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Differential Recognition of Liganded and Unliganded Thyroid Hormone Receptor by Retinoid X Receptor Regulates Transcriptional Repression

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Thyroid hormone receptor (TR) functions as part of multiprotein complexes that also include retinoid X receptor (RXR) and transcriptional coregulators. We have found that both the TR CoR box and ninth heptad are required for RXR interaction and in turn for interaction with corepressor proteins N-CoR and SMRT. Remarkably, the recruitment of RXR to repression-defective CoR box and ninth-heptad mutants via a heterologous dimerization interface restores both corepressor interaction and repression. The addition of thyroid hormone obviates the CoR box requirement for RXR interaction, provided that the AF2 activation helix at the C terminus of TR is intact. These results indicate that RXR differentially recognizes the unliganded and liganded conformations of TR and that these differences appear to play a major role in the recruitment of corepressors to TR-RXR heterodimers.

Thyroid hormone receptor (TR) is a multifunctional protein. It is a transcriptional repressor in the absence of ligand and a transcriptional activator in the presence of thyroid hormone (T3). Repression is mediated by interaction with a family of corepressor proteins, including N-CoR and SMRT (10, 17, 34, 45). A ligand-induced conformational change causes dissociation of the corepressor and recruitment of a transcriptional coactivator to the DNA-bound TR. TR binds DNA most effectively as a heterodimer with retinoid X receptor (RXR) (6, 19, 22, 23, 25, 44, 47). Although RXR itself is a retinoid receptor (24), its main function in TR action does not appear to require retinoid binding (14). The TR-RXR interaction is stable in solution in both the presence and absence of ligand, and it has previously been suggested that the primary role of RXR is to increase the affinity and specificity of TR for T3 response elements, which often consist of two TR half-sites separated by 4 bp (39).

The primary region of importance for the TR-RXR interaction in solution is the region which is generally known as the ninth heptad, corresponding to helices 10 and 11 of the crystal structure of the TR ligand binding domain (LBD) (40). The importance of this domain explains why C-terminal deletions of TR and the C-terminal variant TRα2 do not interact with RXR in solution (31, 42). Interestingly, although the interaction between TR and RXR is ligand independent, two groups have previously described TR ninth-heptad point mutants which interact with RXR only in the presence of T3 (1, 28). Although the TR LBD structure has been solved only in the presence of ligand, a comparison with the unliganded RXR suggests that one of the effects of ligand binding is conformational change in the region between helices 10 and 11 in addition to the turning back of the amphipathic AF2 helix (helix 12) towards the core of the TR (5, 40).

In contrast to the importance of C-terminal domains for RXR interaction, previous studies of TR interaction with N-CoR and SMRT have focused on the CoR box within the hinge region of TR (10, 17). Like the ninth heptad, the CoR box is highly conserved among receptors that interact with RXR, and mutations within the CoR box motif prevent corepressor interaction. Notably, early studies of repression by TR that were conducted prior to the cloning of N-CoR and SMRT indicated that in addition to the CoR box, the ninth heptad was required for repression by TR (2, 8).

We found that ninth-heptad mutants of TR are not transcriptional repressors, although they contain a CoR box that is identical to that of other TRs. As predicted, CoR box mutants of TR did not interact with corepressors. Surprisingly, however, they also did not interact with RXR in the absence of T3. T3 binding obviated the need for an intact CoR box for TR-RXR interaction in vivo. This suggested that RXR differentially recognizes the liganded and unliganded conformations of TR. We therefore investigated the effects of mutations in the C-terminal amphipathic helix (AF2), whose movement comprises the most significant structural change associated with ligand binding. The data indicate that in the absence of T3, the CoR box and the ninth heptad of TR contribute to RXR interactions and that these interactions correlate functionally with the ability to bind N-CoR and SMRT and repress transcription. In the presence of T3, CoR box and ninth-heptad mutants were able to bind RXR, but this was dependent upon AF2. Thus, the liganded state of TR qualitatively regulates its interaction with RXR and these differential interactions modulate TR function in vivo.

MATERIALS AND METHODS

Vectors. The plasmid vectors used in this study were pCMX (gift of R. Evans), pCMX-Gal4 (1-147) (45), pGEX-2T (Pharmacia), pBluescript (pBS; Stratagene), and pSG5 (Stratagene).

Receptor expression constructs. Rat TRα1, TRα1Δ347 and TRα2 in pCMX have been previously described (31). pSG5-RXRα was a gift of P. Chambon. pCMX-Gal4-RXRα, which contains human RXRα LBD (positions 203 to 462) fused to the Gal4 DNA binding domain (DBD) (positions 1 to 147), and pCMX-VP16-RXRα, which contains the same region of human RXRα fused to the VP16 activation domain, were gifts of R. Evans. pCMX-TRα1(9Hα2), pCMX-TRα2(9Hα1), and pCMX-TRα1Δ378 were made from pBS-TRα1(9Hα2), pBS-TRα2(9Hα1), and pBS-TRα1Δ378 (gifts of R. Koenig), respectively. pCMX-TRα1(AHT) was made by overlapping PCR, first by using primer 5′ATCGCT

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GTGGGCATGGCCATGGACCTGGTTCTAGAC3′ (p1) with 5′TTTCCAAT GGCTGCCCTGGGCATTGGCGCTGCGACCGCCCTCTGTAGCAACGTG GATCAGATC3′ and primer 5′GATCTGATCCACGTTGCTACAGAGGGCG GTCGCAGCGCCAATGCCCAGGGCAGCCATTGGAAA3′ with 5′CCTCT GGCCGCCTGAGGCTTTAGACTTCCTGATCCT3′ (p4) with pCMX-TRα1 as the template and then by using primers p1 and p4 with purified products from the first PCR as the template. pCMX-Gal4-TRα1 and pCMX-Gal4-TRα1(AHT) were made by PCR with primers 5′GCGGATCCGCCATGGACCTGGTTCTA GACGATTCAAAGCGG3′ (p5) and 5′CCGGATCCGTCCGCACACCCTCT GGCCGCCTGAGGC3′ with pCMX-TRα1 and pCMX-TRα1(AHT) as templates, respectively. pCMX-Gal4-TRα2 and pCMX-Gal4-TRα1Δ347 were made by PCR with primers p5 and 5′GCGGATCCTTCCTTCACAAAGATCCTCTA GCTACCTAGC3′ with pCMX-TRα2 and pCMX-TRα1Δ347 as templates, respectively. pCMX-Gal4-TRα1(9Hα2), pCMX-Gal4-TRα2(9Hα1), and pCMX-Gal4-TRα1Δ378 were made by replacing the *Bst*XI-*Sal*I fragment of pCMX-Gal4-TRα1 with the corresponding fragments from pCMX-TRα1(9Hα2), pCMX-TRα2(9Hα1), and pCMX-TRα1Δ378, respectively. pCMX-Gal4-TRα1 (P160R) was made by PCR with primers 5′CCGTGCAGCAACGACCAGAG CGCACTCCTGAAGAG3′ and 5′GCCTGCAGCAGAGCCACTTCCGTATC ATCC3′ with pCMX-TRα1 as the template and then inserted into the *Pst*I fragment of pCMX-Gal4-TRα1. pCMX-Gal4-TRα1(L367R) and pCMX-Gal4-TRα1(L374R) were made by overlapping PCR with common external primers 5′CATCCTCCTGAAGGGCTGCTGCATG3′ (p12) and 5′CGCCCTGTCCAA GGGCTGGAGGTTC3′ (p13) and the following internal primers: 5′GAGGTC AGTCACCTTCATCAGCCGCTTGGGCCAGAAGTGCGGAAT3′ and 5′AT TCCGCACTTCTGGCCCAAGCGGCTGATGAAGGTGACTGACCTC3′ for L367R and 5′GTGGCAGGCCCCGATCATGCGGCGGTCAGTCACCTTCA TCAGCAGC3′ and 5′GCTGCTGATGAAGGTGACTGACCGCCGCATGAT CGGGGCCTGCCAC3′ for L374R. The following constructs were made by PCR to amplify the LBD. For pCMX-Gal4-TRα1ΔAF2(120-401) and pCMX-Gal4-TRα1(AHT)ΔAF2(120-401), the primers were p5 and 5′CAGGATCCTT AGAAGAGTGGGGGGAAG3′, with pCMX-TRα1 and pCMX-TRα1(AHT) as templates, respectively; for pCMX-Gal4-TRα1(AHT,E403A), the primers were p5 and 5′CCGGATCCTTAGACTTCCTGATCCTCAAAGACCGCCAG GAAGAGTGG3′, with pCMX-TRα1(AHT) as the template.

pCMX-VP16-TRα1 and pCMX-VP16-TRα1(AHT) were made by inserting PCR products into the *Kpn*I-*Nhe*I fragment of pCMX-VP16-RXRα with primers 5′CGGTACCGCCATGGACCTGGTTCTAGACG3′ and 5′CGCTAGCGTCCG CACACCCTCTGGCCGCC3′. pCMX-VP16-TRα1(9Hα2), pCMX-VP16-TRα2, pCMX-VP16-TRα1Δ378, and pCMX-VP16-TRα2(9Hα1) were made by replacing the *Bst*XI-*Nhe*I fragment of pCMX-VP16-TRα1 with the corresponding fragments from pCMX-TRα1(9Hα2), pCMX-TRα2, pCMX-TRα1Δ378, and pCMX-TRα2(9Hα1), respectively. pCMX-VP16-TRα1(L367R) and pCMX-VP16-TRα1(L374R) were made by replacing the *Eco*NI-*Bsu36*I fragment of pCMX-VP16-TRα1 with the corresponding fragments from pCMX-Gal4-TRα1(L367R) and pCMX-Gal4-TRα1(L374R), respectively. pCMX-VP16-TRα1(P365S) and pCMX-VP16-TRα1(KL366EF) were made by overlapping PCR with common external primers p12 and p13 and the following internal primers: 5′CACCTTC ATCAGCAGCTTGGACCAGAAGTGCGGAATGTTGT3′ and 5′ACAACAT TCCGCACTTCTGGTCCAAGCTGCTGATGAAGGTG3′ for P365S and 5′C AGTCACCTTCATCAGGAACTCGGGCCAGAAGTGCGGAAT3′ and 5′AT TCCGCACTTCTGGCCCGAGTTCCTGATGAAGGTGACTG3′ for KL366EF. pCMX-VP16-TRα1Δ347 and pCMX-VP16-TRα1Δ209 were made by blunt-end ligating the *Nhe*I-*Bsp*MI and *Nhe*I-*Acc*I fragments of pCMX-VP16-TRα1, respectively.

Corepressor constructs. pCMX-Gal4-N-CoR, pCMX-Gal4-SMRT were made by shuttling the *Bam*HI fragments of pGEX-2T-N-CoR (1944-2453) and pGEX-2T-SMRT (982-1495) into pCMX-Gal4. pCMX-VP16-N-CoR was made by PCR with primers 5′CCGGTACCACTGCAGCTAACTTCATAGACGTGATCATC ACC3′ and 5′CCGGTACCTCAGTCGTCACTATCAGACAGTGTCTCATAC TG3′ with pCMX-N-CoR (gift of M. Rosenfeld) as the template and then inserted into the *Kpn*I fragment of pCMX-VP16-RXRα. pCMX-VP16-SMRT was made by shuttling the *Kpn*I-*Nhe*I fragment of pCMX-SMRT (gift of R. Evans) into pCMX-VP16-RXRα. All PCR products, mutations, and fusion junctions were confirmed by sequencing.

Constructs for GST fusion proteins. pGEX-2T-N-CoR (1944-2453) has been previously described (45). pGEX-2T-SMRT (982-1495) was made by PCR with primers 5′CTCGGATCCCACCACGCCAGCCCGGACCC3′ and 5′CGCGGA TCCCTCGCTGTCGGAGAGTGTCT3′. pGEX-2T-RXRα was made by PCR with primers 5′CCGAATTCTAGCCATGGGCATGAAGCGG3′ and 5′CCGA ATTCTAAGTCATTTGGTGCGG3′.

In vitro interaction assays. Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C. Cell pellets were lysed and subsequently sonicated. GST fusion proteins (~10 μg) bound to GST beads (50 μl) in GST binding buffer (50 mM KCl, 20 mM HEPES [pH 7.9], 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 0.5% nonfat dry milk, and 5 mM dithiothreitol) were mixed with 5 μl of in vitro-translated proteins of interest and incubated at 4°C for 1 h. Beads were then washed five times with 1 ml of the same buffer. Bound proteins were eluted by boiling in 20 μl of 2× sodium dodecyl sulfate loading buffer and analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. The input lane in each experiment represented 20% of the total amount used. GST fusion proteins were stained with Coomassie blue to ensure equal loading, and bound proteins were visualized by autoradiography.

Cell culture and transfection. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and changed to Dulbecco's modified Eagle's medium with 10% stripped bovine calf serum 2 h prior to transfection. Cells were transfected by the calcium phosphate precipitation method. For each 60-mm-diameter dish, we used 1 μg of reporter vector, 0.5 μg of β-galactosidase (β-Gal) expression vector, and 1 μg of other expression vectors unless otherwise indicated. Equivalent amounts of an empty expression vector (pCMX or pSG5) were included for cells transfected with submaximal amounts of receptor or corepressor vectors. The Gal4 UAS_5-simian virus 40 luciferase reporter contains five copies of the Gal4 17-mer binding site and has been previously described (15). Cells were lysed in Triton X-100 buffer, and β-Gal and luciferase assays were carried out as described previously (15). Light units were normalized to β-Gal activity, which served as an internal control for transfection efficiency. For the mammalian two-hybrid assay, fold activation was calculated as the activity relative to the activity observed in the transfection of an individual Gal4 fusion protein expression vector alone and/or in the absence of T3 as indicated. Fold repression was calculated as the normalized luciferase activity of the empty expression vector [pCMX-Gal4 (1-147)] divided by the activity in the presence of receptor expression vectors. The data shown are the means and ranges of duplicate samples in representative experiments. Each experiment was repeated two to five times. The expression of all loss-of-function mutants was confirmed by gel shift of nuclear extracts from transfected cells with the Gal4 binding site as the probe as previously described (15) (data not shown).

EMSA. Electrophoretic mobility shift assay (EMSA) was performed by incubating 5 μl of in vitro-translated proteins (TNT kit; Promega) of interest with 100,000 cpm of ^{32}P-labeled probe at room temperature for 20 min in 30 μl of binding buffer. The binding buffer contained 40 mM HEPES (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mg of bovine serum albumin per ml, 1 μM $ZnCl_2$, 6% glycerol, 1 μg of poly(dI-dC), 750 ng of denatured salmon sperm DNA, and 1 μg of bromphenol blue. Reaction mixtures were separated on a 5% polyacrylamide gel with 1% glycerol included on the gel. After electrophoresis, gels were dried and subjected to autoradiography. The Gal4 17-mer probe sequence was 5′CG GAGTACTGTCCTCCG3′.

T3 binding assay. Binding reactions were performed by mixing equal amounts (3 to 10 μl) of in vitro-translated proteins with equal amounts of ^{125}I-T3 (NEN) in the absence or presence of increasing concentrations of unlabeled T3 in T3 binding buffer (50 mM NaCl, 10% glycerol, 2 mM EDTA, 5 mM β-mercaptoethanol, 20 mM Tris-HCl [pH 7.6]) at 4°C overnight. Parallel reactions including a 500-fold excess of cold T3 were set to determine nonspecific binding. Free and bound T3 were separated by gel filtration on a Sephadex G-25/PD-10 column as previously described (16). Dissociation constants (K_d) were obtained by Scatchard plots.

RESULTS

Ninth-heptad mutations that eliminate RXR interaction also abolish corepressor interaction and repression function. We were initially interested in mapping the C-terminal domain of TR that is involved in corepressor interaction (17, 45) with the mutants shown in Fig. 1A. Figure 1B shows the results of a mammalian two-hybrid experiment in which VP16-TR constructs were cotransfected with RXR or corepressors fused to the DBD of Gal4. In this assay, interaction between the Gal4 fusion protein and the VP16 chimera is detected by an increase in transcription from a reporter gene containing Gal4 binding sites. The TRα1 C terminus interacted with both N-CoR and SMRT, as well as with RXR as previously shown (45). In contrast, Fig. 1B shows that deletion mutants ending before the ninth heptad (TRα1Δ209 and TRα1Δ347), previously shown not to interact with RXR (31), failed to interact with N-CoR or SMRT. However, a deletion just distal to the ninth heptad (TRα1Δ378) retained the ability to interact with a corepressor and RXR (Fig. 1B). Figure 1C demonstrates that the C terminus of TRα1 contains an inherent repression domain, since fusion to the Gal4 DBD created a potent repressor of transcription on Gal4 binding sites, and that the ninth-heptad-containing TRα1Δ378 mutant was nearly equally effective at transcriptional repression. However, TRα1Δ347, which lacks the ninth heptad, was not able to repress transcription, consistent with its inability to interact with corepressors in vivo. We next explored the relationship between RXR interaction and repression in ninth-heptad point mutants. Figure 1D shows that one point mutant (P365S) that retained the ability

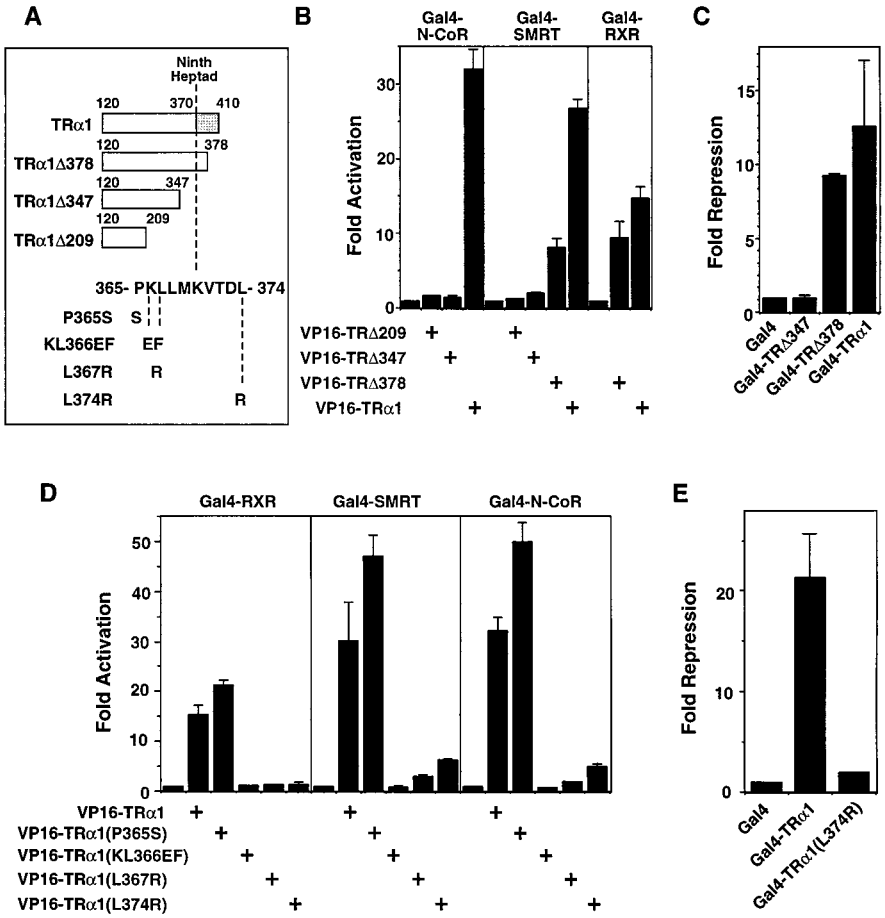

FIG. 1. Role of the ninth heptad in repression and interactions of TR with corepressors and RXR. (A) C-terminal deletions of TRα1. (B) Mammalian two-hybrid assay for interactions of various TR C-terminal deletion mutants with corepressors and RXR. (C) TR C-terminus is required for transcriptional repression by TRα1. Expression vectors for TR C-terminal deletions fused to the Gal4 DBD were transfected into 293T cells and assayed for their abilities to repress transcription. (D) Effects of ninth-heptad point mutations on interactions among TR, corepressors, and RXR. Mammalian two-hybrid assays were performed as described for panel B. (E) Ninth-heptad region is required for transcriptional repression of TRα1. Expression vectors for TR ninth-heptad mutants fused to the Gal4 DBD were transfected into 293T cells and assayed for their abilities to repress transcription. The fold activation of transfection with the individual Gal4 fusion protein expression vector alone was normalized to 1.

to interact with RXR also interacted with N-CoR and SMRT. This mutant also repressed transcription (not shown). In contrast, ninth-heptad mutations which abolished RXR interaction (KL366EF, L367R, and L374R) also abolished interaction with N-CoR and SMRT (Fig. 1D). The inability to interact with RXR, N-CoR, and SMRT correlated with loss of repression (Fig. 1E).

TRα2, a naturally occurring ninth-heptad variant, is also unable to interact with corepressors and repress transcription in vivo. Although the ninth-heptad mutants described above do not occur naturally, TRα2 is an abundant TR isoform that contains an intact CoR box but a variant C terminus which interrupts the ninth heptad (Fig. 2A). Figure 2B demonstrates that in contrast to the potent repression domain of TRα1, which could be further potentiated by exogenous N-CoR, the C terminus of TRα2 was not sufficient for repression and that N-CoR had no effect upon the transcriptional activity of a Gal4-TRα2 fusion protein. Figure 2C shows the result of a ninth-heptad swap of six amino acids between TRα1 and TRα2, creating TRα1(9Hα2) and TRα2(9Hα1) (Fig. 2A). These swaps have previously been shown to transfer the ability to interact with RXR (42). The C terminus of TRα1(9Hα2), which is identical to TRα1 except for six amino acids, was a poor transcriptional repressor. Conversely, TRα2(9Hα1) was a gain-of-function mutation for repression by TRα2. Figure 2D shows the results of a mammalian two-hybrid experiment in which Gal4-TR constructs were cotransfected with RXR or corepressors fused to the transcriptional activation domain of VP16. In this assay, interaction between the Gal4 fusion protein and the VP16 chimera is detected by an increase in transcription from a reporter gene containing Gal4 binding sites. The TRα1 C terminus interacted with both N-CoR and SMRT, as well as with RXR as previously shown (45). In contrast, the TRα2 C terminus did not interact with RXR, as previously shown (42), or with N-CoR or SMRT, which was consistent with its lack of repression function (Fig. 2B). Since the fusion proteins that interacted with corepressors and RXR were also strong repressors, interactions were also studied with TR mutants fused to VP16 and either RXR or SMRT fused to the Gal4 DBD. As shown in Fig. 2E, TRα1 and TRα2(9Hα1) interacted with corepressor and RXR, whereas TRα2 and the ninth-heptad mutant of TRα1 did not, which is consistent with the results shown in Fig. 2D.

CoR box regulates TR interactions with RXR. Since our analysis of the ninth heptad of TR indicated a correlation between the abilities to interact with corepressors and with

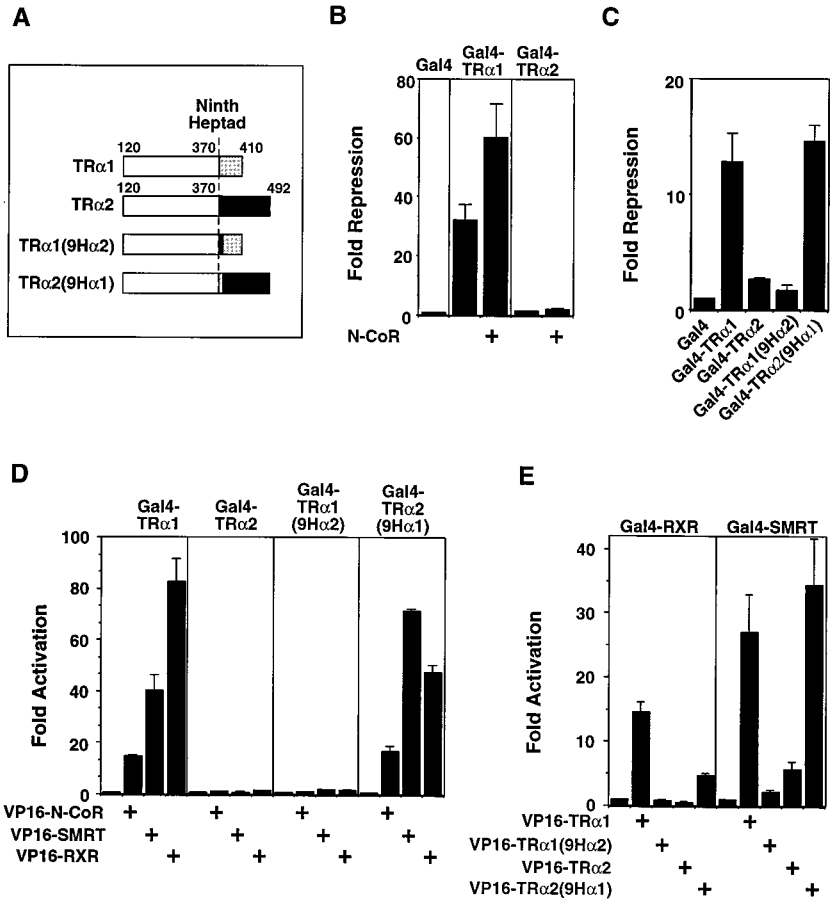

FIG. 2. Repression and functional interactions of TRα1, TRα2, and their ninth-heptad chimeras with corepressors and RXR. (A) Structures of TRα1, TRα2, and their ninth-heptad chimeras. (B) The C terminus of TRα1, but not TRα2, represses transcription. Three micrograms of pCMX-N-CoR (full length) was used in transfection. (C) Swap of the ninth heptad transfers repression function. Expression vectors for the Gal4 DBD, Gal4-TRα1, Gal4-TRα2, Gal4-TRα1(9Hα2) and Gal4-TRα2(9Hα1) were transfected into 293T cells and assayed for their abilities to repress transcription. (D and E) Mammalian two-hybrid assays showing interactions of C termini of TRα1, TRα2, and their ninth-heptad chimeras with corepressors and RXR.

RXR, we next explored the effects of CoR box mutations upon the interaction between TR and RXR. Mutations of amino acids A174, H175, and T178 [TRα1(AHT)] prevented the interaction of a Gal4-TRα1 fusion protein with N-CoR (Fig. 3A) and SMRT (data not shown) and eliminated the repression function of TR (Fig. 3B) as previously described (17). Figures 3A and B show that another CoR box mutation, P160R (10), behaved similarly. Remarkably, Fig. 3A shows that neither TRα1(AHT) nor TRα1(P160R) interacted with RXR. Both Gal4-TRα1(AHT) and Gal4-TRα1(P160R) were expressed and able to activate transcription in the presence of T3 (not shown).

The strong similarities between the abilities of CoR box and ninth-heptad mutants to interact with RXR, N-CoR, and SMRT were surprising since these regions of the protein are distinct. One possible explanation is that the CoR box and ninth heptad are equally important for interaction with corepressors. To investigate this, we compared these TR mutants for their abilities to interact with N-CoR and SMRT in GST pulldown assays. Figure 3C shows that wild-type TR, but not TRα1(AHT), interacted with both N-CoR and SMRT in this assay. However, a variety of ninth-heptad mutants, including the L367R and L374R mutants, were able to interact strongly with the corepressors in the GST pulldown assay, even though they failed to repress or interact with N-CoR and SMRT in vivo (see above). The discrepancy between the GST pulldown and mammalian two-hybrid assay results is most likely due to increased sensitivity of the GST pulldown assay for weak interactions. Clearly, however, the differential interactions of the CoR box and ninth-heptad mutants with corepressors, as revealed by the GST pulldown assay, are indicative of a fundamental difference in the conformations of these mutants.

TR, RXR, and corepressor proteins form a ternary complex in vivo. As noted above, the ability to detect interactions between ninth-heptad mutants in the GST pulldown assay but not in vivo suggested that the interaction was relatively weak and therefore not detectable in vivo. However, the ability of ninth-heptad mutants to interact with corepressors in vitro was similar to that observed for wild-type TR, whose interactions with corepressors are readily detected by the mammalian two-hybrid assay. The most obvious difference between the ninth-heptad mutants and wild-type TR is that the ninth-heptad mutants are defective in RXR heterodimerization. Therefore, we hypothesized that the failure of ninth-heptad mutants to interact with corepressors or to repress transcription in vivo was related to their inability to interact with endogenous RXR. The formation of ternary complexes containing TR, RXR, and a corepressor protein has already been shown to occur both on and off DNA in vitro (35, 46). In the following experiments, we

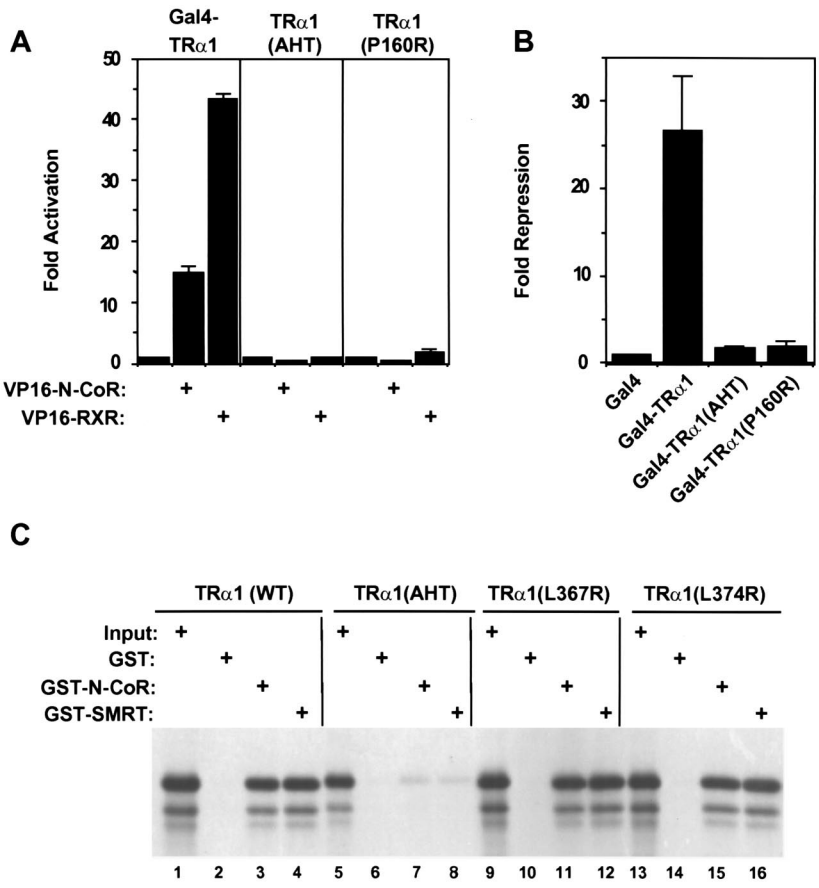

FIG. 3. TR CoR box mutants do not interact with RXR in vivo. (A) Mammalian two-hybrid assay for interactions of C termini of TR CoR box mutants with N-CoR and RXR. (B) Transfection assays indicating that the C terminus of an TR CoR box mutant is unable to repress transcription. (C) Wild-type (WT) TR and its ninth-heptad mutants, but not CoR box mutant TR(AHT), strongly interact with corepressors in vitro. The indicated VP16 receptor fusion proteins were translated in vitro in the presence of [^{35}S]methionine and assayed for their abilities to interact with corepressors in a GST pulldown assay.

examined whether TR, RXR, and corepressors could form a ternary complex in vivo.

Figure 4A confirms that Gal4-RXR interacted with VP16-TRα1. Neither VP16-RXR nor VP16–N-CoR interacted with Gal4-RXR. Coexpression of VP16-RXR actually reduced Gal4-RXR activation by VP16-TRα1, presumably by competing for interaction with VP16-TRα1 off DNA. In contrast, coexpression of VP16–N-CoR increased activation due to cotransfection of Gal4-RXR and VP16-TRα1, most likely because of the formation of a ternary complex among TR, RXR, and N-CoR. Similar results were obtained with SMRT (not shown). To test whether RXR binding actually increased or stabilized the interaction between TR and corepressor, wild-type RXR (lacking VP16) was cotransfected with Gal4–N-CoR and VP16-TRα1. Figure 4B shows that exogenous RXR markedly potentiated the interaction between TR and N-CoR, strongly supporting the conclusion that RXR stabilized the TR-corepressor interaction.

Complementation of repression-defective mutants of TR by RXR. We next tested the hypothesis that the defect in repression of the ninth-heptad mutants was due to their inability to heterodimerize with RXR. This hypothesis predicts that allowing RXR to heterodimerize with a ninth-heptad mutant by a heterologous dimerization interface should restore the abilities to interact with corepressors and to repress transcription. To test this, we took advantage of the dimeric nature of DNA

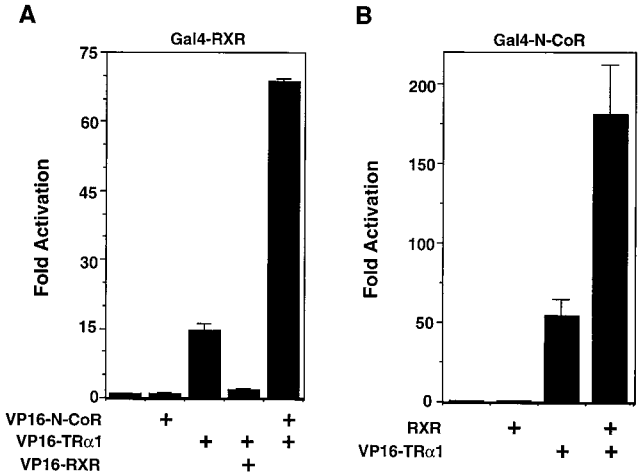

FIG. 4. TR, RXR, and N-CoR form ternary complex in vivo. (A) Mammalian two-hybrid assay showing that VP16–N-CoR, but not VP16-RXR, potentiates the interaction between TRα1 LBD and RXR. (B) RXR potentiates the interaction between TRα1 LBD and N-CoR. Mammalian two-hybrid assay showing the effects of exogenous wild-type RXR. Full-length RXR expression vector pSG5-RXRα was used.

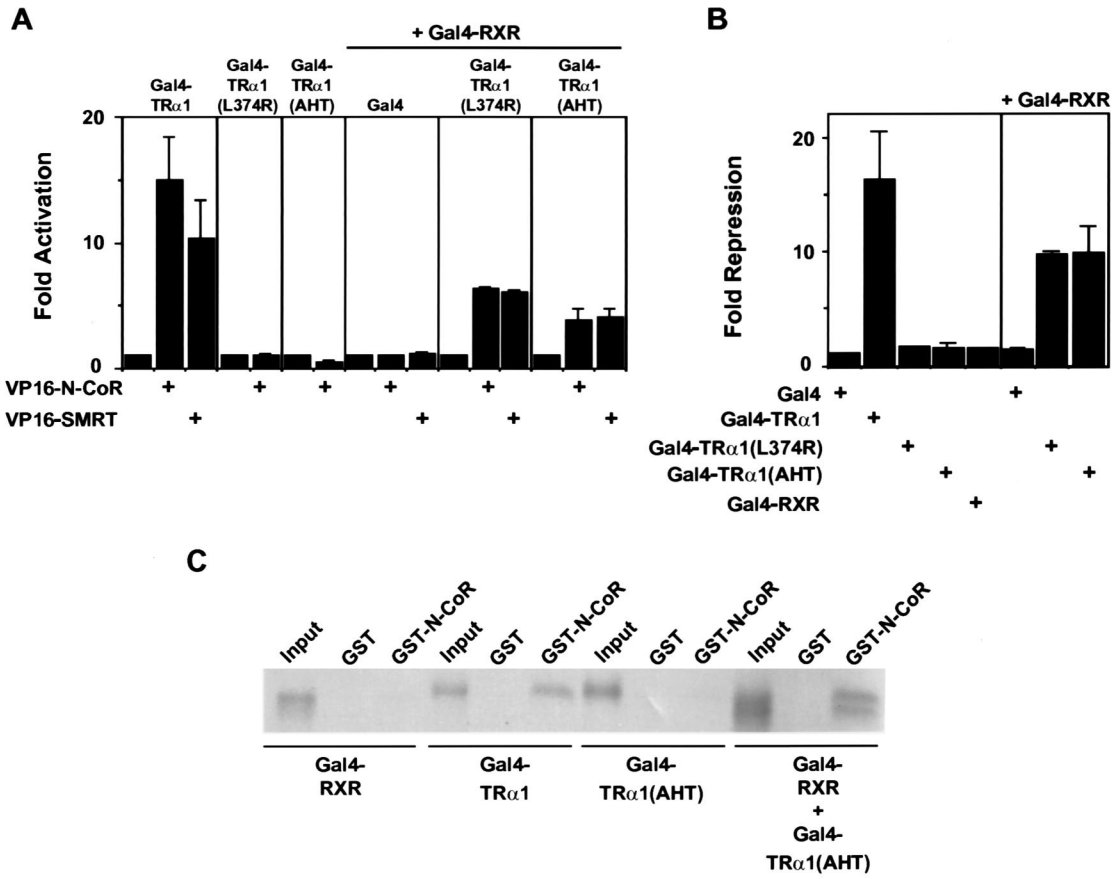

FIG. 5. Complementation of repression-defective mutants of TR by RXR. (A) Mammalian two-hybrid assay of interactions of Gal4-RXR, Gal4-TRα1, Gal4-TRα1(AHT), and Gal4-TRα1(L367R) with corepressors in the absence or presence of Gal4-RXR, as indicated. The fold activations of transfections with Gal4 fusions in the absence of VP16 constructs were normalized to 1. (B) Transcriptional repression of Gal4-RXR, Gal4-TRα1, Gal4-TRα1(AHT), and Gal4-TRα1(L367R) in the absence or presence of Gal4-RXR. Expression vectors for Gal4-RXR, Gal4-TRα1, or its mutants were transfected into 293T cells in the absence or presence of Gal4-RXR expression vector, as indicated. (C) Heterodimer of Gal4-TR(AHT)–Gal4-RXR, but not Gal4-TRα1(AHT) or Gal4-RXR alone, interacts with N-CoR. Gal4 fusion proteins were translated in vitro in the presence of [^{35}S]methionine and assayed for their abilities to interact with N-CoR in a GST pulldown assay. To form a heterodimer of Gal4-TR(AHT)–Gal4-RXR, Gal4-TR(AHT) was cotranslated with Gal4-RXR.

binding by Gal4 and coexpressed Gal4-RXR with ninth-heptad TR mutants fused to Gal4. The results are shown in Fig. 5 A and B. In the absence of RXR, Gal4-TRα1 repressed transcription and interacted with VP16–N-CoR and VP16-SMRT but Gal4-TRα1(L374R) did not. Gal4-RXR was unable to repress transcription on its own (Fig. 5B) and in this assay did not interact at all with N-CoR or SMRT (Fig. 5A), which is consistent with the results of others (17, 20, 26, 34, 37). However, coexpression of Gal4-TRα1(L374R) with Gal4-RXR resulted in potent transcriptional repression (Fig. 5B) and the ability to interact with N-CoR or SMRT (Fig. 5A). Other ninth-heptad TR mutants were similarly complemented by coexpression of Gal4-RXR (not shown). The ability of RXR to complement the defect in ninth-heptad mutants of TR strongly suggests that the presence of RXR, recruited either by the normal TR heterodimerization domain or (as in these experiments) by the Gal4 dimerization domain, is required for repression by TR.

We also tested the ability of RXR to complement the repression defect of TRα1(AHT), whose inherent affinity for corepressors was shown (Fig. 3C) to be considerably less than that of the ninth-heptad mutants. Figures 5A and B show that like the corresponding ninth-heptad TR mutant, Gal4-TRα1(AHT) was able to repress transcription and interact with corepressors in the presence of RXR. To explain this result, we hypothesized that TRα1(AHT) and RXR were able to bind a corepressor only weakly separately but together would bind a corepressor to a degree that would be functional in vivo. We tested this in an in vitro GST pulldown experiment. Figure 5C shows that neither Gal4-RXR nor Gal4-TRα1 (AHT) bound N-CoR effectively on its own. However, cotranslation of these two Gal4 fusion proteins allowed both to interact with N-CoR in this assay. Cotranslation of the two Gal4 proteins was required, which is consistent with previous work suggesting that Gal4 DBD dimers are quite stable in solution (7). Therefore, it is likely that the corepressor interaction domains in TRα1(AHT) and RXR, although they were weak on their own, were complementary. Thus, bringing them together with a heterologous dimerization interface allowed physical and functional interactions with corepressors.

TR CoR box is not required for RXR interaction in the presence of T3. Thus far, we had determined that CoR box and ninth-heptad mutants of TR are functionally similar with regard to in vivo RXR interaction, corepressor interaction, and repression function. Since corepressor interaction is abolished by the addition of T3, we next turned our attention to the effects of T3 on RXR interactions with TR mutants. Although, as shown earlier, the TRα1(AHT) mutant was unable to interact with RXR in the absence of T3 in the context of the mammalian two-hybrid assay, Fig. 6A shows that T3 remark-

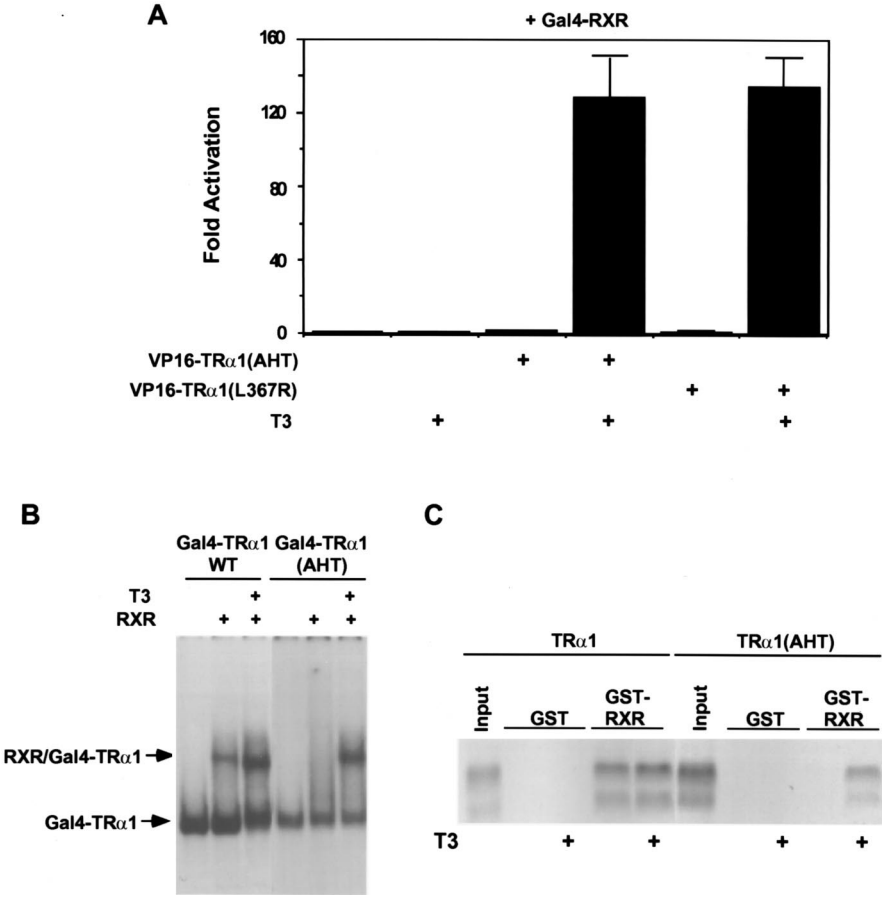

FIG. 6. The TR CoR box is not required for RXR interaction in the presence of T3. (A) Mammalian two-hybrid assay for T3-dependent interaction between the LBD of TRα1(AHT) or TRα1(L367R) with RXR. The Gal4-RXR expression vector was transfected into 293T cells in the absence or presence of VP16-TRα1(AHT) or VP16-TRα1(L367R) expression vector and in the absence or presence of 100 nM T3, as indicated. (B) EMSA analysis showing binding of Gal4-TRα1 and Gal4-TRα1(AHT) to a Gal4 17-mer probe in the absence or presence of RXR or 1 μM T3, as indicated. pBS-RXRα (full length) was used to make RXR protein. The probe is not shown. (C) GST pulldown assay showing that TRα1 interacts with GST-RXR in the presence or absence of 1 μM T3, whereas interaction between TRα1(AHT) and GST-RXR is T3 dependent.

ably stimulated the ability of the CoR box mutant to interact with RXR. The behavior of the CoR box mutant in this assay was strikingly similar to that of the ninth-heptad mutant TRα1(L367R). In agreement with earlier reports by other groups (1, 28), Fig. 6A shows that the interaction of this ninth-heptad mutant with RXR was also T3 dependent. Thus, mutations in either the CoR box or ninth heptad of TR that prevent RXR interaction in the absence of ligand do not interfere with RXR binding to TR in the presence of T3.

Since in vivo interactions in the mammalian two-hybrid assay may be indirect, putative interactions were also assessed in vitro by using Gal4 fusion proteins in a gel shift analysis on the Gal4 binding site (Fig. 6B). Gal4-TR bound strongly to this site, but since RXR does not contact the Gal4 binding site, the binding of RXR on DNA was noncooperative. RXR clearly bound to wild-type Gal4-TRα1 in both the presence and absence of T3. In contrast, TRα1(AHT) interacted with RXR only in the presence of T3. Thus, we observed the CoR box dependency of TR-RXR heterodimerization in the absence, but not the presence, of T3 in two different assays. Both assays, however, utilized Gal4-TR chimeras and required DNA binding by Gal4-TR. Therefore, we examined the abilities of full-length TRα1 and TRα1(AHT) to interact with GST-RXR in the absence of DNA. Figure 6C shows that wild-type TRα1 interacted nearly equally with RXR in the presence and absence of T3. In contrast, TRα1(AHT) interacted with GST-RXR only in the presence of T3. Thus, three lines of evidence strongly suggest that T3 is required for the TR CoR box mutant to interact with RXR.

AF2 activation helix of TR regulates RXR interaction in the presence, but not the absence, of T3. We continued to explore the role of T3 binding in TR-RXR interactions by studying the effects of mutations in the TR AF2 activation helix, whose conformation markedly changes when TR binds T3. We first studied TRα1ΔAF2, which lacks the last nine amino acids of TRα, corresponding to the C-terminal six amino acids of TRβ. Figure 7A shows that TRα1ΔAF2 did not activate transcription in the presence of T3 but retained the ability to interact with RXR, as described above. However, in the context of TRα1(AHT), the ΔAF2 mutation completely eliminated the RXR interaction in the absence or presence of T3, despite the fact that this mutant retained the ability to bind T3 with an affinity similar to that of TRα1ΔAF2. The concentration of T3 used (1 μM) was well above the K_d of wild-type and mutant TRs, although the ΔAF2 mutation modestly decreased the affinity for T3, as previously described (3) [the measured K_d were as follows: the wild type, 1 nM; TRα1ΔAF2, 35 nM; and TRα1(AHT)ΔAF2, 27 nM]. It is known that the AF2 amphi-

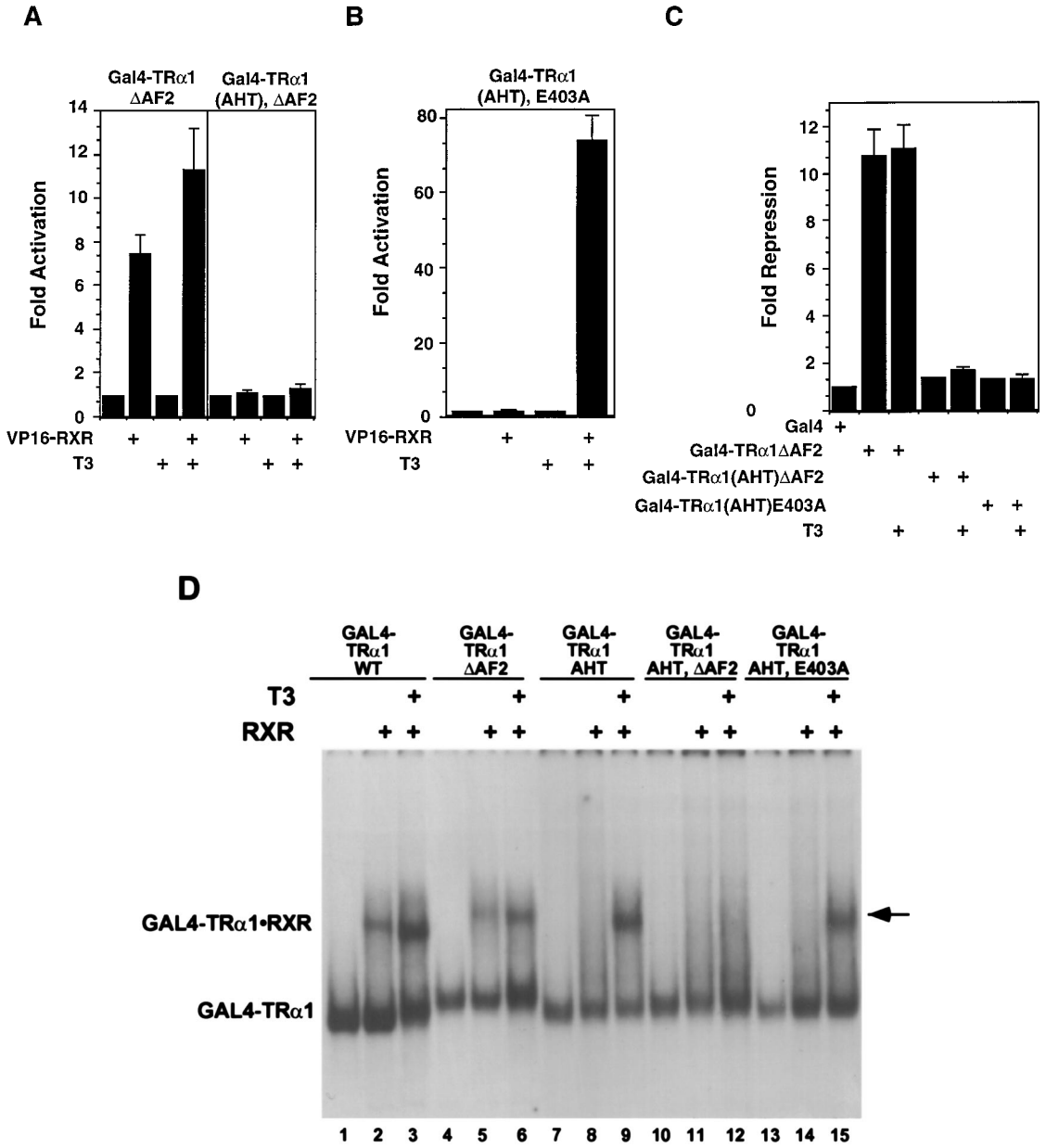

FIG. 7. The TR AF2 helix is important for RXR interaction in the presence of T3. (A) Deletion of the AF2 region abolishes transactivation and RXR interaction with the LBD of TRα1(AHT), but not wild-type TR, in the presence of T3. Expression vectors for Gal4-TRα1ΔAF2 and Gal4-TRα1(AHT)ΔAF2 were transfected into 293T cells in the absence or presence of VP16-RXR expression vector or 1 μM T3, as indicated. (B) AF2 point mutation E403A eliminates transactivation but has no effect on T3-dependent RXR interaction with the TRα1(AHT) LBD. pCMX-Gal4-TRα1(AHT,E403A) was transfected into 293T cells in the absence or presence of VP16-RXR expression vector or 1 μM T3, as indicated. (C) Expression vectors for the Gal4 DBD, Gal4-TRα1ΔAF2, Gal4-TRα1(AHT)ΔAF2, and Gal4-TRα1(AHT)E403A were transfected into 293T cells and assayed for their abilities to repress transcription in the absence or presence of 1 μM T3, as indicated. Fold activation of Gal4 fusions alone in the absence of T3 were normalized to 1. (D) EMSA analysis showing the binding of wild-type (WT) and mutant Gal4-TRα1 to a Gal4 17-mer probe (free probe migrated much faster and is not shown) in the absence or presence of RXR or 1 μM T3, as indicated. The Gal4-TRα1(WT) and Gal4-TRα1(AHT) lanes are identical to those in Fig. 6B and are shown for comparison with AF2 mutants.

pathic helix switches back towards the core and contacts the ligand in the crystal structure of TR. Therefore, we reasoned that deletion of the AF2 helix might well prevent the T3-induced conformational change from occurring but that a more subtle mutation in the amphipathic helix which still interfered with transcriptional activation might preserve T3 binding and the conformational change which allows RXR interaction in the presence of T3.

One such mutant involves conversion of E403 to alanine, which has previously been shown to interfere with transcriptional activation and coactivator interaction without a large change in affinity for T3 (38). Figure 7B shows that when this substitution was made in the context of TRα1(AHT), the mutant TR was unable to activate transcription in the presence of T3. However, unlike other amino acids in the AF2 helix, such as F401 and F405, E403 is on the surface of TR and does not contact the bound thyroid hormone (40). Indeed, Figure 7B shows that unlike in the ΔAF2 mutant, the presence of T3

caused a conformational change in the TRα1(AHT)E403A mutant that allowed it to interact with RXR. Despite its ability to interact with RXR and inability to activate transcription in the presence of T3, the TRα1(AHT)E403A mutant remained defective in repression (Fig. 7C) and corepressor interaction (data not shown). This suggests that although it does not support coactivator interaction, the E403A AF2 helix can assume the liganded conformation that prevents corepressor interaction, as does the wild type. TRα1(AHT)ΔAF2, which cannot interact with RXR (or a corepressor [data not shown]) was not functional as a transcriptional repressor in the presence or absence of T3. In contrast, TRα1ΔAF2 functioned as a repressor even in presence of T3, as noted previously (3, 10).

The results indicating that the AF2 helix of TR regulated RXR interaction in the presence of T3 were obtained in vivo by the mammalian two-hybrid assay. Next, the ability of T3 to promote RXR interaction with TRα1(AHT)E403A, but not TRα1(AHT)ΔAF2, was confirmed in the gel shift assay. Figure 7D shows that Gal4-TRα1 and Gal4-TRα1ΔAF2 were able to interact with wild-type RXR on the Gal4 site in the presence or absence of T3 (lanes 1 through 6), whereas, as shown earlier, Gal4-TRα1(AHT) interacted with RXR only in the presence of T3 (lane 7 through 9). As predicted from the results of mammalian two-hybrid experiments, Gal4-TRα1(AHT)ΔAF2 did not interact with RXR in the presence or absence of T3 (Fig. 7D, lanes 10 through 12). However, consistent with the results shown in Fig. 7B, the activation-deficient E403A AF2 helix did allow the Gal4-TRα1(AHT) mutant to interact with RXR in the presence of T3 (Fig. 7D, lanes 13 through 15). Together, these results strongly support a role for the AF2 helix of TR in RXR interaction only in the presence of T3.

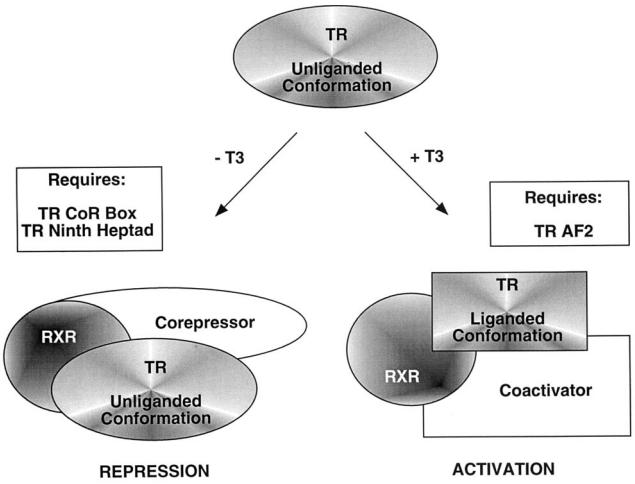

FIG. 8. Differential recognition of liganded- and unliganded-TR conformation by RXR. In the absence of T3 (−T3), RXR recognizes the unliganded conformation of TR, which requires an intact CoR box and ninth heptad of TR. TR AF2 is not required for RXR interaction with unliganded TR. The TR-RXR heterodimer is capable of recruiting corepressors N-CoR and SMRT and therefore represses transcription. In the presence of T3 (+T3), the conformation of TR changes (indicated by the change from an oval to a rectangle) primarily due to a turning back of the AF2 helix towards the ligand binding pocket. RXR differentially recognizes the liganded conformation of TR; therefore, this interaction requires the TR AF2 helix but not the TR CoR box or the ninth heptad (in the case of the L367R mutant). The T3-TR-RXR complex is unable to interact with corepressors but is capable of recruiting coactivators, therefore activating transcription. By analogy with corepressor interactions, the coactivator is depicted as interacting with both TR and RXR, although this has not yet been shown.

DISCUSSION

We have shown that in the unliganded state, both the CoR box and ninth heptad are required for the interaction of TR with RXR and for functional interactions with corepressors N-CoR and SMRT. These polypeptide stretches are in very different regions of TR, but from the crystal structure of TR (or from the related structures of RAR and RXR) neither appears to be readily available for direct intermolecular interactions with other proteins (5, 32, 40). One fundamental difference between CoR box and ninth-heptad mutants is the observation that ninth-heptad mutants interact with corepressors in vitro, whereas CoR box mutants do not. Therefore, we favor the interpretation that CoR box mutations lead to a major change in conformation of unliganded TR that prevents interactions with both RXR and corepressors in solution. In contrast, ninth-heptad mutations have a more direct and specific effect upon RXR heterodimerization, preventing corepressor interaction in vivo because TR primarily interacts with corepressors in the form of a TR-RXR heterodimer.

Productive interactions with corepressors require two nuclear receptor C termini (46), and TR heterodimerizes with RXR but does not homodimerize in solution and in vivo in the absence of DNA (31). Hence, the ability of TR to squelch repression in solution is likely due to its ability to form heterodimers with endogenous RXRs. Furthermore, the inability of ninth-heptad mutants of TR to repress or to squelch the repressive function of wild-type TR (2, 8) is likely due to the inability of these mutants to heterodimerize with endogenous RXR. This also helps to explain why TRα2 is a weak dominant negative (18, 33), whereas mutants associated with thyroid hormone resistance syndromes retain the ability to interact with RXR and corepressors (43). These results indicate that RXR recognition of the unliganded conformation of TR requires the CoR box and ninth heptad and plays a major role in the recruitment of corepressors to TR-RXR heterodimers. Interestingly, we have shown that interaction with RXR via a heterologous interface can functionally complement TR mutants that are repression defective due to either CoR box or ninth-heptad mutations. In some contexts, the weak, DNA-dependent interaction between the TR and RXR DBDs (21, 29–31) may suffice for heterodimerization and perhaps functional repression despite a defective TR ninth heptad.

Although an intact CoR box is required for TR-RXR heterodimerization in the absence of T3, it is not required for RXR interaction with ligand-bound TR. A similar observation that confirms these results was reported while this paper was under review (11). Thus, we propose that RXR differentially interacts with the unliganded and liganded conformations of TR. In the model shown in Fig. 8, RXR binding to unliganded TR supports corepressor interaction and repressive function. Both N-CoR and SMRT can interact with the TR-RXR heterodimer and require the presence of two receptor C termini for functional interactions (46). The ability of RXR to recognize this conformation of TR requires the CoR box and ninth-heptad regions of TR. In contrast, the liganded conformation of TR is recognized by RXR independently of the TR CoR box and in some cases (such as the L367R mutant) independently of the TR ninth heptad. On the other hand, although the TR AF2 helix is not required for interaction with RXR in the absence of ligand, it plays a major role in regulating the interaction between RXR and liganded TR. The liganded TR-RXR heterodimer is in a conformation receptive to coactivator interaction, resulting in positive transcriptional regulation. Although it is not yet known whether coactivator binding requires two receptor molecules, recent studies have indicated that allosteric interactions between RXR and its heterodimer partner

play a major role in transcriptional activation (36, 41). Unlike RAR and LXR (36, 41) the presence of RXR ligand did not alter the transcriptional properties of the TR-RXR heterodimers studied in these experimental paradigms (data not shown).

The role of the TR AF2 helix in RXR interaction with liganded TR had not been predicted from prior studies. Our finding that RXR interacts with the TRα1ΔAF2 mutant in the presence or absence of ligand is consistent with earlier studies of v-erbA, which naturally lacks AF2 (4, 13), and is likely to be a general rule, since the AF2 helix is important for ligand-induced corepressor dissociation from TR as well as RAR (9). We propose that the ΔAF2 mutation prevents corepressor release from the TR-RXR heterodimer because in this context RXR interacts with the unliganded conformation of TR, which favors corepressor association. In contrast, CoR box mutants require T3 for RXR interaction, and RXR binding to the liganded conformation favors corepressor dissociation. Only in the context of a CoR box mutation does the AF2 helix of TR become necessary for RXR interaction, because the CoR box ΔAF2 mutant is defective in assuming both unliganded- and liganded-RXR binding conformations.

The E403A mutation within the TR AF2 helix is particularly interesting. This mutation prevents activation by wild-type TR and TR CoR box mutants, probably because it is unable to interact with putative coactivators, such as SRC-1 and RIP140 (12). Although the AF2 helix makes contacts with thyroid hormone, E403 is not involved in hormone binding; therefore, the E403A mutation has little effect upon T3 binding, suggesting that the E403A AF2 helix does fold back into the core of TR upon ligand binding. In support of this is the observation that RXR can bind to the E403A mutant in the presence of T3, despite the inability of this mutant to support coactivator interaction or transcriptional activation. Thus, TR mutants can achieve ligand-bound conformations that are intermediate between those of unliganded and T3-bound wild-type TR. This is similar to the effects of partial agonists and antagonists on steroid receptor function (27). The ability to uncouple T3 regulation of TR-RXR interaction from T3-dependent activation suggests the existence of multiple TR conformations that recruit specific cofactors with different functions.

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