MOLECULAR AND CELLULAR BIOLOGY, Sept. 1994, p. 5756–5765
0270-7306/94/$04.00+0

Vol. 14, No. 9

Functional Evidence for Ligand-Dependent Dissociation of Thyroid Hormone and Retinoic Acid Receptors from an Inhibitory Cellular Factor

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Received 19 May 1994/Accepted 31 May 1994

The ligand-binding domains of thyroid hormone (L-triiodothyronine [T3]) receptors (T3Rs), all-*trans* retinoic acid (RA) receptors (RARs), and 9-*cis* RA receptors (RARs and RXRs) contain a series of heptad motifs thought to be important for dimeric interactions. Using a chimera containing amino acids 120 to 392 of chicken T3Rα (cT3Rα) positioned between the DNA-binding domain of the yeast GAL4 protein and the potent 90-amino-acid transactivating domain of the herpes simplex virus VP16 protein (GAL4-T3R-VP16), we provide functional evidence that binding of ligand releases T3Rs and RARs from an inhibitory cellular factor. GAL4-T3R-VP16 does not bind T3 and does not activate transcription from a GAL4 reporter when expressed alone but is able to activate transcription when coexpressed with unliganded T3R or RAR. This activation is reversed by T3 or RA, suggesting that these receptors compete with GAL4-T3R-VP16 for a cellular inhibitor and that ligand reverses this effect by dissociating T3R or RAR from the inhibitor. A chimera containing the entire ligand-binding domain of cT3Rα (amino acids 120 to 408) linked to VP16 [GAL4-T3R(408)-VP16] is activated by unliganded receptor as well as by T3. In contrast, GAL4-T3R containing the amino acid 120 to 408 ligand-binding region without the VP16 domain is activated only by T3. The highly conserved ninth heptad, which is involved in heterodimerization, appears to participate in the receptor-inhibitor interaction, suggesting that the inhibitor is a related member of the receptor gene family. In striking contrast to T3R and RAR, RXR activates GAL4-T3R-VP16 only with its ligand, 9-*cis* RA, but unliganded RXR does not appear to be the inhibitor suggested by these studies. Further evidence that an orphan receptor may be the inhibitor comes from our finding that COUP-TF inhibits activation of GAL4-T3R-VP16 by unliganded T3R and the activation of GAL4-T3R by T3. These and other results suggest that an inhibitory factor suppresses transactivation by the T3Rs and RARs while these receptors are bound to DNA and that ligands act, in part, by inactivating or promoting dissociation of a receptor-inhibitor complex.

Thyroid hormone (L-triiodothyronine [T3]) receptors (T3Rs), all-*trans* retinoic acid (RA) receptors (RARs), and 9-*cis* RA receptors (RXRs as well as the RARs) exhibit several unique properties that distinguish them as a subfamily within the steroid receptor superfamily (14, 22, 25). First, T3R, RAR, and RXR can bind to response elements as monomers, as homodimers (24, 67), and as heterodimers (12, 24, 32, 44, 45, 48, 50, 73, 74). Homodimerization and heterodimerization of these receptors appear to be primarily mediated by a region within their ligand-binding domains that contains a series of hydrophobic heptad motifs (3, 24, 25, 27, 32, 50). In contrast, the steroid receptors for glucocorticoids, androgens, and progestins bind to their response elements as homodimers through dimerization mediated by a region within their DNA-binding domains (18, 35, 49). Second, unliganded T3Rs and RARs are thought to be bound to DNA in the cell (22, 56). In the unliganded state, T3Rs and RARs can suppress or silence the basal activity of active promoters containing hormone response elements, while ligand-occupied receptors can mediate strong stimulation of gene expression (5, 6, 10, 20). Suppression and activation probably proceed by distinct mechanisms, since certain mutants of T3R do not mediate suppression but give normal levels of T3-mediated stimulation (3). In contrast with the T3Rs and RARs, unliganded steroid receptors are present in an oligomeric complex with hsp90 that does not bind DNA (14) and do not appear to suppress gene expression. Steroid hormones are thought to promote transcription both by dissociating their cognate receptors from hsp90 to permit DNA binding and by modifying the conformation of DNA-bound receptor to a transcriptionally active form (1, 14). hsp90 also appears to act as a chaperone protein that directs folding of the glucocorticoid receptor into the ligand-binding conformation (11, 69). In contrast, T3R and RAR do not appear to require hsp90 (19) or other eukaryotic proteins to direct proper folding (24).

Although several isolated examples of hormone-independent activation by the T3Rs have been reported (27, 28, 60), transcriptional activation by these receptors is primarily ligand dependent. Binding of T3 to T3R leads to significant conformational changes that alter the electrophoretic mobility of T3R-DNA complexes (2, 24, 55, 58) and the circular dichroism spectrum of T3R (64). Although T3 alters the conformation of T3R, the functional role of ligands in the activation of these receptors is unknown. A ligand-mediated conformational change might expose a transactivating domain of the receptor which allows for interaction with the transcriptional apparatus and/or the release of the receptor from an inhibitory cellular

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factor. In this study, we provide evidence that an inhibitory factor interacts with the C-terminal region of T3R and that its inhibitory effect is reversed by ligand. This interaction appears to occur when the receptor is bound to DNA and to involve the C-terminal region of T3R containing the highly conserved ninth heptad, suggesting that this inhibitory factor may be related to the thyroid hormone/retinoid receptor gene subfamily.

MATERIALS AND METHODS

Cell culture and transfection. HeLa cells and GH4C1 rat pituitary cells were cultured and transfected by electroporation as previously described (3, 23, 27, 63). The chloramphenicol acetyltransferase (CAT) reporter vector and other plasmids used are described below. After incubation for 40 h with or without the indicated ligand(s), cells were harvested for assays of CAT activity (23, 27). Because of the marked changes in CAT expression found in these studies, many of the results are depicted as autoradiograms of thin-layer chromatograms showing unreacted (lower spot) and acetylated (upper spots) [^{14}C]chloramphenicol. CAT activity was quantitated in Tables 1 and 2 and Fig. 4, 5, and 7B by excising the acetylated and unacetylated [^{14}C]chloramphenicol from the chromatograms and determining the amount of radioactivity in a liquid scintillation counter. The results are presented as percent acetylation of [^{14}C]chloramphenicol per the indicated amount of cell protein in a 15-h incubation at 37°C.

Plasmids. Plasmids expressing full-length wild-type chicken T3Rα(1-408) (cT3Rα; numbers in parentheses indicate amino acids) and the deletion mutants cT3Rα(1-392), cT3Rα(1-346), cT3Rα(120-392), and cT3Rα(120-408) have been previously described (27, 63). cT3Rα(120-408) contains the entire ligand-binding domain and binds T3 with an affinity similar to that of wild-type cT3Rα. cT3Rα(1-392), cT3Rα(1-346), and cT3Rα(120-392) have an affinity for T3 that is too low to measure accurately and is at least 50- to 100-fold less than that of the wild-type receptor. v-ErbA(122-402) (63), which corresponds to the ligand-binding region of cT3Rα(120-408), also binds T3 with very low affinity. cT3Rα mutants cT3Rα(L365R) and cT3Rα(L372R) each contain the indicated single amino acid change (3). The other T3R-related constructs used were human T3Rβ1 (hT3Rβ1) (29, 70), rat T3Rα1 (rT3Rα1) (3, 52), and rat c-ErbAα2 (r-c-ErbAα2) (3, 46). All receptor expression vectors were regulated by the Rous sarcoma virus (RSV) long terminal repeat (LTR) in a pBR322-based vector (27) or in a pEXPRESS (pEX) vector (26). An RSV vector expressing COUP-TFI and a pMT2 vector expressing COUP-TFII have been previously described (16, 17) and were obtained from Ming-Jer Tsai.

Plasmids expressing human RARα1(155-462) [hRARα1(155-462)] and mouse RXRβ(185-438) [mRXRβ(185-438)] containing the ligand-binding domains of these receptors were constructed by cleaving pEX-cT3Rα (26) with *Nco*I and *Asp* 718 to excise all of the cT3Rα sequences. The plasmid was then blunt ended with Klenow enzyme and ligated to form pEX-0. This regenerates both enzyme restriction sites and places the *Nco*I site downstream of the RSV LTR in pEX. The nucleotide sequences corresponding to amino acids 155 to 462 in hRARα1 (30) and 185 to 438 in mRXRβ (34, 48, 50) were amplified by PCR with the introduction of an *Nco*I site at both 5′ and 3′ ends. The ATG of the 5′ *Nco*I site starts the open reading frame, and the expressed protein starts with the corresponding methionine immediately preceding the indicated receptor amino acid (155 for hRARα1 and 185 for mRXRβ). The PCR products were cleaved with *Nco*I and cloned into the *Nco*I site of pEX-0. Orientation was determined by restriction enzyme analysis and confirmed by dideoxy sequencing.

GAL-VP16 (61), pSG424 (62), and pMC110, a GAL4-regulated CAT reporter, were obtained from Mark Ptashne. GAL4 chimeras containing regions of cT3Rα were constructed from pSG424, which expresses the DNA-binding domain of GAL4 from simian virus 40 (SV40) early promoter sequences and contains a polylinker sequence downstream of the region encoding GAL4 amino acids 1 to 147 (62). GAL4-T3R contains the entire ligand-binding domain (amino acids 120 to 408) of cT3Rα linked in frame to the GAL4 DNA-binding domain. This was constructed by blunt-end ligating an *Nco*I-*Asp* 718 fragment of cT3Rα (27) into the *Sma*I site of the pSG424 polylinker. The GAL4-T3R vector was digested with *Sac*I and *Afl*III to remove DNA encoding the last 16 amino acids of cT3Rα, and this segment was replaced with the *Sac*I-*Afl*III fragment from GAL4-VP16, creating GAL4-T3R-VP16. The chimeric protein expressed from this plasmid contains the GAL4 DNA-binding domain followed by amino acids 120 to 392 of cT3Rα and the 90-amino-acid transactivating domain of the herpes simplex virus VP16 protein. GAL4-T3R(408)-VP16, which contains the entire ligand-binding domain (amino acids 120 to 408) of cT3Rα, was created by linearizing the GAL4-T3R-VP16 vector with *Sac*I, which cleaves at the junction between codon 392 of cT3Rα and VP16 sequences. An oligonucleotide encoding amino acids 393 to 408 of cT3Rα was then cloned into the *Sac*I site to form the plasmid. cT3R-VP16 was constructed from wild-type cT3Rα in pEX by excising the DNA with *Sac*I and *Afl*III corresponding to the last 16 amino acids of cT3Rα and replacing the DNA with the *Sac*I-*Afl*III fragment from GAL4-VP16. This creates cT3R-VP16, which contains amino acids 1 to 392 of cT3Rα fused to the last 90 amino acids of VP16. cT3Rα(408)-VP16, a chimera which contains the entire cT3Rα(1-408) protein followed by the 90-amino-acid VP16 protein, was constructed by cloning the oligonucleotide described above into the *Sac*I site of cT3R-VP16. Because the SV40 early promoter is poorly expressed in GH4C1 cells, a vector expressing GAL4-T3R-VP16 (containing cT3Rα amino acids 120 to 392) from the RSV LTR was constructed by excising the GAL4-T3R-VP16 coding sequences from the pSG424-derived vector by digestion with *Hin*dIII and *Asp* 718. This fragment was then cloned into a *Hin*dIII- and *Asp* 718-cut RSV LTR-regulated pEX vector (26) to create RSV GAL4-T3R-VP16.

RESULTS AND DISCUSSION

The ligand-binding domain of T3R suppresses the activity of the herpes simplex virus VP16 transactivation domain. The GAL4-VP16 protein containing the yeast GAL4 DNA-binding domain (13) and the C-terminal 90-amino-acid transactivating domain of the herpes simplex virus VP16 protein (66) is a potent transcriptional activator of a GAL4 reporter gene (pMC110) (Fig. 1) (61). A similar GAL4-T3R chimera, containing the entire ligand-binding domain (amino acids 120 to 408) of cT3Rα, mediates T3-dependent activation of the GAL4-responsive reporter. Interestingly, the transactivating activity of GAL4-VP16 is suppressed in GAL4-T3R-VP16, which contains amino acids 120 to 392 of cT3Rα inserted between the GAL4 and VP16 domains. The involvement of an inhibitory factor in this suppression is supported by the finding that GAL4-T3R-VP16 becomes transcriptionally active when coexpressed with cT3Rα(120-392) (Fig. 1). Addition of T3 did not alter this transcriptional activity, presumably because the amino acid 120 to 392 region of cT3Rα has a low affinity for T3 (40, 51). Similar transcriptional activation was observed when

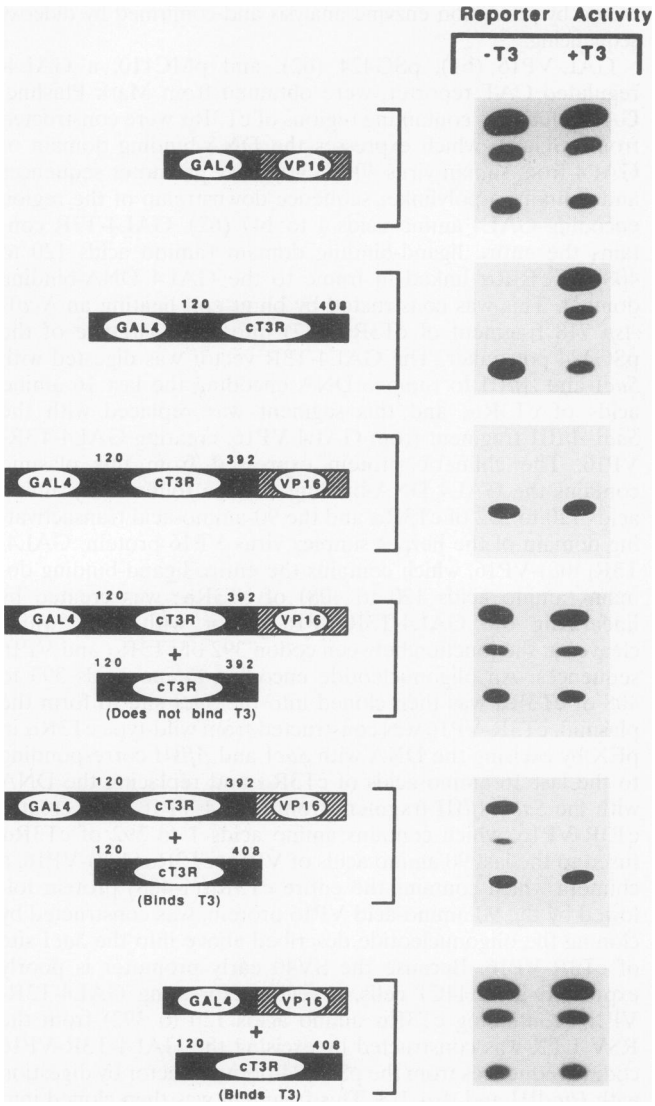

FIG. 1. The C-terminal region of cT3Rα suppresses the activity of GAL4-VP16. HeLa cells were transfected by electroporation with 5 μg of the GAL4-responsive reporter pMC110 along with either GAL-VP16 (1 μg), GAL4-T3R (1 μg), GAL4-T3R-VP16 (1 μg), GAL4-T3R-VP16 (1 μg) and cT3Rα(120-392) (5 μg), GAL4-T3R-VP16 (1 μg) and cT3Rα(120-408) (5 μg), or GAL4-VP16 (1 μg) and cT3Rα(120-408) (5 μg). After incubation for 40 h with or without 100 nM T3, cells were harvested for assays of CAT activity. Depicted are autoradiograms of thin-layer chromatograms showing unreacted (lower spot) and acetylated (upper spots) [^{14}C]chloramphenicol. Basal CAT activity from the pMC110 reporter was low (not shown) and was not affected by cotransfection with GAL4-T3R-VP16 (also see Fig. 5).

the analogous C-terminal region of v-ErbA, amino acids 121 to 402, was coexpressed with GAL4-T3R-VP16 (not shown). Coexpression of the T3-binding fragment cT3Rα(120-408) also activated GAL4-T3R-VP16, but addition of T3 unexpectedly reversed this activation (Fig. 1). This T3-mediated reversal of activation appears to require the C-terminal region of T3R in GAL4-T3R-VP16 since T3 did not inhibit activation by GAL4-VP16 when coexpressed with cT3Rα(120-408). Similar results were also found in transfection experiments using the Rat2 fibroblast cell line (57a).

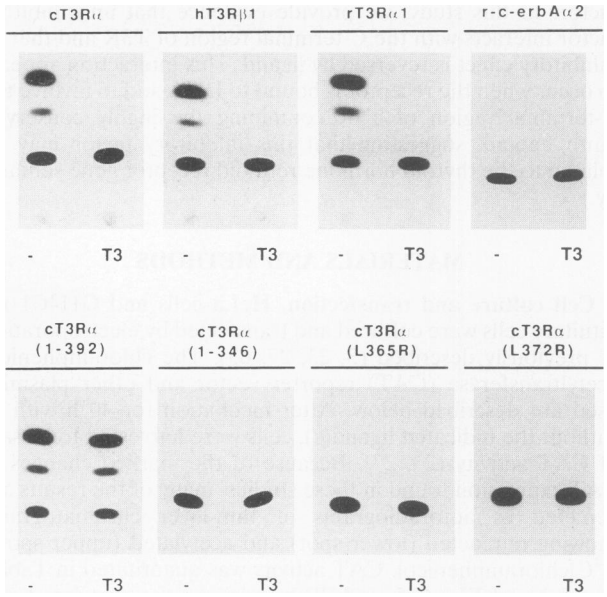

FIG. 2. Activation of GAL4-T3R-VP16 by wild-type and mutant thyroid hormone receptors. HeLa cells were transfected with 5 μg of pMC110 and 1 μg of vector expressing GAL4-T3R-VP16 along with a vector expressing either cT3Rα (5 μg), hT3Rβ1 (10 μg), rT3Rα1 (10 μg), r-c-ErbAα2 (25 μg), cT3Rα(1-392) (5 μg), cT3Rα(1-346) (25 μg), cT3Rα(L365R) (25 μg), or cT3Rα(L372R) (25 μg). After transfection, cells were incubated for 40 h with or without 100 nM T3 and then assayed for CAT activity. Constructs shown not to activate GAL4-T3R-VP16 were also inactive when up to 50 μg of plasmid was used for transfection (not shown).

Full-length cT3Rα, hT3Rβ1, and rT3Rα1 also mediate activation of the GAL4-T3R-VP16 chimera that is reversed by T3 (Fig. 2). However, r-c-ErbAα2, which differs from rT3Rα1 in the C-terminal region as a result of alternative splicing (46, 47), did not activate the chimera. The splice junction in r-c-ErbAα2 disrupts the highly conserved ninth heptad of rT3Rα1. Two cT3Rα ninth-heptad mutants, cT3Rα(L365R) and cT3Rα(L372R) (3), also did not activate GAL4-T3R-VP16 (Fig. 2). Furthermore, cT3Rα(1-392) activated GAL4-T3R-VP16, while cT3Rα(1-346), which lacks the ninth heptad, did not (Fig. 2). The rat glucocorticoid receptor, which does not contain the conserved heptad domain of the thyroid/retinoid receptor subfamily, also did not activate GAL4-T3R-VP16 (not shown). These results suggest that the region containing the ninth heptad, which is known to be involved in heterodimeric interactions of T3Rs with RARs and RXRs (3, 50), may also participate in interactions with an inhibitory factor.

A model to account for activation and T3-dependent reduction of GAL4-T3R-VP16 activity by T3R. These findings support a model in which a cellular factor binds to the region of GAL4-T3R-VP16 containing the ninth heptad of cT3R and inhibits the activity of the chimeric protein (Fig. 3). Although the effect of cT3Rα(120-408) is illustrated in Fig. 3, a similar model applies to wild-type full-length T3Rs. Coexpressed cT3Rα(120-408) competes with GAL4-T3R-VP16 for the inhibitor, and the resulting free chimera is able to activate transcription. T3 reverses the activation of the chimera by dissociating cT3Rα(120-408) from the inhibitor. The inhibitory factor then binds to and suppresses the activity of GAL4-T3R-VP16. Unlike cT3Rα(120-408), the cT3Rα(120-392) protein has a very low affinity for T3; thus, activation by this C-terminal

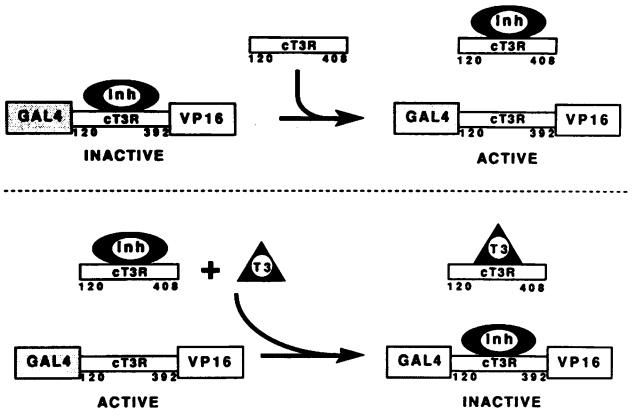

FIG. 3. A model to explain the activation and T3-dependent suppression of GAL4-T3R-VP16 by thyroid hormone receptor. Inh, inhibitor.

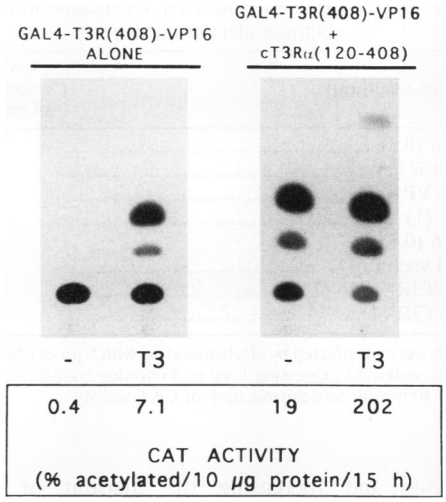

FIG. 4. Activation of GAL4-T3R(408)-VP16 is mediated by either T3 or the C-terminal region of cT3Rα. HeLa cells were transfected with 5 μg of pMC110 and 1 μg of GAL4-T3R(408)-VP16 alone or with 5 μg of vector expressing cT3Rα(120-408). Cells were incubated for 40 h with or without 100 nM T3 prior to assaying for CAT activity. GAL4-T3R(408)-VP16 contains the entire ligand-binding domain of cT3Rα (amino acids 120 to 408) linked in frame on its N-terminal side to the GAL4 DNA-binding domain and on its C-terminal end to the 90-amino-acid transactivating domain of VP16. Depicted are autoradiograms of a thin-layer chromatogram after separation of unacetylated and acetylated [^{14}C]chloramphenicol. For accurate quantitation, various amounts of cell protein were assayed at 37°C to maintain conversion at less than 30%, and the results were normalized to percent acetylated per 10 μg of protein per 15 h.

region is not reversed by T3. Implicit in this model is that the inhibitory factor can interact in a reversible fashion with the C-terminal region of cT3Rα and related proteins.

Additional support for this model comes from studies using GAL4-T3R and a GAL4-T3R(408)-VP16 chimera that contains the entire ligand-binding domain of cT3Rα (amino acids 120 to 408) (Fig. 4 and 5). GAL4-T3R(408)-VP16 would be predicted to be activated either by T3 or by coexpressing the ligand-binding domain of cT3Rα in the absence of ligand. GAL4-T3R(408)-VP16 mediates T3-dependent stimulation and is also activated when coexpressed with cT3Rα(120-408) (Fig. 4). Unlike the case for GAL4-T3R-VP16 (Fig. 1 and 2), activation of GAL4-T3R(408)-VP16 by cT3Rα(120-408) is not inhibited by T3 since the chimera can be converted to a transcriptionally active form by hormone. In other experiments (Fig. 5), GAL4-T3R, containing the entire ligand-binding domain of cT3Rα (amino acids 120-408), was cotransfected with cT3Rα(120-392), v-ErbA(122-402), or cT3Rα(120-408), all of which are expressed at similar levels in HeLa cells (63). Unlike GAL4-T3R-VP16 (Fig. 1 and 2), GAL4-T3R is not activated by cT3Rα(120-392), v-ErbA(122-402), or cT3Rα(120-408) in the absence of T3. This is not unexpected since wild-type T3R generally requires ligand to convert the receptor into a transcriptionally active form. However, expression of cT3Rα(120-392) and v-ErbA(122-402), which poorly bind T3, enhanced the T3-mediated stimulation by GAL4-T3R about 10-fold (Fig. 5). This enhancement suggests that the inhibitor may remain associated with and suppress the activity of a fraction of GAL4-T3R in the presence of T3 and that cT3Rα(120-392) and v-ErbA(122-402) compete for the remaining inhibitor. In contrast, cT3Rα(120-408) does not enhance T3-mediated activation by GAL4-T3R (Fig. 5), presumably because it competes less efficiently for the putative inhibitor in the presence of T3.

The enhancement of GAL4-T3R activity by cT3Rα(120-392) contrasts with its inhibitory effect on wild-type T3Rs or RARs (27, 63). This difference may be explained by the finding that the dimerization domains of the T3Rs and RARs necessary for efficient DNA binding are localized within their C-terminal regions (24, 25), while the GAL4 protein binds as a dimer to its response element through its DNA-binding domain (13). This notion is supported by the finding that the GAL4(1-147) DNA-binding domain binds to its response element as a dimer with the same efficiency as a GAL4–v-ErbA or a GAL4-RAR chimera (5). Thus, cT3Rα(120-392) inhibits transactivation by T3R and RAR by interfering with dimerization and effective DNA binding (24, 27, 63). In contrast, cT3Rα(120-392) would be unable to interfere with dimerization mediated by the GAL4 DNA-binding domain but would enhance the T3-mediated activity of GAL4-T3R by binding to

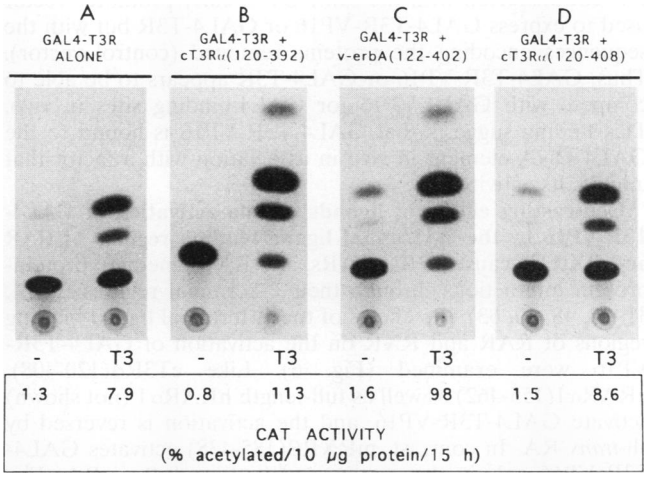

FIG. 5. Activation of GAL4-T3R by T3 is enhanced by the C-terminal regions of cT3Rα or v-ErbA that do not bind ligand. HeLa cells were transfected with 5 μg of pMC110 and 1 μg of GAL4-T3R alone or with 5 μg of vectors expressing cT3Rα(120-392), v-ErbA(122-402), or cT3Rα(120-408). Cells were incubated for 40 h with or without 100 nM T3 prior to assaying for CAT activity. Results were quantitated as described in the legend to Fig. 4.

TABLE 1. GAL4-T3R-VP16 and GAL4-T3R bind to the GAL4 response element in vivo[a]

Transfection (amt [μg])	CAT activity (% acetylated/25 μg of protein/15 h)
GAL4-VP16 (0.5)	9.4
Control vector (1)	0.31
GAL4-T3R-VP16 (1)	0.58
GAL4-T3R (1)	0.42
GAL4-VP16 (0.5)	
+ control vector (1)	10.3
+ GAL4-T3R-VP16 (1)	0.38
+ GAL4-T3R (1)	0.25

[a] HeLa cells were transfected by electroporation with 5 μg of pMC110 and 0.5 to 1 μg of the indicated expression vectors. Following transfection, cells were incubated for 40 h prior to determination of CAT activity.

the inhibitor and increasing the amount of inhibitor-free GAL4-T3R. Consistent with this interpretation are studies with cT3R-VP16, which links the C-terminal region of cT3Rα(1-392) in frame with the 90-amino-acid VP16 transactivation domain. This chimera contains the DNA-binding and RXR heterodimerization domains of cT3Rα and inefficiently binds T3. However, it does not activate a highly responsive thyroid hormone-responsive reporter gene (ΔMTV-TREp-CAT) in HeLa cells when expressed alone, using up to 50 μg of cT3R-VP16 expression vector or when coexpressed with cT3Rα(120-392) with or without T3. In contrast, cT3R(408)-VP16, which contains the wild-type cT3Rα protein (amino acids 1 to 408) linked in frame to the 90-amino-acid VP16 protein, mediated stimulation by T3 but was inactive without ligand (results not shown).

Since T3R is thought to bind to DNA response elements in vivo in the absence of ligand (22, 56), the receptor-inhibitor complex may be bound to DNA in the cell. To examine this possibility, cells were transfected with GAL4-VP16 alone or together with GAL4-T3R-VP16 or GAL4-T3R (Table 1). This study shows that the activity of GAL4-VP16 is inhibited by GAL4-T3R-VP16, or by GAL4-T3R in the absence of T3. No inhibition was found when the GAL4-VP16 expression vector was cotransfected with the same SV40 early promoter vector used to express GAL4-T3R-VP16 or GAL4-T3R but with the sequences encoding the proteins removed (control vector). Thus, GAL4-T3R-VP16 or GAL4-T3R appears to be able to compete with GAL4-VP16 for GAL4-binding sites in vivo. This finding suggests that GAL4-T3R-VP16 is bound to the GAL4 DNA element in vivo in association with a factor that inhibits its activity.

Contrasting effects of ligands on the activation of GAL4-T3R-VP16 by the C-terminal ligand-binding regions of RAR and RXR. Because T3Rs, RARs, and RXRs mediate protein-protein interactions through their C-terminal regions (3, 24, 31, 32, 48, 50, 63), the effects of the C-terminal ligand-binding regions of RAR and RXR on the activation of GAL4-T3R-VP16 were examined (Fig. 6). Like cT3Rα(120-408), hRARα1(155-462) as well as full-length hRARα1 (not shown) activate GAL4-T3R-VP16, and the activation is reversed by all-*trans* RA. In contrast, mRXRβ(185-438) activates GAL4-T3R-VP16 only in the presence of its ligand, 9-*cis* RA (Fig. 6D). This paradoxical effect may reflect a high-affinity interaction involving the C-terminal regions of T3R and RXR that occurs independently of DNA binding (50). This notion is also supported by a recent gel shift study using a GAL4 response element indicating that RXRβ forms a heterodimer with GAL4-rT3Rα1, a chimera consisting of the GAL4 DNA-binding domain linked to the ligand-binding domain of rT3Rα1 (amino acids 121 to 410) (42). Thus, even if RXR is able to compete with GAL4-T3R-VP16 for binding to the putative inhibitor, it might also interact with the T3R region of the chimera with sufficient affinity to repress the activity of the chimera. Stimulation by 9-*cis* RA suggests that the ligand may act to dissociate complexes formed in vivo between RXR and T3R and is compatible with the recent in vitro finding that 9-*cis* RA decreases RXR-T3R heterodimers and increases RXR homodimer formation (75). An alternative explanation for this finding is that GAL4-T3R-VP16 binds to the GAL4 response element in association with RXR and the activation by 9-*cis* RA is mediated by a transactivation domain in the tethered RXR molecule. Such a mechanism has been suggested to explain stimulation of a GAL4 reporter in cells expressing GAL4-RXR and wild-type RAR at low concentrations of all-*trans* RA (53). To determine whether this mechanism might account for the stimulation found with 9-*cis* RA in Fig. 6D, cells were cotransfected with GAL4-T3R and mRXRβ(185-438) (Fig. 6E). In contrast to GAL4-T3R-VP16 and mRXRβ(185-438), no stimulation by 9-*cis* RA was found in cells cotransfected with GAL4-T3R and mRXRβ(185-438), suggesting that the activation found with 9-*cis* RA is mediated by GAL4-T3R-VP16 and not by a transactivation domain in RXR. Addition of 9-*cis* RA to HeLa cells transfected with GAL4-T3R-VP16 alone did not cause significant activation of the chimera (Fig. 6A), indicating that the putative inhibitor in

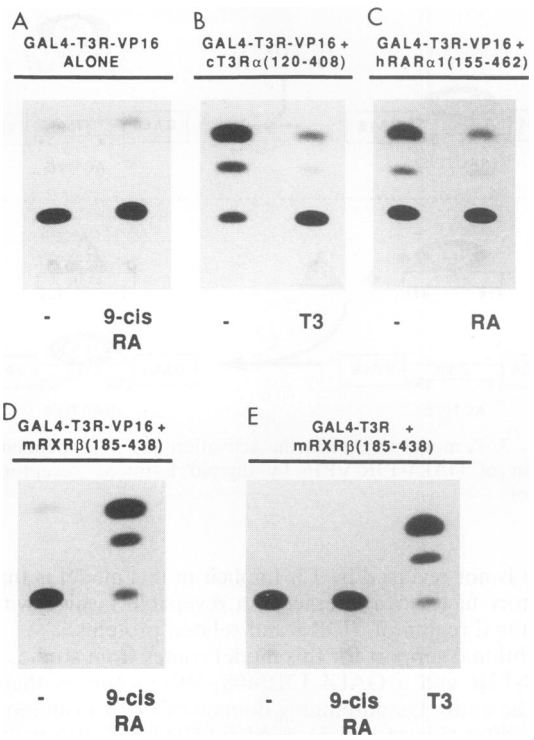

FIG. 6. Influence of the C-terminal ligand-binding region of RAR and RXR on the activation of GAL4-T3R-VP16. (A to D) HeLa cells were transfected with 5 μg of pMC110 and 1 μg of GAL4-T3R-VP16 alone or with 5 μg of the indicated vector expressing the ligand-binding domains of cT3Rα(120-408), hRARα1(155-462), or mRXRβ(185-438). In a parallel experiment (E), cells were transfected with 1 μg of GAL4-T3R and 5 μg of mRXRβ(185-438). Cells were incubated for 40 h with or without 100 nM T3, 1 μM all-*trans* RA, or 1 μM 9-*cis* RA as indicated prior to measurement of CAT activity.

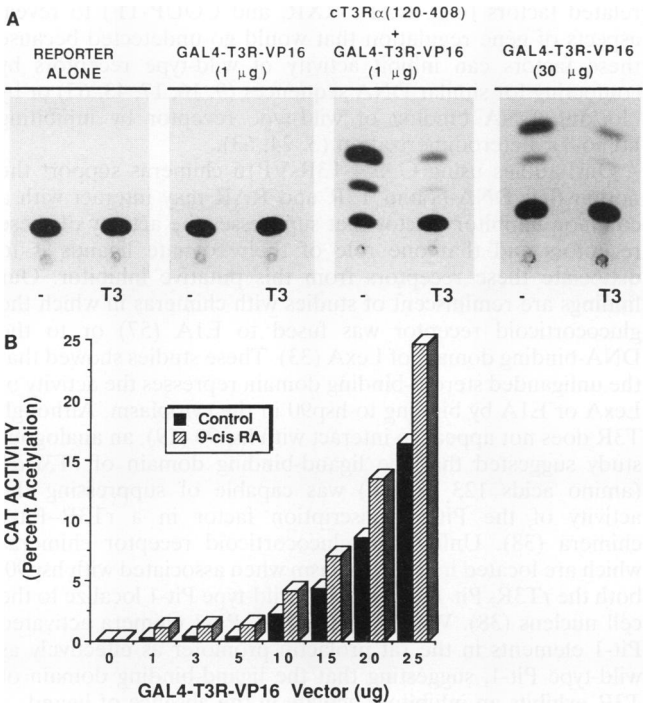

FIG. 7. Activation of GAL4-T3R-VP16 in GH4C1 cells. (A) GH4C1 cells were transfected by electroporation with 5 μg of pMC110 reporter alone or with 1 μg of a plasmid expressing GAL4-T3R-VP16 from the RSV LTR, 1 μg of the RSV GAL4-T3R-VP16 vector plus 5 μg of cT3Rα(120-408), or 30 μg of RSV GAL4-T3R-VP16 vector. Cells were incubated for 40 h with or without 100 nM T3 and then harvested and assayed for CAT activity. (B) GH4C1 cells were transfected with 5 μg of pMC110 alone or with the amounts of RSV GAL4-T3R-VP16 indicated. Cells were then incubated for 40 h with or without 1 μM 9-*cis* RA prior to determination of CAT activity. Following autoradiography, acetylated and unacetylated [^{14}C]chloramphenicol were excised from the thin-layer plates and analyzed in a liquid scintillation counter. The results are presented as percent acetylation of [^{14}C]chloramphenicol, using 25 μg of cell protein in a 15-h incubation at 37°C. Because the SV40 early promoter in the pSG424-based vector expresses poorly in GH4C1 cells, a vector expressing GAL4-T3R-VP16 from the RSV LTR, constructed as described in Materials and Methods, was used.

HeLa cells is not the endogenous hRXRβ expressed in these cells (48).

Effects of endogenous T3Rs and RXRs on the activity of GAL4-T3R-VP16 in GH4C1 cells. Activation of GAL4-T3R-VP16 also occurs in rat GH4C1 pituitary cells, a well-characterized cell line responsive to physiological concentrations of T3 and RA (Fig. 7A) (8, 27, 28). Since the SV40 promoter used to express GAL4-T3R-VP16 is minimally active in GH4C1 cells, GAL4-T3R-VP16 was cloned into an RSV LTR expression vector which is highly active in these cells (23, 27, 28). When GAL4-T3R-VP16 is expressed at lower levels by transfecting GH4C1 cells with 1 μg of the RSV vector, coexpression of cT3Rα(120-408) activates the chimera, and this activation is repressed by T3 (Fig. 7A). With 30 μg of the RSV-GAL4-T3R-VP16 vector, activation occurs without cotransfection of a vector expressing a T3R derivative but is still repressed by T3 (Fig. 7A). This coexpression-independent activation of the chimera in GH4C1 cells and its subsequent inhibition by ligand may result from low levels of an inhibitor and/or from competition for the putative inhibitory factor by rT3Rα1, rT3Rβ1, and rT3Rβ2 which are expressed in these cells (15, 36, 37, 39). In contrast, 30 to 50 μg of vector expressing GAL4-T3R-VP16 alone from either SV40 or RSV sequences did not activate gene expression in HeLa cells (not shown).

Since GH4C1 cells and related cell lines also express RXR (73), which might contribute to the inhibition of GAL4-T3R-VP16, the effects of 9-*cis* RA were assessed in these cells following transfection with various amounts of the RSV GAL4-T3R-VP16 vector (Fig. 7B). Transfection with 1, 3, or 5 μg of RSV-GAL4-T3R-VP16 resulted in a low level of activation of the GAL4 reporter gene that was increased about threefold by 9-*cis* RA. This level of activation of GAL4-T3R-VP16 by 9-*cis* RA represents only about 10% of the level of activation following cotransfection of 1 μg of RSV-GAL4-T3R-VP16 and 5 μg of vector expressing cT3Rα(120-408) (Fig. 7A). This result again indicates that although RXR is capable of inhibiting GAL4-T3R-VP16, RXR is probably not the major inhibitor indicated by these studies. Higher levels of expression of GAL4-T3R-VP16, following transfection with 10 to 25 μg of RSV-GAL4-T3R-VP16, resulted in a marked dose-dependent increase in CAT activity (Fig. 7B), suggesting that the activity of GAL4-T3R-VP16 increases as its level exceeds that of the putative inhibitory factor.

COUP-TF inhibits activation of gene expression by GAL4-T3R-VP16 and GAL4-T3R. The finding that the highly conserved ninth heptad in the ligand-binding domain of T3R may be involved in the interaction of receptor with an inhibitory factor (Fig. 2) suggests that the inhibitor may a related member of the thyroid/retinoid receptor subfamily. The COUP-TFs (COUP-TFI and COUP-TFII) are widely expressed orphan receptor members of this subfamily (14, 16, 17, 68). COUP-TFs are thought to bind their cognate response elements as homodimers and have been shown to inhibit the activity of the T3Rs, RARs, and RXRs on both optimized and native hormone receptor response elements (9, 16, 17, 43, 65). Several mechanisms for this inhibition, including active silencing of transcription, sequestration of RXR, or competition for DNA binding, have been proposed (16, 17, 43, 65). Heterodimeric interactions between COUP-TF and T3R or RAR were not thought to be a mechanism for inhibition (16, 17, 65), although one report indicated that COUP-TFI could bind to DNA as a heterodimer with rT3Rα1 (9). Since the major inhibitory factor in HeLa cells does not appear to be RXR and the COUP-TFs are expressed in a wide variety of cells, including HeLa cells (68), we examined whether COUP-TFI might act to inhibit activation of GAL4-T3R-VP16 by cT3R(120-408) and GAL4-T3R by T3 (Table 2). COUP-TFI does not inhibit activation of GAL4-VP16, indicating that it does not bind to the GAL4-activating sequence or inhibit transcriptional activation of the promoter. In contrast, COUP-TFI inhibits activation of GAL4-T3R-VP16 by cT3R(120-408) and the T3 stimulation of GAL4-T3R. Thus, unlike mRXRβ (compare Fig. 5A and Fig. 6E), COUP-TFI inhibits T3-dependent activation of GAL4-T3R. Similar results were found for COUP-TFII (not shown).

Implications for ligand-dependent regulation of gene expression. Ligands mediate significant changes in the conformation of the thyroid/retinoid receptor subfamily. In addition to T3-mediated changes in the circular dichroism spectrum of T3R (64), ligand increases the electrophoretic mobility of certain DNA-T3R complexes (2, 24, 55, 58). T3 also decreases the extent of T3R homodimer bound to certain direct repeat elements (2, 21, 71). In contrast, 9-*cis* RA has been reported to enhance RXR homodimer formation (75). T3 does not alter the abundance of T3R-RXR heterodimers bound to DNA elements, although the ligand may alter the contact sites of interaction between these two proteins (3). In contrast, T3 can

TABLE 2. COUP-TFI inhibits activation by GAL4-T3R-VP16 and GAL4-T3R[a]

Transfection (amt [μg])	CAT activity (% acetylated/15 μg of protein/16 h)	
	Basal	+T3
pMC110 (5)	0.2	0.2
+ GAL4-T3R-VP16 (1)	0.2	0.2
+ GAL4-T3R-VP16 (1) + cT3R(120-408) (5)	26	3.1
+ GAL4-T3R-VP16 (1) + COUP-TFI (4)	0.4	0.3
+ GAL4-T3R-VP16 (1) + cT3R(120-408) (5) + COUP-TFI (4)	2.0	0.8
+ GAL4-T3R-VP16 (1) + cT3R(120-408) (5) + control vector (4)	24	2.9
+ GAL4-T3R (1)	0.2	58
+ GAL4-T3R (1) + COUP-TFI (4)	0.2	13
+ GAL4-T3R (1) + control vector (4)	0.2	63
+ GAL4-VP16 (1)	35	36
+ GAL4-VP16 (1) + COUP-TFI (4)	33	35

[a] HeLa cells were transfected by electroporation with 5 μg of the GAL4 CAT reporter plasmid pMC110 alone and with the indicated amounts of expression vectors. The GAL4 chimeras are expressed from the SV40 early promoter, while the other proteins, including COUP-TFI, are regulated by an RSV expression vector. The indicated control vector is an RSV vector lacking a cDNA. Following transfection, cells were incubated for 40 h without (basal) or with (+T3) 1 μM T3 prior to determination of CAT activity.

decrease the amount of DNA bound T3R-RAR heterodimers on certain response elements (3, 72). Thus, the binding of ligand can alter homo- or heterodimeric interactions and mediate conformational changes which may lead to the formation of a transactivating domain or structure. If transactivation is mediated by a heterodimer between T3R and RXR, it is not yet apparent whether this transactivation function results solely from a T3-mediated conformational change in T3R or whether the unliganded heterodimeric partner contributes an activation function in addition to enhancing DNA binding.

Although the in vitro binding studies described above identify effects of T3 on the conformation of T3R and its DNA binding, an important question relates to how these conformational changes lead to transcriptional activation. In this study, we used GAL4-receptor chimeras to probe the role of ligand on transcriptional activation. Several lines of evidence indicate that GAL4-receptor chimeras provide a model for activation of wild-type receptor by ligand. First, GAL4-T3R transcriptionally activates gene expression only in the presence of ligand (7, 42) (Fig. 1 and 5; Table 2). Second, other effects of wild-type T3R or RAR, such as the ligand-independent reduction in basal gene expression (active silencing), are also mediated by GAL4-T3R or GAL4-RAR (5, 7). This silencing effect is thought to result from the interaction of unliganded receptor with one or more of the basal core transcription factors (4). Therefore, two of the most-studied actions of the T3Rs and RARs, ligand-dependent activation and silencing by unliganded receptors, are also mediated by GAL4-receptor chimeras. A potential advantage of GAL4-receptor chimeras is that mammalian cell transcription factors do not appear to interact with GAL4 response elements or interfere with the activation of GAL4-responsive genes by GAL4 proteins (54). Furthermore, GAL4 binds to its response element as a dimer through a dimerization domain in its DNA-binding domain (13). In contrast, the dimerization domains of the T3Rs, RARs, and RXRs necessary for efficient homo- and heterodimeric DNA binding are localized within their ligand-binding domains (24, 25). Thus, GAL4-T3R chimeras permit the use of T3R and related factors (e.g., RAR, RXR, and COUP-TF) to reveal aspects of gene regulation that would go undetected because these factors can inhibit activity of wild-type receptors by competing for similar DNA sequences (9, 16, 17, 43, 65) or by blocking DNA binding of wild-type receptor by inhibiting homo- or heterodimerization (3, 24, 63).

Our studies using GAL4-T3R-VP16 chimeras support the notion that DNA-bound T3R and RAR may interact with a common inhibitory factor that suppresses the activity of these receptors and that one role of their cognate ligands is to dissociate these receptors from this putative inhibitor. Our findings are reminiscent of studies with chimeras in which the glucocorticoid receptor was fused to E1A (57) or to the DNA-binding domain of LexA (33). These studies showed that the unliganded steroid-binding domain represses the activity of LexA or E1A by binding to hsp90 in the cytoplasm. Although T3R does not appear to interact with hsp90 (19), an analogous study suggested that the ligand-binding domain of rT3Rα1 (amino acids 123 to 410) was capable of suppressing the activity of the Pit-1 transcription factor in a rT3R–Pit-1 chimera (38). Unlike the glucocorticoid receptor chimeras which are located in the cytoplasm when associated with hsp90, both the rT3R–Pit-1 chimera and wild-type Pit-1 localize to the cell nucleus (38). With T3, the rT3R–Pit-1 chimera activated Pit-1 elements in the rat prolactin promoter as effectively as wild-type Pit-1, suggesting that the ligand-binding domain of T3R exhibits an inhibitory activity in the absence of ligand.

Our results of studies using GAL4 chimeras provide additional evidence for an inhibitory factor that interacts with T3R and suggest that the same factor also interacts with RAR (Fig. 6). Although RXR appears to exhibit weak inhibitory effects under the conditions of these experiments (Fig. 7B), our results suggest that RXR is not the major inhibitory factor in the two cell lines studied. Our studies also provide certain functional insights into the role of ligands in the interactions of T3R and RXR in cells. The finding that mRXRβ activates GAL4-T3R-VP16 only in the presence of 9-*cis* RA (Fig. 6) provides functional evidence that this ligand may reduce an inhibitory heterodimeric interaction between RXR and the T3R sequences in GAL4-T3R-VP16. This observation is consistent with the suggestion of Zhang et al. (75) that 9-*cis* RA-mediated stimulation of RXR homodimerization may lead to a decrease in the amount of T3R-RXR or RAR-RXR heterodimers. Lastly, the observation that r-c-ErbAα2 as well as cT3Rα(L365R) and cT3Rα(L372R) do not activate GAL4-T3R-VP16 suggests that the highly conserved ninth heptad is involved in the interaction of T3R with this inhibitory factor. Because the region containing the ninth heptad is known to play a role in heterodimeric interactions between related members of the thyroid/retinoid receptor gene subfamily (3, 50, 63), the inhibitor may be a related factor or an orphan receptor member of this family. In support of this notion is our finding that the related orphan receptors COUP-TFI and COUP-TFII inhibit activation of GAL4-T3R by T3 or GAL4-T3R-VP16 by cT3Rα(120-408) (Table 2). These inhibitions appear to be mediated through the ligand-binding domain of T3R since the COUP-TFs do not inhibit activation by GAL4-VP16, which is known to interact with similar core transcription factors (e.g., TFIIB) as COUP-TF (41, 59). Although several studies have suggested that COUP-TF does not form heterodimers with T3R but inhibits by competing for DNA binding, by titration of RXR, or by active silencing of gene promoters (16, 17, 43, 65), Berrodin et al. (9) reported that COUP-TF could form heterodimers with rT3Rα1. Our finding that COUP-TF inhibits activation by GAL4-T3R-VP16 and GAL4-TR suggests that the ligand-binding domain of T3R

associates with COUP-TF independently of DNA binding and that such an interaction may lead to silencing or suppression of transcriptional activity that is mediated by the ligand-binding domain of T3R. Although these studies do not document that COUP-TF is the putative inhibitor, they support the notion that the inhibitor of activation detected in these studies is a related factor and further emphasize the complex functional interactions mediated by members of the steroid/thyroid hormone receptor gene family.

ACKNOWLEDGMENTS

We thank J. Toney and M. Marks for helpful suggestions and comments on the manuscript. We also thank M. Marks and K. Ozato for the wild-type mRXRβ cDNA clone, M. Ptashne for pMC110, pSG424, and GAL4-VP16, Ming-jer Tsai for RSV-COUP-TFI and pMT2-COUP-TFII, and A. Levin and J. Grippo (Hoffmann La Roche) for 9-*cis* RA.

This study was supported by NIH grant DK16636 (H.H.S.), NYU GCRC grant NCRR M0100096, a Hascoe Foundation grant to NYU, NIH Medical Scientist Training Program grant GM-07308 (M.A.-F.), The Aaron Diamond Foundation (V.D.-Y.), and a postdoctoral fellowship from the Philippe Foundation and Association pour la Recherche sur le Cancer (S.S.-R.).

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