# Interaction of Human β1 Thyroid Hormone Receptor and its Mutants with DNA and Retinoid X Receptor β

## $T_3$ Response Element–dependent Dominant Negative Potency

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## Abstract

**Mutations in the human beta thyroid hormone receptor (h-TRβ) gene are associated with the syndrome of generalized resistance to thyroid hormone. We investigated the interaction of three h-TRβ1 mutants representing different types of functional impairment (kindreds ED, OK, and PV) with different response elements for 3,3′,5-triiodothyronine ($T_3$) and with retinoid X receptor β (RXRβ). The mutant receptors showed an increased tendency to form homodimers on a palindromic $T_3$-response element (TREpal), a direct repeat (DR + 4), and an inverted palindrome (TRElap). On TRElap, wild type TR binding was decreased by $T_3$, while the mutant receptors showed a variably decreased degree of dissociation from TRElap in response to $T_3$. The extent of dissociation was proportional to their $T_3$ binding affinities. RXRβ induced the formation of h-TRβ1:RXRβ heterodimers equally well for mutants and the wild type h-TRβ1 on these $T_3$ response elements. However, the $T_3$-dependent increase in heterodimerization with RXRβ was absent or reduced for the mutant TRs.**

**Transient transfection studies indicated that the dominant negative potency was several-fold more pronounced on the TRElap as compared to TREpal or DR + 4. In CV-1 and HeLa cells, transfection of RXRβ could not reverse the dominant negative action. These results demonstrate that the binding of mutant h-TRs to DNA, as well as their dominant negative potency, are TRE dependent. In addition, competition for DNA binding, rather than for limiting amounts of RXRβ, is likely to mediate the dominant negative action. (*J. Clin. Invest.* 1993. 92:1986–1993.) Key words: thyroid hormone receptor • generalized resistance to thyroid hormone • $T_3$-response element • retinoid X receptor • dominant negative action**

## Introduction

Mutations in the ligand-binding domain (LBD)[1] of one allele of the human β1 thyroid hormone receptor (h-TRβ1) gene can cause the clinical syndromes of generalized resistance to thyroid hormone (GRTH) (1–3). This disease is characterized by the refractoriness of tissues to the action of thyroid hormone. However, a marked variability exists in the organ resistance within individuals with GRTH (3–5). Intriguingly, the dominant inheritance of the disease is not caused by a lack of active h-TRβ1 protein, since heterozygotes with a deleted h-TRβ1 allele are clinically and biochemically normal (6). It has been hypothesized that the mutant h-TRβ1 inhibits the function of the normal h-TR by a dominant negative mechanism. This was confirmed by in vitro studies demonstrating that h-TRβ1 from patients with mutations in the carboxy-terminal part of the LBD have not only reduced $T_3$-binding and transcriptional activity, but are also able to inhibit normal h-TRα1 and h-TRβ1 function in transient transfection systems (7–9).

We propose to classify the function of mutant TRs into three different phenomenological types. The type I mutations, represented by kindred ED in this study, have a reduced $T_3$-binding affinity and transcriptional capacity. High levels of $T_3$ can completely normalize TR function and reverse the dominant negative potency. The recently described kindred OK represents a type II mutation. Its $T_3$-binding affinity and transcriptional capacity are reduced, but $T_3$ can neither restore normal TR activity nor reverse the dominant negative potency (10). This mutation therefore impairs independently the $T_3$-binding as well as presumably a transactivating domain, although this latter point needs to be mechanistically proven. Kindred PV represents a type III mutation, which has neither $T_3$-binding nor transcriptional activity, and the dominant negative potency is not reversed by $T_3$.

Three main mechanisms have been proposed to account for the inhibitory property of the three types of mutant h-TRβ1: (*a*) formation of inactive dimers between mutant and normal h-TRs; (*b*) competition of normal and mutant h-TRs for binding to $T_3$-response elements; and (*c*) competition for limiting amounts of nuclear auxiliary factors. While the first hypothesis could not be experimentally confirmed, substantial evidence supports a mechanism of competition between mutant and normal receptors for DNA-binding while the role of limiting accessory factors remains equivocal (9, 11). One class of these nuclear TR accessory proteins (TRAP) was recently cloned (retinoid X receptor, subtypes α, β, and γ) and shown to be expressed in a tissue-specific manner. RXRs are capable of homodimerizing, as well as of heterodimerizing with TRs, thereby modifying their DNA-binding and transcriptional capacity several-fold (12–21). The natural ligand of RXR was recently shown to be an isomer of all-*trans*-retinoic acid, 9-*cis*-retinoic acid (22, 23).

The present study was designed to compare the interaction of various mutants and normal h-TRβ1 with different positive TREs in the presence and absence of RXRβ. Specifically, we

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1. *Abbreviations used in this paper:* ABCD, avidin-biotin complex DNA binding; GRTH, generalized resistance to thyroid hormone; h-TR, human $T_3$ receptor; LBD, ligand-binding domain; rGH, rat growth hormone; RXR, retinoid X receptor; $T_3$, 3,3′,5-triiodothyronine; TRAP, TR-accessory protein; TRE, $T_3$-response element.

wished to establish whether the dominant negative potency of mutant receptors may be attributed, at least in part, to a preferential binding to DNA and/or RXR$\beta$, as well as to elucidate molecular mechanisms for the intraindividual differences in the tissue-resistance of patients with GRTH. While TRE-specific and mutation-specific differences in the DNA-binding and transcriptional characteristics were found, we also demonstrated that competition for limiting amounts of RXR$\beta$ did not account for the dominant negative mechanism.

## Methods

*Construction of rRXR$\beta$ expression vector.* The full-length cDNA for the rat RXR$\beta$ was a kind gift of Dr. C. K. Glass, University of California (San Diego, CA) (12). After excising the rRXR$\beta$ cDNA from pKS-RXR$\beta$ with EcoRI/BstXI, the ends were blunt-ended with T4 DNA polymerase. HindIII linkers were added and the fragment was ligated into the HindIII site of the pSV2 eucaryotic expression vector (9). The construct was verified by restriction analyses.

*In vitro transcription and translation of receptors.* The construction of pGEM3 vectors containing the mutant cDNAs for the h-TR$\beta$1 from kindreds ED and PV was described elsewhere (9). The vector carrying mutant cDNA from kindred OK was constructed by cloning exon 10 of the h-TR$\beta$1 after amplification by the polymerase chain reaction from genomic DNA into the BglII/HindIII site of pGEM3-h-TR$\beta$1-WT. The subcloned fragment was sequenced to rule out artifactual mutations. The vector containing the normal placental h-TR$\beta$1 cDNA was a kind gift of Dr. C. Weinberger (Salk Institute, San Diego, CA) (24). For in vitro transcription, each cDNA was linearized with HindIII and transcribed with T7 RNA polymerase. [$^{35}$S]methionine labeled and unlabeled receptors were synthesized using rabbit reticulocyte lysate (Promega, Madison, WI). The labeled translation products were analyzed for the appropriate size, as well as for quantitation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The values were in excellent agreement with the quantitation of [$^{35}$S]methionine labeled proteins by the trichloroacetic acid precipitation method (3).

*Preparation of RXR$\beta$ protein.* The protein was obtained by infecting SF9-cells with a recombinant baculovirus (rH-2RIIBP) harboring a mouse RXR$\beta$ (mRXR$\beta$-H-2RIIBP) cDNA, kindly provided by Dr. Keiko Ozato (National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD). The SF9 cells were cultured in suspension in the serum-free medium SF-900 II SFM (Gibco Life Technologies Inc., Gaithersburg, MD) and infected with rH-2RIIBP virus with a moi of 2 for 2 d at 27°C. The nuclear extracts were prepared according to Dignam et al. (25) except that the protease inhibitors were included in all buffers (1 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, and 0.5 mM phenylmethyl-sulfonylfluoride). From $1 \times 10^8$ SF9 cells, 1.5 ml (5 mg/ml protein) of nuclear extracts were obtained. Analysis by gel electrophoresis indicated that RXR$\beta$ accounted for 20–25% of total proteins. The nuclear extracts of wild type SF9 cells were prepared similarly.

*Electrophoretic gel mobility shift assay.* Single stranded oligonucleotides with sequences shown in Table I were synthesized and purified by high-pressure liquid chromatography. The purified complementary strands were annealed and the ends filled in using Klenow DNA polymerase (Stratagene, San Diego, CA) in the presence of [$^{32}$P]CTP. The labeled double-stranded oligonucleotides were separated on a 12% polyacrylamide gel. The radioactive band was excised and electrotransferred onto DE 81 paper (Whatman Ltd., Maidstone, United Kingdom). The paper was washed with a low salt buffer (10 mM Tris/pH 7.5, 1 mM EDTA, and 0.1 M LiCl), and thereafter, the labeled oligonucleotides were eluted by a high salt buffer (10 mM Tris/pH 7.5, 1 mM EDTA, and 1 M LiCl). After ethanol precipitation, the labeled oligomers were dissolved in a buffer containing 10 mM Tris/pH 7.5 and 1 mM EDTA and stored at −20°C.

The gel mobility shift assay was carried out similarly as described by Yen et al. (26), except that poly(dI-dC) and bovine serum albumin were omitted, and the concentration of the sheared salmon DNA was increased to 0.8 $\mu$g/$\mu$l. Briefly, equal amounts of either in vitro–translated wild type or mutant receptor (1–2 $\mu$l) were first incubated with the labeled probe (10–12,000 cpm) with or without $T_3$ in the binding buffer containing 25 mM Hepes/pH 7.5, 5 mM $MgCl_2$, 4 mM EDTA, 10 mM DTT, 0.11 M NaCl, and 0.8 $\mu$g/$\mu$l sheared salmon DNA). Where appropriate, RXR$\beta$ (200 ng of SF9 nuclear extracts) was added. For the control experiments, the nuclear extracts of SF9 cells (200 ng) were added. After incubation for 30 min at 25°C, the mixture was loaded onto a 5.2% polyacrylamide gel and electrophoresed at 4°C for 2–3 h at a constant voltage of 230 V. The gel was dried and subsequently autoradiographed.

*Avidin biotin complex DNA binding (ABCD) assay.* The protocol is a modification of a previously published method (27). 2–4 $\mu$l of reticulocyte lysate containing [$^{35}$S]methionine-labeled receptors were incubated for 40 min at 4°C with 1–200 ng of biotinylated oligonucleotide in a buffer containing 50 mM KCl, 20 mM Hepes pH 7.8, 1 mM $\beta$-mercaptoethanol, 1 mM EDTA, 0.1% Nonidet-40, and 20% glycerol (vol/vol). An aliquot of the incubation mixture was transferred to a tube containing 30 $\mu$l streptavidin-agarose slurry. After a 10-min incubation at 4°C, the streptavidin-agarose was quickly washed three times with the above binding buffer at 4°C. The pellet was resuspended in scintillation cocktail and counted.

*Transient transfection assay.* The construction of the wild-type h-TR$\beta$1 and mutant pSV2 expression vectors, as well as the chloramphenicol acetyltransferase reporter plasmid containing a TREpal (pMTV-TREpal-CAT) was described before (9, 28). The pSV2-OK plasmid was obtained by subcloning the insert from pGEM3-OK into the pSV2-WT vector. The pMTV-TRElap-CAT and pMTV-DR+4-CAT plasmids were constructed by cloning a TRElap (5′-AGCTTGACCTGACGTCAGGTCAAGCTT-3′) or DR+4 (5′-AGCTTAGGTCACTGGAGGTCAAGCTT-3′) oligonucleotide into the HindIII site of pMTV-CAT. The constructs were verified by restriction analyses and sequencing.

*Table I. Oligonucleotides Used in the DNA-Binding Experiments*

| TRE | Sequence |
|---|---|
| rGH | 5′ AAGGGGATCAGGTAAGATCAGGGACGCGACCGCAGG<br>TCCATTCTAGTCCCTGCGCTGGCGTCCTCTAGAAGGA 5′ |
| TREpal | 5′ AAGGGGATCCAGCTTCAGGTCATGACCTGAGAGCT<br>TCCAGTACTGGACTCTCGATCTAGAAGGA 5′ |
| DR + 4 | 5′ AAGGGGATCCAGCTTCAGGTCACAGGAGGTCAGAGAGCT<br>TCGAAGTCCAGTGTCCTCCAGTCTCTCGACTAGAAGGA 5′ |
| TRElap | 5′ AAGGGGATCCAGCTTGACCTGACGTCAGGTCAAGCT<br>TCGAACTGGACTGCAGTCCAGTTCGACTAGAAGGA 5′ |

*Table II. Classification of Mutant h-TRβ1*

| Mutation (kindred/codon) | AA changed | $T_3$-binding (relative $K_d$) | Transcription capacity, dominant negative potency | Restoration of h-TR activity by $T_3$ |
|---|---|---|---|---|
| *h*-TRβ1-WT | — | 1 | Normal/none | — |
| Type I mutation | | | | |
| ED/312 | Ala/Thr | 0.2 | Reduced/yes | Full |
| Type II mutation | | | | |
| OK/437 | Met/Val | 0.2 | Reduced/yes | Partial |
| Type III mutation | | | | |
| PV/443 | Frame shift | <0.01 | Absent/yes | Absent |

Cells (CV-1, HeLa) were plated 24 h before transfection in Dulbecco's modified Eagle's medium containing 10% (vol/vol) hormone-depleted fetal calf serum (29), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in 10-cm petri dishes (HeLa) or six-well plates (CV-1) at a density of $1.3 \times 10^6$ cells/dish and $0.5 \times 10^6$ cells/well, respectively. The medium was changed 4 h before transfection. Using the calcium-phosphate method (CellPhect kit; Pharmacia-LKB, Piscataway, NJ) the cells were transfected with the appropriate plasmids. 24 h later, the plates were washed once with phosphate-buffered saline, and fresh medium was added together with the appropriate $T_3$ concentration. After another 24 h, the cells were harvested, lysed, and the chloramphenicol acetyltransferase activity determined in the extract as described (30). Chloramphenicol acetyltransferase activity was normalized for the protein concentration as measured by the Coomassie blue method. No substantial differences in the transfection efficiency were present as assessed by the cotransfection of a growth hormone expression vector (pXGH5).

*Statistics.* Where not indicated otherwise, results were expressed as means±SEM. To examine statistical significance of the Scatchard analysis data in Fig. 8, the slopes of the least square regression lines (with and without $T_3$) were compared by multiple regression analysis with a categorical variable and using an interaction term (SYSTAT 5.03; Systat Inc., Evanston, IL).

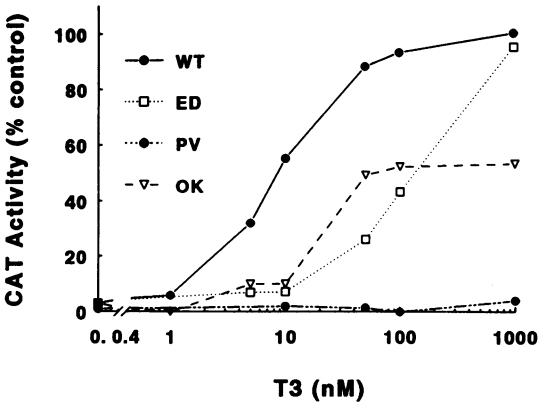


*Figure 1.* $T_3$-dependent transcriptional responses of wild type (*WT*) and mutant β1 $T_3$ receptors assessed by CAT activity. After transfection of HeLa cells (10-cm dishes) with 1 μg of the appropriate receptor expression vector for each kindred and with 5 μg pMTV-TREpal-CAT reporter/plate, the cells were cultured for 24 h in the presence of various $T_3$ concentrations. The results were normalized to the CAT activity observed with normal h-TRβ1 at 100 nM $T_3$.

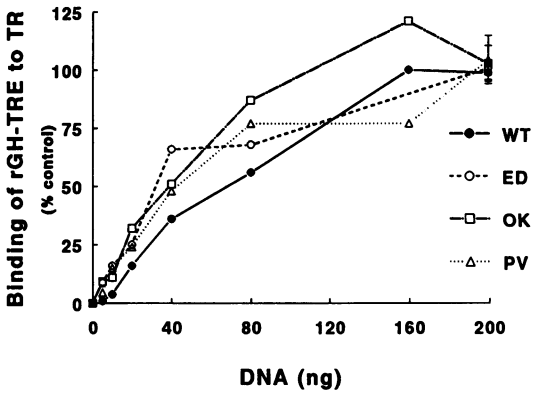


*Figure 2.* Binding of wild type (*WT*) and mutant h-TRβ1 to rGH-TRE (−186/−158). Equal amounts of in vitro–translated receptors were bound to various amounts of biotinylated rGH-TRE. Similar results were obtained in the absence or presence of 100 nM $T_3$.

## Results

*Transcriptional capacity of h-TRβ1-OK.* The characteristics of the mutant receptors are summarized in Table II (5). The transcriptional capacity of the mutant receptors on a TREpal in response to $T_3$ is depicted in Fig. 1. While the $T_3$-response of the receptors from kindreds ED and PV representing type I and III mutations were reported previously (9), this figure describes the novel functional characteristics of the h-TRβ1 from kindred OK, whose mutation was published recently (5). The transcriptional function of all previously studied mutant h-TRβ1 with residual $T_3$-binding could be fully restored by high

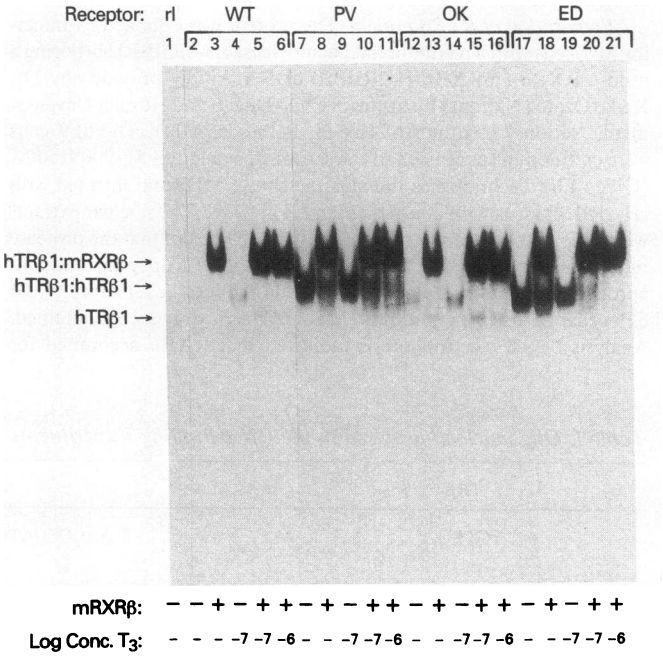


*Figure 3.* Effect of $T_3$ and RXRβ on h-TRβ1 binding to TREpal. Equal amounts of in vitro translated receptors were bound to $^{32}$P-labeled TREpal oligonucleotide in the presence or absence of $T_3$ and mRXRβ and then electrophoresed as described in Methods.

levels of $T_3$, while the mutant h-TR$\beta$1-OK could not achieve full transcriptional activity. In addition, the dominant negative potency of h-TR$\beta$1-OK could not be reversed by high levels of $T_3$ (data not shown). This suggests that the single point mutation in this kindred impairs independently a $T_3$-binding domain, as well as a trans-activating domain (type II mutation).

*Binding of mutant h-TR to rat growth hormone (rGH) (−186/−158), TREpal, DR+4, and TRElap.* The binding of mutant TRs to the rGH promoter (−186/−158) assessed by the ABCD assay is shown in Fig. 2. The Scatchard analysis of the binding curves revealed a small increase in the DNA-binding affinity of all mutant receptors studied ($K_d$: wildtype, 2.4±0.4 nM; OK, 1.5±0.2 nM; ED, 1.5±0.5 nM; and PV, 1.2±0.2 nM). The binding of TRs to the rGH was not influenced by $T_3$ at concentrations ≤ 100 nM (data not shown).

In the gel mobility shift assay using the TREpal (Fig. 3) or DR+4 (Fig. 4) as a probe, mutant receptors showed an increased tendency to form homodimers, as summarized in Table III. While $T_3$ did not alter the binding of normal and mutant TRs to the TREpal, a small decrease (20%) of wild-type homodimer binding was observed on the DR+4 at high levels of $T_3$ (Fig. 4, *A* and *B*). RXR$\beta$ equally heterodimerized with normal and mutant h-TR$\beta$1 and $T_3$ did not influence this interaction.

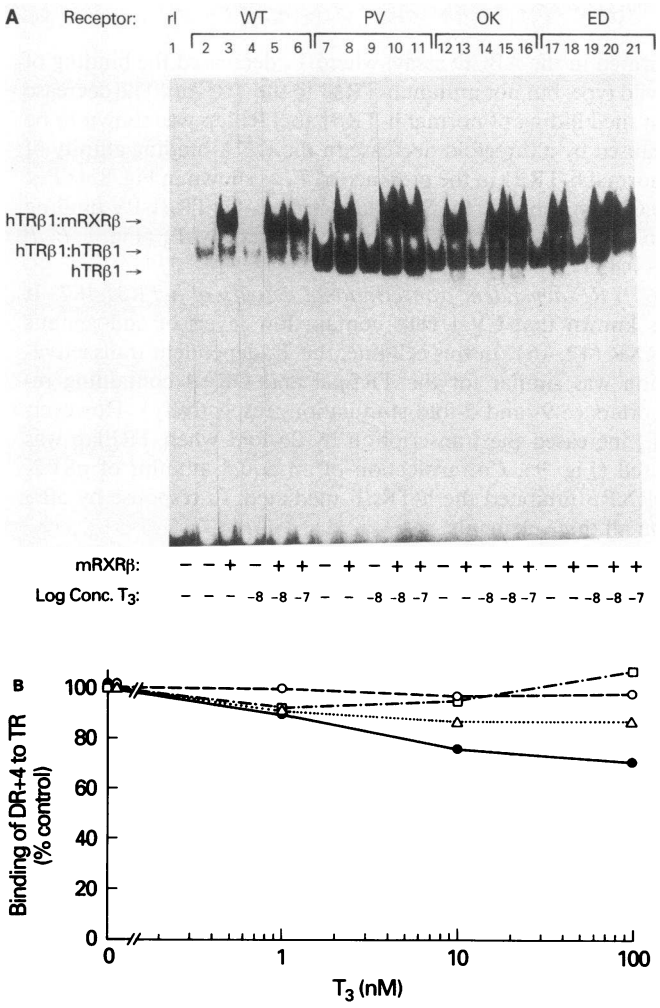


*Figure 4.* Effect of $T_3$ and RXR$\beta$ on h-TR$\beta$1 binding to DR + 4. Equal amounts of in vitro–translated h-TR$\beta$1 proteins were bound to $^{32}$P-DR+4 oligonucleotide in the presence or absence of $T_3$ and mRXR$\beta$ and electrophoresed as described in Methods. (*A*) Autoradiogram. (*B*) After electrophoresis the appropriate dimer bands from four independent experiments were quantitated. Shown is the effect of $T_3$ on the binding of wild type (●), OK (△), ED (□), and PV (○) h-TR$\beta$1 to DR+4 in the absence of RXR$\beta$. The data are averages of four experiments.

*Table III. Homodimer Formation of WT and Mutant hTR$\beta$1**

| | Relative binding | | | |
|---|---|---|---|---|
| TRE | WT | PV | ED | OK |
| PAL | 1 | 5 | 4 | 1.5 |
| DR + 4 | 1 | 2.3 | 2 | 1.5 |
| LAP | 1 | 4 | 2.8 | 1.5 |

* Data were averages of four independent experiments.

Yen et al. have recently shown that on the TRElap derived from the chicken lysozyme silencer (31), $T_3$ decreased TR$\alpha$1 and TR$\beta$1 homodimer binding in a dose dependent manner (26). Fig. 5 illustrates the binding of normal and mutant h-TR$\beta$1 to TRElap in the presence of various concentrations of $T_3$, as well as RXR$\beta$. Addition of nanomolar concentrations of $T_3$ dissociated the WT homodimers very effectively (Fig. 6 *A*). However, no effect of $T_3$ on the binding of h-TR$\beta$1-PV, which is characterized by a non-detectable $T_3$-binding affinity, was observed. The mutants with detectable, but reduced $T_3$-binding showed a moderate degree of dissociation at high levels of $T_3$. When RXR$\beta$ was added, the normal h-TR$\beta$1 forms a heterodimer (TR:RXR) and a weaker homodimer (TR:TR) band. Upon addition of $T_3$, the homodimer band further decreases while the heterodimer species increased in a dose-dependent manner (Fig. 6 *B*). This increase in the relative amount of heterodimers was virtually absent with the mutant h-TR$\beta$1. The possibility of h-TR$\beta$1 interacting with an endoge-

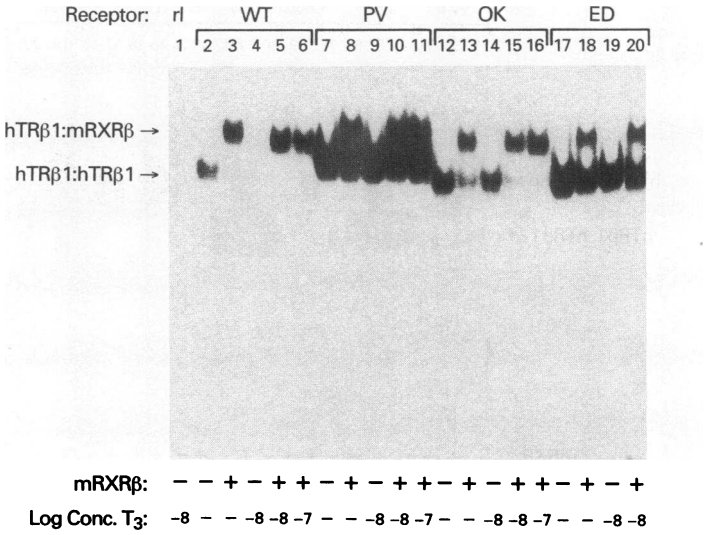


*Figure 5.* Effect of $T_3$ and RXR$\beta$ on h-TR$\beta$1 binding to TRElap. Equal amounts of in vitro–translated receptors were bound to $^{32}$P-labeled TRElap oligonucleotide in the presence or absence of $T_3$ and mRXR$\beta$ and then electrophoresed as described in Methods.

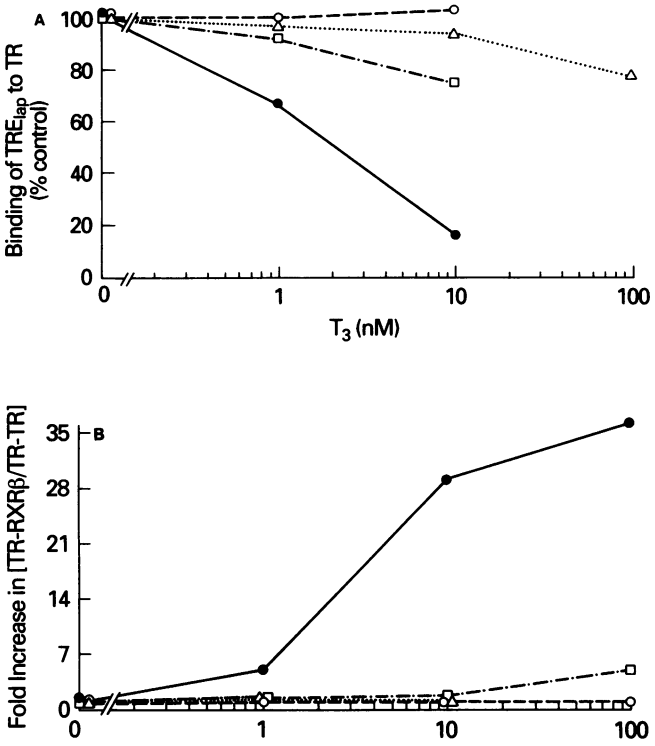


*Figure 6.* Quantitation of three independent experiments similar to that described in Fig. 5. (A) Effect of $T_3$ on h-TR$\beta$1 homodimer binding to TRElap in the absence of RXR$\beta$. (B) Effect of $T_3$ on the ratio of [h-TR$\beta$1:RXR$\beta$] heterodimers to [h-TR$\beta$1:h-TR$\beta$1] homodimers; wild type (●), OK (□), ED (△), and PV (○).

nous SF9 protein was excluded. Fig. 7 shows that in lanes *3, 6, 9,* and *12* for TRElap and lanes *14, 16, 18,* and *20* for DR+4, no heterodimer was detected when the nuclear extracts from the wild-type SF9 cells was used. The above results were con-

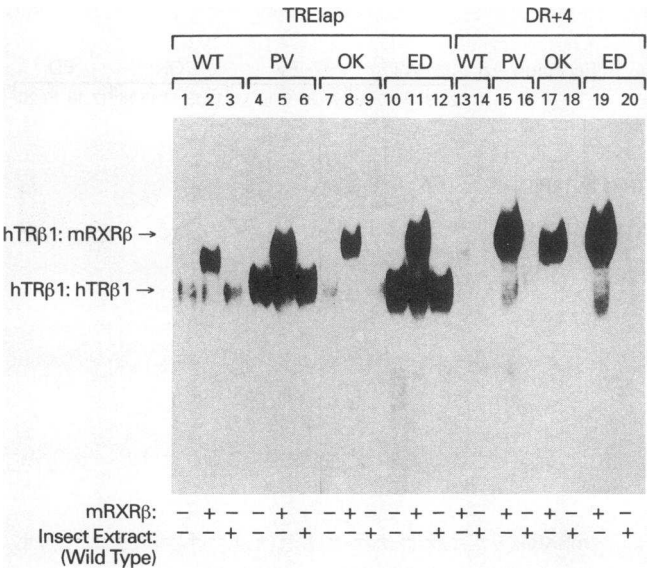


*Figure 7.* Lack of interaction of SF9 cellular extract with in vitro translated h-TR$\beta$1 protein on TRElap and DR+4. Equal amounts of in vitro–translated receptors were bound to $^{32}$P-labeled TRElap and DR+4 in the presence of an equal amount of nuclear extracts of wild type SF9 cells or SF9 cells expressing RXR$\beta$.

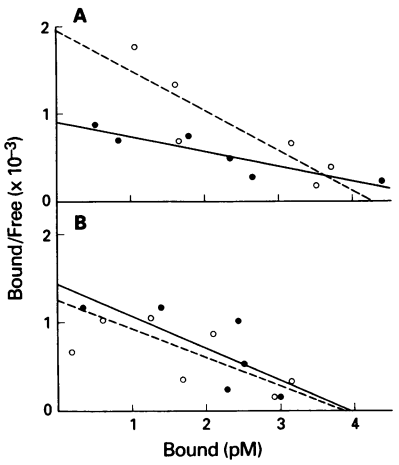


*Figure 8.* Determination of the dissociation constant of wild type and mutant h-TR$\beta$1 to TRElap using the ABCD assay and scatchard analysis. (A) Binding of wild type receptor in the presence (●) and absence (○) of 100 nM $T_3$. The affinity of wild type h-TR$\beta$1 is threefold higher in the absence of $T_3$ ($K_d$ = 2nM) than in its presence ($K_d$ = 6nM; $P \leq 0.05$). (B) Binding of h-TR$\beta$1-PV in the presence (●) and absence (○) of 100 nM $T_3$. No significant difference exists between the slopes of the two binding curves.

firmed in the ABCD assay, where $T_3$ decreased the binding of wild type, but not mutant h-TR$\beta$1 to the TRElap. The decrease in the binding of normal h-TR$\beta$1 to TRElap was shown to be caused by a threefold decrease in the DNA-binding affinity of normal h-TR$\beta$1 in the presence of $T_3$ as shown in Fig. 8 *A* ($P \leq 0.05$). In contrast, the Scatchard curves of h-TR$\beta$1-PV binding to TRElap were not significantly altered by $T_3$ (Fig. 8 *B*, $P > 0.45$).

*TRE-dependent transcriptional activity of h-TR$\beta$1-WT.* It is known that CV-1 cells contain low levels of endogenous RXR (12, 16). In this cell line, the $T_3$-dependent transactivation was similar for the TREpal and DR+4 containing reporters (5.9- and 5-fold stimulation, respectively). However, $T_3$ increased the transcription by 25-fold when TRElap was used (Fig. 9). Cotransfection of an equal amount of pSV2-rRXR$\beta$ inhibited the h-TR$\beta$1–mediated $T_3$ response by 50% on all three elements.

*TRE-dependent dominant negative potency and effect of RXR$\beta$.* To explain the dominant negative potency of mutant TRs, it has been proposed that mutant and normal receptors may compete for limiting amounts of a nuclear accessory fac-

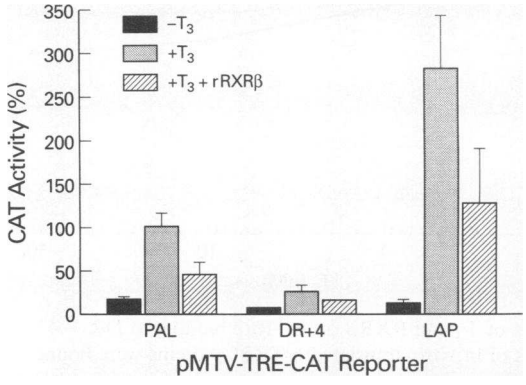


*Figure 9.* Transcriptional capacity of transfected wild type h-TR$\beta$1 on TREpal, DR+4, and TRElap in the presence or absence of rRXR$\beta$ in CV1 cells. pSV2-WT (200 ng) and 1,000 ng of the appropriate pMTV-CAT reporter were transfected with or without 200 ng pSV2-rRXR$\beta$ into CV-1 cells in six-well plates. The results were normalized to the CAT activity from h-TR$\beta$1-WT in the presence of 500 nM $T_3$.

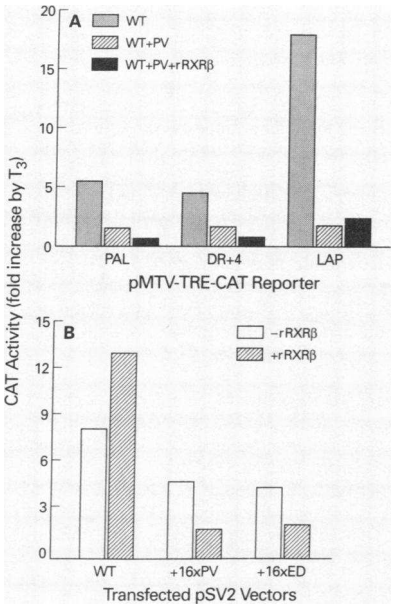


*Figure 10.* Transfection of rRXR$\beta$ does not reverse the TRE-dependent dominant negative potency of mutant h-TR$\beta$1. (A) CV-1 cells in six-well plates were transfected with pSV2-WT (200 ng) alone or with a fivefold excess of pSV2-PV in the presence or absence of 1 $\mu$g pSV2-rRXR$\beta$. The results are expressed as fold increase of CAT activity in response to $T_3$. (B) HeLa cells in 10-cm dishes were transfected with pSV2-WT (250 ng) and pMTV-TREpal-CAT (5 $\mu$g) alone or with a 16-fold excess of pSV2-PV or pSV2-ED in the presence (*hatched bars*) or absence (*open bars*) of 5 $\mu$g pSV2-rRXR$\beta$. The results are expressed as fold increase of CAT activity in response to $T_3$.

tor (32). Since the h-TR$\beta$1 mutants were able to interact with RXR$\beta$ as discussed above, and RXR$\beta$ was shown to account for most of the TRAP activity in HeLa cells, this hypothesis could be directly tested. Transfection of a fivefold excess of h-TR$\beta$1-PV expression plasmid into CV-1 cells resulted in a fourfold decrease in $T_3$-dependent transcription on the TREpal and DR+4. In contrast, ligand dependent transcription on the TRElap was inhibited by 10-fold (Fig. 10 *A*), thereby establishing the TRE-dependence of the dominant negative potency of mutant h-TRs. Cotransfection of rRXR$\beta$ did not reverse this inhibition. Similarly, as shown for HeLa cells in Fig. 10 *B*, the transfection of 5 $\mu$g pSV2-rRXR$\beta$ increased slightly the transcriptional capacity of normal h-TR$\beta$1 in response to $T_3$ on the TREpal, whereas rRXR$\beta$ was also not able to reverse the dominant negative effect of the mutant TRs from kindreds PV and ED.

## Discussion

We investigated the interaction of naturally occurring h-TR$\beta$1 mutants with various TREs and RXR$\beta$. The three different mutations from kindreds with GRTH were chosen to represent three distinct types of functional impairment. The mutant receptor from kindred ED was previously shown to have a fivefold reduction in $T_3$-binding affinity and a correspondingly shifted $T_3$-dose response curve in a transient transfection assay; as expected, at high levels of $T_3$ normal receptor function was restored and the dominant negative potency was abolished (type I mutation) (9). Although this mutation is located in the $\tau_i$ subdomain that is thought to be involved in the interaction with TRAPs (33, 34), the h-TR$\beta$1-ED protein interacted normally with RXR$\beta$. The point mutation in the h-TR$\beta$1 of kindred OK reduced $T_3$-binding also fivefold, but high levels of $T_3$ could neither restore full receptor activity nor reverse the dominant negative potency (type II mutation) (10). Although a defect in the dimerization with RXR$\beta$ might explain these characteristics, we now show that this is not the case. Therefore, it can be speculated that this single mutation not only affects the $T_3$-binding, but also a trans-activating domain. The type III mutation studied, h-TR$\beta$1-PV, had a frame-shift mutation in the carboxy terminus of the molecule, which is thought to form an amphipathic alpha helix involved in protein–protein contacts (35, 36). Nevertheless, this mutation which did neither bind $T_3$ nor trans-activate, could interact normally with RXR$\beta$. Together with a report that RXR$\beta$ may be a limiting nuclear factor for TR action (15, 37), these findings suggested, that the binding to RXR$\beta$ might be crucial for the mediation of the dominant negative action of these receptors. The hypothesis of competition of mutant and normal h-TRs for limiting accessory factors has been proposed before (8, 9, 32). However, we now report that in a transient transfection system using cell lines with low (CV-1) and high (HeLa) levels of endogenous RXR (12, 14, 16), the cotransfection of various amounts of RXR$\beta$ could not reverse the dominant negative effect of mutant h-TR$\beta$1.

The DNA-binding affinity of all three mutant receptors was slightly increased on the rGH promoter. Similarly, these mutants demonstrated a variably increased tendency for homodimerization on the three TREs examined in gel-shift experiments. The degree of increased homodimer formation was not dependent on the type of TRE, but was rather influenced by the different mutations in the h-TR$\beta$1; the frame-shifted h-TR$\beta$1-PV exhibited the most pronounced increase in homodimerization. The influence of $T_3$ on homodimer formation was dependent on the TRE. While ligand did not influence h-TR$\beta$1 binding to TREpal, a small or a marked effect was observed on the DR+4 or TRElap, respectively. The binding of TRs to the latter was previously shown to be $T_3$-dependent by Yen et al. (26). Data from the same group suggested that a type III mutation in codon 345 (kindred Mf) did not dissociate from TRElap in response to $T_3$ (38). This observation was confirmed for the h-TR$\beta$1-PV using the ABCD assay, where the binding of ligand to normal h-TR$\beta$1 decreased the binding affinity for TRElap by threefold, while the affinity of h-TR$\beta$1-PV remained unchanged. The type I and II mutations, however, were able to partially dissociate from TRElap in the presence of high levels of $T_3$. In contrast, $T_3$ could only minimally increase the RXR:TR heterodimer to TR:TR homodimer ratio for these two types of mutations.

It has recently been shown that DNA-binding is required for the dominant negative effect of certain mutations to occur (11). The increased tendency for homodimerization as found in the present study may render the mutant receptors more efficient competitors on dimer-permissive TREs. It can be hypothesized that the dominant negative potency on TRElap-like enhancers such as the myelin basic protein TRE (26, 31, 39–41), is even more pronounced because of the lack of ligand-induced dissociation of homodimers. This hypothesis was tested and confirmed by transfection studies. While the $T_3$-dependent transcriptional activity of wild-type h-TR$\beta$1 was fivefold higher on the TRElap than on TREpal or DR+4, cotransfection of mutant h-TR$\beta$1 repressed $T_3$-dependent trans-activation 2.5-fold more efficiently on the TRElap than on the TREpal or DR+4.

Taken together, our observations suggest that mutant h-TR$\beta$1 inhibit the action of wild-type h-TR mainly by competing for DNA-binding. On TREs where the dissociation of the

TRs is ligand induced, the mutant h-TRβ1 are more effective dominant negative regulators, since they have a higher affinity for the TRE in the presence of $T_3$ than the wild-type h-TRβ1. While we used the TREpal, DR+4, and TRElap elements as models to study the presumably different binding arrangements of h-TRβ1:RXRβ homo- and heterodimers on DNA (head-to-head, head-to-tail, and tail-to-tail, respectively), it can be speculated that endogenous TREs represent quantitative variations of these themes, subject to the same basic rules established in the present study.

In summary, the functional characteristics of a particular mutant h-TR are not only determined by the type of the mutation itself (type I, II, or III), but in addition by the type of the $T_3$-regulated enhancer these receptors are acting on. Assuming that the TREs in the promoter of various organ-specific genes are of different types, it is tempting to speculate that the clinically observed intraindividual differences in organ resistance could be explained in part by the TRE-dependent interaction of mutant receptors with DNA and RXRβ.

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