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# Interactions between the retinoid X receptor and a conserved region of the TATA-binding protein mediate hormone-dependent transactivation

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ABSTRACT **The retinoid X receptor (RXR) participates in a wide array of hormonal signaling pathways, either as a homodimer or as a heterodimer, with other members of the steroid and thyroid hormone receptor superfamily. In this report the ligand-dependent transactivation function of RXR has been characterized, and the ability of RXR to interact with components of the basal transcription machinery has been examined. *In vivo* and *in vitro* experiments indicate the RXR ligand-binding domain makes a direct, specific, and ligand-dependent contact with a highly conserved region of the TATA-binding protein. The ability of mutations that reduce ligand-dependent transcription by RXR to disrupt the RXR–TATA-binding protein interaction *in vivo* and *in vitro* suggests that RXR makes direct contact with the basal transcription machinery to achieve activation.**

Members of the steroid and thyroid hormone receptor (TR) superfamily regulate expression of complex gene networks involved in vertebrate development, differentiation, and homeostasis. A defining characteristic of these receptors lies in part in their ability to function as ligand-activated transcription factors. Retinoid X receptors (RXRs) occupy a central position in the function and activity of receptors for thyroid hormones and vitamins. By forming heterodimers with retinoic acid receptors (RARs), TRs, vitamin D receptors (VDRs), peroxisome proliferator activated receptors (PPARs), and several orphan receptors, RXRs participate in a diverse array of signaling pathways (1). The ability of RXR homodimers to respond to 9-*cis*-retinoic acid identifies still another signaling pathway influenced by this nuclear receptor. The critical role for RXRs in the function of nuclear receptors is further highlighted by the structural and functional conservation between vertebrate RXRs and the *Drosophila* nuclear receptor ultraspiricle (2).

The mechanism by which RXR (and other nuclear receptors) activates transcription is poorly understood. Numerous studies have defined two independent transactivation functions (tau domains, $\tau$) in most members of the steroid and TR superfamily. These activation functions include a constitutive activation function ($\tau$1 or AF-1) present in the amino-terminal region and a ligand-dependent activation function ($\tau$c or AF-2) present in the carboxyl-terminal 200–250 amino acids. The carboxyl-terminal domain of nuclear receptors is complex, mediating ligand-dependent activation, receptor homo- and heterodimerization, and ligand binding. Binding of ligand is thought to induce a conformational change in receptors that leads to activation of transcription (3, 4).

How activated receptors propagate their signals to the basal transcription machinery is not known. Direct interactions between the basal transcription factor TFIIB and several nuclear receptors have been reported (5–9). The nuclear receptor–TFIIB interaction does not appear to be influenced by ligand, and it has been suggested that interaction between TR and TFIIB may be associated with transcriptional repression (6, 7). The identification of several proteins suggested to be involved in ligand-activated transcription by nuclear receptors (10–14) suggests that coactivators or bridging factors may also be involved in transmitting the signal from ligand-activated receptors to the basal transcription apparatus.

Recently, a small region at the carboxyl terminus of RXR has been identified that is required for ligand-activated transcription (refs. 15 and 16; I.G.S., unpublished data). This activation domain ($\tau$c), which is conserved among most members of the steroid and TR superfamily (17), functions as a constitutive activator when fused to a heterologous DNA-binding domain. The ability of the RXR $\tau$c domain to function in both mammalian and *Saccharomyces cerevisiae* cells suggests that the pathway of activation mediated by RXR is conserved. In this report, both *in vivo* and *in vitro* experiments indicate that the RXR $\tau$c domain mediates an interaction between the RXR ligand-binding domain and the conserved carboxyl-terminal domain of the TATA-binding protein (TBP). Mutations in either the RXR $\tau$c domain or in TBP disrupt this interaction, suggesting that the RXR–TBP interaction plays a functional role in transactivation by RXR.

## MATERIALS AND METHODS

**Plasmids.** For integration in *S. cerevisiae*, plasmid pRS305CYH was constructed by cloning a *Bgl* II–*Sal* I restriction fragment from pAS1-CYH2 (gift of S. Elledge, Baylor College of Medicine, Houston) into *Bam*HI/*Sal* I-digested pRS305 (18). For expression of GAL4 DNA-binding domain fusions of human TBP mutants in *S. cerevisiae* (see below), plasmid pG6H was constructed by PCR amplification of the GAL4 DNA-binding domain from pAS1-CYH2. The amplified product was ligated into *Bam*HI-digested pG-1 (19). For expression of GAL4 DNA-binding domain fusions in CV-1 cells, the plasmid pCMXG4epi was constructed by PCR amplification of the GAL4 DNA-binding domain and ligation with *Hin*dIII/*Bam*HI-digested pCMX (20).

Receptor ligand-binding domain fusions were cloned by PCR amplification of human RXR$\alpha$ (amino acids 197–462 and 197–443), human RAR$\alpha$ (amino acids 186–462), and human TR$\alpha$ (amino acids 121–410) DNA. For $\tau$c domain fusions, DNA encoding amino acids 444–462 of human RXR$\alpha$ and 391–410 of human TR$\alpha$ was amplified by PCR. Point mutations were introduced into the RXR $\tau$c domain by PCR using oligonucleotides with the appropriate base changes. Amplified products were ligated into *Nco* I/*Bam*HI-digested pRS305CYH. For expression in CV-1 cells, appropriate restriction fragments from the pRS305CYH clones were isolated and ligated into pCMXG4epi.

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Abbreviations: RXR, retinoid X receptor; RAR, retinoic acid receptor; TR, thyroid hormone receptor; TBP, TATA-binding protein; GST, glutathione *S*-transferase; TK, thymidine kinase.

For two-hybrid assays, GAL4-activation domain fusions of RXR, RAR, and TR were constructed by cloning the same amplification products described above into *Nco* I/*Bam*HI-digested pACTII (S. Elledge). GAL4 DNA-binding domain fusions expressing the carboxyl-terminal domain of human TBP (pAS+h180c), full-length *Drosophila* TAF110 (pAS+dTAF110), or full-length *Drosophila* TAF40 (pAS+dTAF40) were provided by G. Gill and R. Tjian (University of California, Berkeley). Human TBP DNA (encoding amino acids 155–335) was amplified by PCR in two fragments. Point mutations were introduced into the appropriate oligonucleotides. After PCR, the two fragments were cloned by ligation into *Nco* I/*Bam*HI-digested pG6H.

Glutathione *S*-transferase (GST)–RXR197–462 was constructed by PCR amplification of the appropriate sequences from human RXRα. The amplification products were cloned into *Eco*RI/*Bam*HI-digested pGEX2TK. All PCR-derived constructs were verified by DNA sequencing. The plasmid pGEX-TBP was the kind gift of I. Verma (The Salk Institute). Mammalian expression constructs expressing the ligand-binding domains of human RXRα, human RARα, and human TRβ have been described elsewhere (21). The luciferase reporter construct GAL3-TK-LUC containing three binding sites for GAL4 upstream of the thymidine kinase (TK) promoter luciferase fusion was the gift of P. N. Rangarajan (The Salk Institute).

**Yeast Strains and Methods.** The strain Y190 (MATa *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 cyh*$^r$ *URA3::GAL → lacZ LYS2::GAL → HIS3*; gift of S. Elledge) was used for all experiments. For β-galactosidase assays, a minimum of three independent transformants were grown overnight at 30°C in minimal medium [0.66% yeast nitrogen base, 2% (wt/vol) glucose] supplemented with the appropriate amino acids. Cells were diluted 1:20 into fresh medium, and 9-*cis*-retinoic acid or 3,3′,5-triiodthyroacetic acid (TRIAC) was added if required, as described in the figure legends. β-Galactosidase activity was measured after 16 h of growth at 30°C, as described by Rose *et al.* (22).

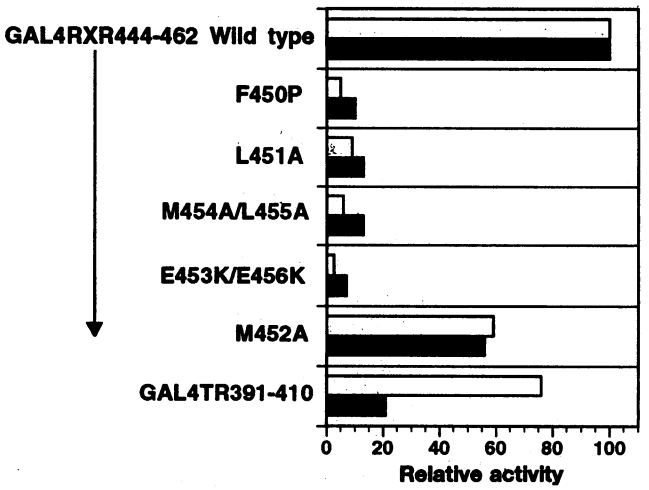


FIG. 1. Point mutations in the RXR τc domain reduce transactivation. CV-1 cells were transfected with DNA encoding fusions between the GAL4 DNA-binding domain and either the last 19 amino acids of human RXRα (amino acids 444–462) or the last 20 amino acids of human TRα (amino acids 391–410), along with the reporter construct GAL3-TK-LUC (solid bars), or the same fusion constructs were integrated into the genome of *S. cerevisiae* strain Y190 (open bars) containing an integrated GAL1-lacZ reporter (see *Materials and Methods*). CV-1 cell transfection results were normalized by cotransfection with a β-galactosidase expression plasmid (see *Materials and Methods*). Single point mutations were made in GAL4RXR444–462. The activity of wild-type GAL4RXR444–462 was set at 100%. Western blotting of *S. cerevisiae* extracts indicates the GAL4 fusions are expressed at similar levels.

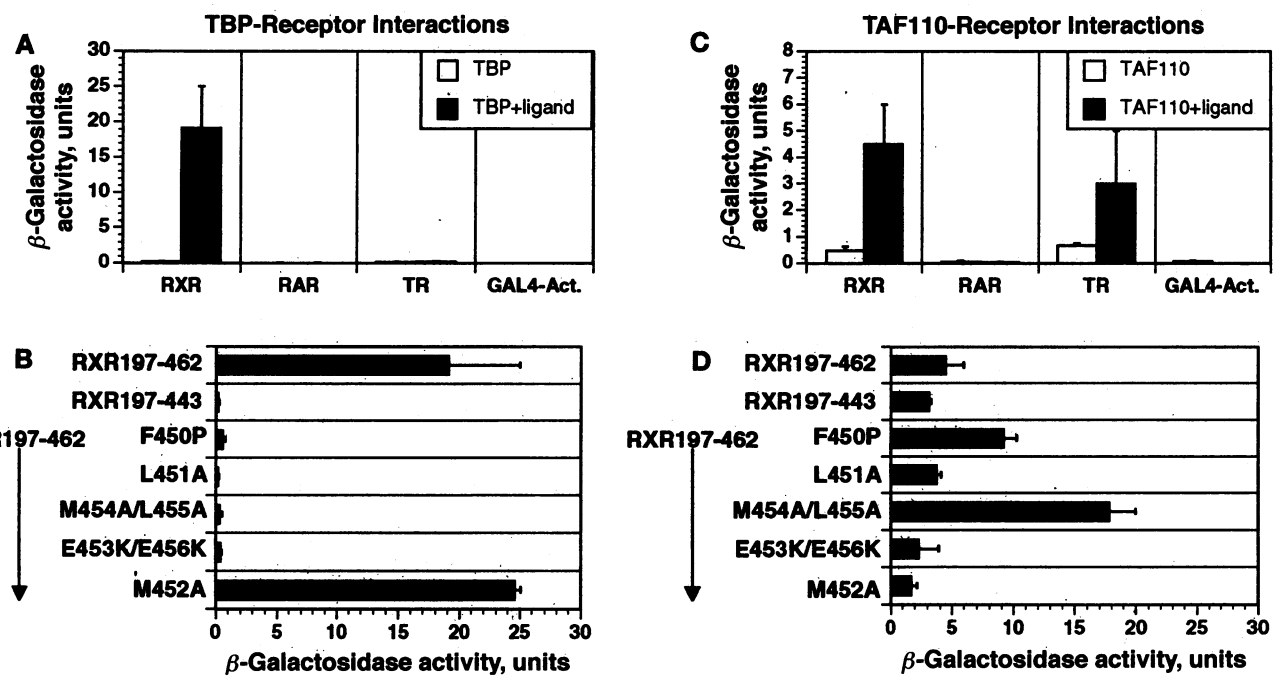


FIG. 2. The yeast two-hybrid assay detects ligand-dependent interactions between RXR and TBP. DNAs encoding fusions between the GAL4-activation domain and the ligand-binding domains of RXR, RAR, TR, and RXR τc mutants (see *Materials and Methods*) were introduced into the strain Y190, along with DNA encoding fusions between the GAL4 DNA-binding domain and the conserved carboxyl-terminal domain of human TBP (*A* and *B*) or full-length *Drosophila* TAF110 (*C* and *D*). β-Galactosidase activity was measured after growth for 16 h in the presence (solid bars) or absence (open bars) of receptor ligand [1 μM 9-*cis*-retinoic acid (RXR and RAR) or 1 μM 3,3′,5-triiodothyroacetic acid (TR)]. (*A* and *C*) Interaction between receptor ligand-binding domains and either TBP (*A*) or TAF110 (*C*). The activity of the GAL4-activation domain alone was measured only in the absence of ligand. (*B* and *D*) Interaction between RXR ligand-binding-domain mutants and either TBP (*B*) or TAF110 (*D*). Only activity in the presence of 9-*cis*-retinoic acid is shown. Point mutants consist of amino acids 197–462 of RXR. RXR197–443 represents the τc truncation. Western blotting of *S. cerevisiae* extracts indicates that the GAL4-activation domain fusions are expressed at similar levels.

**Transfection.** CV-1 cells were plated in 48-well plates at a density of $2 \times 10^4$ cells per well in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) charcoal-resin split fetal bovine serum. After growth at 37°C for 12–16 h, cells were transfected by using the DOTAP transfection reagent following the manufacturer's (Boehringer Mannheim) instructions. For each well, cells were transfected with 12 ng of GAL3-TK-LUC reporter, 36 ng of the appropriate expression constructs, and, as an internal control, 60 ng of pCMX-βgal DNA. After 5 h at 37°C, the medium was removed, the cells were washed once, and 200 μl of fresh medium was added. Cells were harvested after 36 h of growth at 37°C. Luciferase activity of each sample was normalized by the level of β-galactosidase activity. Each transfection was carried out in duplicate and repeated at least three times. The fold induction reported is relative to the activity of the GAL3-TK-LUC reporter alone included in each experiment.

**RXR–TBP Interaction Assay.** GST-fusion proteins were induced, solublized, and bound to glutathione beads following the manufacturer's procedures (Pharmacia LKB). After binding to glutathione beads, 15 μl of the suspension was incubated with 1–2 μl of the appropriate $^{35}$S-labeled, *in vitro* translated protein for 1 h in 500 μl of NETN (20 mM Tris·HCl, pH 7.5/100 mM KCl/0.7 mM EDTA/0.5% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride). Following incubation, the beads were washed three times with NETN. Bound proteins were eluted with 20 μl of 1× SDS/PAGE buffer and electrophoretically separated in a SDS/10% polyacrylamide gel. The interaction of *in vitro* translated GAL4 fusions with GST–TBP was carried out by using the above procedure with the following modifications. The initial interaction was carried out in NETN in which KCl was replaced with 0.3 M NaCl and nonfat dry milk was added to final concentration of 0.5%. Following incubation, the beads were washed three times with NETN in which the NaCl concentration was increased to 0.5 M and nonfat dry milk was added to final concentration of 0.5%.

## RESULTS

The carboxyl-terminal 19 amino acids of RXR and 20 amino acids of TR have been shown to activate transcription when fused to heterologous DNA-binding domains (Fig. 1; refs. 15, 16, and 23–25). This region has been proposed to form an amphipathic α-helix with hydrophobic and negatively charged faces (17). Mutation of the carboxyl-terminal 19 amino acids of RXR (Fig. 1) indicates that, like several other transactivation domains, the hydrophobic and acidic amino acids are critically important for function (26). Within the hydrophobic face of the helix, individual changes of phenylalanine at position 450 to proline (F450P), leucine at position 451 to alanine (L451A), and the double mutant methionine 454 to alanine/leucine 455 to alanine (M454A/L455A) severely reduce the ability of GAL4 fusions to activate transcription when assayed in the context of the isolated τc domain in both mammalian and *S. cerevisiae* cells. The double mutant glutamic acid 453 to lysine/glutamic acid 456 to lysine (E453K/E456K) on the charged face of the helix also eliminates the ability of the isolated τc domain to activate transcription (Fig. 1). The single mutations E453K and E456K reduce transcription ≈60–70% (data not shown). Mutation of methionine 452 to alanine (M452A), however, has little effect. Incorporation of these same mutations into the complete ligand-binding domain or into full-length receptors reduces the ability of these mutant RXRs to activate transcription in response to RXR-specific ligands. Removal of the 19 amino acids (RXR197–443) also produces a receptor that fails to activate transcription (I.G.S., unpublished data). Taken together, the results confirm that the last 19 amino acids of RXR are both necessary and sufficient for transactivation and indicate that both the hydrophobic and charged faces of the helix residues are important for this function.

The finding that mutations in the RXR τc domain have qualitatively similar effects in mammalian and *S. cerevisiae* cells (Fig. 1) suggests that RXR directly contacts a structurally and functionally conserved component of the transcription machinery. This observation is consistent with the finding that several other transcription factors, including members of the steroid and TR superfamily, interact with components of the basal transcription machinery (5–9). Therefore, we examined the interactions between RXR and several basal transcription factors, including the TBP, TAF110, TAF40, and TFIIB, by using both the yeast two-hybrid system (27, 28) and *in vitro* protein–protein interaction assays. As shown in Fig. 2*A*, the two-hybrid assay detects a specific and ligand-dependent interaction between RXR and the conserved carboxyl-terminal domain of TBP. Mutations in the RXR τc domain that eliminate the ability of RXR to activate transcription (Fig. 1) eliminate a detectable interaction between RXR and TBP (Fig. 2*B*). Although TR and RAR have τc domains that exhibit significant sequence similarity to the RXR τc domain (17), an interaction between TR or RAR and TBP is not detected (Fig.

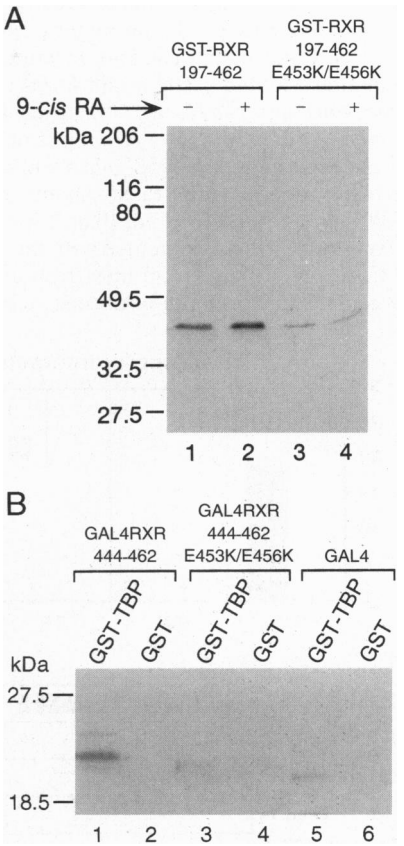


FIG. 3. RXR and TBP interact *in vitro*. (*A*) TBP was *in vitro* translated as described in *Materials and Methods* and incubated with equal amounts of immobilized GST–RXR197–462 (lanes 1 and 2) or GST–RXR197–462-E453K/E456K (lanes 3 and 4), as determined by Coomassie-stained gels. Following extensive washing of the beads, bound proteins were eluted and resolved by SDS/PAGE, and the gel was processed for autoradiography. For the samples in lanes 2 and 4, 1 μM 9-*cis*-retinoic acid (9-*cis* RA) was included in all buffers. Exposure time was 2 h. Little or no interaction between TBP and GST alone is detected under these conditions. (*B*) Equal amounts of *in vitro* translated GAL4RXR444–462, GAL4RXR444–462-E453K/E456K, or GAL4(1–147), as determined by phosphorimaging analysis, were incubated with immobilized GST–TBP (lanes 1, 3, and 5) or with immobilized GST (lanes 2, 4, and 6). Following extensive washing of the beads, bound proteins were eluted and resolved by SDS/PAGE, and the gel was processed for autoradiography. Exposure time was 7 h.

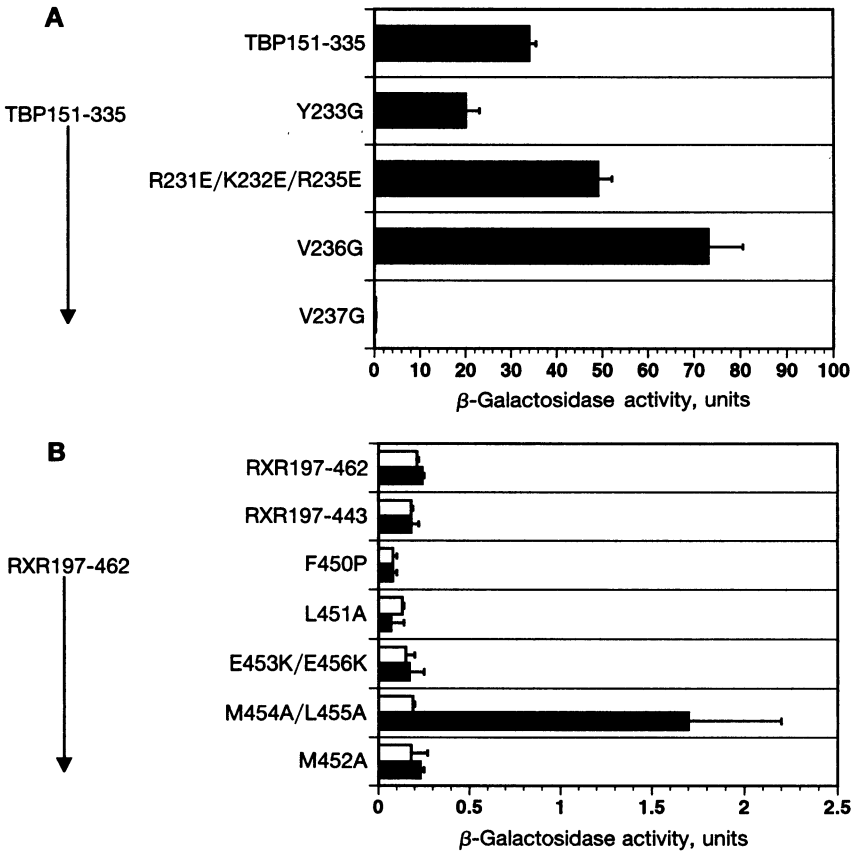


FIG. 4. A point mutation in the basic repeat of TBP disrupts the interaction with RXR *in vivo*. (*A*) DNA encoding a fusion between the GAL4-activation domain and RXR (amino acids 197–462) was introduced into the strain Y190 along with DNA encoding fusions between the GAL4 DNA-binding domain and human TBP (the conserved carboxyl-terminal domain; amino acids 151–335). Y233G, R321E/K232E/R235E, V236G, and V237G identify the amino acid changes introduced into TBP. β-Galactosidase activity was measured after growth for 16 h in the presence of 1 μM 9-*cis* retinoic acid, as described in *Materials and Methods*. Western blotting of *S. cerevisiae* extracts indicates the GAL4–TBP fusions are expressed at similar levels. (*B*) DNA encoding fusions between the GAL4-activation domain and RXR τc mutants (amino acids 197–462 of RXR) was introduced into the strain Y190, along with DNA encoding a fusion between the GAL4 DNA-binding domain and TBP-V237G. β-Galactosidase activity was measured after growth for 16 h in the presence (solid bars) or absence (open bars) of 1 μM 9-*cis* retinoic acid, as described in *Materials and Methods*. RXR197–443 represents the τc truncation.

2*A*). Nevertheless, the same region of TR activates transcription in *S. cerevisiae* when fused to the GAL4 DNA-binding domain (data not shown). The failure to detect an interaction between RAR and TBP or between TR and TBP suggests that transactivation by RXR homodimers may utilize different components of the transcription machinery than transactivation by RAR and TR heterodimers.

Fig. 2*C* also shows that RXR can make a ligand-dependent interaction with a second component of the TFIID complex, TAF110. The interaction between RXR and TAF110 is detectable even when τc domain mutants are analyzed, indicating that the functional state of the τc domain is not important for the interaction (Fig. 2*D*). Nevertheless, the ability to detect ligand-dependent interactions between transcriptionally defective RXR mutants and TAF110 suggests that mutations in the RXR τc domain do not have large effects on ligand binding. The observation that TR also interacts with TAF110 (Fig. 2*C*) suggests that this basal factor may be a common target for multiple nuclear receptors.

Although the results of the two-hybrid assay suggest that RXR makes a direct protein–protein interaction with TBP, the possibility that this interaction is mediated by a conserved coactivator cannot be ruled out by this assay. To further characterize the interaction between RXR and TBP, the ability of TBP to interact *in vitro* with bacterially expressed GST–RXR fusion proteins was examined. The GST pull-down experiment shown in Fig. 3*A* reveals a strong interaction between *in vitro* translated TBP and GST–RXR197–462 (lanes 1 and 2). An *in vitro* interaction between GST–RXR197–462 and TAF110 is also observed (data not shown). A mutation of the RXR τc domain (E453K/E456K) that eliminates the RXR–TBP interaction in the two-hybrid assay (Fig. 2*B*) reduces the *in vitro* interaction between RXR and TBP by ≈85% (Fig. 3*A*, compare lane 2 with lane 4). Similar results are observed when a full-length GST–RXR fusion is used (D.C. and R.M.E., unpublished data). A direct *in vitro* interaction between TBP and the τc domain itself (GAL4RXR444–462) that is sensitive to the functional state of the τc domain can also be detected (Fig. 3*B*). The sensitivity of the *in vitro* interactions to mutations in the RXR τc domain strongly suggests that the τc domain mediates a direct interaction between RXR and TBP.

Unlike the two-hybrid assay, an RXR–TBP interaction *in vitro* can be detected in the absence of ligand. Addition of ligand stimulates the interaction 3- to 5-fold when quantitated by phosphorimaging (Fig. 3*A*, compare lanes 1 and 2). The detection of ligand-independent interactions *in vitro* may result from the ability of the large amounts of protein used *in vitro* to stabilize a weak interaction that cannot be detected in the two-hybrid assay.

To further define the RXR–TBP interaction, mutations were introduced into well-conserved amino acids present in the basic repeat of TBP and analyzed for interaction with RXR in the two-hybrid assay. This domain of TBP has been shown to be a common target of several transcription factors (29, 30). Fig. 4*A* shows that the TBP mutant V237G eliminates a detectable RXR–TBP interaction. Several other mutations in this region of TBP, including V236G, have no effect (Fig. 4*A*).

The finding that a single point mutation in TBP could disrupt the interaction with the wild-type RXR ligand-binding domain prompted an examination of the ability of TBP-V237G to interact with the RXR τc mutants. As shown in Fig. 4*B*, a positive and ligand-dependent interaction can be detected between TBP-V237G and a single RXR τc-domain mutant, M454A/L455A. Although the interaction detected between TBP-V237G and RXR-M454A/L455A is weak relative to the wild-type interaction, a ≈10-fold, ligand-dependent induction of the interaction is observed (Fig. 4*B*). Rescue of the RXR–TBP interaction by combining an RXR τc-domain mutant with a TBP mutant strongly suggests the RXR–TBP interaction detected in the two-hybrid assay results from a direct protein–protein interaction and is not mediated by a third factor.

## DISCUSSION

In this study, we have taken advantage of mutations of the ligand-dependent activation function (τc) of RXR to examine the role of this domain in ligand-dependent transactivation. The τc domain encodes a potential amphipathic α-helix with hydrophobic and negatively charged faces. This domain is necessary for ligand-dependent activation of transcription by RXR and is sufficient to activate transcription when fused to a heterologous DNA-binding domain in both mammalian cells and *S. cerevisiae* (Fig. 1). By using both the yeast two-hybrid assay and *in vitro* GST pull-down experiments, the RXR ligand-binding domain has been shown to make a direct and specific contact with the basic repeat present in the conserved carboxyl-terminal domain of the TBP (Figs. 2–4). The ability of mutations in the τc domain that reduce the transactivation ability of RXR to disrupt the RXR–TBP interaction *in vivo* and *in vitro* suggests that this interaction has functional significance.

In the two-hybrid assay,the RXR–TBP interaction is ligand dependent. The two-hybrid assays are carried out with ligand concentrations well above the $K_d$ value of 9-*cis*-retinoic acid (31), so that small changes in ligand affinity would not be expected to have significant effects. The ability of RXR τc-domain mutants to interact in a ligand-dependent fashion with TAF110 (Fig. 2*D*) and to bind ligand *in vitro* (I.G.S., unpublished data) indicates that the absence of an interaction between the mutants and TBP does not result from a defect in ligand binding. Taken together, these results suggest that the RXR τc domain directly interacts with TBP and that this interaction is regulated by ligand. This conclusion is supported by the *in vitro* interaction between a GAL4–τc domain fusion and TBP (Fig. 3*B*). Finally, the ability to recover an interaction between a RXR τc-domain mutant and TBP by introducing a second site mutation in TBP (Fig. 4*B*) further supports the conclusion that the τc domain makes a direct interaction with TBP. Interestingly, the activation domains of several other transcription factors, including Fos and E1A, have been shown to interact with the same region of TBP (29, 30). The ability of multiple factors to contact the basic repeat of TBP suggests that interaction with this domain of TBP may represent a common mechanism for transactivation.

The observation that RAR and TR do not interact with TBP (Fig. 2*A*) suggests that different RXR/nuclear receptor heterodimers may activate transcription by contacting different components of the transcriptional machinery. This conclusion is consistent with our recent results showing that the ligand responsiveness of RXR can be modified by heterodimeric pairing (21). The ability of mutations in the RXR τc domain to adversely affect transactivation by heterodimers (I.G.S., unpublished data) suggests that when complexed as a heterodimer the RXR τc domain may be redirected to a different coactivator or component of the basal transcription machinery.

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