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Research Article

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Thyroid Hormone Resistance Syndrome

Inhibition of Normal Receptor Function by Mutant Thyroid Hormone Receptors

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Abstract

Thyroid hormone (T_3) resistance is inherited in most cases in an autosomal dominant manner. The disorder is characterized by elevated free thyroid hormone levels and partial resistance to thyroid hormone at the cellular level. Distinct single amino acid substitutions in the ligand binding domain of the β form of the thyroid hormone receptor have been described in two kindreds with this disorder. We used transient expression assays to characterize the functional properties of these receptor mutants, one containing a Gly to Arg change at amino acid 340 (G340R) and the other a Pro to His change at amino acid 448 (P448H). A nine amino acid carboxy terminal deletion (Δ 448-456), analogous to an alteration that occurs in *v-erbA*, was also studied for comparison with the mutations that occur in the T_3 resistance syndrome. None of the receptor mutants were able to mediate thyroid hormone dependent activation (TreTKCAT) or repression (TSH α CAT) of reporter genes when compared with the wild type receptor. In addition, the mutants inhibited the activity of normal α and β receptor isoforms when examined in co-expression assays. This activity, referred to as dominant negative inhibition, was manifest with respect to both the positively and negatively regulated reporter genes. Although mutant receptor binding to DNA was unaffected, ligand binding studies showed that the G340R and Δ 448-456 mutants failed to bind T_3 , whereas the P448H mutant bound hormone with reduced affinity (\sim 10% of normal) compared to the wild type receptor. Consistent with this finding, the P448H mutant receptor was partially active at higher T_3 concentrations. Furthermore, the dominant negative inhibition elicited by the P448H receptor mutant at higher T_3 concentrations was reversed in the presence of high doses of T_3 . These findings indicate that mutant β receptors in patients with thyroid hormone resistance have reduced affinity for T_3 and are functionally deficient, but impair the activity of normal receptors, thereby providing a mechanism for the dominant mode of inheritance in this disorder. (*J. Clin. Invest.* 1991. 87:1977-1984.) **Key words:** transcriptional regulation • *erbA* • thyroid-stimulating hormone • thyroid hormone resistance • thyroid hormone

Introduction

The syndrome of generalized thyroid hormone resistance (GTHR)¹ was first described by Refetoff et al. (1), and is characterized by elevated circulating levels of free thyroxine (T_4) and triiodothyronine (T_3) and inappropriately normal or increased levels of thyroid-stimulating hormone (TSH). Although patients with GTHR are relatively refractory to the metabolic effects of increased thyroid hormone, there is variability in the degree of resistance in different target tissues within an individual and the clinical phenotype varies in different kindreds (2). An autosomal recessive mode of inheritance was suggested in original GTHR kindred, but the disorder has been shown to segregate in an autosomal dominant manner in most families (1, 3).

Although both quantitative (4) and qualitative defects (5) in thyroid hormone binding to fibroblast nuclear receptors from GTHR patients have been described, these studies did not firmly establish a defect at the level of the thyroid hormone receptor. Recently, two thyroid hormone receptor genes, designated *hTR α* and *hTR β* , have been identified, encoding highly homologous proteins with different tissue distributions (6, 7). Genetic analysis has now shown linkage between the GTHR syndrome and the β receptor gene locus in three kindreds (8, 9). These observations have been extended by sequencing β receptor cDNAs from two separate families with the disorder. In both cases, affected individuals have a point mutation in one allele of the β receptor gene together with a second normal allele, whereas their unaffected relatives have two normal alleles. In each kindred, the point mutation alters a single amino acid codon in the carboxy-terminal ligand binding domain of the receptor. In one kindred, the mutation is a Glycine to Arginine substitution at amino acid 340 (G340R) (10). In the other kindred, a Proline to Histidine substitution was identified at amino acid 448 (P448H) (9).

Although ligand binding studies have shown that these mutant proteins either fail to bind T_3 (10) or bind hormone with reduced affinity (11), their ability to modulate target gene expression has yet to be defined. Moreover, because the affected individuals also possess a second normal β receptor allele and presumably two normal α receptor alleles, the mechanism by which the mutant receptor causes resistance to thyroid hormone action is unclear. It has been hypothesized that the mutant receptor might block the activity of normal receptors (9, 10). In support of this concept, receptor homologues (*v-erbA*; splicing variant α 2) that are unable to bind thyroid hormone

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1. *Abbreviations used in this paper:* ABCD, avidin-biotin complex DNA; CAT, chloramphenicol acetyltransferase; GTHR, generalized thyroid hormone resistance; K_d , receptor affinity constant; RSV, Rous sarcoma virus; TRE, thyroid response element; nTRE, negative TRE; TREp, positive TRE.

have been shown to inhibit the action of the wild type receptors (12–14).

Transient expression assays provide a convenient model for examining the functional properties of thyroid hormone receptors. We have previously used a receptor deficient human cell line (JEG-3) for studies of the functional properties of human α and β receptor isoforms (15, 16). Both types of thyroid hormone receptor are capable of activating expression of a reporter gene (TreTKCAT) that contains a palindromic positive TRE (TREp) and repressing the activity of a reporter gene (TSH α CAT) that contains a negative thyroid response element (nTRE) (15, 16). Transcriptional stimulation and repression each require receptor activation by thyroid hormone.

In this study, we used this transient assay system to characterize the functional properties of the mutant receptors from two GTHR kindreds (G340R and P448H) as well as a carboxy-terminal truncated form of the β receptor (Δ 448-456) that corresponds to the deletion in *v-erbA* (13). We demonstrate that in addition to being nonfunctional, the receptor mutants inhibit the action of their wild type receptor counterparts. This dominant negative action by the mutant receptors may explain the dominant mode of inheritance as well as resistance to hormone action.

Methods

Plasmid constructions and receptor mutagenesis. TSH α CAT contains 846 bp, of 5' flanking sequence and 44 bp of exon I from the human TSH α subunit gene, linked to the gene encoding chloramphenicol acetyltransferase (CAT) (15). TreTKCAT (provided by G. Brent and D. Moore, Massachusetts General Hospital, Boston, MA) contains two copies of a palindromic TRE (5' gatc TCAGGTCATGACCTGAgatc-3'; linker sequences are shown in small case letters) inserted upstream of the thymidine kinase (TK) promoter and CAT gene (17).

For site directed mutagenesis (18), the human thyroid hormone β receptor cDNA was subcloned into M13mp18. The following oligonucleotides were used to direct second strand synthesis: G340R, 5' CAGCTGAAAAATGGGCGTCTTGGGGTGGTG 3', P448H, 5' ACA-GAACTCCTCCCCATTTGTTCTCGGAA 3', β Δ 448-456, 5'-TGCCCCACAGAACTCCTCCCC Δ TAGACTGACTGGATTCT 3'. Recombinant phage containing the desired mutations were identified by sequence analysis. Both mutant and wild type receptor cDNAs were then subcloned into a vector in which expression is driven by the Rous sarcoma virus (RSV) promoter (14). The mutations are illustrated schematically in Fig. 1 A.

Cell culture and transient expression assays. JEG-3 cells (HTB 36, American Type Culture Collection, Rockville, MD) were grown in Optimem (BRL-Gibco Laboratories, Grand Island, NY) containing 2% (vol/vol) FCS, penicillin (100 U/ml) and streptomycin (100 μ g/ml). The cells were trypsinized 18 h before transfection and plated into medium containing 2% FCS depleted of T₃ by treatment with activated charcoal. Triplicate plates of cells were transfected with 5 μ g reporter plasmid (TreTKCAT or TSH α CAT) together with 0.2–4 μ g of wild type or mutant receptor expression vector with the addition of RSVLUC plasmid as necessary to maintain a constant amount of RSV-containing plasmid. Following a 16-h exposure to a calcium phosphate-DNA precipitate (19), fresh serum-stripped media were added with or without T₃. After an additional 48 h, the cells were harvested and CAT enzyme activity was measured by quantitating the acetylation of [¹⁴C]chloramphenicol (20). The intraassay variation in CAT activity in triplicate transfections was typically < 10%. Absolute CAT assay values varied two to threefold in separate experiments, presumably reflecting differences in transfection efficiency. Representative experiments from a single group of transfections are therefore presented, but similar results were obtained in independent experiments.

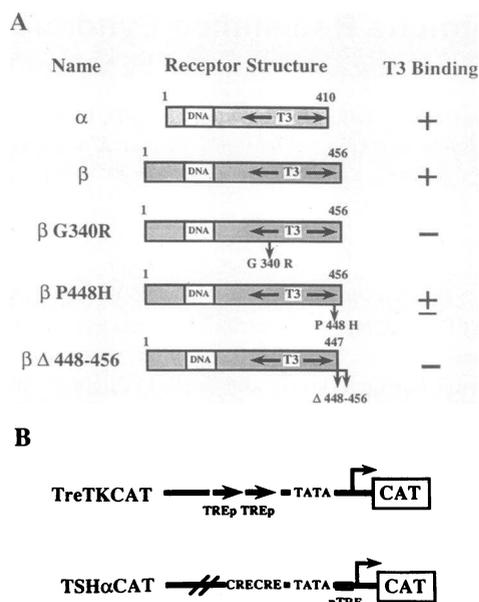


Figure 1. Structures of wild type and mutant T₃ receptors and reporter genes. (A) Schematic representation of human α and β receptor isoforms and β receptor mutants. Putative DNA (open boxes) and T₃ binding domains are shown schematically. The positions of amino acid substitutions as well as a nine amino acid deletion in the β receptor are indicated. The T₃ binding properties of these receptors are summarized at the right of the figure (+, normal binding; -, absent binding; ±, reduced binding affinity). (B) Reporter gene constructs used in transient expression assays. In TreTKCAT, the location of two copies of a positive TRE (TREp) is indicated by arrows. In TSH α CAT, the positions of two cAMP response elements (CREs) and the putative negative TRE (nTRE, hatched box) are diagrammed. The locations of the TATA box, transcriptional start site, and the CAT gene are also indicated for both constructs.

Receptor binding studies. Avidin-biotin complex DNA (ABCD) binding assays were used to assess receptor binding to DNA as described previously (15). Both wild type and mutant β receptor cDNAs were subcloned into pGEM 7 (Promega Biotec, Madison, WI) for in vitro transcription and translation. The transcribed and capped mRNAs were used to program translation in a rabbit reticulocyte lysate system in the presence of [³⁵S]radiolabelled methionine according to the protocol of the supplier (Promega). Aliquots of labeled protein were analyzed using SDS polyacrylamide gel electrophoresis to confirm receptor integrity and to demonstrate that comparable amounts of the different mutants were synthesized (data not shown). Double stranded TREp (16) or a control oligonucleotide (-72 to -32 bp in the human TSH α promoter) that does not bind to the thyroid receptor (15) were annealed, and the 3' recessed ends were filled in with biotin-11-dUTP (Enzo Biochem, Inc., New York, NY), dATP, dCTP, and dGTP using Klenow polymerase. Biotinylated DNA (500 fmol) was incubated with [³⁵S]methionine labelled receptor (100,000 cpm of TCA precipitable protein) for 40 min at 24°C. The receptor-DNA complexes were precipitated with streptavidin-agarose beads (Bethesda Research Laboratories, Gaithersburg, MD) and quantitated by scintillation counting.

Receptor interactions with DNA were also analyzed using gel mobility shift assays in which nuclear receptor extracts were prepared from COS-1 cells (16). COS-1 cells (CRL 1650, American Type Culture Collection) were transferred to Optimem with 2% charcoal stripped fetal bovine serum before transfection. Cells were transfected with CDM8 expression vectors (16) encoding human α 1, β , or different site-directed mutants of the β receptor. After 36 h, cells were harvested and extracts

were prepared as described by Damm et al. (13). Binding reactions (50 μ l) contained 32 P-labelled TREp (50 fmol) or a control oligonucleotide (-72 to -32 bp in the human TSH α promoter) incubated with transfected COS cell nuclear extract (7.5 μ g protein) in binding buffer (20 mM Hepes pH 7.9, 120 mM KCl, 2 mM dithiothreitol, 0.1% NP-40, 100 μ g/ml poly [d(I-C)], and 100 nM T₃). The DNA-protein mixture was incubated at room temperature for 60 min before electrophoresis through a 5% nondenaturing acrylamide gel.

For hormone binding assays, unlabelled *in vitro* translation products (5 μ l) were incubated with 0.01 nM [125 I]T₃ and increasing amounts (0.001–0.4 nM) of T₃ (21). The reactions were carried out at 4°C for 16 h in a final vol of 200 μ l of binding buffer (10% (vol/vol) glycerol, 15 mM Tris pH 7.6, 50 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol). Nonsaturable binding was measured in the presence of a 1,000-fold excess of unlabeled T₃. Bound and free hormone was separated by filtration (HAWP 2540; Millipore/Continental Water Systems, Bedford, MA) (22). Receptor affinity constants (K_d) were calculated using Scatchard plot analyses.

Results

Functional properties of mutant receptors. To assess the ability of wild type and mutant receptors to activate or repress transcription, TreTKCAT and TSH α CAT (Fig. 1 B) were used as reporter genes to measure transcriptional activation and repression, respectively, in thyroid hormone receptor deficient JEG-3 choriocarcinoma cells. Receptors and mutants were subcloned into an RSV expression vector. Using this system, maximal receptor activity is elicited using 100 ng of the receptor expression vector and 1 nM T₃ (16). In these experiments, supramaximal doses (200 ng) of wild type or mutant receptor and T₃ (5 nM) were used to ensure maximal receptor activity.

In the absence of cotransfected receptor, T₃ treatment repressed TSH α CAT activity (20–50% suppression in different experiments) (Fig. 2 B). In other experiments, we have also observed minimal induction of TreTKCAT expression in the absence of transfected receptor (one to threefold, data not shown). These T₃-mediated effects may reflect the presence of

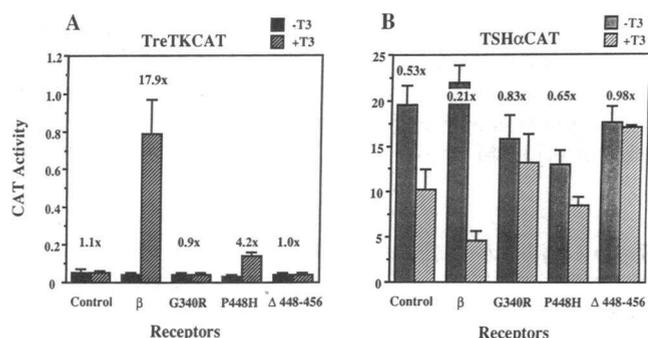


Figure 2. Functional properties of the wild type and mutant β receptors. Control plasmid (RSVLUC) or RSV expression vector (200 ng) encoding either wild type or mutant β receptors were transfected into JEG-3 cells with 5 μ g of the indicated TreTKCAT or TSH α CAT reporter genes. Triplicate transfections were incubated in either the absence (*dark bars*) or presence (*hatched bars*) of 5 nM T₃. CAT activity is expressed as percent chloramphenicol acetylation per milliliter of cell extract per hour. The data shown is the mean \pm SD of triplicate transfections. Relative stimulation or repression in the presence of T₃ treatment is indicated above the bars. (A) Positive regulation of TreTKCAT by T₃. (B) Negative regulation of TSH α CAT by T₃.

low amounts of endogenous receptor in these cells. Coexpression of wild type β receptor conferred a marked increase in T₃ responsiveness. TreTKCAT expression was induced 18-fold and TSH α CAT was suppressed by 79% (Fig. 2 A). Under the same conditions, the mutant receptors exhibited markedly diminished activity. The β Δ 448–456 mutant failed to mediate activation of TreTKCAT or repression of TSH α CAT. The GTHR receptor mutants (G340R and P448H) were also functionally deficient, but differed in their activities. Whereas G340R was essentially inactive (0.9-fold induction of TreTKCAT and 17% repression of TSH α CAT), P448H exhibited partial T₃ responsiveness (4.2-fold induction of TreTKCAT and 35% repression of TSH α CAT).

Dominant negative action of mutant receptors. Having established the functional capabilities of the individual receptor mutants, their ability to modulate the activity of wild type α and β receptors was examined. Wild type β receptor (200 ng) was cotransfected with or without a 10-fold excess (2 μ g) of each of the mutant receptors. Under these conditions, the receptor mutants markedly inhibited both positive and negative transcriptional responses mediated by the wild type receptor (Fig. 3). For example, T₃-dependent activation of TreTKCAT was reduced from 73-fold with β receptor alone to 21-fold and 24-fold in the presence of coexpressed G340R or P448H, respectively. The Δ 448–456 mutant was more potent and reduced activation to sixfold. A similar pattern of results was obtained with respect to negative regulation using TSH α CAT. Repression of TSH α CAT activity in the presence of wild type receptor alone (80%) was blunted to 16% by G340R, 50% by P448H, and repression was eliminated using Δ 448–456.

Dose response studies were carried out to determine the amount of mutant receptor expression vector required to inhibit wild type receptor activity. Increasing concentrations of the G340R mutant receptor progressively reduced transcriptional activation of TreTKCAT from 22- to 7-fold (Fig. 4 A). Similarly, the degree of repression of TSH α CAT was reduced from 62% with wild type β receptor alone to 30% with a 10-fold excess of G340R (Fig. 4 B). In both cases, half-maximal inhibition of wild type receptor activity occurred using a four to fivefold excess of mutant receptor.

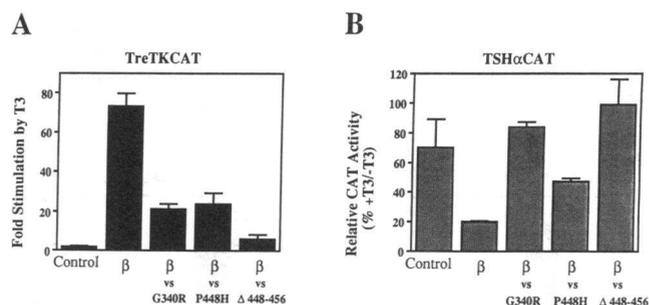


Figure 3. Effect of receptor mutants on positive and negative transcriptional regulation by wild type β receptor. JEG-3 cells were transfected with 5 μ g of TreTKCAT or TSH α CAT together with either 2,200 ng of RSVLUC (Control), or 200 ng of β receptor and a 10-fold excess (2,000 ng) of RSVLUC or mutant β receptors. Triplicate transfections were incubated in either the absence or presence of 5 nM T₃ and the results are presented as the relative activity of +T₃ / -T₃ treated plates. (A) Positive regulation of TreTKCAT by T₃. (B) Negative regulation of TSH α CAT by T₃.

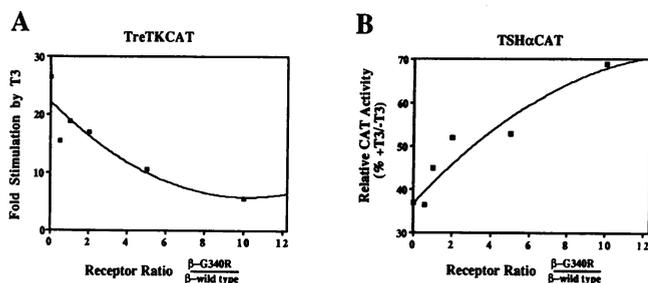


Figure 4. Dose response for inhibition of wild type β receptor activity by mutant receptor. Transfections were performed with 200 ng of wild type β receptor and increasing amounts (0–2,000 ng) of the G340R receptor mutant. RSVLUC was added as required to keep the total amount of expression vector constant. Triplicate transfections were incubated with 5 nM T_3 and CAT activity is expressed as the ratio of $+T_3/-T_3$ treated transfections. (A) Positive regulation of TreTKCAT by T_3 . (B) Negative regulation of TSH α CAT activity by T_3 .

The human α isoform of the thyroid hormone receptor is highly homologous (> 85%) to the β receptor in the central DNA binding and carboxy-terminal ligand binding domains (Fig. 1 A). Consistent with this observation, the α receptor isoform binds T_3 and can activate and repress target reporter genes in JEG-3 cells in a manner similar to the β receptor (12, 16). To test the ability of the mutant receptors to inhibit wild type α receptor activity, experiments analogous to those with the β receptor were performed (Fig. 5). T_3 -dependent activation of TreTKCAT by the α receptor was reduced from 118-fold to 36- and 43-fold with the G340R and P448H mutants, respectively. $\Delta 448-456$ was a more potent inhibitor and reduced stimulation to 11-fold. The receptor mutants were less effective in their inhibition of T_3 dependent suppression by α receptor. After treatment with T_3 , TSH α CAT was repressed by 84% in the presence of α receptor alone and the degree of repression was reduced to 61%, 64%, and 43% in the presence of excess G340R, P448H, and $\Delta 448-456$ mutants, respectively.

Ligand and DNA binding properties of the receptor mutants. As shown in the foregoing experiments, the mutant receptors exhibited somewhat different functional properties. When tested alone, the P448H mutant was capable of modulating target gene activity slightly, whereas G340R and $\Delta 448-456$ were completely inactive (Fig. 2). Likewise, when listed in

order of potency as dominant negative inhibitors of wild type receptor activity (Fig. 3, 5), the rank order was $\Delta 448-456 > G340R > P448H$. One potential explanation for these results could involve differences amongst the receptors in their ligand binding properties. Therefore, each of the receptors was generated by in vitro transcription and translation to allow measurements of the affinity of T_3 binding (Table I). The wild type receptor binding affinity ($1.46 \times 10^{10} M^{-1}$) was similar to values reported previously (21). The P448H mutant bound T_3 , but with an affinity that was reduced by a log order ($1.52 \times 10^9 M^{-1}$). Binding to G340R and $\Delta 448-456$ mutants, if it occurs, was below detection in these assays.

Two different assays were employed to assess the ability of receptor mutants to bind to DNA. In the ABCD binding assay, [^{35}S]methionine-labelled receptors were prepared by in vitro translation, and binding of labelled receptors to biotinylated DNA was measured after precipitation with streptavidin agarose beads (Fig. 6 A). Using a known receptor binding site (TREP), the binding of each of the ligand domain receptor mutants (G340R, P448H, $\Delta 448-456$) was comparable to that of the wild type β receptor, whereas a mutation in the DNA binding domain of the β receptor (C122S) (15) resulted in negligible interaction with TREP.

Receptors were also expressed in COS-1 cells and used to assess DNA binding properties using gel mobility shift assays (Fig. 6 B) (16). In addition to providing an independent measurement of DNA binding, this assay has the potential advantage of detecting major alterations in mRNA or protein stability that might result from receptor mutations. As shown previously (16), untransfected COS cells contain little receptor binding activity (lane 3). However, after transfection with either wild type $\alpha 1$ or β receptors, distinct receptor-DNA complexes were formed using nuclear extracts and TREP (lanes 4 and 5), but not with control DNA that does not bind to receptor (15) (lanes 1 and 2). Expression and DNA binding of the G340R mutant (lane 6) and the $\Delta 448-456$ mutant (lane 7) were similar to the wild type β receptor. These assays indicate that the ligand binding domain mutations have not altered the ability of the receptors to interact with DNA.

T_3 dose dependence of mutant receptor action. In light of its ability to bind T_3 with reduced affinity, the properties of the P448H mutant receptor were examined at low and high T_3 concentrations (Fig. 7). Activation and repression of reporter gene expression by wild type β receptor was similar in the presence of 0.75 nM or 100 nM T_3 concentrations. The mutant

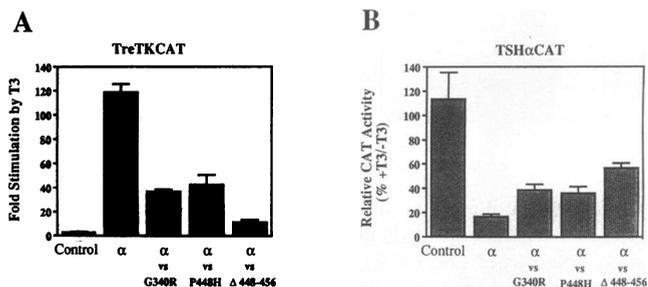


Figure 5. Effect of receptor mutants on positive and negative transcriptional regulation by wild type α receptor. Transfections were carried out as described in Fig. 3 except that wild type human α receptor expression vector was used. (A) Positive regulation of TreTKCAT by T_3 . (B) Negative regulation of TSH α CAT by T_3 .

Table I. T_3 Binding to Mutant β Receptors*

Receptor	K_d (M^{-1})
β wild type	1.46×10^{10}
β G340R	Below detection
β P448H	1.52×10^9
β $\Delta 448-456$	Below detection

* β receptors were transcribed and translated in vitro as described in Methods. T_3 binding was measured using a filter binding assay, and the affinity constants (K_d) were determined by Scatchard analysis. Below detection ($\sim 5 \times 10^8 M^{-1}$) refers to binding measurements that were indistinguishable from control reactions using unprogrammed reticulocyte lysates.

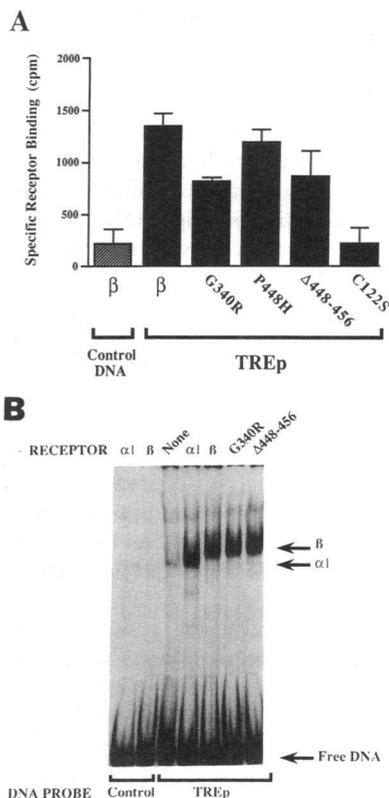


Figure 6. Expression and DNA binding properties of mutant β receptors. (A) ABCD receptor-DNA binding assays. In vitro translated, ^{35}S methionine-labeled wild type β or β receptor mutants (G340R, P448H, Δ 448-456, C122S) were bound to biotinylated TREp and the receptor-DNA complexes precipitated with streptavidin agarose beads. Results are the mean \pm SD of triplicate assays. Background binding in the absence of added DNA was subtracted to yield specific binding. Binding to a control DNA fragment (human TSH α promoter -72 to -32 bp) that does not bind receptor with high affinity is indicated as control DNA. (B) Gel mobility shift assays. Wild type and mutant receptors were transfected into COS-1 cells to attain high levels of expression. Nuclear extracts containing transiently expressed receptors were analyzed in gel mobility shift assays using either ^{32}P -labeled TREp (lanes 3-7) or a control DNA fragment derived human TSH α promoter (-72 to -32 bp) that does not bind receptors (lanes 1 and 2). Expressed receptors (α 1, β , G340R, Δ 448-456) are indicated at the top of the gel. An extract from untransfected cells is denoted as none (lane 3). Because it had not been subcloned into a CDM8 vector, mutant P448H was omitted from this experiment. The locations of free DNA and receptor-bound DNA complexes are indicated with arrows.

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P448H was only weakly active (fourfold activation of TreTK-CAT, 40% repression of TSH α CAT) at the low T_3 concentration, but became fully functional at the higher T_3 concentration. The apparent increase in the activity of the P448H mutant relative to the wild type β receptor at high T_3 doses was not

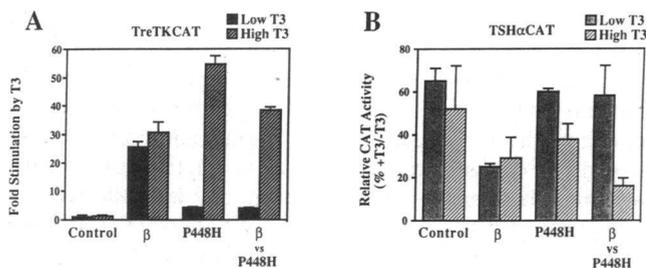


Figure 7. Effect of T_3 concentration on mutant receptor function and dominant negative activity. Transfections were carried out as described in previous figures except that the cells were treated with either low (0.75 nM) or high (100 nM) T_3 concentrations. (A) Positive regulation of TreTKCAT by T_3 . (B) Negative regulation of TSH α CAT by T_3 .

observed in other experiments. Thus, although P448H binds T_3 with reduced affinity, it appears to function normally in the presence of saturating doses of hormone. As expected based upon the T_3 binding data, the reduced activity of mutants G340R and Δ 448-456 was unaffected by increasing the dose of T_3 (data not shown).

The dominant negative effects of the P448H receptor mutant were also dependent on T_3 concentration (Fig. 7). TreTK-CAT activation by wild type β receptor was reduced from 25-fold to 4-fold by mutant P448H at 0.75 nM T_3 , but TreTK-CAT activation was not inhibited at 100 nM T_3 . Similarly, repression of TSH α CAT activity was effectively blocked by mutant P448H at a low T_3 concentration, but full repression was retained at the high T_3 concentration.

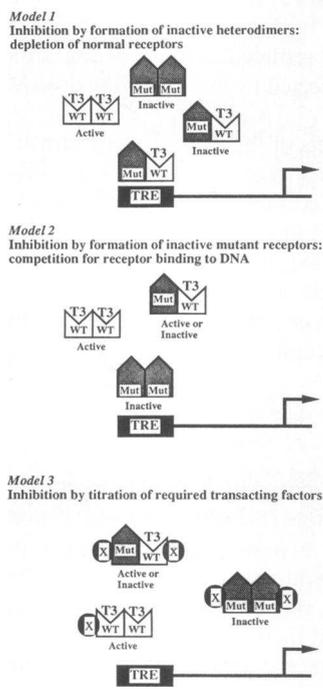
Discussion

In this study, we have characterized the functional properties of mutant thyroid hormone receptors (G340R and P448H) that were identified previously in two separate kindreds with GTHR syndrome (9, 10). Several main conclusions can be drawn from these studies. First, the mutant receptors bind T_3 with greatly reduced affinity and their ability to transcriptionally activate or repress target genes in response to T_3 is therefore impaired. This observation is in keeping with previous studies showing T_3 receptor isoforms (e.g., v-erbA, α 2) that cannot bind T_3 are transcriptionally inactive (12, 13, 16) and emphasizes the fact that transcriptional regulation is a ligand dependent process. Second, the different receptor mutants have distinct functional properties. Whereas G340R and Δ 448-456 exhibit no T_3 binding and are completely inactive in transfection assays, P448H binds T_3 with reduced affinity, and consequently is partially active in response to T_3 . Third, we find that the mutant receptors inhibit the transcriptional activity of the wild type thyroid hormone receptor. Notably, the inhibition by mutant receptors applies to the activity of both β and α receptors and is manifest using both positively and negatively regulated target genes.

Two major mechanisms might be considered to explain the fact that GTHR can be inherited in an autosomal dominant manner. Given that the mutant receptors are transcriptionally inactive, one possibility is that the amount of functional receptor is reduced as a consequence of having the mutation occur in one of two autosomal alleles. Alternatively, it is possible that the mutant receptor in some manner interferes with the function of normal receptors, thereby accounting for its phenotypic expression in the heterozygous state. The receptor deficiency mechanism assumes that the quantity of functional receptor within the cell is a limiting step for hormone action and that the normal allele is not upregulated to compensate for the mutant allele. There is currently no evidence that excludes this mechanism. However, the observation that mutant receptors can block the transcriptional regulation of two different reporter genes supports an interference mechanism as the basis for resistance to thyroid hormone action in heterozygotes.

Given the apparent complexity of transcriptional regulation by thyroid hormone receptors, it is useful to consider potential cellular mechanisms by which mutant receptors could inhibit wild type receptors. One mechanism could involve the formation of nonfunctional heterodimers between wild type and mutant receptor proteins (Fig. 8, model 1). This type of

Potential Mechanisms for LBD Mutants to Inhibit T₃ Receptor Action



required for the function of normal receptors. The formation of dimers is shown, but is not required to invoke mechanisms 2 or 3. These models are not mutually exclusive and it is possible that dominant negative inhibition involves several different mechanisms.

mechanism has been documented with other DNA binding transcription factors. For example, in the CREB/ATF family of transcription factors, homodimers and heterodimers form via a conserved leucine zipper motif (23) and mutant CREB proteins function in a dominant negative manner by dimerizing with the wild type protein to inhibit transcriptional capability (24). Another such example is provided by the Id protein which inhibits the transcriptional activity of the muscle differentiation factor MyoD by forming heterodimers via a shared helix-loop-helix (25). In the case of thyroid hormone receptors, deletion mutants of the chicken α receptor repress wild type receptor function and this inhibitory property has been localized to the carboxy-terminal domain that may contain a dimerization interface (14, 26). In support of this concept, the thyroid hormone receptor has recently been shown to form heterodimers with the retinoic acid receptor via a shared structural motif within the carboxy-terminal region (27). Both of the GTHR receptor mutations occur outside of this putative dimerization domain, raising the possibility that they could still form dimers. Further structure-function analyses within this region will be required to precisely delineate the receptor sequences that are required for receptor-receptor interactions.

A second mechanism for dominant negative activity would be for the transcriptionally defective mutants to compete with the wild type receptor for binding to a common DNA target sequence, which in this case is the TRE (Fig. 8, *model 2*). The finding that a mutation in the DNA binding domain of the viral oncogene, v-erbA, abolishes its dominant negative properties, lends support to this type of mechanism (13). The introduction of DNA binding domain mutations into the GTHR

Figure 8. Potential mechanisms for dominant negative inhibition. Three potential models for inhibition by ligand binding domain (LBD) mutants of T₃ receptor action are presented. Wild type receptors (WT) can bind T₃, whereas mutant receptors (Mut) are unable to bind T₃. The formation of homodimers and heterodimers of wild type and mutant receptors is hypothetical. Model 1 suggests that mutant receptor could form non-functional heterodimers with the wild type receptor to deplete the amount of residual active receptor. Model 2 suggests that mutant receptors could block wild type activity by competing for binding to a common target TRE sequence. Model 3 indicates a mechanism in which functionally inactive receptors interact with and deplete a limiting cellular factor (X) that is re-

ceptors may provide further insight regarding the contribution of this mechanism to their dominant negative activity. It is interesting to note that in another receptor resistance syndrome (hereditary resistance to vitamin D), the disorder is inherited in an autosomal recessive manner and heterozygotes are phenotypically normal (28). In this instance, the mutations occur within the DNA binding domain of the vitamin D receptor, consistent with the hypothesis that binding to DNA may be required for receptors to exert inhibitory activity in the heterozygous state.

The mutant receptors could also inhibit the action of normal receptors by sequestering a shared accessory factor that is necessary for wild type receptor modulation of gene transcription (Fig. 8, *model 3*). This property, sometimes referred to as "squenching" (29), has been postulated to explain transcriptional interference between coexpressed estrogen and progesterone receptors (30), and recent analyses of transcriptional regulation by the adenovirus E1a protein also support this concept (31). Because inhibition by the GTHR and $\Delta 448-456$ mutants occurs with relatively low doses of coexpressed mutant receptors, we do not favor such a mechanism for these mutants, but it cannot be excluded at this point. These three potential pathways for transcriptional interference by the mutant receptors are not mutually exclusive and inhibition may well result from a combination of several different mechanisms.

In many respects, the dominant negative properties that we have observed with the GTHR receptor mutants are analogous to the findings with other transcriptionally inactive thyroid hormone receptor variants (12, 13, 16). For example, both the v-erbA oncogene and the $\alpha 2$ isoform have been shown to act as dominant negative inhibitors when coexpressed with the wild type receptor in transient expression assays. The GTHR mutants, v-erbA, and $\alpha 2$ receptors share in common the fact that they contain sequence alterations in the extreme carboxy terminus of the receptor. Although these alterations eliminate or greatly reduce hormone binding in each of these mutants, it may be premature to conclude that this is the only modification in receptor function that is required for dominant negative activity. For example, the carboxy-terminal nine amino acid deletion that occurs in v-erbA has been proposed to serve as a transactivation domain (32). Thus, its deletion could potentially eliminate both T₃ binding and the potential for transactivation. As suggested in Fig. 8, the activity of heterodimers formed between v-erbA and a normal cellular receptor could potentially be influenced by the presence or absence of a transactivation domain within v-erbA. This feature might account for the somewhat greater inhibition that is observed with the v-erbA homologue ($\Delta 448-456$) than with the GTHR mutants (G340R, P448H) (Figs. 3 and 5), or with the $\alpha 2$ isoform (16).

If an interference mechanism is the basis for thyroid hormone resistance in the heterozygous state, then in the simplest case it might be proposed that the normal and mutant alleles would be expressed equally resulting in a 1:1 ratio of normal and mutant receptor. The fact that mutant and wild type sequences comprise a similar number of isolated cDNA clones (10) provides some data to support this assumption. However, several lines of evidence suggest that the situation in vivo may be complex. For example, T₃ can regulate the expression of its own receptors (33, 34) and the impact of thyroid hormone resistance on this process is currently unknown. Furthermore, the levels of expressed α and β receptors varies widely in different tissues (34, 35) and a number of receptor splicing variants

have been recognized for both forms of receptor (36–38). Thus, if the mutation occurs in one of the β receptor genes, the degree of resistance may reflect the composition of receptor isoforms within a given cell as well as compensatory responses of the various receptor isoforms to the T_3 resistant state. Future studies of receptor expression and regulation in vivo may help to clarify the relative proportions of different receptor isoforms.

We examined the issue of the stoichiometry of mutant receptor inhibition in vitro by altering the ratio of mutant to wild type receptors in transient expression assays (Fig. 4). Although some degree of dominant negative activity was apparent at low mutant/wild type receptor ratios (1:1 or 2:1), it was somewhat surprising that 50% inhibition required at least a fourfold excess of mutant receptor. The basis for the requirement for excess mutant receptor and its relevance to clinical thyroid hormone resistance are unclear. It is possible that clinical measurements of resistance (e.g., elevated thyroid hormone levels) are more sensitive indicators of receptor inhibition than our in vitro assays of transient gene expression. A more likely basis for the requirement for excess mutant receptor may involve the artificial nature of the transient expression assay. Although the predicted ratios of expressed receptor are likely to correlate with the amounts of transfected plasmid, we do not have a quantitative measure of the expressed receptor proteins. Based upon their levels of expression in transfected COS cells (Fig. 6 B), there is some evidence that the stabilities of the mutant and wild type receptor mRNAs and proteins are similar in transfected cells. It is possible that quantitative differences in dominant negative activity would be found under different conditions such as performing the experiments at a lower or higher level of expressed reporter gene or using a different level of wild type receptor. In addition, the current studies have been performed in only a single receptor deficient cell line (JEG-3 cells). A number of recent reports indicate that T_3 receptors interact with other cellular proteins (27, 39–41) and the levels and compositions of such factors may alter dominant negative regulation in a cell type specific manner. One must also consider that the inhibitory effects of the mutant receptors may be dependent upon the target gene for thyroid hormone receptor action. For example, coexpressed thyroid hormone and retinoic acid receptors act cooperatively to induce the expression of a target gene containing a palindromic TRE, but inhibit the expression of a gene containing TREs from the myosin heavy chain gene (27). Because of these issues, it is apparent that one should interpret transient receptor assays of receptor function cautiously, particularly with respect to quantitative effects. Despite these reservations, this technique appears to provide a valuable in vitro assay for the transcriptional activity of receptor mutants and to serve as an index of dominant negative activity.

The observation that different kindreds with GTHR display distinct clinical features is a particularly intriguing aspect of this disorder (2, 3, 9). We have shown that the receptor mutants from two kindreds differ in their functional properties. At a low T_3 concentration, the properties of P448H and G340R are essentially indistinguishable in vitro. However, at a T_3 concentration which saturates P448H, this mutant is transcriptional active and loses its dominant negative activity. However, we note that individuals with the P448H mutation (9) and the G340R mutation (10) have similar circulating thyroid hormone levels and the clinical features of resistance are different, but not necessarily less severe, in individuals with the P448H

mutation. Thus, it is unclear at this stage whether differences in ligand affinity or some other aspect of mutant receptor function account for phenotypic differences in this disorder. To better understand the clinical features that result from thyroid hormone resistance, it will be necessary to acquire more information about the in vivo distribution, regulation, and mechanisms of action of normal and mutant receptors. Recent studies (9, 42) suggest that a number of different mutations may exist in other kindreds with GTHR. Analyses of their functional properties will be of interest to further elucidate the pathophysiology of this disorder.

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References

1. Refetoff, S., L. T. DeWind, and L. J. DeGroot. 1967. Familial syndrome combining deaf-mutism, stippled epiphyses, goiter and abnormally high PBI: possible target organ refractoriness to thyroid hormone. *J. Clin. Endocrinol. & Metab.* 27:279–294.
2. Magner, J. A., P. Petrick, M. M. Menezes-Ferreira, M. Stelling, and B. D. Weintraub. 1986. Familial generalized resistance to thyroid hormones: report of three kindreds and correlation of affected tissues with the binding of ^{125}I triiodo-L-thyronine to fibroblast nuclei. *J. Endocrinol. Invest.* 9:459–470.
3. Refetoff, S. 1982. Syndromes of thyroid hormone resistance. *Am. J. Physiol.* 243:E88–E98.
4. Ichikawa, K., I. A. Hughes, A. L. Horwitz, and L. J. DeGroot. 1987. Characterization of nuclear thyroid hormone receptors of cultured skin fibroblasts from patients with resistance to thyroid hormone. *Metab. Clin. Exp.* 36:392–399.
5. Menezes-Ferreira, M. M., C. Eil, J. Wortsman, and B. D. Weintraub. 1984. Decreased uptake of ^{125}I triiodo-L-thyronine in fibroblasts from patients with peripheral thyroid hormone resistance. *J. Clin. Endocrinol. & Metab.* 59:1081–1087.
6. Weinberger, C., C. C. Thompson, E. S. Ong, R. Lebo, D. J. Gruol, and R. M. Evans. 1986. The c-erbA gene encodes a thyroid hormone receptor. *Nature (Lond.)* 324:641–646.
7. Nakai, A., A. Sakurai, G. I. Bell, and L. J. DeGroot. 1988. Characterization of a third human thyroid hormone receptor co-expressed with other thyroid hormone receptors in several tissues. *Mol. Endocrinol.* 2:1087–1092.
8. Usala, S. J., A. E. Bale, N. Gesundheit, C. Weinberger, R. W. Lash, F. E. Wondisford, O. W. McBride, and B. D. Weintraub. 1988. Tight linkage between the syndrome of generalized thyroid hormone resistance and the human c-erbA β gene. *Mol. Endocrinol.* 2:1217–1220.
9. Usala, S. J., G. E. Tennyson, A. E. Bale, R. W. Lash, N. Gesundheit, F. E. Wondisford, D. Accili, P. Hauser, and B. D. Weintraub. 1990. A base mutation of the c-erbA β thyroid hormone receptor in a kindred with generalized thyroid hormone resistance: molecular heterogeneity in two other kindreds. *J. Clin. Invest.* 85:93–100.
10. Sakurai, A., K. Takeda, K. Ain, P. Ceccarelli, A. Nakai, S. Seino, G. I. Bell, S. Refetoff, and L. J. DeGroot. 1989. Generalized resistance to thyroid hormone associated with a mutation in the ligand-binding domain of the human thyroid hormone receptor β . *Proc. Natl. Acad. Sci. USA.* 86:8977–8981.
11. Usala, S. J., F. E. Wondisford, T. L. Watson, J. B. Menke, and B. D. Weintraub. 1990. Thyroid hormone and DNA binding properties of a mutant c-erbA β receptor associated with generalized thyroid hormone resistance. *Biochem. Biophys. Res. Commun.* 171:575–580.
12. Koenig, R. J., M. A. Lazar, R. A. Hodin, G. A. Brent, P. R. Larsen, W. W. Chin, and D. D. Moore. 1989. Inhibition of thyroid hormone action by a non-hormone binding c-erbA protein generated by alternative mRNA splicing. *Nature (Lond.)* 337:659–661.
13. Damm, K., C. C. Thompson, and R. M. Evans. 1989. Protein encoded by v-erbA functions as a thyroid hormone receptor antagonist. *Nature (Lond.)* 339:593–596.
14. Forman, B. M., C. Yang, M. Au, J. Casanova, J. Ghysdael, and H. H. Samuels. 1989. A domain containing a leucine-zipper like motif mediates novel in vivo interactions between the thyroid hormone and retinoic acid receptors. *Mol. Endocrinol.* 3:1610–1626.

15. Chatterjee, V. K. K., J-K. Lee, A. Rentoumis, and J. L. Jameson. 1989. Negative regulation of the thyroid-stimulating hormone α gene by thyroid hormone: receptor interaction adjacent to the TATA box. *Proc. Natl. Acad. Sci. USA.* 86:9114-9118.
16. Rentoumis, A., V. K. K. Chatterjee, L. D. Madison, S. Datta, G. D. Gallagher, L. J. DeGroot, and J. L. Jameson. 1990. Negative and positive transcriptional regulation by thyroid hormone receptor isoforms. *Mol. Endocrinol.* 4:1522-1531.
17. Brent, G. A., J. W. Harney, Y. Chen, R. L. Warne, D. D. Moore, and P. R. Larsen. 1989. Mutations of the rat growth hormone promoter which increase and decrease response to thyroid hormone define a consensus thyroid response element. *Mol. Endocrinol.* 3:1996-2004.
18. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82:488-492.
19. Graham, B., and A. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus DNA. *Virology.* 52:456-467.
20. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
21. Scheuler, P. A., H. L. Schwartz, K. A. Strait, C. N. Mariash, and J. H. Oppenheimer. 1990. Binding of 3,5,3'-triiodothyronine (T3) and its analogs to the in vitro translational products of the c-erbA protooncogenes: differences in the affinity of the α and β forms for the acetic acid analog and failure of the human testis and kidney α -2 products to bind T3. *Mol. Endocrinol.* 4:227-234.
22. Inoue, A., J. Yamakawa, M. Yukioka, and S. Morisawa. 1983. Filter-binding assay procedure for thyroid hormone receptors. *Anal. Biochem.* 134:176-183.
23. Abel, T., and T. Maniatis. 1989. Action of leucine zippers. *Nature (Lond.)* 341:24-25.
24. Dwarki, V. J., M. Montminy, and I. M. Verma. 1990. Both the basic region and the leucine zipper domain of CREB protein are essential for transcriptional activation. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:225-232.
25. Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell.* 61:49-59.
26. Forman, B. M., and H. H. Samuels. 1990. Minireview: interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol. Endocrinol.* 4:1293-1301.
27. Glass, C. K., S. M. Lipkin, O. V. Devary, and M. G. Rosenfeld. 1989. Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. *Cell.* 59:697-708.
28. Hughes, M. R., P. J. Malloy, D. G. Kieback, R. A. Kesterson, J. W. Pike, D. Feldman, and B. W. O'Malley. 1988. Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. *Science (Wash. DC)* 242:1702-1705.
29. Ptashne, M. 1988. How eukaryotic transcriptional activators work. *Nature (Lond.)* 335:683-689.
30. Meyer, M., H. Gronemeyer, B. Turcotte, M. Bocquel, D. Tasset, and P. Chambon. 1989. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell.* 57:433-442.
31. Martin, K. J., J. W. Lillie, and M. R. Green. 1990. Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. *Nature (Lond.)* 346:147-152.
32. Zenke, M., A. Munoz, J. Sap, B. Vennstrom, and H. Beug. 1990. v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA. *Cell.* 61:1035-1049.
33. Lazar, M. A., and W. W. Chin. 1988. Regulation of two c-erbA messenger ribonucleic acids in rat GH3 cells by thyroid hormone. *Mol. Endocrinol.* 2:479-484.
34. Hodin, R. A., M. A. Lazar, B. J. Wintman, D. S. Darling, R. J. Koenig, P. R. Larsen, D. D. Moore, and W. W. Chin. 1989. Identification of a thyroid hormone receptor that is pituitary specific. *Science (Wash. DC)* 244:76-79.
35. Sakurai, A., A. Nakai, and L. J. DeGroot. 1989. Expression of three forms of thyroid hormone receptor in human tissues. *Mol. Endocrinol.* 3:392-399.
36. Mitsuhashi, T., G. E. Tennyson, and V. M. Nikodem. 1988. Alternative splicing generates messages encoding rat c-erbA proteins that do not bind thyroid hormone. *Proc. Natl. Acad. Sci. USA.* 85:2781-2785.
37. Izumo, S., and V. Mahdavi. 1988. Thyroid hormone receptor α isoforms generated by alternative splicing differentially activate myosin HC gene transcription. *Nature (Lond.)* 334:539-542.
38. Yaoita, Y., Y. Shi, and D. D. Brown. 1990. *Xenopus laevis* α and β thyroid hormone receptors. *Proc. Natl. Acad. Sci. USA.* 87:7090-7094.
39. Murray, M. B., and H. C. Towle. 1989. Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. *Mol. Endocrinol.* 3:1434-1442.
40. Burnside, J., D. S. Darling, and W. W. Chin. 1990. A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. *J. Biol. Chem.* 265:2500-2504.
41. Lazar, M. A., and T. J. Berrodin. 1990. Thyroid hormone receptors form distinct nuclear protein-dependent and independent complexes with a thyroid hormone response element. *Mol. Endocrinol.* 4:1627-1635.
42. Takeda, K., S. Balzano, A. Sakurai, L. J. DeGroot, and S. Refetoff. 1990. Screening of 19 unrelated families with generalized resistance to thyroid hormone (GRTH) for known point mutations in the thyroid receptor (TR) β and the detection of a new mutation. The 72nd Annual Meeting of The Endocrine Society, 20-23 June 1990, Atlanta, GA. (3):25 (Abstr.)