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Ligand-dependent interaction of nuclear receptors with potential transcriptional intermediary factors (mediators)

B. LE DOUARIN, E. VOM BAUR, C. ZECHEL, D. HEERY*, M. HEINE, V. VIVAT, H. GRONEMEYER, R. LOSSON AND P. CHAMBON‡

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, 67404 ILLKIRCH-CEDEX, France

SUMMARY

The activity of the ligand-inducible activation function 2 (AF-2) contained in the ligand binding domain (LBD) of nuclear receptors (NRs) is thought to be mediated by transcriptional intermediary factors (TIFs). We have recently reported the isolation and characterization of two novel mouse proteins, designated TIF1 and mSUG1, that interact in a ligand-dependent fashion with the LBD (region E) of several NRs *in vivo* as well as *in vitro*. Remarkably, these interactions require the conserved core motif of the AF-2 activating domain (AF-2 AD) and can be blocked by AF-2 antagonists. TIF1 and mSUG1 might therefore represent TIFs/mediators for the ligand-dependent AF-2 of NRs. By comparing the interaction properties of these two putative TIFs with different NRs including the oestrogen (ER), thyroid hormone (TR), vitamin D3 (VDR), retinoic acid (RARα) and retinoid X (RXR) receptors, we demonstrate that: (i) RXRα efficiently interacts with TIF1, but not with mSUG1, whereas TRα interacts much more efficiently with mSUG1 than with TIF1, and RARα, VDR and ER efficiently interact with both TIF1 and mSUG1; (ii) the amphipathic α helix core of AF-2 AD is differentially involved in the interactions of RARα with TIF1 and mSUG1; and (iii) the AF-2 AD cores of RARα and ER are similarly involved in their interaction with TIF1, but not with mSUG1. Thus the interaction interfaces between the various NRs and either TIF1 or mSUG1 may vary depending on the nature of both the receptor and the putative mediator of its AF-2 function. We discuss the possible roles of TIF1 and mSUG1 as mediators of the transcriptional activity of the AF-2 of NRs.

1. INTRODUCTION

Nuclear receptors (NRs) represent a large family of ligand-inducible transcription factors that 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. Like other transcriptional regulators, NRs display a modular structure. Five to six distinct regions (denoted A to F) exhibit different degrees of evolutionary conservation. The N-terminal A/B region contains an autonomous activation function (AF-1), which can activate transcription constitutively in the absence of the ligand binding domain (LBD). The highly conserved region C encompasses the DNA binding domain (DBD), which recognizes cognate cis-acting response elements (REs). In addition to the LBD, region E contains a homo- and/or a heterodimerization surface, and the ligand-dependent transcriptional activation function AF-2 (Green & Chambon 1988; Gronemeyer 1991; Leid *et al.* 1992; Chambon 1994; Giguere 1994; Mangelsdorf *et al.* 1994). An autonomous activating domain (AF-2 AD) has been characterized in the C-terminal part of the E region of several NRs and shown to contain a core motif (AF-2 AD core) corresponding to an amphipathic α-helix which is an essential conserved element of the ligand-inducible AF-2 function (Zenke *et al.* 1990; Danielan *et al.* 1992; Barettino *et al.* 1994; Durand *et al.* 1994; Bourguet *et al.* 1995).

How the AFs of NRs control initiation of transcription is still obscure. The central question is whether and how these AFs function at the level of the transcription machinery itself and/or at the level of the chromatin template. Some of the underlying molecular mechanisms must be highly conserved in eukaryotic evolution, because NRs have been shown to stimulate transcription in yeast (Metzger *et al.* 1988; Heery *et al.* 1993; and references therein). The observation that the activity of AF-2 of a given receptor can be inhibited or squelched by overexpression of the AF-2-containing region E of the same or another receptor (auto- or hetero-interference) in the presence of the cognate ligand has suggested that transcriptional intermediary factors (TIFs), also referred to as bridging factors, mediators or adaptors, are required for activation

* Present address: Imperial Cancer Research Fund, Molecular Endocrinology Laboratory, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K.
‡ Author to whom correspondence should be addressed.

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(Meyer *et al.* 1989; Tasset *et al.* 1990). We and others have recently isolated and characterized proteins that interact in a ligand-dependent manner with AF-2 of several steroid and non-steroid NRs and which may fullfill a cofactor-like function (Halachmi *et al.* 1994; Cavailles *et al.* 1995; Le Douarin *et al.* 1995; Lee *et al.* 1995; vom Baur *et al.* 1996). We review here the main properties of two of these potential TIFs, namely TIF1 and mSUG1 (Le Douarin *et al.* 1995; vom Baur *et al.* 1996).

2. RESULTS

(*a*) *Isolation of TIF1 as an activity that enhances AF-2 of RXR in yeast*

TIF1 has been cloned in a yeast 'multicopy suppressor' screen designed to isolate cDNAs whose overexpression increases the AF-2 activity of RXR. This screen was based on the observation that a chimeric receptor containing the DE region of RXR fused to a DNA binding domain was able to transactivate in a 9-cis-retinoic acid (9C-RA)-dose-dependent manner expression of a URA3 reporter gene in yeast (Heery *et al.* 1993). Activation of the reporter in response to 100 nM 9C-RA was sufficient to rescue the Ura$^-$ phenotype of the cells on medium lacking uracil. In contrast, no growth was observed at 10 nM. A high-level expression library of mouse embryonal carcinoma cell cDNAs was therefore screened for cDNAs encoding proteins that complement the growth defect of the yeast cells grown in the presence of 10 nM 9C-RA. Among the receptor-dependent Ura$^+$ clones isolated, one partial cDNA clone, designated TIF1.22, was further characterized because it specifically enhanced the ligand-dependent activity of RXR on a reporter containing a RXRE in yeast (figure 1) (Le Douarin *et al.* 1995).

(*b*) *TIF1 contains multiple evolutionary conserved domains present in several regulatory proteins*

The full-length cDNA encoding TIF1 was cloned, and its nucleotide and deduced amino acid sequence revealed a protein of 1017 amino acids with a predicted molecular mass of 112 kDa and a number of sequence motifs characteristic of several families of regulatory proteins (figure 2) (Le Douarin *et al.* 1995). The N-terminal part of TIF1 contains three Cys/His-rich clusters, an evolutionary conserved C3HC4 zinc finger motif or RING finger preceding two B-box type

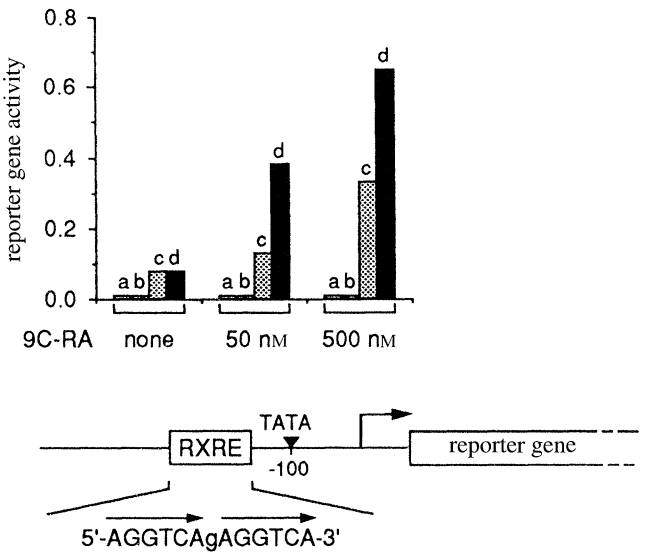

Figure 1. TIF1.22 stimulates ligand-induced transactivation by RXRα in yeast. High copy number plasmids expressing RXRα (c and d), TIF1.22 which encodes residues 396–1017 (b and d), or no insert (a) were introduced into a yeast strain that contains an integrated URA3 reporter gene driven by a RXRE. Cells were grown in liquid medium containing uracil +/− 9C-RA as indicated. Reporter gene activity on cell-free extracts was determined by measuring the specific activity of the URA3 gene product, OMPdecase, which is expressed in nmols substrate/min/mg protein (for details see Le Douarin *et al.* 1995). Open area = control; hatched area = TIF1.22; dotted area = RXRα; filled area = RXRα + TIF1.22.

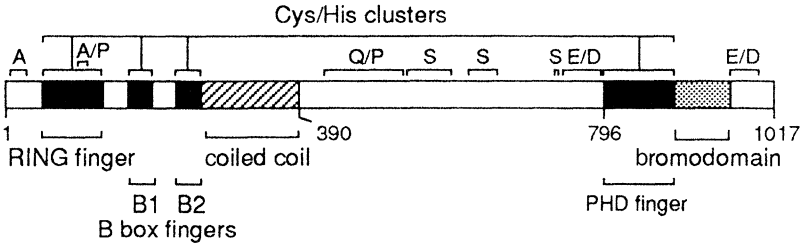

Figure 2. Structural features of TIF1. The conserved motifs present in TIF1 are schematically represented. Regions rich in particular amino acids (abundance > 20%) are indicated (see text and Le Douarin *et al.* 1995).

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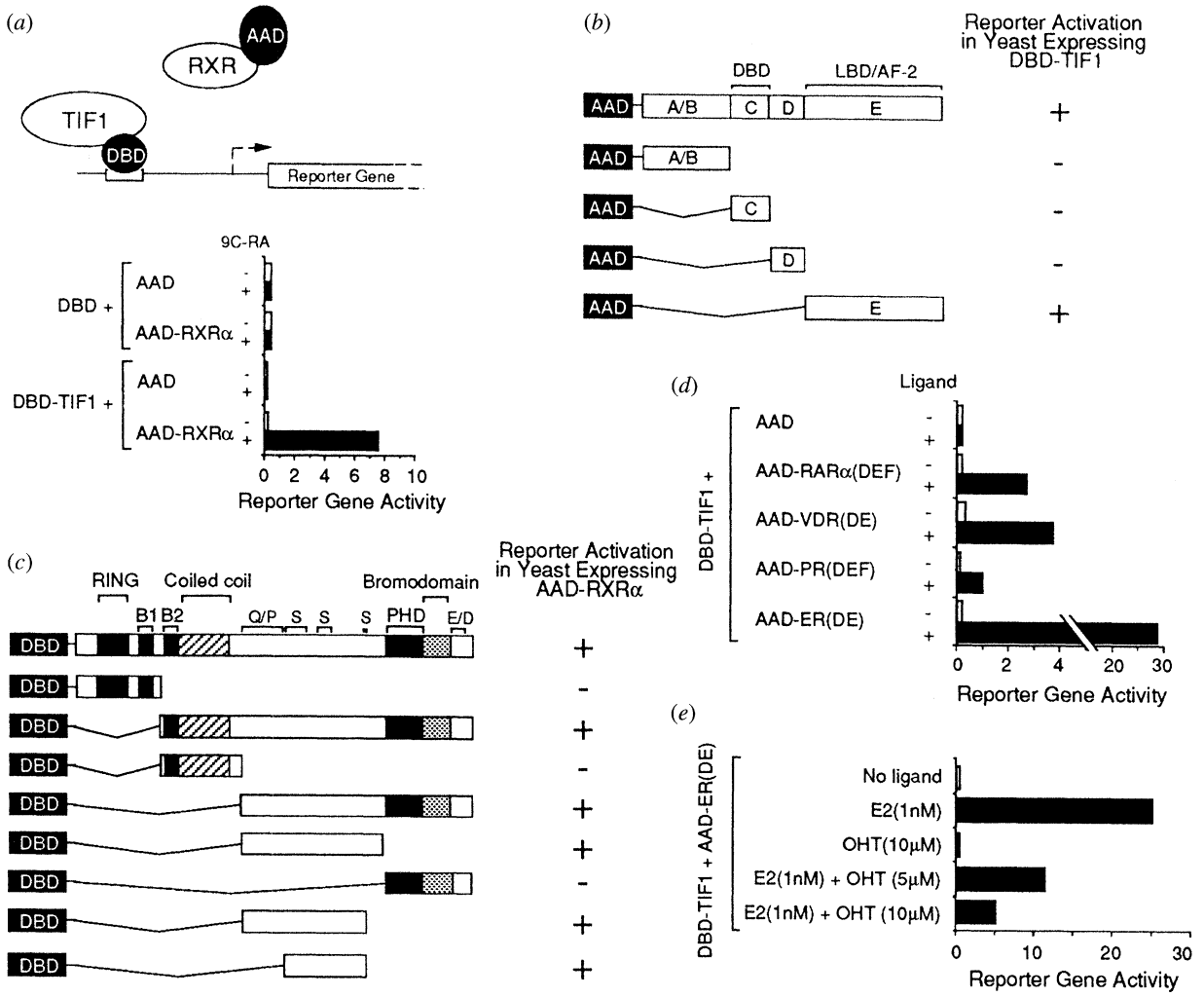


Figure 3. TIF1 and NRs functionally interact in yeast. (*a*) Yeast interaction assay showing a 9C-RA-dependent interaction between TIF1 and RXRα. The DBD of ER (aa 176–282) and the acidic activation domain (AAD) of VP16 (aa 411–490) fused to the complete coding sequences of TIF1 and RXRα respectively, are schematically represented as well as the URA3 reporter gene whose expression is regulated by three EREs in the yeast reporter strain PL3 (Le Douarin *et al.* 1995). Plasmids expressing the ER DBD or DBD-TIF1 were introduced into PL3 together with the AAD or AAD-RXRα fusion construct. Transformants were grown in liquid medium containing uracil in the presence or absence of 500 nM of 9C-RA. Reporter gene activities are expressed as in figure 1. (*b*) The AF-2/LBD-containing region E of RXRα is sufficient for interaction with TIF1. Various regions of RXRα were fused to the VP16 AAD and assayed for activation with DBD-TIF1 in PL3 grown in the presence of 9C-RA. +, binding; −, no binding. (*c*) The serine-rich region of TIF1 (aa 539–750) is sufficient for interaction with RXRα. A series of TIF1 N- and C-terminal deletion mutants were fused to the ER DBD and assayed for activation with AAD-RXRα in PL3 grown in the presence of 9C-RA. (*d*) Functional interaction between TIF1 and various NRs. The indicated AAD fusion receptors were coexpressed with DBD-TIF1 in PL3. Transformants were grown in the presence (+) or absence (−) of the cognate ligand [500 nM T-RA for RAR, 5 μM vitamin D3 for VDR, 10 μM R5020 for PR, 1 μM estradiol for ER]. Reporter gene activities are expressed as in figure 1. (*e*) No interaction is seen between TIF1 and the DE region of ER in the presence of the AF-2 antagonist hydroxytamoxifen (OHT). The PL3 reporter strain was cotransformed with plasmids expressing DBD-TIF1 and AAD-ER(DE) fusion constructs. Transformants were grown in the presence or absence of estradiol (E2) and OHT as indicated. For further details, see Le Douarin *et al.* (1995).

fingers, which are followed by a putative coiled coil domain. This tripartite motif, designated RBCC (Le Douarin *et al.* 1995), has been found in the N-terminal part of several nuclear proteins, some of which are involved in the control of transcription (Freemont 1993). Interestingly, three of these RBCC proteins, TIF1, PML, and RFP, have been identified in the context of oncogenic fusion proteins. The nature of these fusions that all include the entire RBCC motif recombined with other genes, namely B-raf, RARα, and ret, respectively, has suggested that this motif may play an important role in cell transformation (Kastner *et al.* 1992; Freemont 1993; Le Douarin *et al.* 1995 and references therein). Another highly conserved sequence, the bromodomain motif, is present in the C-terminal region of TIF1 (figure 2). This motif of unknown function is conserved from yeast to man (Haynes *et al.* 1992), and has been found in several

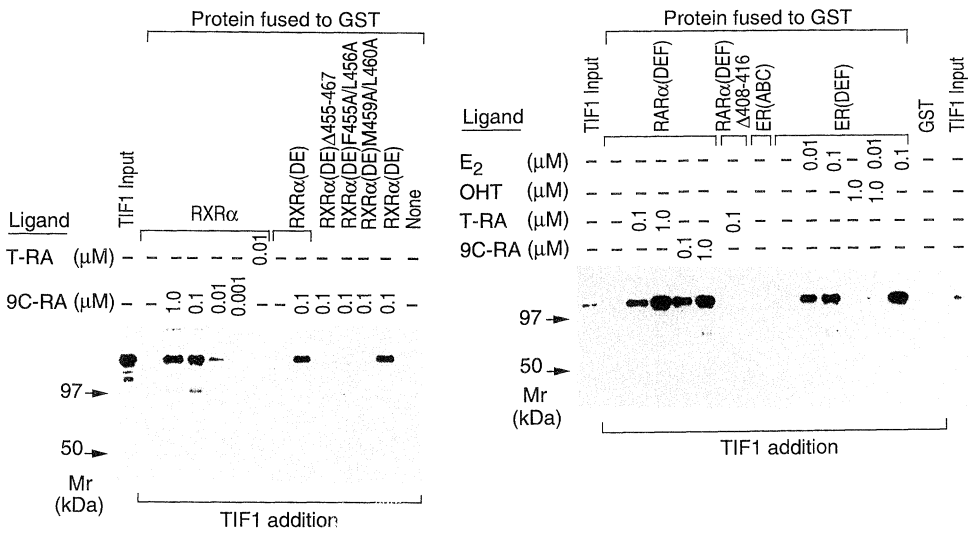
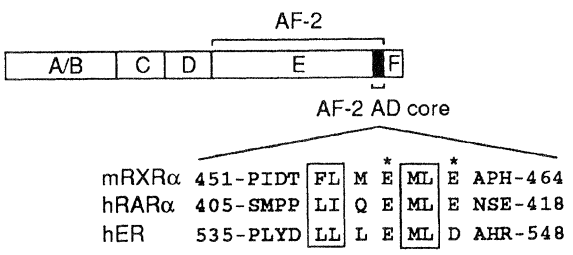


Figure 4. TIF1 interacts *in vitro* with the liganded LBD of NRs. The indicated GST fusion proteins or GST alone bound to glutathione-Sepharose beads were incubated with purified histidine-tagged TIF1 in the presence or absence of ligands, as indicated. Bound proteins were identified by Western blotting (see also Le Douarin *et al.* 1995).



	interaction with TIF1	transcriptional activation
RXRαWT	++	++
RXRαΔ455-467	-	-
RXRαE461Q	+	+
RXRαF455A/L456A	-	-
RXRαM459A/L460A	-	-

Figure 5. Point mutations in the AF-2 AD of RXRα-eliminate interaction with TIF1 and block transactivation by RXRα.-The sequence of the AF-2 AD core motifs of RXRα, RARα, and ER is shown. The conserved hydrophobic and acidic residues are indicated by a box and a star, respectively. Interactions between TIF1 and the indicated mutants of RXRα-were assayed both in yeast and *in vitro* as described in figure 3 and 4. To measure transcriptional activation, 100 ng receptor expression vector was cotransfected into Cos-1 cells with 2 μg RXRE(DR1T)-tk/CAT reporter gene. Cells were treated with EtOH or 100 nM 9C-RA for 24 h and CAT activity was determined. The results are indicated relative to the wild type RXRα, which was attributed the arbitrary value ++. +, less than 50%; −, less than 1% of wild type values (see Le Douarin *et al.* 1995 for details).

proteins implicated as transcriptional adaptors or coactivators. Included in this group of polypeptides are the yeast and human coactivator SWI2/SNF2 (Carlson & Laurent 1994), the yeast transcriptional coactivator GCN5 (Georgakopoulos & Thireos 1992), the human and Drosophila TBP-associated factor $TAF_{II}250$ (Hisataka *et al.* 1993), and the human CREB binding protein CBP (Chrivia *et al.* 1993). Interestingly, two of these bromodomain proteins, TIF1 and CBP, contain adjacent to this domain a new kind of zinc finger motif, the C4HC3 motif or PHD finger (Aasland *et al.* 1995). The presence of this motif in several chromatin-'related' proteins, including trithorax (trx) and Polycomblike (Pcl) which have antagonistic effects on chromatin structure and function, has suggested that it may be involved in chromatin interactions (Aasland *et al.* 1995).

In addition to these conserved domains, TIF1 contains: (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 region; and (iii) several regions with high contents of serine (S) or acidic residues (E/D) in the C-terminal moiety (figure 2), which are also characteristic of transcriptional activators (Tjian & Maniatis 1994). Thus, in view of its amino acid sequence, TIF1 is likely to play a role in transcriptional regulation.

(*c*) *TIF1 functionally interacts with several members of the NR superfamily in yeast*

Using the yeast two-hybrid system (Fields & Sternglanz 1994), TIF1 was shown to functionally interact in a retinoid-dependent manner with RXRα (figure 3*a*). Deletion analysis revealed that the AF-2/LBD-containing region E of RXRα and the serine-rich region of TIF1 were sufficient for the interaction (figure 3*b* & *c*) (Le Douarin *et al.* 1995). Interactions between TIF1 and four other members of the NR superfamily, the vitamin D3 (VDR), oestrogen (ER), progesterone (PR) and retinoic acid (RARα) receptors, have also been detected in yeast (figure 3*d*). All of these interactions are strictly dependent on the presence of the cognate ligand, and appear to be specifically induced by ligands which activate AF-2, because no interaction with the ER was observed in the presence of the AF-2 antagonist hydroxytamoxifen (OHT; figure 3*e*). Several reports have shown that estrogen and OHT induce distinct conformational changes in

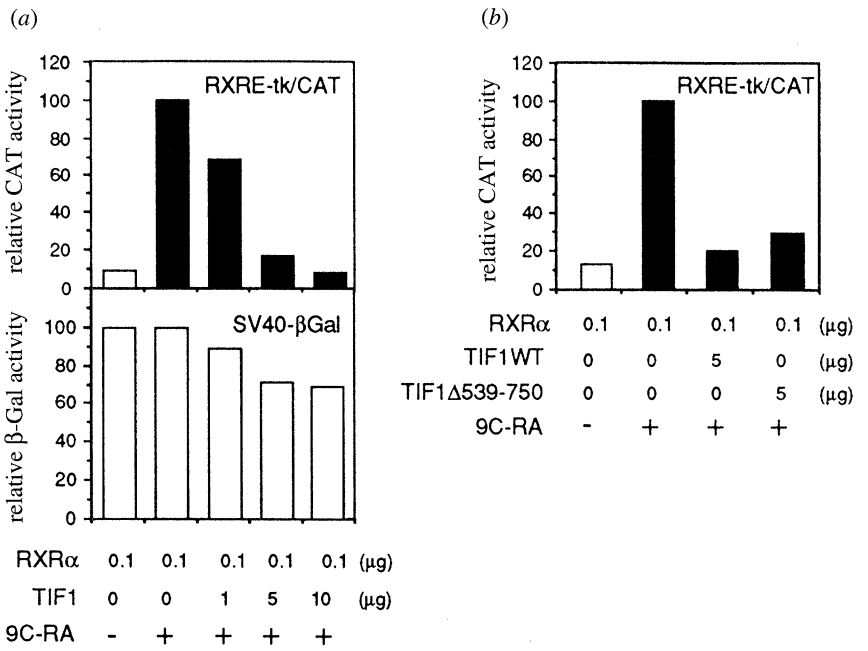


Figure 6. TIF1 interferes with transactivation by RXRα. (*a*) Overexpression of TIF1 inhibits RXRα-mediated transactivation. Cos-1 cells were transiently cotransfected with 4 μg RXRE(DR1T)-tk/CAT reporter, 100 ng RXRα, 1 μg pCH110 (expressing β-galactosidase under the control of the SV40 promoter) and increasing amounts of TIF1 expression vector, as indicated, in the absence (−) or presence (+) of 1 μM 9C-RA. Enzyme activities are expressed relative to those seen in the absence of TIF1 with added 9C-RA (taken as 100%). See also Le Douarin *et al.* (1995). (*b*) TIF1 inhibition does not require the domain interacting with RXRα.-Cos-1 cells were transfected with either wild type TIF1 or a TIF1 mutant lacking the nuclear receptor interacting domain along with the RXRα-expression vector and the RXRE(DR1T)-tk/CAT reporter gene in the presence of 9C-RA ligand. Enzyme activities are expressed as in panel A.

the ER (Beekman *et al.* 1993). This may explain why these coumpounds can or cannot induce the AF-2 function in mammalian cells, as well as the interaction of the receptor with TIF1.

(*d*) *Ligand-dependent Interaction between TIF1 and the LBD of NRs* in vitro

To further characterize the interaction between TIF1 and the NRs, the ability of GST-NR fusion proteins to interact *in vitro* with Baculovirus-expressed histidine-tagged TIF1 was examined (Le Douarin *et al.* 1995). The GST pull-down experiments shown in figure 4 revealed a ligand-dependent interaction between TIF1 and the LBD of RXRα, RARα, and ER. Hydroxytamoxifen (OHT) failed to induce the binding of TIF1 to GST-ER(DEF) and blocked estradiol-dependent interaction of the receptor with TIF1 in a dose-dependent manner (figure 4). These results are therefore in complete agreement with the functional interactions detected in yeast, indicating that TIF1 and liganded LBDs of NRs can directly interact with each other.

(*e*) *Conserved amino acid residues of the AF-2 AD of RXRα are required for the interaction with TIF1*

To test the correlation between NR activation function and TIF1 binding, several RXRα receptors bearing mutations in the AF-2 AD core were tested for their ability to transactivate in Cos-1 cells and to interact with TIF1 both in yeast and *in vitro* (figure 5) (Le Douarin *et al.* 1995). In contrast to the wild type, a RXRα mutant lacking the core motif of the AF-2 AD, but still binding 9C-RA, could neither bind TIF1 *in vitro* (figure 4 & 5) nor transactivate *in vivo* (figure 5).

Receptor mutants with point mutations in this core motif showed reduced interaction and transactivation, or had lost both functions (figure 5). A similar correlation has been observed between the ability of RARα and ER mutant receptors to stimulate transcription with their ability to interact with TIF1 (vom Baur *et al.* 1996), suggesting that this interaction has functional significance.

(*f*) *TIF1 interferes with transactivation by RXRα*

Further support for TIF1 playing an important role in mediating the ligand-induced AF-2 activity was provided by transient cotransfection assays in Cos-1 cells, which showed that an overexpression of TIF1 had no effect on basal transcription, but specifically inhibited the ligand-induced transcription by RXRα (figure 6*a*). Interestingly, this inhibitory activity of TIF1 did not depend on its ability to interact with the receptor, because a TIF1 mutant lacking the nuclear receptor interacting domain was still able to inhibit transactivation by RXRα in transfected cells (figure 6*b*). This inhibition or transcriptional interference may reflect the squelching of a limiting factor that interacts 'downstream' of TIF1 to further transduce AF-2 activity.

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(*g*) *mSUG1 is the mouse homologue of the yeast transcription factor SUG1*

The mSUG1 cDNA has been isolated in a yeast two hybrid screen designed to clone mouse cDNAs encoding proteins that specifically interact with the DEF regions of RARα in the presence of all-trans retinoic acid (T-RA) (vom Baur *et al.* 1996). Sequence analysis identified the protein mSUG1 as a member of a large, highly conserved family of putative ATPases, which are involved in diverse cellular functions (Confalonieri & Duguet 1995). Within this family, mSUG1 showed a particularly high degree of sequence identity with the yeast protein SUG1 (Swaffield *et al.* 1992; hereafter designated ySUG1) (figure 7). Given the ability of mSUG1 to complement the lethal phenotype of a ySUG1 mutant (figure 7), this sequence conservation must probably reflect a conserved function.

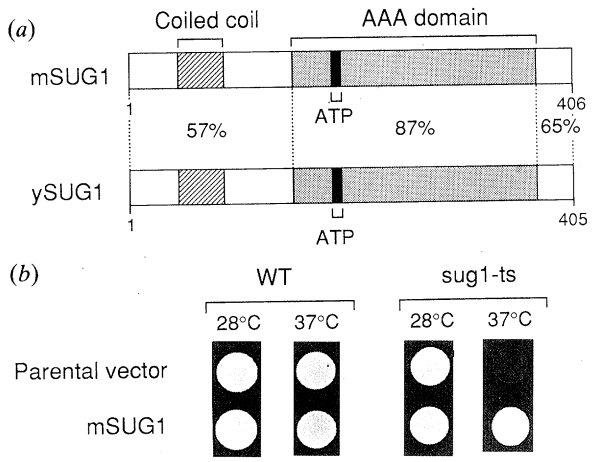

Figure 7. mSUG1 is the mouse homologue of the yeast protein SUG1 (ySUG1). The upper part of the figure shows a schematic alignment of mouse and yeast SUG1. The putative coiled coil and conserved AAA (for ATPases Associated to a variety of cellular Activities; see Confalonieri & Duguet 1995) domains are represented as well as the conserved ATP binding site motif. The percentage of amino acid identity is given for the N- and C- terminal regions, as well as the AAA domain. The lower part of the figure shows the yeast complementation assay indicating that mSUG1 can rescue the conditional lethality of a sug1 mutant. Isogenic yeast strains carrying the wild type (WT) SUG1 allele or a thermosensitive sug1-ts allele were transformed with a multicopy expression vector containing no insert or the coding sequence of mSUG1. The growth phenotypes of the transformants were assayed by using a spot test on agar medium at 30 °C and 37 °C. Plates were incubated for 3 days and photographed (see also vom Baur *et al.* 1996).

(*h*) *TIF1 and mSUG1 differ in their ability to interact with RARα*

By using both the yeast two hybrid assay and *in vitro* GST pull-down experiments, mSUG1 has been shown to contact directly the AF-2/LBD-containing region DEF of RARα (figure 8). In both systems, a T-RA-independent interaction was evident, which was further stimulated by the presence of T-RA, whereas under the same conditions the interaction between TIF1 and RARα was strictly ligand-dependent (figure 3*d* and 4), thus indicating that TIF1 and mSUG1 interact differently with RARα.

Consistent with this idea, the core motif of the AF-2 AD of RARα was found to be differentially required for interaction with both TIF1 and mSUG1. Several receptor mutants with the AF-2 AD core deleted or mutated in the conserved residues were tested for their ability to interact with TIF1 and mSUG1 (vom Baur *et al.* 1996). All mutants were defective for TIF1 binding, but some of them were still able to interact with mSUG1 (figure 9*a*). Thus not all the conserved residues of the AF-2 AD of RARα which are important for transactivation are required for the interaction with mSUG1, indicating that the binding of RARα to mSUG1 may be necessary, but not sufficient for transactivation.

Dissociated synthetic retinoids that do not induce RARα AF-2 transcriptional activity (BMS411 and BMS 453; Chen *et al.* 1995) were compared with T-RA for their ability to induce a functional interaction between RARα and either mSUG1 and TIF1 in yeast. These compounds were only two- to fourfold less efficient than T-RA at stimulating the interaction

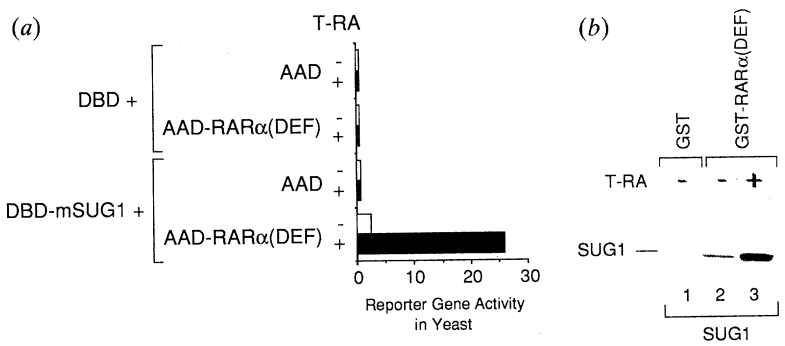

Figure 8. mSUG1 interacts with the DEF region of RARα both in yeast and *in vitro*. (*a*) A plasmid expressing mSUG1 fused to the ER DBD was introduced into the yeast reporter strain PL3 together with VP16 AAD or the AAD-RARα(DEF) fusion construct. Transformants were grown in liquid medium containing uracil in the presence or absence of 500 nM T-RA. Reporter gene activities are expressed as in figure 1. (*b*) *In vitro* interaction between ^{35}S-labeled mSUG1 and GST alone (lane 1) or a GST-RARα(DEF) fusion protein (lanes 2 and 3) was tested using the pull-down assay in the absence or presence of 1 μM T-RA (see also vom Baur *et al.* 1996).

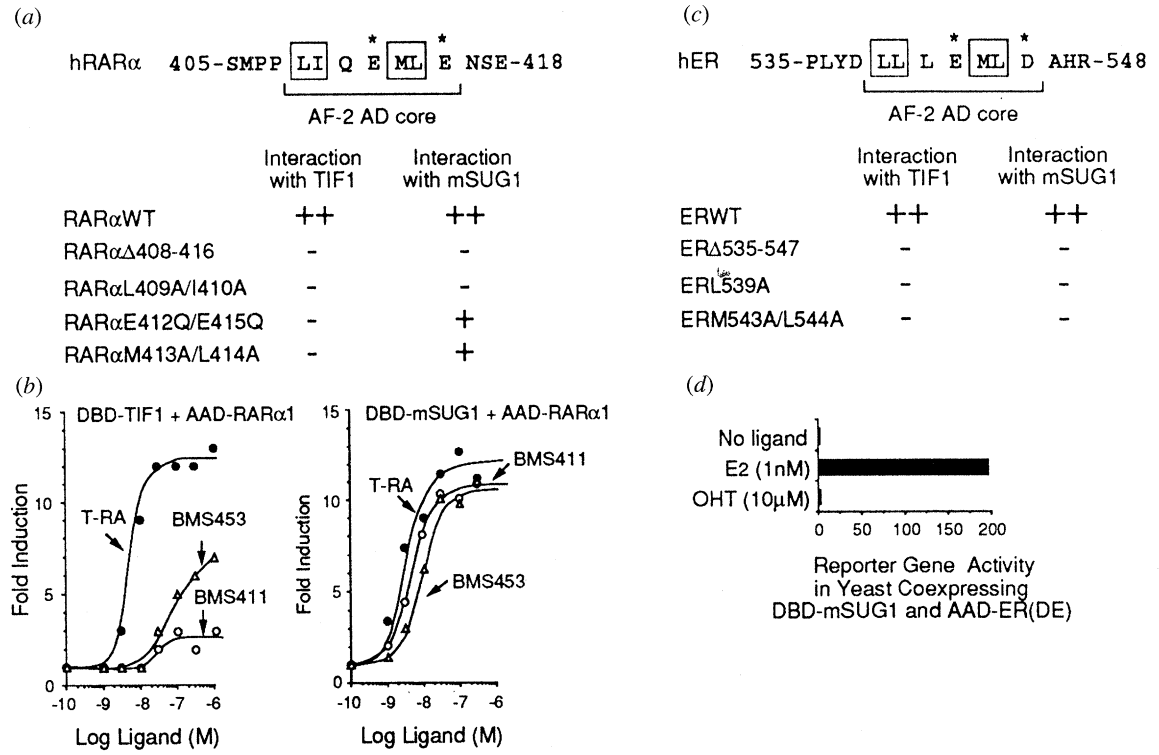

Figure 9. TIF1 and mSUG1 differentially interact with RARα and ER. (*a*) Differential requirements of conserved residues of the AF-2 AD core motif of RARα for interaction with TIF1 and mSUG1. The sequence of the AF-2 AD core motif of RARα is shown. The conserved hydrophobic and acidic residues are indicated by a box and a star, respectively. The DEF regions of the indicated mutants of RARα were fused to the AAD of VP16 and assayed for interaction with either DBD-TIF1 or DBD-mSUG1 in the yeast reporter strain PL3 grown in the presence of 1 μM T-RA (see also vom Baur *et al.* 1996) (*b*) RARα-antagonists differentially affect the binding of TIF1 and mSUG1 to RARα. AAD-RARα1 was coexpressed with either DBD-mSUG1 or DBD-TIF1 in the yeast reporter strain PL3. Transformants were grown in the absence or presence of T-RA (●), BMS411 (○) and BMS453 (△) at the concentrations indicated. Transcription of the reporter gene was determined by measuring OMPdecase activity, and is represented as fold induction above the level of OMPdecase activity observed in the absence of ligand. (*c*) Point mutations in the AF-2 AD core motif of ER prevent interaction with both TIF1 and mSUG1. The DEF regions of the indicated mutants of ER were fused to the AAD of VP16 and assayed for interaction with either DBD-TIF1 or DBD-mSUG1 in yeast (see vom Baur *et al.* 1996 for details). (*d*) mSUG1 and ER do not interact in the presence of the antagonist hydroxytamoxifen (OHT) (see also vom Baur *et al.* 1996).

between RARα and mSUG1, whereas they were far less efficient than T-RA for inducing interaction with TIF1 (figure 9*b*). This finding is consistent with the above conclusion that the structural requirements for the interaction of RARα with mSUG1 and TIF1 are different.

(*i*) *The various NRs differentially interact with mSUG1 as well as TIF1*

mSUG1 also interacted with the DEF region of the ER in the presence of estradiol both in yeast and *in vitro* (vom Baur *et al.* 1996). However, in marked contrast with RARα, no interaction was detected in the absence of ligand or in the presence of the antiestrogen hydroxytamoxifen (figure 9*d*). Furthermore, all point mutations in the AF-2 AD core of ER which abrogated the binding of TIF1 to the receptor, also prevented mSUG1 binding (figure 9*c*), indicating that RARα and ER interact differently with mSUG1.

Other nuclear receptors were tested for their ability to interact with mSUG1 (vom Baur *et al.* 1996). For comparison, binding assays were also performed with

receptor	SUG1	TIF1
RARα	++	+
VDR	++	+
TRα	++	ε
RXRα	ε	++
ER	++	++

Figure 10. Preferential binding of mSUG1 and TIF1 to different subsets of NRs. The DE/F regions of the indicated NRs were assayed for interaction with mSUG1 and TIF1 both in yeast and *in vitro*. ++, +: significant or intermediate interaction; ε, little or no interaction (see vom Baur *et al.* 1996 for details).

TIF1. The results are summarized in figure 10. mSUG1 showed interactions with VDR and the thyroid hormone receptor, TRα, in the presence of their cognate ligand. In contrast, it interacted only weakly with RXRα under conditions where an efficient

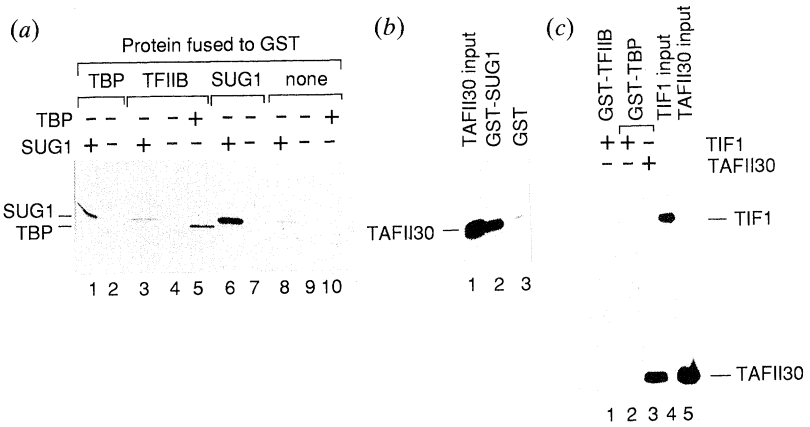

Figure 11. *In vitro* interaction of mSUG1 and TIF1 with components of the transcriptional machinery. (*a*) mSUG1 dimerization and interaction with TBP. Interaction of ^{35}S-labeled mSUG1 with GST alone (lane 8) and GST fusion proteins comprising GST-TBP (lane 1), GST-TFIIB (lane 3), and GST-mSUG1 (lane 6) was tested in a pull-down assay. As a positive control, ^{35}S-labelled TBP was also incubated with GST-TFIIB (lane 5). (*b*) mSUG1 interacts *in vitro* with a component of the human TFIID complex. GST or GST-mSUG1 were incubated with purified histidine-tagged $TAF_{II}30$. $TAF_{II}30$ bound to the glutathione beads was detected by Western blotting (*c*) TIF1 does not interact with TBP or TFIIB. Purified histidine-tagged TIF1 was incubated with either GST-TBP (lane 2) or GST-TFIIB (lane 1). As a control, histidine-tagged $TAF_{II}30$ was also incubated with GST-TBP (lane 3) (see also Le Douarin *et al.* 1995; vom Baur *et al.* 1996 for details).

binding to TIF1 could be detected (vom Baur *et al.* 1996). On the other hand, the ability of TIF1 to interact with TRα was much weaker than that of mSUG1, whereas an efficient binding to the other receptors i.e. RAR, ER, VDR and RXR, was observed (vom Baur *et al.* 1996). Thus mSUG1 and TIF1 clearly display a preferential binding to certain receptors. Whether this reflects differences in the non-conserved residues of the AF-2 AD core of the various receptors and/or additional specific interactions with other region(s) of the LBD remains to be investigated. In any event, because RAR, TR and VDR function as heterodimers with RXR (Leid *et al.* 1992; Chambon 1994; Mangelsdorf *et al.* 1994), our data suggest that the transactivation function(s) of RXR/RAR, RXR/TR or RXR/VDR heterodimers may be synergistically mediated by TIF1 and mSUG1 interacting with RXR and its heterodimeric partner, respectively. In the case of ER which functions as a homodimer, it is conceivable that one monomer may interact with mSUG1, the other one interacting concomitantly with TIF1.

(*j*) *mSUG1, but not TIF1 interacts with components of the transcriptional machinery*

The ability of mSUG1 and TIF1 to interact with components of the basal transcription machinery, namely TBP and TFIIB with which a number of activators, including NRs (Ing *et al.* 1992; Sadovsky *et al.* 1995; Schulman *et al.* 1995), have been shown to interact directly *in vitro*, has been investigated in GST pull-down experiments (vom Baur *et al.* 1996). As indicated in figure 11*a* and *c*, mSUG1 but not TIF1 was specifically retained on GST-TBP beads and weakly on GST-TFIIB beads, suggesting that mSUG1 and TIF1 may mediate the transcriptional activity of the AF-2 of NRs through distinct mechanims. Interestingly, mSUG1 did also interact with the TBP-associated factor, TAFII30, which is present in the TFIID complex and has been shown to bind to the N-terminal part of the E region of the ER (Jacq *et al.* 1994) (figure 11*b*). Thus mSUG1 may mediate the activation function(s) associated with the ER LBD to both TBP and TAF-containing TFIID complexes.

3. DISCUSSION

The activity of the ligand-dependent activation function AF-2 of NRs is believed to be mediated to the transcriptional machinery by intermediary factors/mediators. Our data suggest that TIF1 and mSUG1, two mouse proteins that directly interact in a ligand-dependent fashion with the AF-2/LBD-containing region E of several NRs *in vivo* as well as *in vitro*, served as such intermediary factors. The significance of these interactions for the AF-2 function is indicated by: (i) the potential of AF-2 antagonists to prevent these interactions; (ii) a correlation between effects of AF-2 AD mutations on these interactions and on the levels of NR-dependent transcription; and (iii) effects of overexpression of these proteins on NR-dependent, but not basal transcription in transfected cells (Le Douarin *et al.* 1995; vom Baur *et al.* 1996).

TIF1 is a novel widely expressed nuclear protein (see Le Douarin *et al.* 1995) which in addition to the region that has been shown to be sufficient for the interaction with NRs, contains several conserved domains found in a number of regulatory proteins, some of them are known to function as coactivators (e.g. the bromodomain and the PHD finger). The evidence presented here identified mSUG1 as the counterpart in mammalian cells of ySUG1 in yeast: both the mouse and the yeast proteins share ~ 75% sequence identity; the mouse protein can functionally substitute for ySUG1 in yeast; both proteins bind directly and specifically to activators, including NRs; both interact *in vitro* with TBP; both are subunits of the PA700 regulatory

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complex of the 26S proteasome (Ghislain *et al.* 1993; Akiyama *et al.* 1995; vom Baur *et al.* 1996). The yeast protein may be also a component of the recently discovered mediator complex (Kim *et al.* 1994), whose interaction with the C-terminal repeat of RNA polymerase II enables transcriptional activation (Carey 1995).

By comparing the interaction properties of a number of steroid and non-steroid receptors with both TIF1 and mSUG1, we demonstrated that: (i) the liganded RXRα and TRα exhibited a preferential binding to TIF1 and mSUG1 respectively, whereas RARα, VDR, and ER efficiently interacted with both mSUG1 and TIF1; (ii) the amphipatic α-helix core of the AF-2 AD was differentially involved in the interactions of RARα with TIF1 and mSUG1; and (iii) the AF-2 AD cores of RARα and ER were similarly involved in their interaction with TIF1, but not mSUG1. Thus the interaction interfaces between the various NRs and either TIF1 or mSUG1 may vary depending on the nature of both the receptor and the putative mediator. Moreover, in view of the conservation of the AF-2 AD core among the receptors, there are probably additional specific interactions with other region(s) of the LBD. In support of this idea, a dominant negative mutant bearing a point mutation in the N-terminal region of the LBD of RXRα has recently been identified that can bind ligand or dimerize, but can neither bind TIF1 *in vitro* nor transactivate *in vivo* (Renaud *et al.* 1996).

The possibility that mSUG1 and TIF1 may mediate the transcriptional activity of the AF-2 AD of NRs through distinct mechanisms (vom Baur *et al.* 1996) is supported by the evidence that mSUG1, but not TIF1 is able to contact directly TBP and less efficiently TFIIB *in vitro*. Although the relevance of these interactions to the NR function *in vivo* is not demonstrated, it suggests that mSUG1 might be a mediator linking the ligand-dependent AF-2 of NRs to the basal transcriptional machinery, possibly through a multiprotein complex similar to the yeast RNA polymerase II-associated mediator (Carey 1995). In view of its similarity to members of a large family of ATPases (Confalonieri & Duguet 1995), and the requirement of integrity of its putative ATP binding site for the interaction with the NRs (vom Baur *et al.* 1996), it is proposed that mSUG1 functions in an ATP-dependent fashion. As shown for other components of the PA700 proteosomal complex, NR-bound mSUG1 might act as an ATP-dependent molecular chaperone catalysing either conformational changes in the region E of the NRs or rearrangements (assembly/disassembly) of transcriptional protein complexes (e.g. RNA polymerase II and/or TFIID), that may be required for initiation of transcription. In marked contrast with mSUG1, no physical interaction has been detected between TIF1 and the general transcription factors TBP and TFIIB. The possibility that TIF1 is itself a TAF that does not directly bind TBP, but belongs to the TFIID complex, was investigated, because components of this complex have been shown to function as coactivators (Tjian & Maniatis 1994; Jacq *et al.* 1994). However, antibodies against TIF1 failed to cross-react with immunopurified TFIID, indicating that TIF1 is not associated with this complex (Le Douarin *et al.* 1995). Thus, it is conceivable that TIF1 may contact basal factors other than TBP and TFIIB, and/or may not directly contact the basal machinery, but instead chromatin-associated targets, as suggested for other global transcriptional regulators, such as the SWI2/SNF2 factors (Côté *et al.* 1994).

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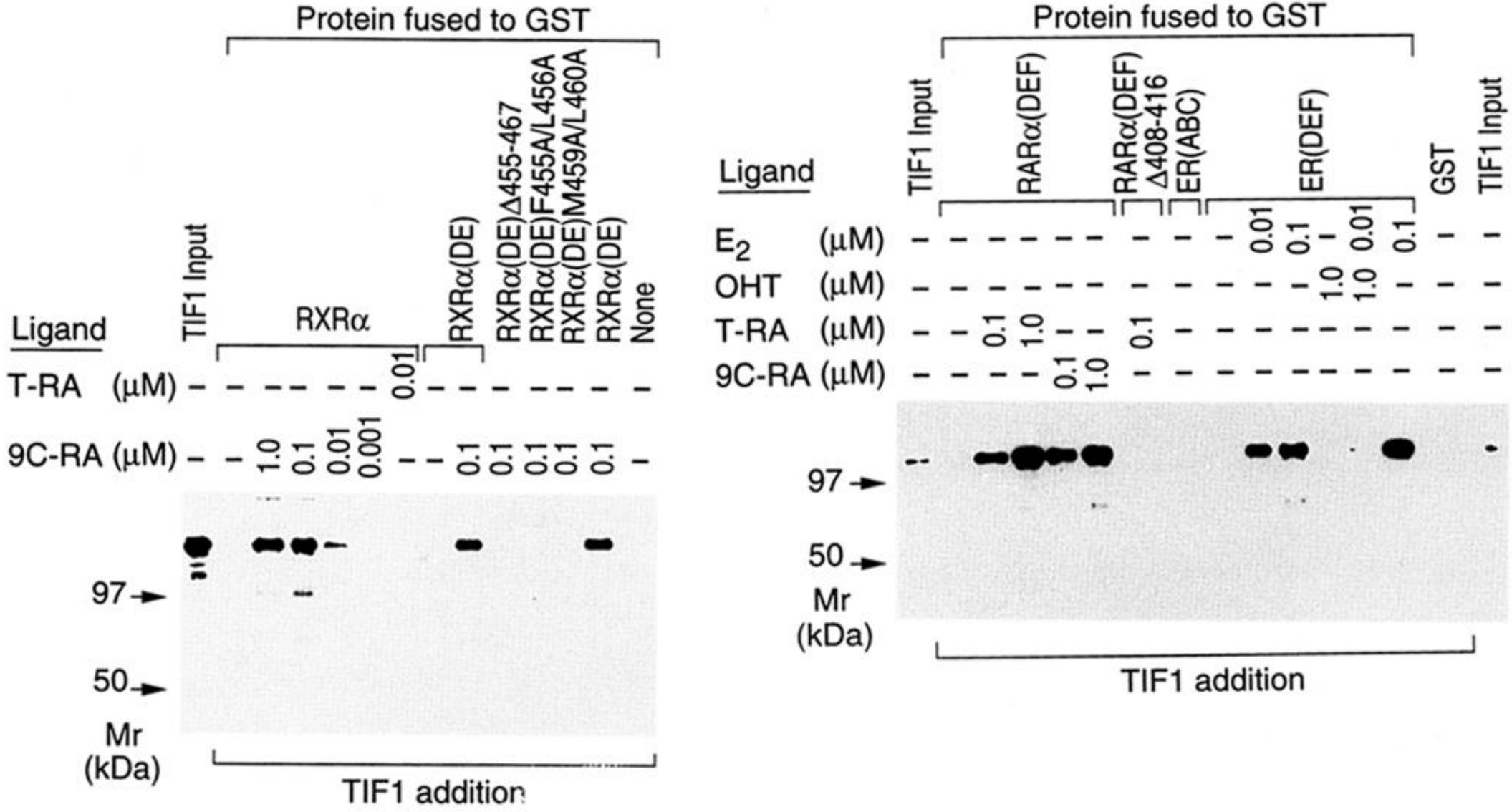
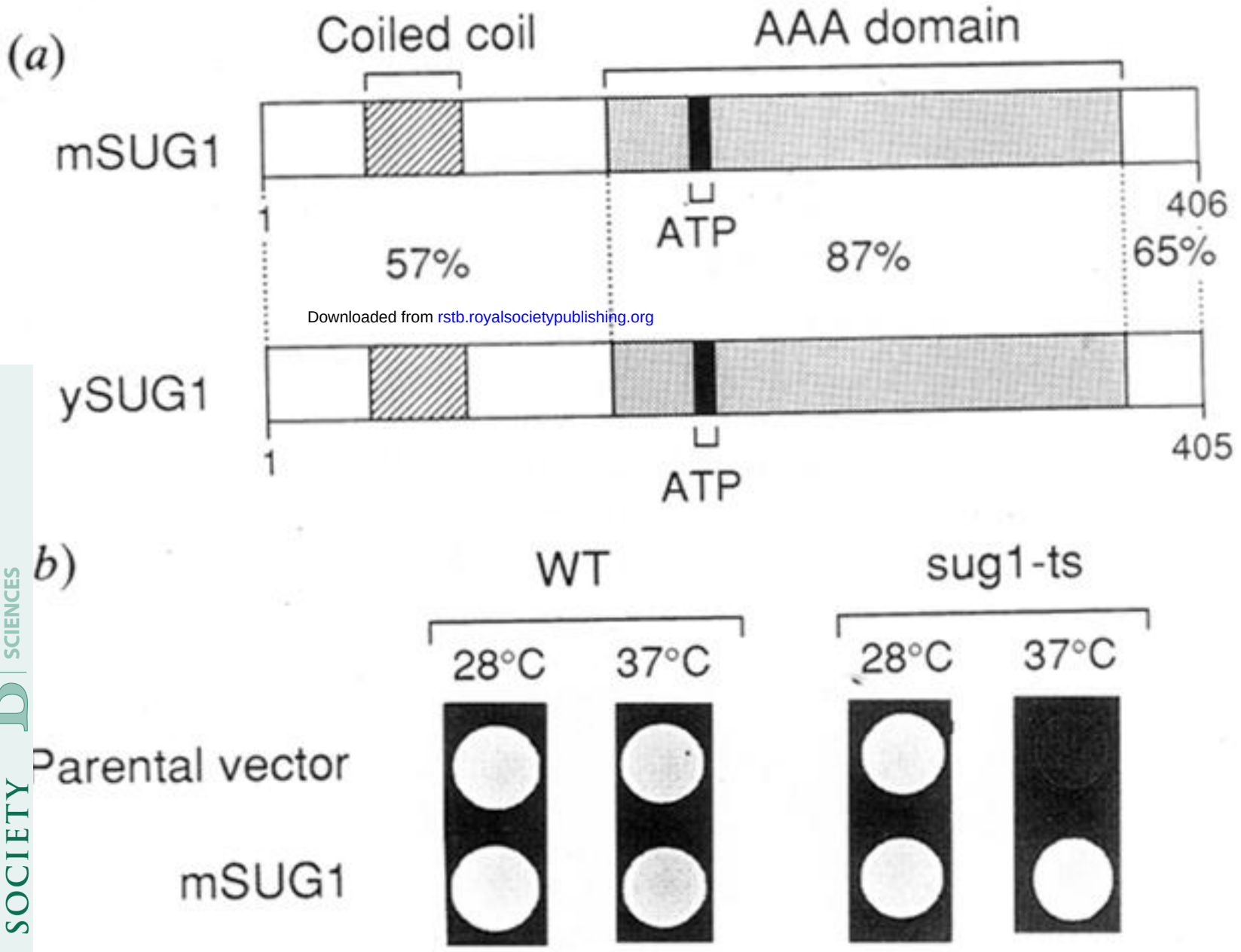
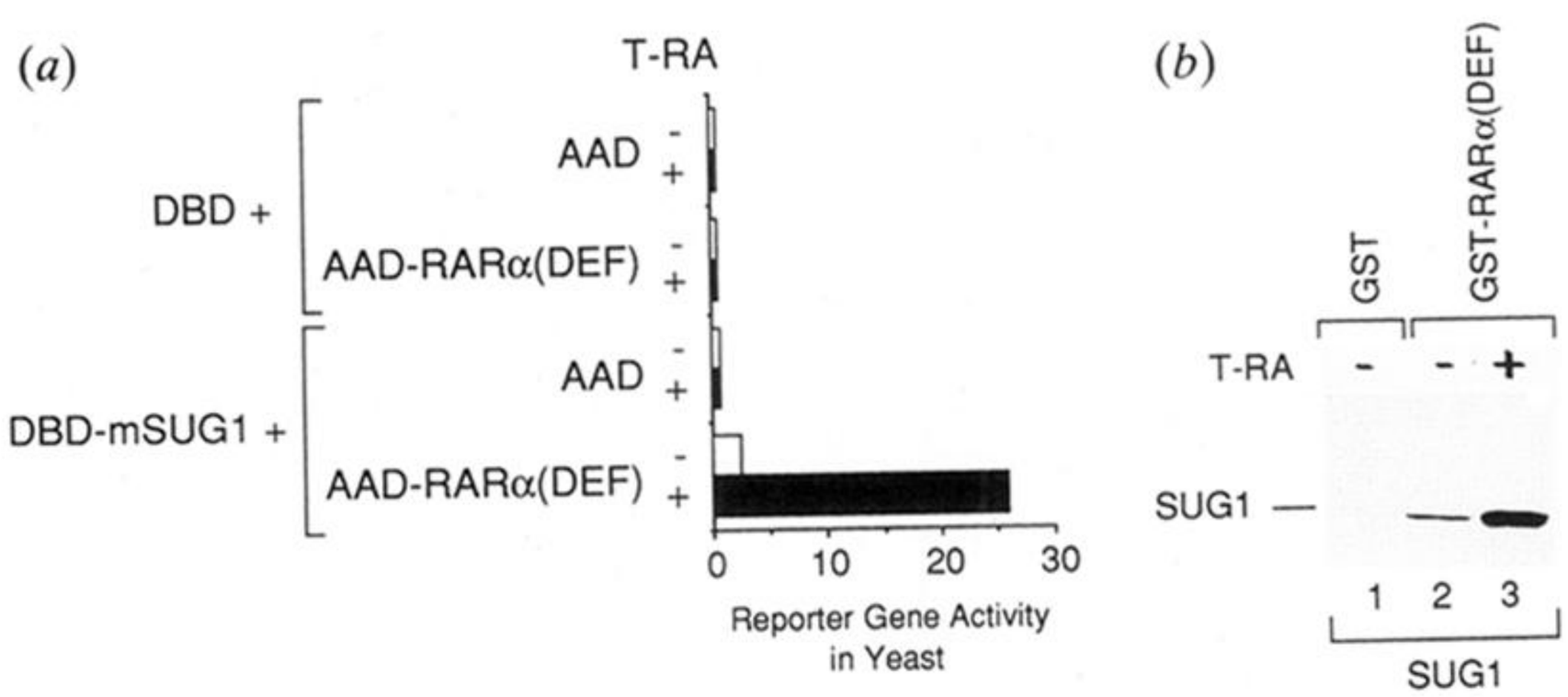


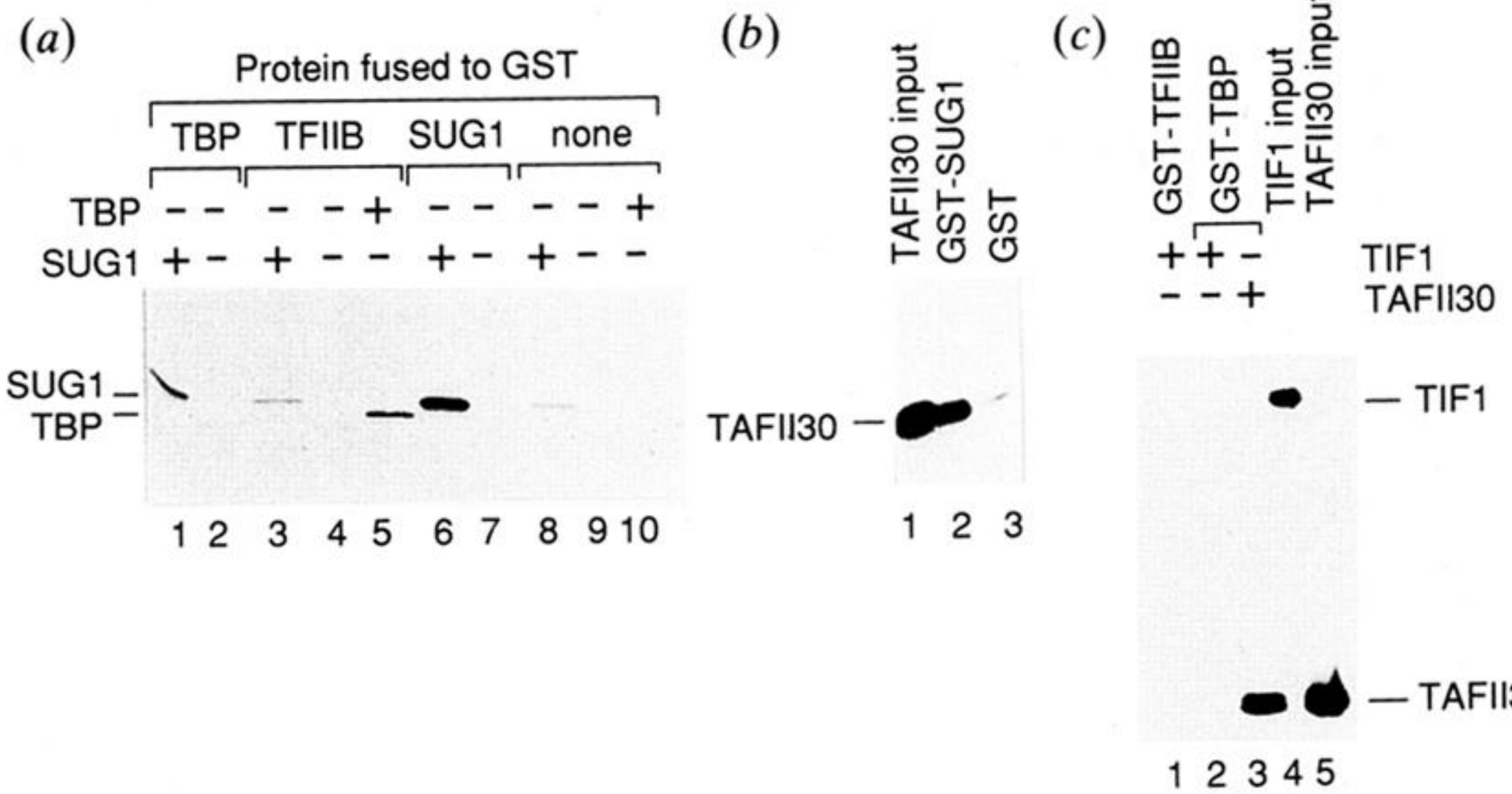
gure 4. TIF1 interacts *in vitro* with the liganded LBD of NRs. The indicated GST fusion proteins or GST alone ound to glutathione-Sepharose beads were incubated with purified histidine-tagged TIF1 in the presence or absence ligands, as indicated. Bound proteins were identified by Western blotting (see also Le Douarin *et al.* 1995).



gure 7. mSUG1 is the mouse homologue of the yeast otein SUG1 (ySUG1). The upper part of the figure shows schematic alignment of mouse and yeast SUG1. The tative coiled coil and conserved AAA (for ATPases sociated to a variety of cellular Activities; see Confalonieri Duguet 1995) domains are represented as well as the nserved ATP binding site motif. The percentage of amino id identity is given for the N- and C- terminal regions, as ell as the AAA domain. The lower part of the figure shows e yeast complementation assay indicating that mSUG1 can scue the conditional lethality of a sug1 mutant. Isogenic ast strains carrying the wild type (WT) SUG1 allele or a ermosensitive sug1-ts allele were transformed with a ulticopy expression vector containing no insert or the ding sequence of mSUG1. The growth phenotypes of the ansformants were assayed by using a spot test on agar edium at 30 °C and 37 °C. Plates were incubated for 3 days d photographed (see also vom Baur *et al.* 1996).



gure 8. mSUG1 interacts with the DEF region of RARα both in yeast and *in vitro*. (*a*) A plasmid expressing mSUG1 sed to the ER DBD was introduced into the yeast reporter strain PL3 together with VP16 AAD or the AAD-ARα(DEF) fusion construct. Transformants were grown in liquid medium containing uracil in the presence or sence of 500 nM T-RA. Reporter gene activities are expressed as in figure 1. (*b*) *In vitro* interaction between ^{35}S-beled mSUG1 and GST alone (lane 1) or a GST-RARα(DEF) fusion protein (lanes 2 and 3) was tested using the ll-down assay in the absence or presence of 1 μM T-RA (see also vom Baur *et al.* 1996).



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