicted molecular mass of 159 160 Da (1464 amino acids), which includes N-terminal putative nuclear localization signals (NLSs), one Gln- and three Ser/Thr-rich regions, and two charged clusters (Figure 2). In one of the isolated cDNA clones, most probably resulting from alternative splicing, the coding region for amino acids Thr869–Thr938 was missing and amino acid Gly939 was changed to Arg (see Figure 2). Some regions of TIF2 exhibit sequence similarities with the recently described steroid receptor coactivator SRC-1 (Onate *et al.*, 1995) (Figure 2), indicating the existence of a novel gene family of mediators for AF-2 of NRs (see below).

The TIF2 protein appears to be widely expressed, since the corresponding transcript was found in several human tissues (pancreas, skeletal muscle, liver, lung, placenta, brain, heart), albeit at a much lower level in kidney (Figure 1c, and data not shown). In keeping with the presence of putative NLS(s) in the TIF2 coding sequence, and as expected for a protein interacting with NRs, transiently expressed full-length TIF2 was nuclear (Figure 3a). It was mainly associated with discrete bodies, but was also present throughout the nucleus with the exclusion of nucleoli.

Immunodepletion studies strongly support that TIF2 is the 160 kDa protein species that interacts in an agonist-dependent manner with NR LBDs (see above and Cavaillès *et al.*, 1994; Halachmi *et al.*, 1994; Kurokawa *et al.*, 1995). Western blotting with a rabbit antiserum (pα-TIF2), raised against bacterially expressed TIF2.1, revealed predominantly a 160 kDa HeLa cell protein that interacted with agonist-bound GST–ER(DEF) (Figure 1b, lanes 1 and 2; see also legend to Figure 1b). Immunodepletion with a mouse monoclonal TIF2 antibody (mα-TIF2) before affinity purification resulted in a specific decrease of TIF2, but not TIF1 (Le Douarin *et al.*, 1995), retained on E2-bound GST–ER(DEF) (Figure 1b, compare lanes 2 with 4 and 6 with 8). Importantly, a subsequent Far-Western analysis with an E2-bound ^{32}P-GST–ER(DEF) probe revealed the 160 kDa species only in control, but not in TIF2-immunodepleted extracts (Figure 1b, compare lanes 10 and 12).

Agonist-dependent interaction between TIF2 and nuclear receptors in vivo

Since the overexpressed TIF2.1 fragment was, in contrast to the nuclear full-length TIF2 protein, essentially cytoplasmic (supporting the above assignment of a N-terminal TIF2 NLS), the interaction of TIF2.1 with NRs could be studied in mammalian cells using nuclear co-translocation assays. In the absence of ligand, TIF2.1 (green fluorescence) remained cytoplasmic and NRs (red fluorescence) were nuclear [for RARα, ER and progesterone receptor (PR), see Figure 3b, d and g]. Addition of an agonist, however, resulted in all three cases in nuclear co-localization of TIF2.1 and NR (yellow staining), indicating NR–TIF2 interaction *in vivo* (Figure 3c, e and h). Agonist-dependent interaction of TIF2.1 with NRs was observed for all other nuclear receptors analysed [retinoid X (RXR), thyroid (TR), vitamin D3 (VDR), glucocorticoid (GR) and androgen (AR) receptors; data not shown]. Interestingly, no interaction was detected between ER and TIF2.1 in presence of the ER AF-2 antagonist hydroxytamoxifen (OHT) (Figure 3f), and the PR AF-2 antagonist RU486 reversed the R5020-induced PR-TIF2.1 interaction (Figure 3i).

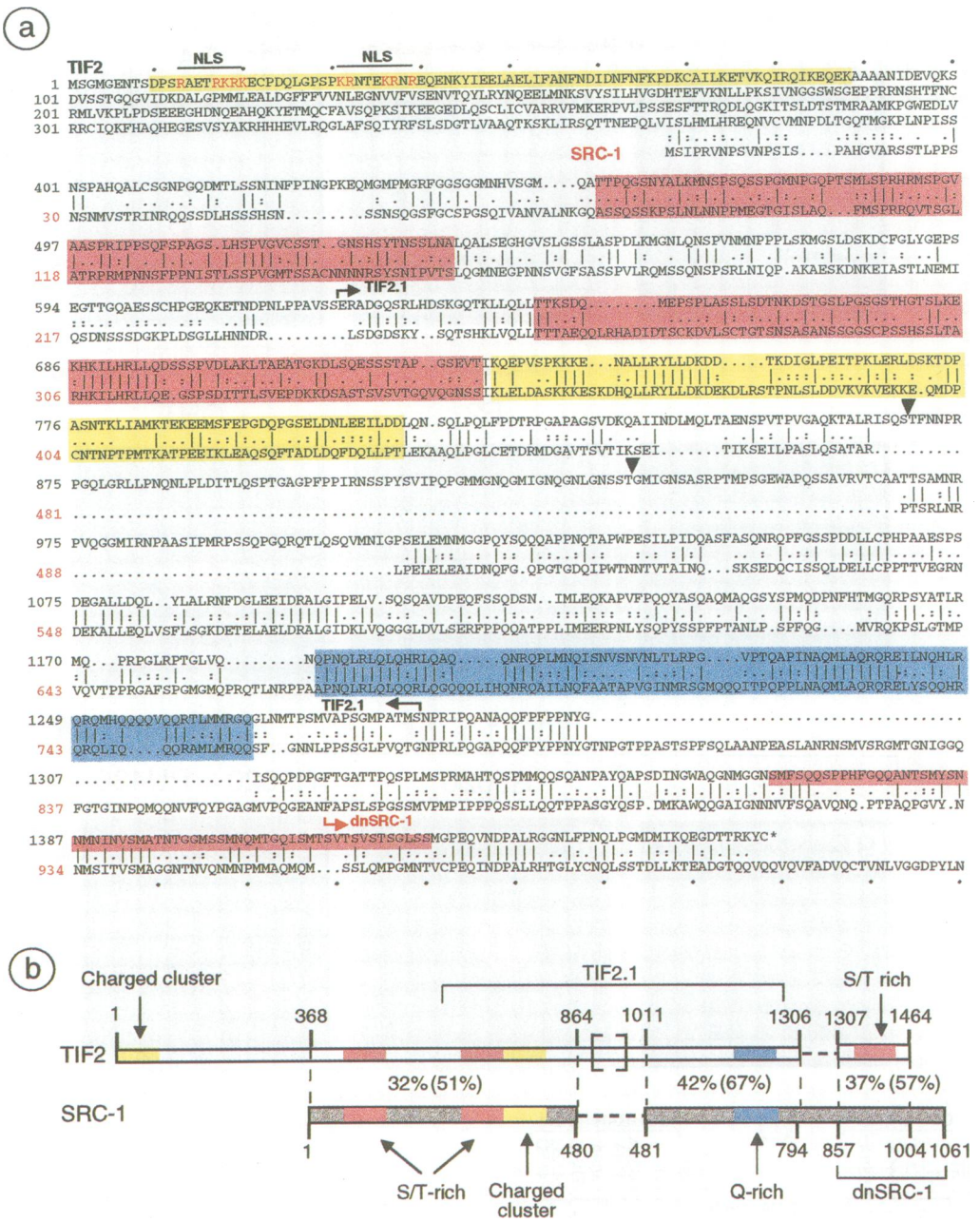
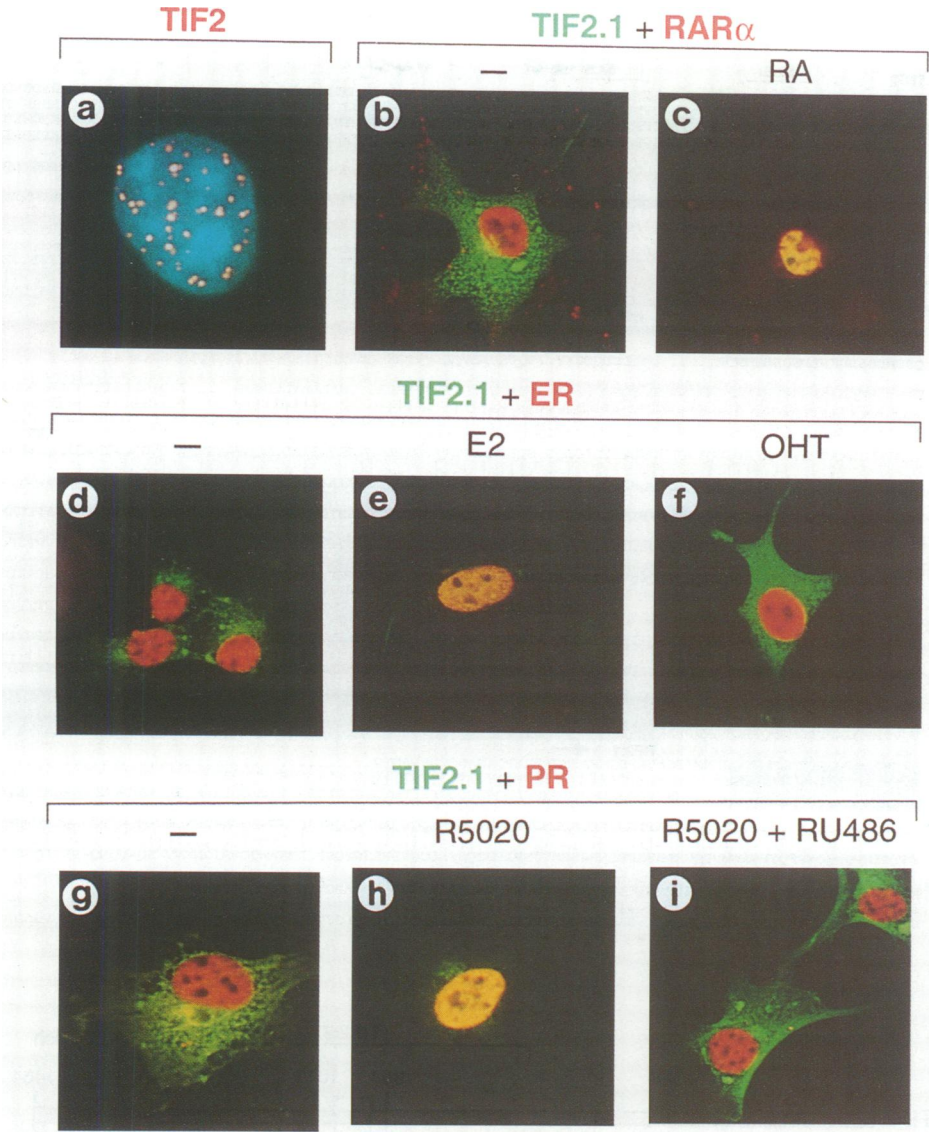


Fig. 2. Sequence of TIF2: homology with SRC-1 indicates the existence of a novel family of NR mediators. (**a**) Alignment and amino acid sequences of TIF2 and the steroid receptor coactivator SRC-1. Two charged clusters (yellow) rich in acidic and basic amino acids, three S/T-rich (red) and one Q-rich region (blue) are highlighted. The N-terminal charged cluster harbours putative bipartite NLSs (red lettering, overlined). The regions encoding TIF2.1 (amino acids 624–1287; functional coactivator fragment) and dnSRC-1 (amino acids 865–1061; dominant-negative fragment) are indicated. Two closed triangles indicate the endpoints of the region missing in one of the isolated cDNA clones, generated most probably by alternative splicing of the TIF2 mRNA. An asterisk identifies the TIF2 stop codon. (**b**) Schematic comparison of TIF2 and SRC-1. Percent identities (similarities in parentheses) of homologous regions are indicated. The N-terminal charged cluster harbouring the putative NLS and the C-terminal S/T-rich region of TIF2 are not, or only weakly, conserved in SRC-1. The coding region missing in one cDNA clone, most probably generated by alternative splicing, is indicated with brackets.

Agonist- and AF-2-integrity-dependent interaction between TIF2 and the LBD of nuclear receptors in vitro

In agreement with the Far-Western blot experiments NRs and TIF2 interacted directly, as purified TIF2.1 protein bound *in vitro* in the presence of an agonist to GST–ER(DEF), GST–RARα(DEF), GST–RXRα(DE) and GST–TR(DE) (Figure 3k–m, lanes 3 and 4; Figure 3n, lanes 5 and 6). As expected, TIF2 binding to GST–RXRα(DE) occurred with 9*cis*-RA (9C-RA) but not all-*trans*-RA (T-RA) (Figure 3n, lanes 1 and 2), and OHT prevented E2-dependent binding of TIF2 to GST–ER(DEF) (Figure 3n, lanes 7–9). The integrity of the conserved core of the ER, RARα and RXRα AF-2



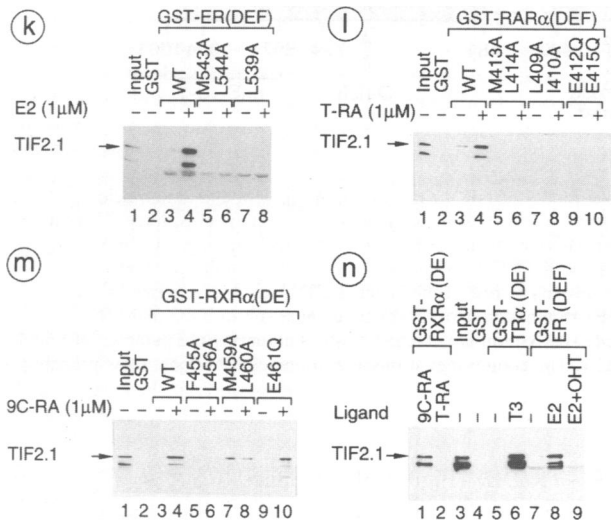


Fig. 3. *In vivo* and *in vitro* interactions of TIF2 with nuclear receptors. (**a**) Overexpressed TIF2 protein is mostly localized in discrete nuclear bodies and excluded from nucleoli. Superposed images of Hoechst DNA staining (blue) and TIF2 immunostaining (red) are shown. (**b–i**) Cytoplasmic TIF2.1 interacts in an agonist-dependent manner with nuclear receptors in mammalian cells. Yellow staining (resulting from superposition of green TIF2.1 and red NR fluorescence) indicates TIF2.1-NR colocalization. (**k–n**) TIF2.1 interacts directly *in vitro* in an agonist-dependent manner with nuclear receptors, and point mutations within the AF-2 AD core abolish this interaction. WT, wild-type. Ligand concentrations for *n*: 9C-RA, T-RA and T3, 10^{-6} M; E2, 5×10^{-8} M; OHT, 5×10^{-6} M. The smaller immunodetected polypeptide is a degradation product of TIF2.1. Note that the anti-TIF2 serum cross-reacts weakly with GST–hER(DEF).

activating domains (AF-2 AD), which was shown to be critical for AF-2 activity (Zenke *et al.*, 1990; Danielian *et al.*, 1992; Barettino *et al.*, 1994; Durand *et al.*, 1994; Le Douarin *et al.*, 1995; Leng *et al.*, 1995; vom Baur *et al.*, 1996), was required for TIF2 interaction *in vitro*. Most AF-2 AD core mutants that have lost AF-2 activity

(ER, Figure 3k, lanes 5–8; RARα, Figure 3l, lanes 5–10; RXRα, Figure 3m, lanes 5 and 6) associated only very weakly, if at all, with TIF2, whereas the GST–LBD fusion of the RXRα mutant E461Q, whose AF-2 is only partially impaired (Le Douarin *et al.*, 1995), still exhibited a significant RA-dependent interaction with TIF2.1 *in vitro* (Figure 3m, lanes 9 and 10). Note that we have no satisfactory explanation for the weak ligand-independent interaction of TIF2 with GST–RXR(M459A/L460A) (Figure 3m, lanes 7 and 8). No significant *in vitro* interaction of TIF2.1 was observed with GST–VP16 (acidic activating domain), GST–fusions of the basal transcription factors TBP and TFIIB, or a number of GST–TAFs ($hTAF_{II}18$, $hTAF_{II}20$, $hTAF_{II}28$ and $hTAF_{II}55$; see Jacq *et al.*, 1994; Mengus *et al.*, 1995; data not shown). Taken all together, the results of these *in vivo* and *in vitro* interaction studies indicated a possible role of TIF2 as a mediator for the ligand-dependent AF-2 located in the LBD of NRs.

TIF2 contains an autonomous activation function, relieves squelching and stimulates nuclear receptor AF-2 activity in an agonist-, promoter- and cell-dependent manner

Conceptually, a TIF capable of mediating the transcriptional activity of a cognate AF to the transcription machinery, could itself be an activator when fused to a heterologous DNA-binding domain. Interestingly, in transiently transfected HeLa cells, TIF2.1 fused to the GAL4(1-147) DNA-binding domain strongly transactivated a GAL4 reporter (Figure 4a). Thus, TIF2 may correspond to one of the hypothetical limiting factor(s) previously proposed to be involved in NR transcriptional interference/squelching (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tasset *et al.*, 1990). Supporting this possibility, 'anti-squelching' experiments showed that the expression of TIF2.1 in ER-transfected cells could, at least partially, reverse the transcriptional autointerference generated by expressing increased amounts of ER (Bocquel *et al.*, 1989) (Figure 4b; note the marked shift of the bell-shaped activation curve to higher ER concentrations in the presence of TIF2.1). At high ER expression levels, the TIF2.1-stimulated transactivation decreased, possibly due to the squelching of other putative mediators (Jacq *et al.*, 1994; Cavaillès *et al.*, 1995; Le Douarin *et al.*, 1995; Lee *et al.*, 1995a,b; Onate *et al.*, 1995; vom Baur *et al.*, 1996) and/or transcriptional factors.

As expected, coexpression of TIF2.1 with antagonist-bound NR did not lead to any stimulation of the transactivation brought about by AF-1 in the presence of pure AF-2 antagonists (Berry *et al.*, 1990; Meyer *et al.*, 1990), further supporting that TIF2 is AF-2-specific (Figure 4b and e for ER, OHT; Figure 4d for PR, RU486). TIF2 expression also increased AF-2/agonist-mediated transactivation by the androgen (AR) and progesterone (PR) receptors, but not transactivation by GAL–VP16 and GAL–AP2 (Seipel *et al.*, 1992) (Figure 4c). Under similar conditions transactivations by GAL–RAR, GAL–RXR, GAL–VDR, GAL–TR and GAL–GR were unaffected by TIF2 (Figure 4c, and data not shown), suggesting that for these NRs either TIF2 is not critically involved in mediating their AF-2 activities or endogenous TIF2 amounts are sufficient to optimally support transactivation, for instance because TIF2 has a higher affinity for these receptors.

TIF2 stimulation was to some extent affected by the promoter environment of the responsive gene, as the TIF2 effect on PR/R5020-induced transactivation was greater for a complex (MMTV) than for a minimal (GRE–TATA) promoter (Figure 4d), although the latter was also reproducibly stimulated. This suggests that TIF2 may exert a stimulatory effect on its own, in the absence of other transactivators bound to the same promoter region.

As expected from the distinct levels of TIF2 transcripts in different tissues (see Figure 1c), the effect of TIF2 was cell type-dependent, since TIF2 had a much stronger effect on PR- and ER-induced transactivations in Cos-1 than in HeLa cells (Figure 4d and e). Note that the weak, seemingly ligand-independent, TIF2-induction of ER (Figure 4e, compare lanes 1 with 2 and 7 with 8) is due to residual oestradiol in the culture medium and was abolished by the addition of the ER antagonist OHT (Figure 4e, lanes 6 and 12).

Under similar conditions no significant influence of TIF2 on transcriptional activation by RXR homo- and heterodimers (with RAR, TR and VDR) could be detected in HeLa or Cos-1 cells. Whether this could be due to higher affinities of TIF2 for these receptors *in vivo* (see above), or whether TIF2, despite its agonist-dependent interaction with these NRs, is not critical for their transactivation activities, remains to be established.

Discussion

Differential interactions of putative TIFs with the LBD/AF-2 of liganded nuclear receptors in vitro

Several putative mediators for the ligand-dependent activation function AF-2 of nuclear receptors have been cloned and their *in vitro* interactions with NR LBDs have been analysed (Cavaillès *et al.*, 1995; Le Douarin *et al.*, 1995; Lee *et al.*, 1995a,b; Onate *et al.*, 1995; vom Baur *et al.*, 1996). Common to many of these proteins are agonist-dependent or agonist-enhanced interactions with several wild-type NR LBDs. However, several of these factors show markedly different affinities for various NR LBDs. TIF1, for example, interacts strongly with the LBDs of ER, RAR and RXR, whereas only a very weak interaction is detected with the TR LBD (vom Baur *et al.*, 1996). SUG1 on the other hand, interacts well *in vitro* with ER, RAR and TR, but no significant interaction is observed with RXR (vom Baur *et al.*, 1996). Furthermore, *in vitro* interactions of SUG1 are not strictly ligand-dependent but rather ligand-enhanced. The behaviour of TIF2 is novel, as it interacts in a strictly ligand-dependent manner with all four of these LBDs (ER, RAR, RXR and TR). For all three putative mediators, SUG1, TIF1 and RIP140 the observed *in vitro* interactions are dependent on the integrity of the AF-2 AD core (Cavaillès *et al.*, 1995; Le Douarin *et al.*, 1995; vom Baur *et al.*, 1996). Mutations in this motif which lead to transcriptionally inactive NRs, also abolish NR–TIF interaction. With one exception, the RXRα mutant M459A/L460A, we observe similar results with TIF2. This mutant shows a weak ligand-independent interaction with TIF2.1 *in vitro* whereas it is transcriptionally inactive (Figure 3m, lanes 7 and 8). We have no satisfactory explanation for this result, but it is possible

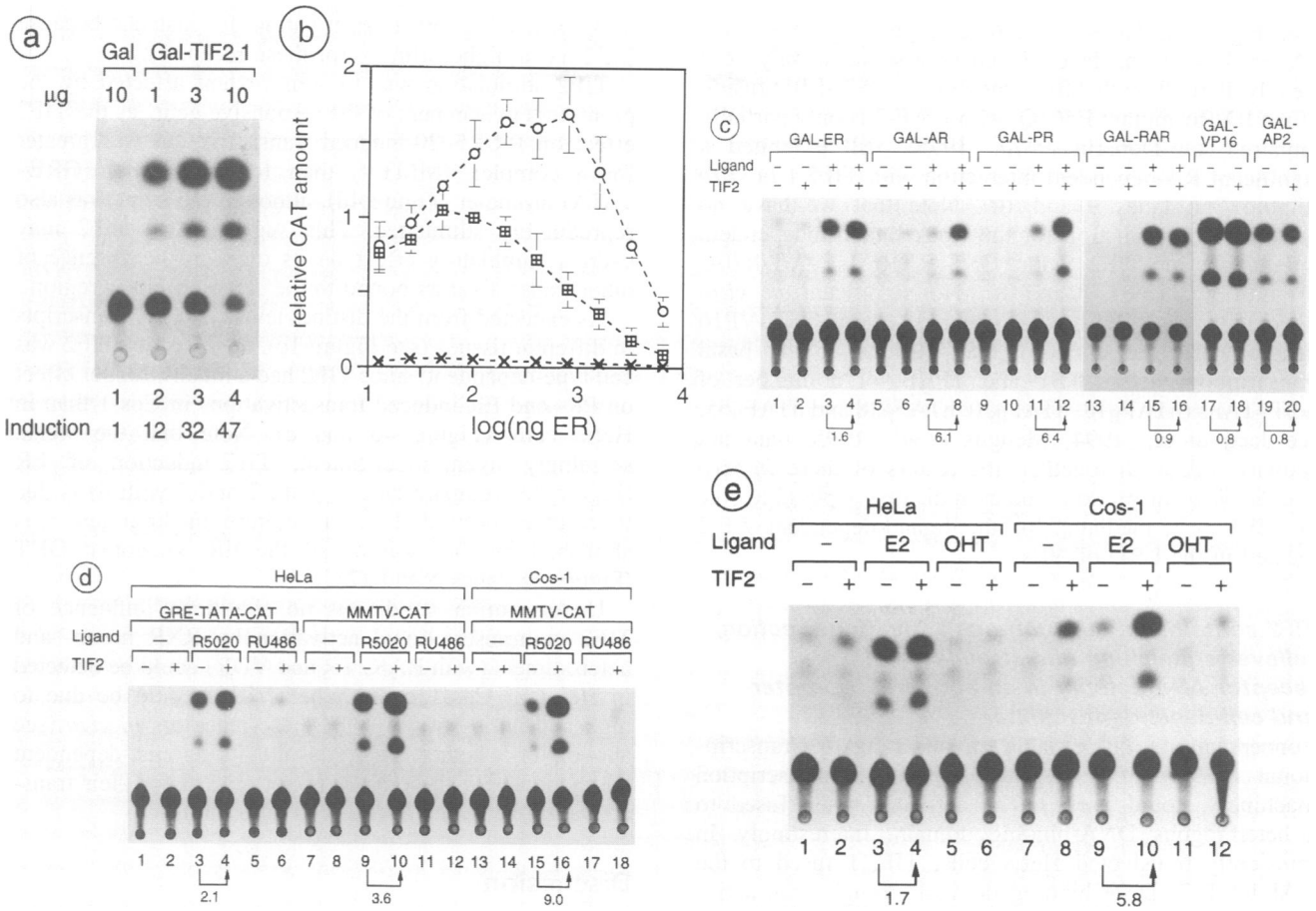


Fig. 4. TIF2 contains an autonomous AF, relieves squelching and stimulates NR-AF2 activity in an agonist-, promoter- and cell-dependent manner. (**a**) Increasing amounts of GAL–TIF2.1 fusion protein (lanes 2–4) activate transcription of a cognate reporter in transfected cells. HeLa cells were co-transfected with 1 µg $(17m)_5$-βG-CAT and 10 µg GAL4(1-147) or 1, 3 and 10 µg GAL4(1-147)-TIF2.1, respectively. Fold-induction is given below the CAT assays. (**b**) TIF2.1 partially reverses transcriptional autointerference of ER. HeLa cells were co-transfected with 5 µg Vit-tk-CAT and the indicated amount of HEG0, with or without 5 µg TIF2.1 in the presence of 10^{-6} M E2 or OHT. CAT activity is given relative to that induced by 100 ng HEG0 in presence of E2. Normalized CAT expression (mean ± SE of four independent experiments) is shown. Open circles, +E2,+TIF2; squares, +E2,–TIF2; crosses, +OHT,+TIF2. (**c**) TIF2 enhances transactivation mediated by some NR AF-2s but not that mediated by other transcription factors. HeLa cells were co-transfected with 1 µg 17m-tk-CAT and 1 µg of the indicated GAL–fusion vectors with or without the addition of 3 µg TIF2 expression vector in the presence or absence of 10^{-6} M ligand. Mean TIF2 stimulations of three independent experiments are given (variation ≤13%). Ligands: lanes 3–4, E2; lanes 7–8, DHT (dihydrotestosterone); lanes 11–12, R5020; lanes 15–16, T-RA. (**d**) TIF2 enhances PR-mediated transcriptional activation from both a minimal (GRE–TATA) and a complex (MMTV) promoter; this stimulation is significantly greater in Cos-1 than in HeLa cells. HeLa (lanes 1–12) or Cos-1 (lanes 13–18) cells were transfected with 5 µg GRE–TATA–CAT (lanes 1–6) or 1 µg MMTV–CAT (lanes 7–18) together with 1 µg hPR with or without 3 µg TIF2 in the presence or absence of 10^{-6} M of the indicated ligand. (**e**) TIF2 greatly enhances agonist-induced activation by ER in Cos-1 and more weakly in HeLa cells. HeLa or Cos-1 cells were co-transfected with 1 µg Vit-tk-CAT and 1 µg HEG0 with or without 3 µg TIF2 in the presence or absence of 10^{-6} M of the indicated ligands. In (d) and (e), TIF2 inductions of three or more experiments are shown (variation ≤10%).

that this mutation creates an artificial surface for TIF2 interaction which does not exist in the wild-type receptor. Interestingly, the RXRα mutant E461Q, whose AF-2 is partially active (Le Douarin *et al.*, 1995), still exhibits a RA-dependent interaction with TIF2.1 *in vitro* (Figure 3m, lanes 9 and 10). For SRC-1 only the interaction with PR has been investigated and a comparison with TIF2 is therefore not possible. Note in this context, that the functional mediator fragment TIF2.1 (see Figure 4a and b) and the dominant negative mediator fragment dnSRC-1 (Onate *et al.*, 1995) do not overlap (see Figure 2b), indicating that SRC-1 may possibly contain a NR-interacting region distinct from that of TIF2. Future studies will show whether TIF2 and/or SRC-1 contain a second NR-interacting domain and whether the C-terminal region of TIF2 may act as a dominant negative repressor of transactivation.

Among the putative mediators, only TIF2 and SRC-1 efficiently stimulate nuclear receptor AF2 activity in vivo

The steroid receptor coactivator SRC-1 was the first NR mediator which was reported to efficiently stimulate AF-2 activity (Onate *et al.*, 1995). The present report demonstrates similar effects for the SRC-1-related TIF2. However, whereas SRC-1 stimulates the AF-2 activity of ER, GR, PR, RXR and TR, TIF2 enhances only transactivation by AR, ER and PR, but not of GR, RXR, RAR, TR or VDR under the experimental conditions used. Further studies are necessary to investigate why certain NRs

interact with a given TIF without exhibiting enhanced AF-2 activity when exogenous TIF is coexpressed. Conceptually several possibilities exist: (i) NRs may have distinct TIF affinities and even low endogenous levels of a given TIF may suffice for maximal AF-2 activity; (ii) as it is the case for TIF2, the TIF levels may vary in a cell-specific manner, so that in certain cells the overexpression of TIFs may not or may less efficiently stimulate AF-2 activity; and (iii) a given TIF may be critical for AF2 activity of certain NRs, while for other NRs two or more TIFs may have to synergize (possibly with other promoter-bound, transcription factor-associated proteins) to exert a measurable effect.

We show here that TIF2 contains an autonomous activation function (see Figure 4a). It will be interesting to see also whether SRC-1 contains such an independent activation function, and whether this function resides in (one of) the regions conserved between TIF2 and SRC-1. Neither for SUG1, TIF1 nor RIP140 an efficient stimulation of NR mediated transactivation has been reported, and the existence of an autonomous activation function has not been demonstrated in these cases (Cavaillès *et al.*, 1995; Le Douarin *et al.*, 1995; Lee *et al.*, 1995a,b; vom Baur *et al.*, 1996).

Understanding the chain of events finally leading to enhancement of transcription will require the identification of the downstream partners interacting with TIFs. We have performed GST fusion protein pull-down experiments using the active mediator fragment TIF2.1 (see Figure 4a and b) and available candidate downstream factors (TBP, TFIIB, $hTAF_{II}18$, $hTAF_{II}20$, $hTAF_{II}28$ and $hTAF_{II}55$; see Jacq *et al.*, 1994; Mengus *et al.*, 1995). However, no significant interaction was observed. Further studies are necessary to investigate whether TIF2 can interact with other components of the transcription machinery or alternatively with components of the chromatin.

In conclusion, squelching/transcriptional interference (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tasset *et al.*, 1990) and structural studies (Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995; Wurtz *et al.*, 1996) have supported a model in which binding of the ligand to the ligand-binding domain of nuclear receptors results in conformational changes generating the surface(s) required for interaction with transcriptional intermediary factors (TIFs/mediators) which transduce the AF-2 activity to the transcription machinery. Conceptually such mediators should exhibit the following properties: (i) they should be localized in the nucleus of the cells; (ii) they should bind to agonist-, but not antagonist-bound NR LBDs; (iii) their binding should be prevented by mutations abolishing AF-2 activity; (iv) they should collectively exhibit a transactivation function(s); (v) their expression should relieve AF-2 autosquelching; and (vi) their overexpression should stimulate the activity of AF-2, whenever they are present in limiting amounts. The present study is the first report of a putative mediator of NR AF-2s for which all these properties have been demonstrated. Disruption of the TIF2 gene or inactivation of its expression products is required to prove that TIF2 is in fact critically involved in mediating the NR AF-2 activity *in vivo*.

Materials and methods

All recombinant DNA work was performed according to standard procedures (Ausubel *et al.*, 1993). Details concerning the plasmid constructions, which were all verified by sequencing, are available on request.

Library screening

Some 340 000 clones of a human placenta cDNA λEXlox expression library were screened with a ^{32}P-labelled GST–hER(DEF) probe in presence of 10^{-6} M E2 using the Far-Western technique (as described by Cavaillès *et al.*, 1994). The 1992-bp insert corresponding to the initial clone (TIF2.1) was used to re-screen the same library. Five highly overlapping cDNA inserts covered a region of 6 kb containing a 1464-amino acid ORF. All inserts were sequenced on both strands. Transient expression of the assembled cDNA inserts encompassing the predicted ORF yielded a 160 kDa protein.

In vitro binding assays

With in vivo [^{35}S]methionine-labelled MCF7 whole-cell extracts. Extracts prepared as described (Cavaillès *et al.*, 1994) were twice precleared with GST-loaded glutathione–Sepharose, and incubated as described (vom Baur *et al.*, 1996) with GST, GST–hER(DEF) or GST–hRARα(DEF), in the presence or absence of 10^{-6} M E2 or T-RA. Bound proteins were recovered with SDS sample buffer and revealed by fluorography (Amplify, Amersham) of SDS–polyacrylamide gels.

With HeLa whole-cell extracts (followed by Western and Far-Western detection). Extracts (2 ml in 500 mM NaCl, 250 mM Tris–HCl pH 7.5, 20% glycerol, 5 mM DTT), were precleared with protein G–Sepharose (400 µl) and treated with protein G–Sepharose (3×400 µl) loaded with mα-TIF2 or non-specific mouse IgG serum. After further clearing with protein G–Sepharose (400 µl), the supernatant was incubated as described (vom Baur *et al.*, 1996) with GST–hER(DEF) in the presence or absence of E2 (10^{-6} M). Retained proteins were recovered with SDS sample buffer, separated by SDS–PAGE and electroblotted on nitrocellulose membranes. Far-Western blotting was as described (Cavaillès *et al.*, 1994). For ECL-based Western blotting (Amersham) pα-TIF2 and rabbit polyclonal pα-TIF1 were diluted 1:2000.

With Escherichia coli-expressed recombinant TIF2.1. Assays were performed as described (vom Baur *et al.*, 1996), using purified (as described by Chen *et al.*, 1994) His-tagged TIF2.1 protein. Bound TIF2.1 was revealed by Western blotting with pα-TIF2 antiserum (dilution 1:30 000). Equal loading of affinity matrices was verified by SDS–PAGE and Coomassie staining. In the corresponding figures 'Input' lanes contain one-third of TIF2.1 input.

Antibodies

mα-TIF2 was raised against a synthetic peptide encompassing amino acids E624–Q643 of TIF2 coupled to ovalbumin. pα-TIF2 rabbit polyclonal antiserum was raised against purified (as described by Chen *et al.*, 1994) recombinant *E.coli*-expressed His-tagged TIF2.1.

Northern blotting

Human Northern blot (Clonetech, No. 7760-1; Lot 5x332) was revealed with ^{32}P-labelled TIF2.1. To confirm proportionate loading, the membrane was rehybridized with ^{32}P-labelled β-actin cDNA (Clonetech).

Immunocytofluorescence assays

Cos-1 cells were transiently transfected with TIF2.1 (10 µg) either alone or in addition with the indicated NR expression vectors (10 µg, except RARα, 1 µg) in the absence or presence of the cognate ligand (10^{-6} M, except R5020, 10^{-8} M). For ER, HE0 (Webster *et al.*, 1988) was used. Immunocytofluorescence assays were as described (Kastner *et al.*, 1992). Images were recorded by confocal laser microscopy.

Transient transfection assays

The construction of the used reporter plasmids and expression vectors has been described (Strähle *et al.*, 1988; Webster *et al.*, 1988; Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tasset *et al.*, 1990; Seipel *et al.*, 1992; Nagpal *et al.*, 1993; Chen *et al.*, 1995) or will be provided on request (GAL-TIF2.1, TIF2.1, TIF2). Transfections and CAT assays were performed as described (Bocquel *et al.*, 1989).

Acknowledgements

We thank A.Pornon for excellent technical assistance; Y.Lutz for antibody production and immunocytochemistry studies; J.-M.Garnier for the expression library and valuable advice; J.-L.Vonesch for recording confocal laser microscopy images; G.Eisenmann, D.Bonnier, F.Granger

and C.Erb for technical help; I.Davidson and L.Tora and their collaborators for the generous gifts of GST–TAF fusion proteins; the DNA and peptide synthesis and sequencing, cell culture, photography and artwork services for help; G.Schütz and W.Schaffner for gifts of recombinants; colleagues at the IGBMC for discussion. J.J.V. and M.J.S.H. are recipients of EMBO and Boehringer-Ingelheim fellowships, respectively. This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Ministère de la Recherche et de la Technologie, the Collège de France, the International Human Frontier Science Programme and the EC Biotech Programme (BIO2-CT 93-0473).

References

Ausubel,F.M., Brent,R., Kingston,R., Moore,D., Seidman,J.J., Smith,J. and Struhl,K. (1993) *Current Protocols in Molecular Biology.* John Wiley & Sons, New York.

Barettino,D., Vivanco-Ruiz,M.D.M. and Stunnenberg,H.G. (1994) Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. *EMBO J.*, **13**, 3039–3049.

Beato,M., Herrlich,P. and Schütz,G. (1995) Steroid hormone receptors: many actors in search of a plot. *Cell*, **83**, 851–857.

Berry,M., Metzger,D. and Chambon,P. (1990) Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen hydroxytamoxifen. *EMBO J.*, **9**, 2811–2819.

Bocquel,M.T., Kumar,V., Stricker,C., Chambon,P. and Gronemeyer,H. (1989) The contribution of the N- and C-terminal regions of steroid receptors to activation of transcription is both receptor- and cell-specific. *Nucleic Acids Res.*, **17**, 2581–2595.

Bourguet,W., Ruff,M., Chambon,P., Gronemeyer,H. and Moras,D. (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. *Nature*, **375**, 377–382.

Cavaillès,V., Dauvois,S., Daniellian,P.S. and Parker,M.G. (1994) Interaction of proteins with transcriptionally active estrogen receptors. *Proc. Natl Acad. Sci. USA*, **91**, 10009–10013.

Cavaillès,V., Dauvois,S., L'Horset,F., Lopez,G., Hoare,S., Kushner,P.J. and Parker,M.G. (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J.*, **14**, 3741–3751.

Chen,J.Y., Penco,S., Ostrowski,J., Balaguer,P., Pons,M., Starrett,J.E., Reczek,P., Chambon,P. and Gronemeyer,H. (1995) RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *EMBO J.*, **14**, 1187–1197.

Chen,Z.P., Shemshedini,L., Durand,B., Noy,N., Chambon,P. and Gronemeyer,H. (1994) Pure and functionally homogeneous recombinant retinoid X receptor. *J. Biol. Chem.*, **269**, 25770–25776.

Danielian,P.S., White,R., Lees,J.A. and Parker,M.G. (1992) Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J.*, **11**, 1025–1033.

Durand,B., Saunders,M., Gaudon,C., Roy,B., Losson,R. and Chambon,P. (1994) Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. *EMBO J.*, **13**, 5370–5382.

Gronemeyer,H. and Laudet,V. (1995) Transcription factors 3: nuclear receptors. *Protein Profile*, **2**, 1173–1380.

Halachmi,S., Marden,E., Martin,G., MacKay,H., Abbondanza,C. and Brown,M. (1994) Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science*, **264**, 1455–1458.

Jacq,X., Brou,C., Lutz,Y., Davidson,I., Chambon,P. and Tora,L. (1994) Human $TAF_{II}30$ is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. *Cell*, **79**, 107–117.

Kastner,P., Perez,A., Lutz,Y., Rochette-Egly,C., Gaub,M.P., Durand,B., Lanotte,M., Berger,R. and Chambon,P. (1992) Structure, localization and transcriptional properties of two classes of retinoic acid receptor α fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins. *EMBO J.*, **11**, 629–642.

Kastner,P., Mark,M. and Chambon,P. (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell*, **83**, 859–869.

Kurokawa,R., Söderström,M., Hörlein,A., Halachmi,S., Brown,M., Rosenfeld,M.G. and Glass,C.K. (1995) Polarity-specific activities of retinoic acid receptors determined by a co-repressor. *Nature*, **377**, 451–454.

Le Douarin,B., Zechel,C., Garnier,J.M., Lutz,Y., Tora,L., Pierrat,B., Heery,D., Gronemeyer,H., Chambon,P. and Losson,R. (1995) 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. *EMBO J.*, **14**, 2020–2033.

Lee,J.W., Choi,H.S., Gyurist,J., Brent,R. and Moore,D.D. (1995a) Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. *Mol. Endocrinol.*, **9**, 243–254.

Lee,J.W., Ryan,F., Swaffield,J.C., Johnston,S.A. and Moore,D.D. (1995b) Interaction of thyroid hormone receptor with a conserved transcriptional mediator. *Nature*, **374**, 91–94.

Leng,X., Blanco,J., Tsai,S.Y., Ozato,K., O'Malley,B.W. and Tsai,M.-J. (1995) Mouse retinoid X receptor contains a separable ligand-binding and transactivation domain in its E region. *Mol. Cell. Biol.*, **15**, 255–263.

Mangelsdorf,D.J. and Evans,R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, **83**, 841–850.

Mangelsdorf,D.J., Thummel,C., Beato,M., Herrlich,P., Schütz,G., Umesono,K., Blumberg,B., Kastner,P., Mark,M., Chambon,P. and Evans,R.M. (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.

Mengus,G., May,M., Jacq,X., Staub,A., Tora,L., Chambon,P. and Davidson,I. (1995) Cloning and characterization of $hTAF_{II}18$, $hTAF_{II}20$ and $hTAF_{II}28$: three subunits of the human transcription factor TFIID. *EMBO J.*, **14**, 1520–1531.

Meyer,M.E., Gronemeyer,H., Turcotte,B., Bocquel,M.T., Tasset,D. and Chambon,P. (1989) Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell*, **57**, 433–442.

Meyer,M.E., Pornon,A., Ji,J.J., Bocquel,M.T., Chambon,P. and Gronemeyer,H. (1990) Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. *EMBO J.*, **9**, 3923–3932.

Nagpal,S., Saunders,M., Kastner,P., Durand,B., Nakshatri,H. and Chambon,P. (1992) Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. *Cell*, **70**, 1007–1019.

Nagpal,S., Friant,S., Nakshatri,H. and Chambon,P. (1993) RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization *in vivo*. *EMBO J.*, **12**, 2349–2360.

Onate,S.A., Tsai,S.Y., Tsai,M.J. and O'Malley,B. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science*, **270**, 1354–1357.

Renaud,J.P., Rochel,N., Ruff,M., Vivat,V., Chambon,P., Gronemeyer,H. and Moras,D. (1995) Crystal structure of the RAR-γ ligand-binding domain bound to all-*trans* retinoic acid. *Nature*, **378**, 681–689.

Seipel,K., Georgiev,O. and Schaffner,W. (1992) Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. *EMBO J.*, **11**, 4961–4968.

Strähle,U., Schmid,W. and Schütz,G. (1988) Synergistic action of the glucocorticoid receptor with transcription factors. *EMBO J.*, **7**, 3389–3395.

Tasset,D., Tora,L., Fromental,C., Scheer,E. and Chambon,P. (1990) Distinct classes of transcriptional activating domains function by different mechanisms. *Cell*, **62**, 1177–1187.

Thummel,C.S. (1995) From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. *Cell*, **83**, 871–877.

Tora,L., White,J., Brou,C., Tasset,D., Webster,N., Scheer,E. and Chambon,P. (1989) The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell*, **59**, 477–487.

vom Baur,E., Zechel,C., Heery,D., Heine,M.J.S., Garnier,J.M., Vivat,V., Le Douarin,B., Gronemeyer,H., Chambon,P. and Losson,R. (1996) Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *EMBO J.*, **15**, 110–124.

Wagner,R.L., Apriletti,J.W., McGrath,M.E., West,B.L., Baxter,J.D. and Fletterick,R.J. (1995) A structural role for hormone in the thyroid hormone receptor. *Nature*, **378**, 690–697.

Webster,N.J., Green,S., Jin,J.R. and Chambon,P. (1988) The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell*, **54**, 199–207.

Wurtz,J.M., Bourguet,W., Renaud,J.P., Vivat,V., Chambon,P., Moras,D. and Gronemeyer,H. (1996) A canonical structure for the ligand-binding domain of nuclear receptors. *Nature Struct. Biol.*, **3**, 87–94.
Zenke,M., Munoz,A., Sap,J., Vennström,B. and Beug,H. (1990) v-*erbA* oncogene activation entails the loss of hormone-dependent regulator activity of c-*erbA*. *Cell*, **61**, 1035–1049.

Received on January 29, 1996; revised on March 25, 1996

Note added in proof

While this paper was in press, Hong *et al.* [(1996) *Proc. Natl Acad. Sci. USA*, **93**, 4948–4952] reported the cloning of a partial cDNA for GRIP1, which is apparently the mouse homologue of TIF2. Kamel *et al.* [(1996) *Cell*, **85**, 403–414] recognized a PAS-A domain in the N-terminus of mSRC-1a. Note that sequences homologous to this domain are also present in TIF2 (amino acids 117–176); the PAS-B domain, however, is only weakly conserved (amino acids 250–369). The TIF2 nucleotide sequence has been deposited in the EMBL/GenBank/DDBJ databases (accession No. X97674).