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Trans-activation by thyroid hormone receptors: Functional parallels with steroid hormone receptors

(*erbA* gene/gene regulation/transcription factors)

CATHERINE C. THOMPSON*† AND RONALD M. EVANS*

*Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA 92138; and †University of California at San Diego, Department of Biology, La Jolla, CA 92093

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ABSTRACT **The effects of thyroid hormones are mediated through nuclear receptor proteins that modulate the transcription of specific genes in target cells. We previously isolated cDNAs encoding two different mammalian thyroid hormone receptors, one from human placenta (hTRβ) and the other from rat brain (rTRα), and showed that their *in vitro* translation products bind thyroid hormones with the characteristic affinities of the native thyroid hormone receptor. We now demonstrate that both of the cloned receptors activate transcription from a thyroid hormone-responsive promoter in a hormone-dependent manner, with rTRα eliciting a greater response than hTRβ. The putative functional domains of the thyroid hormone receptors were examined by creating chimeric thyroid hormone/glucocorticoid receptors, producing receptors with hybrid functional properties. These experiments support the proposal that the thyroid hormone receptors are composed of interchangeable functional domains, and indicate that the mechanism of hormone-inducible gene regulation has been conserved in steroid and thyroid hormone receptors.**

The thyroid hormones, thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3), are critical for the development of the central nervous system and maintenance of homeostasis and can influence the synthesis and activity of many important regulatory proteins (1). The actions of thyroid hormones, like those of the steroid hormones, are mediated through an intracellular receptor protein (2, 3).

The structural relationship of the v-*erbA* oncogene product to the human glucocorticoid receptor (hGR) led to the proposal that the cellular homolog of v-*erbA* might encode a ligand-binding transcription factor (4). A cellular homolog of v-*erbA* was isolated, and its product was found to bind thyroid hormones with affinities characteristic of the native thyroid hormone receptor (5, 6). The previous identification of multiple c-*erbA* genes on human chromosomes 3 and 17 (5, 7, 8) predicted the existence of multiple thyroid hormone receptors, which led to the discovery of a second thyroid hormone receptor (rTRα) (9). The existence of at least two mammalian thyroid hormone receptors [α, represented by a rat brain cDNA (rTRα), and β, represented by a human placental cDNA (hTRβ)] poses the question of the physiological significance of multiple receptors for the same ligand and forces a reevaluation of the means by which thyroid hormones exert their effects.

In addition to being highly related to each other, the deduced primary amino acid sequences of both thyroid hormone receptors (hTRβ and rTRα) showed conservation with the steroid hormone receptors, members of a superfamily of ligand-binding transcription factors (10). Extensive analysis of the structural and functional properties of the glucocorticoid and other steroid hormone receptors has led to the identification of discrete functional domains in these molecules (10, 11). A centrally located region of 66–68 amino acids with 9 conserved cysteine residues, thought to form two "zinc finger" structures, is essential for DNA binding and for the specificity of target-gene activation (12–14). The carboxyl-terminal part of the receptors is required for ligand binding and regulates transformation of the receptors to an active form (13, 14). The function of the amino terminus is not clear, although in some cases it appears to determine the efficiency and specificity of trans-activation (13–15). The thyroid and steroid hormone receptors show the greatest similarity in the DNA-binding domain, with 47% amino acid identity (10). The ligand-binding domains have low but measurable homology, whereas the amino termini show no conservation of size or amino acid composition (10).

The structural similarity between the thyroid hormone and steroid hormone receptors, in conjunction with their hormone-dependent regulation of gene expression, suggests that they may be functionally related as well. Therefore, we have addressed the issues of whether both thyroid hormone receptors can act as transcription factors and whether thyroid hormone receptors have the same functional architecture as the steroid hormone receptors.

To analyze thyroid hormone receptor action, we have used a cotransfection assay to demonstrate that both of the cloned thyroid hormone receptors are capable of activating transcription from a thyroid hormone-responsive promoter in a hormone-dependent manner. Further, we have compared the functional properties of thyroid hormone and glucocorticoid receptors by exchanging domains between the human glucocorticoid and thyroid hormone receptors to produce functional hybrids. These experiments identify the domains of the thyroid hormone receptors responsible for promoter recognition and ligand binding and demonstrate that the mechanism of the "hormone switch" for receptor activation is maintained between distantly related members of the steroid hormone receptor superfamily.

MATERIALS AND METHODS

Cell Culture and Transfection. Conditions for growth and transfection of CV-1 (monkey kidney) cells were as previously described (13), except that the calcium phosphate precipitate was left on the cells for 4–8 hr. The medium was then changed to Dulbecco's modified Eagle's medium with 5% T_3-free bovine serum (Scantibodies, Lakeside, CA) plus or minus 0.1 μM T_3. For experiments with hybrid receptors, serum was also treated with charcoal to remove steroids (16)

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Abbreviations: T_3, 3,5,3′-triiodothyronine; T_4, thyroxine; Triac, 3,5,3′-triiodothyroacetic acid; CAT, chloramphenicol acetyltransferase; rTRα, rat α thyroid hormone receptor; hTRβ, human β thyroid hormone receptor; hGR, human glucocorticoid receptor; TRE, thyroid hormone response element; GRE, glucocorticoid response element; RSV, Rous sarcoma virus; MTV, mouse mammary tumor virus; LTR, long terminal repeat.

and T_3 or dexamethasone was added to 0.1 μM. Cells were harvested after 36 hr, and chloramphenicol acetyltransferase (CAT) assays were performed as described (17, 18). Typically, 5 μg of reporter and 2–5 μg of expression vector were cotransfected, with 2.5 μg of RSV-βgal as a control for transfection efficiency. Acetylated and nonacetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography, excised, and quantitated by liquid scintillation counting in Econofluor (DuPont) with 5% dimethyl sulfoxide. β-Galactosidase assays were performed as described (19). CAT activity is expressed as percent conversion divided by β-galactosidase activity.

Construction of Reporter and Expression Plasmids. To create TRE-CAT, a synthetic oligonucleotide corresponding to positions −169 to −200 of the rat growth hormone gene was inserted into a linker scanning mutant of MTV-CAT that has a *Hin*dIII site at −190/−181 (20). To create TREp1 MCAT and GREp1 MCAT, synthetic oligonucleotides corresponding to either a palindromic thyroid hormone response element (TRE) (21) or a palindromic glucocorticoid response element (GRE) (22) were inserted into −190/−88 MTV-CAT, which has a *Hin*dIII site replacing the nucleotides between −88 and −190 (23). Expression vectors were constructed for the thyroid hormone receptors by inserting the full-length cDNAs of pheA12 (5) and rbeA12 (9) between the *Kpn* I and *Bam*HI sites of the pRS vector (13, 24).

Construction of Chimeric Receptors. The construction of hGR_{NX} has been described (24). To construct $hTR\beta_{NX}$, the cDNA insert of pheA12 (5) was subcloned between the *Kpn* I and *Bam*HI sites of M13mp19 and mutagenized by the method of Kunkel (25). The oligonucleotide used to create the *Not* I site changed Asp-97 to Arg, Lys-98 to Pro, and Asp-99 to Pro. The oligonucleotide used to create the *Xho* I site changed Thr-171 to Leu and Asp-172 to Gly. The mutant receptor cDNA was transferred to the pRS vector (13, 24); hybrids were constructed by exchanging *Kpn* I–*Not* I, *Kpn* I–*Xho* I, or *Not* I–*Xho* I restriction fragments between $RShGR_{NX}$ and $RShTR\beta_{NX}$. $RShGR_{NX}$ has about 75% of wild-type activity, and $RShTR\beta_{NX}$ has about 60% of wild-type activity. To add the τ_1 domain of the hGR to rTRα, the unique *Bst*EII site at the codon for rTRα amino acid 21 was changed to a *Bam*HI site by inserting an oligonucleotide adaptor that encoded a *Bam*HI site flanked by *Bst*EII ends. This allowed the in-frame insertion of a *Bam*HI–*Bgl* II fragment encoding amino acids 77–262 of the hGR into this site. ΔTT and ΔGG were constructed by deleting the *Asp*718–*Not* I fragment of $RShTR\beta_{NX}$ and $RShGR_{NX}$, respectively, and replacing it with an oligonucleotide adaptor consisting of a consensus ribosome binding site followed by an initiator codon (23).

RESULTS

Trans-Activation by Thyroid Hormone Receptors. To study transcriptional activation by thyroid hormone receptors, we have adapted the cotransfection assay originally devised to study the function of the hGR (13). In this assay, two plasmids are transfected into a receptor-deficient cell line, the first to express a receptor protein, the other to monitor transcription from a hormone-responsive promoter. For the thyroid hormone receptor assay, the expression plasmid contains the Rous sarcoma virus (RSV) long terminal repeat (LTR) directing the expression of a cDNA encoding a thyroid hormone receptor (Fig. 1*A*). For the hGR, the reporter plasmid has the mouse mammary tumor virus (MTV) LTR fused to the bacterial CAT gene. To convert MTV-CAT to a thyroid hormone-responsive reporter, an oligonucleotide containing a TRE was inserted at position −191 of the MTV LTR to generate TRE-CAT (Fig. 1*A*). This TRE, positions −169 to −200 of the rat growth hormone gene, specifically binds thyroid hormone receptors and can confer T_3 responsiveness on a heterologous promoter (26). Expression and reporter plasmids were cotransfected into CV-1 cells and CAT activity was measured in the absence and presence of T_3. Fig. 1*B* shows that neither thyroid hormone receptor activates transcription from MTV-CAT. However, the presence of a TRE results in a thyroid hormone-responsive MTV promoter. Induction of CAT activity is dependent on the cotransfection of a functional thyroid hormone receptor and the addition of T_3. In the presence of T_3, rTRα induces CAT activity ≈15-fold, while hTRβ induces activity ≈5-fold.

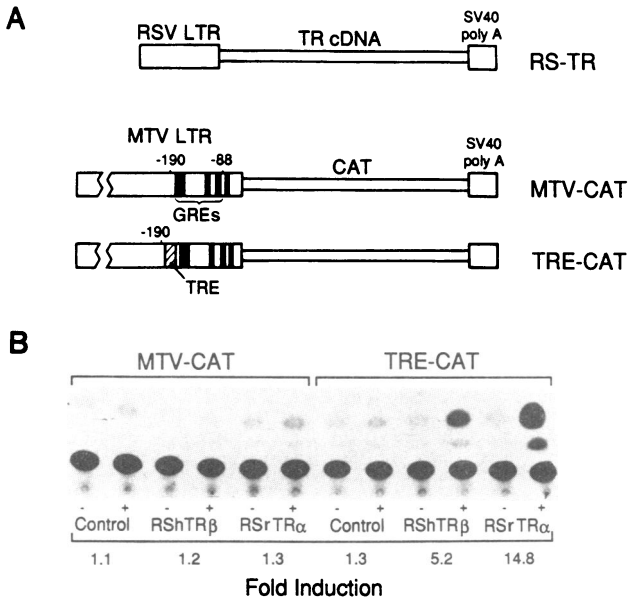


FIG. 1. Trans-activation by thyroid hormone receptors. (*A*) Structure of thyroid hormone receptor expression and reporter plasmids. RS-TR has the RSV LTR directing the expression of either rTRα (9) or hTRβ (5) cDNA followed by a simian virus 40 (SV40) polyadenylylation signal. MTV-CAT, which has the MTV LTR directing expression of the CAT gene, is shown with its GREs indicated as black boxes (20). TRE-CAT is identical to MTV-CAT except that it has a TRE from the rat growth hormone promoter (26) inserted at position −191. (*B*) Induction of CAT activity by thyroid hormone receptors. CV-1 cells were cotransfected with TRE-CAT and either rTRα or hTRβ expression plasmids. An expression vector with the rTRα cDNA in the antisense orientation was cotransfected as a negative control. Cultures were maintained in the absence (−) or presence (+) of 0.1 μM T_3 and assayed for CAT activity. Fold induction, which is CAT activity in the presence of T_3 divided by CAT activity in the absence of T_3, is the average of five experiments.

To evaluate how hormone binding relates to receptor activation, the dose response of the thyroid hormone receptors to T_3 and other thyroid hormones was characterized (Fig. 2). Half-maximal stimulation was observed at ≈6 nM T_3 for rTRα and ≈3 nM T_3 for hTRβ. The concentration of T_3 required for efficient activation is higher than would be predicted based on the dissociation constant of the receptors for T_3, but this is probably due to the presence of serum binding proteins that decrease the concentration of free T_3. While T_4 was capable of activating the thyroid hormone receptors, 20- to 40-fold more T_4 than T_3 was required to elicit a comparable response, reflecting its lower binding affinity for the receptors. Triac showed half-maximal activation at 30 nM for rTRα and ≈4 nM for hTRβ, consistent with the previous observation that rTRα has lower affinity for Triac than hTRβ (9). Binding studies with *in vitro* translated hTRβ had shown that hTRβ has greater affinity for Triac than for T_3 (5). However, approximately the same concentrations of Triac and T_3 are required for equivalent activation, indicating that affinity and activation are not strictly correlated.

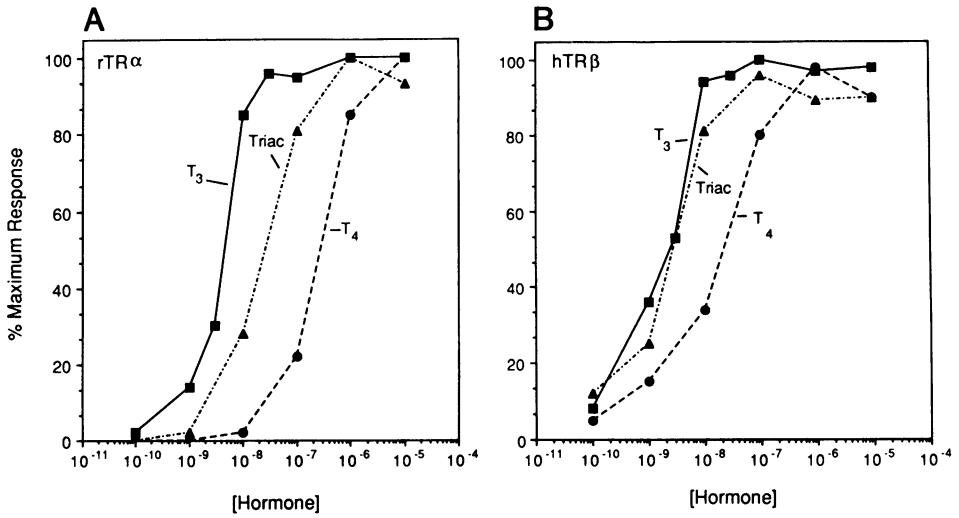


FIG. 2. Dose response of thyroid hormone receptor transcriptional activity. Individual plates of CV-1 cells cotransfected with TRE-CAT and either rTRα (*A*) or hTRβ (*B*) expression plasmid were exposed to various concentrations of T_3 (■), T_4 (●), or 3,5,3'-triiodothyroacetic acid (Triac, ▲). Percent maximum response was calculated separately for each hormone and is the average of at least three experiments. The magnitude of activation is approximately the same for all of the hormones tested. Cells transfected with the reporter plasmid but not with a receptor expression plasmid showed background activity (unpublished observations).

Chimeric Thyroid Hormone/Glucocorticoid Receptors. A domain structure for the thyroid hormone receptors has been assigned based on homology with the steroid hormone receptors (5, 9), but the functions of these putative domains have not been demonstrated directly. We have constructed hybrid thyroid hormone/glucocorticoid receptors to compare the functional properties of the thyroid hormone and glucocorticoid receptors. To construct hybrid receptors, unique sites for the restriction enzymes *Not* I and *Xho* I were introduced into the amino- and carboxyl-terminal sides of the DNA-binding domains of the hGR and hTRβ. These receptors, termed hGR_{NX} (24) and $hTR\beta_{NX}$, were used to create hybrids with all possible combinations of amino terminus, DNA-binding domain, and ligand-binding domain (Fig. 3). The hybrid and parental receptors were assayed using both thyroid hormone- and glucocorticoid-responsive promoters, in the absence or presence of T_3 or the synthetic glucocorticoid dexamethasone. To assess the properties of the DNA-binding domains, promoters that were selectively hormone-responsive and of similar structure were created by replacing the wild-type GREs in MTV-CAT with either a palindromic TRE (21) (TREp1 MCAT) or a palindromic GRE (22) (GREp1 MCAT). With these reporters, $hTR\beta_{NX}$ gives ≈20-fold induction, and hGR_{NX} gives ≈150-fold induction (unpublished observations).

The structures and activities of the hybrid receptors are shown in Fig. 3. The receptors are divided into three sections, so that $hTR\beta_{NX}$ is called TTT, and hGR_{NX} is called GGG. Hybrids are named by letters referring to the origin of the domain; for example, "TGT" has the amino and carboxyl termini of hTRβ (T-,-T) and the DNA-binding domain of the hGR (-G-). Hybrids with a putative hTRβ DNA-binding domain (TTG, GTT, and GTG) activated transcription only from TREp1 MCAT, whereas hybrids with a hGR DNA-binding domain (GGT, TGG, and TGT) activated transcription only from GREp1 MCAT. This demonstrates that this region of hTRβ is analogous to the hGR DNA-binding domain and is responsible for promoter recognition. Hybrid receptors with an hTRβ carboxyl terminus were activated only by T_3, whereas those with an hGR carboxyl terminus were activated only by dexamethasone. This is consistent with the identification of the carboxyl terminus as the part of the receptor that is responsible for hormone binding and activation specificity, and implies that the thyroid and steroid hormone receptors have a common mechanism of activation.

Comparing the activities of the various hybrid receptors provides insight into the regions of the receptors that are responsible for trans-activation. Removal of the amino terminus of either thyroid hormone receptor has little impact on the ability of these receptors to activate this promoter (Fig. 3 and unpublished observations). This suggests that the thyroid hormone receptors do not have a trans-activation function in their amino terminus but may, like the glucocorticoid and estrogen receptors (23, 27), have this function in their carboxyl terminus. The indication that the amino termini of the thyroid hormone receptors lack a trans-activation function that is active on the MTV promoter is supported by the reduced activity of hybrids in which the hTRβ amino terminus replaces that of the hGR. For example, TGG has about 10% of the activity of GGG, or close to the same activity as an hGR with the amino terminus completely removed (ΔGG).

In contrast, hTRβ hybrids with an hGR amino terminus show a significant increase in activity. For example, GTT has ≈7-fold greater activity than TTT. This suggests that the hGR amino terminus possesses a trans-activation function that is not present in the thyroid hormone receptor amino terminus. Previous studies have shown that a discrete region of the hGR amino terminus (τ_1) is responsible for at least part of the transcriptional activity of the hGR (23, 28). To see whether this activity could be transferred to a thyroid hormone receptor, cDNA sequences encoding amino acids 77–262 of the hGR were inserted in-frame after codon 21 of the rTRα cDNA in one or multiple copies (Fig. 4). When the resulting hybrid receptors were assayed for trans-activation, the presence of a single τ_1 domain enhanced activity ≈14-fold. Additional τ_1 domains also increased activity, with maximal activation observed in the presence of two τ_1 domains. These results show that the activity of the thyroid hormone receptor can be augmented by the addition of a trans-activation domain from a heterologous receptor.

DISCUSSION

The action of thyroid hormones is mediated by intracellular receptors, which are associated with chromatin and are believed to be sequence-specific DNA-binding proteins (26, 29–31). These receptors are the primary effectors of thyroid hormone response, presumably by modulating the expression of specific genes in target cells (1, 3, 10). Others have recently shown that the cloned thyroid hormone receptor subtypes can activate transcription from thyroid hormone-responsive promoters (32–34). The results of this study demonstrate that both rTRα and hTRβ can stimulate transcription from a thyroid hormone-responsive promoter, that rTRα produces consistently stronger activation than hTRβ, and that these molecules function in a manner mechanistically similar to that of the steroid hormone receptors. The difference in the response of the two receptors may have important consequences for modulating the transcription of

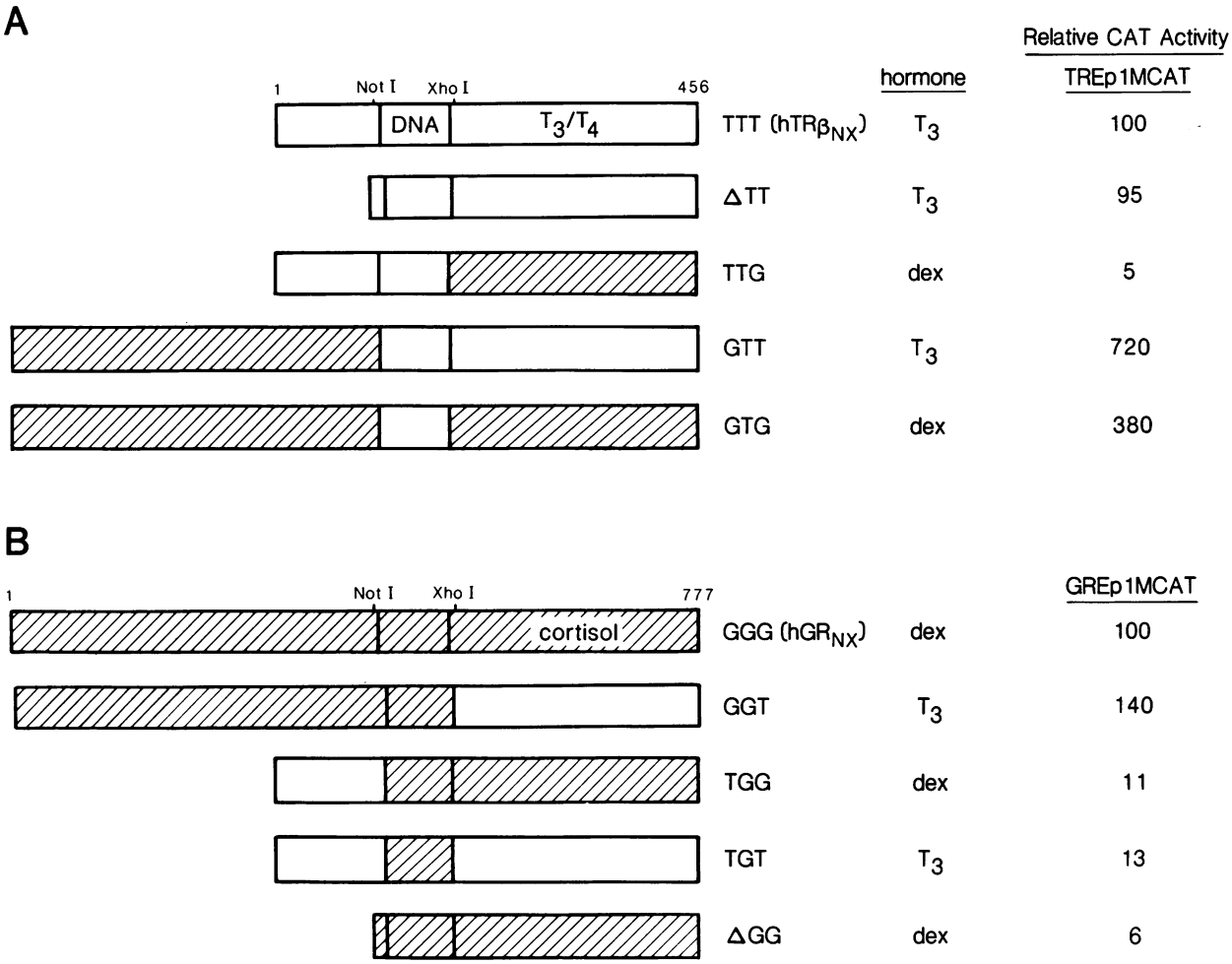


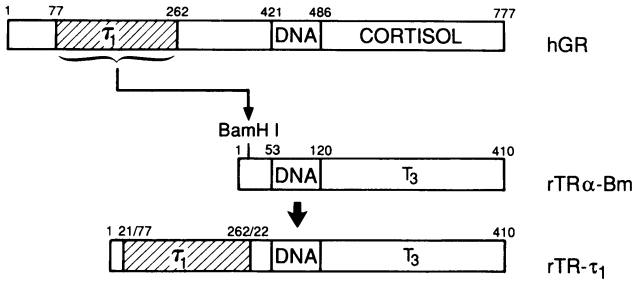
FIG. 3. Structure and activity of chimeric thyroid hormone/glucocorticoid receptors. Unique *Not* I and *Xho* I sites were introduced flanking the sequences encoding the DNA-binding domains of the hGR and hTRβ to create hGR_{NX} (24) and $hTR\beta_{NX}$. "DNA" indicates the DNA-binding domain; "T_3/T_4" and "cortisol" indicate the ligand-binding domains of hTRβ and hGR, respectively. The numbers above the boxes indicate amino acids (5, 18). Hybrids are named by letters referring to the origin of the domain; for example, the TGT hybrid has the amino and carboxyl termini of hTRβ (T-, -T) and the DNA-binding domain of the hGR (-G-). All receptors were assayed on TREp1 MCAT and GREp1 MCAT in the absence or presence of 0.1 μM T_3 or dexamethasone (dex). Values are shown only for receptor/reporter/ligand combinations that gave activation above background, and are the average of at least four experiments. Relative CAT activity is defined as induced CAT activity minus uninduced CAT activity normalized to either $hTR\beta_{NX}$ or hGR_{NX}. Activation is normalized on identical promoters so that activity is measured relative to $hTR\beta_{NX}$ for hybrids with an hTRβ DNA-binding domain (*A*) and relative to hGR_{NX} for hybrids with an hGR DNA-binding domain (*B*).

endogenous thyroid hormone-responsive genes. Although it is not known whether this difference extends to all thyroid hormone-responsive promoters, it implies that these receptors do not merely represent redundant functions but rather are capable of distinct physiological responses.

Although the rTRα cDNA clone was derived from rat brain mRNA, and brain was reported to show high expression of rTRα mRNA (9), the adult brain is not commonly considered a thyroid hormone-responsive tissue. Thyroid hormones are essential for the proper development of the central nervous system (35), yet the adult brain does not show a detectable physiological response to thyroid hormones despite the presence of receptor at levels comparable to T_3-responsive tissues (36). The activity of rTRα indicates that this is not due to the absence of a functional thyroid hormone receptor. However, the recent recognition of multiple alternatively spliced forms of the α receptor, whose function has yet to be determined (refs. 32 and 37–40; unpublished observations), indicates considerable complexity and advises caution for resolving this paradox.

The putative functional domains of the thyroid hormone receptors have been inferred based on structural homology with the glucocorticoid and estrogen receptors (5, 9). By creating hybrid thyroid hormone/glucocorticoid receptors, we were able to experimentally confirm these assignments. Thus, as in the steroid receptors, the cysteine-rich region of the thyroid hormone receptors is also a DNA-binding domain, since a hybrid glucocorticoid receptor harboring this domain activates transcription from a thyroid hormone-responsive promoter. Previous studies had indicated that the carboxyl terminus was required for hormone binding (41), and consistent with this result, in hybrid receptors this region is sufficient to transfer the specificity of hormone activation. The observation that the ligand-binding domain is responsible for the inducibility of the thyroid hormone receptors, and can be interchanged with that of a steroid hormone receptor, points out a striking conservation of function between these receptors. Since thyroid and steroid hormones are neither structurally nor biosynthetically related, it is not surprising that the ligand-binding regions of their receptors are only distantly related. However, the degree of structural conservation present in these molecules is sufficient to maintain the mechanism of the "hormone switch" for receptor activation.

Comparison of the transcriptional activities of the hybrid receptors indicates some differences as well as similarities between the thyroid hormone and glucocorticoid receptors. As is the case for the glucocorticoid and estrogen receptors (23, 27), the carboxyl terminus of the thyroid hormone



Receptor	# of τ_1 domains	Relative CAT Activity
hGR	1	0
rTRα-Bm	0	100
rTR-τ_1-1	1	1400
rTR-τ_1-2	2	4900
rTR-τ_1-3	3	2600
rTR-τ_1-4	4	1600

FIG. 4. Addition of τ_1 increases trans-activation by rTRα. A segment of hGR cDNA encoding amino acids 77–262 (τ_1) (23) was inserted in the rTRα cDNA in one or multiple copies, and the resulting hybrids were assayed using TREpl MCAT as the reporter plasmid. The activity of rTRα-Bm is ≈90% that of rTRα and represents an induction of about 25-fold. Relative CAT activity was determined as in Fig. 3.

receptors appears to play a role in trans-activation. However, the amino terminus of the glucocorticoid receptor contains an additional activation function (23, 28). Surprisingly, this activity can be transferred to a thyroid hormone receptor to produce a receptor with superphysiological activity. This suggests that the τ_1 domain of the hGR can function in the context of the thyroid hormone receptor in a manner analogous to its action within the glucocorticoid receptor, perhaps by its association with other transcription factors.

The absence of a detectable function in the thyroid hormone receptor amino terminus does not mean that this region has no role in trans-activation. The amino terminus is important for the activation efficiency and promoter specificity of some steroid hormone receptors. For example, both the A and B forms of the progesterone receptor, which differ only in the amino terminus, can activate transcription of the MTV promoter but only the A form can activate the ovalbumin promoter (15). Similarly, an amino-terminally deleted estrogen receptor can regulate a vitellogenin–thymidine kinase hybrid promoter construct with the same efficiency as the wild-type receptor but activates the pS2 promoter (an estrogen-responsive promoter isolated from a human breast cancer cell line) only 10% as well (14). It is interesting that the amino terminus is the only part of the thyroid hormone receptors in which they are not homologous to each other. Like the amino termini of the estrogen and progesterone receptors, the amino terminus of the thyroid hormone receptors may have a function in the specificity or efficiency of activation.

Analysis of thyroid hormone receptor-mediated trans-activation reveals striking similarities with steroid hormone receptor action. Based on our studies, structural domains for promoter recognition, hormone binding, and trans-activation have been conserved. The ability to interchange domains between thyroid and steroid hormone receptors to produce functional hybrids indicates that the mechanisms involved in hormone-inducible gene regulation have been conserved in these molecules.

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