Cell Type-Dependent Modulation of the Dominant Negative Action of Human Mutant Thyroid Hormone β1 Receptors

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ABSTRACT

Background: Mutations in the ligand-binding domain of the thyroid hormone receptor β (TRβ) gene cause the syndrome of resistance to thyroid hormone (RTH). The clinical phenotype results from the antagonism of the normal TRα and the non-mutated TRβ alleles by the TRβ1 mutants, via a dominant negative effect. There is, however, marked heterogeneity of organ resistance within and among kindreds with RTH. This study examines the potential role of cell type in modulating the dominant negative potency of human TRβ1 (h-TRβ1) mutants.

Materials and Methods: Transient transfections were performed in HeLa and NIH3T3 cells, using a wild type (WT) and three naturally occurring mutant h-TRβ1 constructs, and three natural thyroid hormone response elements (TREs). Immunocytochemistry was performed to detect levels of TRβ1 expression in these two cell types. In order to determine how TRβ1 interacts with other cellular partners, gel-shift analyses using HeLa and NIH3T3 nuclear extracts were performed.

Results: Transfection studies using WT h-TRβ1 in HeLa and NIH3T3 cells, showed that the 3,3',5-triiodothyronine (T_3)-induced transactivation of the different TREs varied between cell types. Unlike the non-T_3-binding h-TRβ1 mutant, PV, mutants ED and OK displayed the expected T_3-induced dose responsiveness in these two cell types. For each TRE examined, the magnitude of the dominant negative effect varied between the cell types. The levels of receptor expression in HeLa and NIH3T3 cells were identical, as determined by immunocytochemistry. Gel-shift analyses showed differences in the formation of hetero- and homodimers depending on both the cell type and TRE motif.

Conclusions: The cell type in which a mutant receptor operates affects the relative amounts of hetero- and homodimers. Together with the nature of the mutation and the TRE-motif, this could modulate the dominant negative action of mutant receptors in different tissues, which, in turn, could contribute to the variable phenotypic characteristics of RTH.

INTRODUCTION

Resistance to thyroid hormone (RTH) is a syndrome characterized by refractoriness of the pituitary and peripheral tissues to the action of thyroid hormone and is usually transmitted in an autosomal dominant fashion. There is an inappropriately elevated level of thyroid-stimulating hormone in the face of elevated levels of circulating free thyroid hormones, together with clinical features of variable degrees of thyroid hormone resistance action in peripheral tissues and the associated condition of attention-deficit hyperactivity disorder (1–3). The disease is caused by mutations in the ligand-binding domain of the thyroid hormone receptor β (h-TRβ) gene, which result in variable reductions in the affinity

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of h-TRβ1 for its ligand, 3,3',5-triiodothyronine (T_3), and impaired transcriptional capacity (4,5). The mutant h-TRβ1 inhibits the function of normal h-TRβ1 and α1 via a dominant negative effect, thereby mediating the abnormal phenotype (6–9). Kindreds with RTH display a remarkable heterogeneity in organ resistance within an individual and among kindreds harboring identical mutations (10), and there are ongoing attempts to correlate genotype with the phenotype of these patients.

The difficulty in establishing such correlations stems from the complex molecular nature of the interactions of the thyroid hormone receptor. Not only are there four isoforms of the thyroid hormone receptor, which are expressed in a tissue-specific and development-stage-specific manner, but they may also heterodimerize with multiple cellular partners, most prominently the retinoid X-receptors (RXRs), to create unique transcriptional responses of T_3-regulated genes (11–16). Published examples of genotype-phenotype correlations include an association between language abnormalities with mutations in exon 9 (17), and a high ratio of mutant:normal h-TRβ mRNA during a period of bone resistance with growth retardation, attenuated in teenage years with a concomitant improvement in growth rate (18). In general, however, neither the T_3-binding impairment of h-TRβ1 mutants, as reflected by blood levels of thyroid hormones, nor the location of mutations predicts a phenotype.

Until recently, studies of mutant h-TRβ1 have employed mostly artificial thyroid response elements (TREs) (7,19–21). Moreover, there has not been a systematic comparison of the effects of mutant h-TRβ1 on various natural positive TREs in different cell types. Furthermore, there have been conflicting data regarding the relative roles of heterodimers versus homodimers in mediating the dominant negative effect of these mutant receptors (22,23). These studies have employed transfections in a single cell type, utilizing mostly artificial TREs and exogenous RXRs to characterize the nature of protein-protein complexes. We were interested in determining the extent to which the cellular environment contributes to the dominant negative action of mutant receptors. Thus, we performed transient transfection assays using naturally occurring TREs in two different cell lines, HeLa and NIH3T3, and gel-shift studies using these nuclear extracts to establish if there are differences in protein-protein interactions. These proteins may

include those that interact either directly with mutant h-TRβ1, so called thyroid hormone receptor auxiliary proteins (TRAPs), or indirectly to bring the TR-complex into contact with the transcriptional machinery, so called adaptor or coactivator proteins (24). Our results demonstrated that the extent of the transactivation by wild-type (WT) h-TRβ1 on the three natural TREs studied is different in the two cell types and that the three mutant receptors display different dominant negative potencies in the two cell types despite equivalent expression levels of receptor in these cells, as demonstrated by immunocytochemistry. Furthermore, the pattern of hetero- and homodimerization differs when nuclear extracts from these two cell types are used in gel-shift studies. The cell type in which the mutant receptors act, together with the type of TRE motif, most probably determines the patterns of hetero- and homodimerization of these receptors, the relative abundance of which, in turn, contributes to the strength of their dominant negative effect.

MATERIALS AND METHODS

Construction of Plasmids

Construction of the WT h-TRβ1 and mutant pSV2 expression vectors -ED, -OK, and -PV, and the corresponding pGEM 3Z-vectors for in vitro translation has been described before (19,20,25). The TK-TRE constructs, -Lys, -ME, and -GH, were a kind gift of Dr. G. Brent, Harvard Medical School, Boston, MA, U.S.A.; the construction of these has been described previously (26).

Transient Transfection

Cells (HeLa, NIH3T3) were plated 24 hr before transfection in Dulbecco's modified Eagle's medium containing 10% (v/v) hormone-depleted fetal calf serum (27), 100 μ/ml penicillin, 100 μg/ml streptomycin and 0.25 μl/ml amphotericin B in 10-cm petri dishes. The medium was changed 4 hr prior to transfection. Transfections were performed in pairs (CellPhect kit; Pharmacia-LKB, Piscataway, NJ, U.S.A.) using the CaPO$_4$ method. Each plate received 5.0 μg TRE-containing TK-CAT plasmid, 3.0 μg pXGH5 plasmid as a transfection efficiency control, 1.0 μg pSV$_2$-WT h-TRβ1, 0-5.0 μg mutant h-TRβ1, and 0-5.0 μg pSV2 to keep the amount of transfected DNA constant. After 24 hr, the plates were

washed once with phosphate-buffered saline and fresh medium added together with the appropriate T_3 concentration. CAT assays were performed 24 hr later, after harvesting and lysing the cells, as previously described (28). CAT activity was normalized for protein concentration as measured by the Coomassie blue method. No substantial differences in transfection efficiency were present as assessed by the cotransfection of a growth hormone expression vector pXGH5.

In Vitro Transcription and Translation of Receptors

For in vitro transcription, WT and mutant h-TRβ1 cDNAs were linearized with HindIII and used as a template for transcription with T7-RNA Polymerase. [^{35}S]-labeled and unlabeled receptor proteins were synthesized from the transcribed RNA using the rabbit lysate kit according to the manufacturer's manual (Promega, Madison, WI, U.S.A.). The translated proteins were analyzed for their molecular weights by 10% SDS-PAGE, and quantitation was performed by the trichloroacetic acid precipitation method.

Electrophoretic Gel Mobility Shift Assay

Complementary oligonucleotides with 5′ overhangs were annealed and filled-in in the presence of ^{32}P-dCTP with Klenow DNA Polymerase. The labeled double-stranded oligonucleotides were purified on a 12% polyacrylamide gel. The gel-mobility shift assay was carried out in a 10 μl volume: equal amounts of receptor were incubated with the purified double-stranded oligonucleotides with or without T_3 in binding the buffer (25 mM Hepes/pH 7.5, 5 mM $MgCl_2$, 4 mM EDTA, 10 mM DTT, 0.11 M NaCl, and 0.8 μg/μl sheared salmon DNA). Where appropriate, RXRβ was added. After incubation for 30 min at room temperature, the reaction mixture was loaded onto a 5% polyacrylamide gel and electrophoresed at 4°C. for 2.5 hr. The gel was dried and autoradiographed.

Preparation of Nuclear Extracts

HeLa and NIH3T3 cells were grown as above. Nuclear extracts were prepared according to the Schaffner method (29). Briefly, the scraped cells were pooled, spun at 1500 rpm, 4°C. for 5 min and then resuspended in cold Buffer A (10 mM HEPES/pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). The cells

were then lysed with Nonidet-40 at a final concentration of 0.5%. After inverting the tube several times, it was spun at 1500 rpm, 4°C. for 5 min, and the supernatant decanted leaving a whitish nuclear pellet, which was resuspended in 400 μl Buffer C (20 mM HEPES/pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). This was thoroughly vortexed for 5 min at 4°C., placed on a rotating shaker for 2 hr, then spun at full speed, precipitating the nuclear membranes and leaving nuclear extract in the supernatant. Protein concentration was determined by the Bradford method.

Immunocytochemistry

Cultured HeLa and NIH3T3 cells were transfected as described above. After 2 days, the cells were processed for immunological studies as previously described (30). Briefly, the cells were fixed with 3.7% formaldehyde in PBS for 5 min at 23°C. and incubated with 10 μg/ml monoclonal antibody C4 (30) in PBS containing 0.1% saponin and 4 mg/ml normal goat globulin for 30 min at room temperature. After being washed with PBS, the cells were incubated with affinity-purified goat antimouse immunoglobulin G conjugated to rhodamine (25 μg/ml) for 30 min. The cells were viewed, and representative fields were photographed using a microscope equipped with rhodamine epifluorescence optics.

Statistical Analysis

Transfection experiments were performed at least twice, with duplicate determinations. Statistical analyses were performed by Student's t test; $p < 0.05$ was considered significant. Note that in Figs. 3 and 4, separate values were obtained for the dominant negative potencies in both HeLa and NIH3T3 cells at the designated WT:mutant receptor ratio and the resultant values obtained for each cell were then compared by t test to obtain a p value. Thus, the asterisk refers to this second determination.

RESULTS

The characteristics of the h-TRβ1 mutants (5) are shown in Table 1. ED and OK have point mutations leading to reduced T_3-binding affinities and reduced transcriptional capacities; PV has a frameshift mutation at the carboxy-terminus which abolishes both T_3-binding activity and transcrip-

TABLE 1. Characteristics of wild-type and mutant h-TRβ1

Receptor	Codon	Amino Acid	Exon	Affinity Constant of T_3 Binding (Ka $\times$ 10^9 M^{-1})
WT-TRβ1	—	—	—	1.50
ED	317	Ala$\rightarrow$Thr	9	0.22
OK	442	Met$\rightarrow$Val	10	0.20
PV	448	Frameshift	10	0.01

tional capacity. These mutations display dominant negative potencies on idealized TREs. While T_3 was able to fully restore transcriptional activity of the ED mutant, there was only partial restoration for OK and, as expected, none with PV (31).

Transcriptional Capacity of WT h-TRβ1 on Natural TREs Was Cell Type Specific

In order to establish the rank order of T_3-induced transactivation by WT h-TRβ1 on natural TREs in HeLa and NIH3T3 cells, transient transfection studies were performed. The natural TREs, with the sequences shown in Table 2, were placed upstream of the TK-promoter to drive the expression of the CAT gene. In HeLa cells, maximal T_3-stimulation of 10-fold occurred on the Lys-TRE, followed by a 7-fold stimulation on ME-TRE, using 100 nM T_3 and taking into account the basal activities in the absence of T_3. However, there was very low T_3-dose responsiveness on the GH-TRE (Fig. 1A). In the mouse fibroblast NIH3T3 cell line, T_3-induced CAT activities occurred in the following rank order: Lys > ME = GH (Fig. 1B), where there was a 5-fold stimulation on the Lys-TRE and 4-fold on the ME- and GH-TREs. It is to be noted that for the GH-TRE, there was no transactivation by WT h-TRβ1 in HeLa cells, while in NIH3T3 cells a 4-fold stimulation was observed. Thus, the extent to which these TREs were transactivated differed between the two cell types, suggesting that there are cell-specific factors that affect the TRE-dependent transactivation by WT h-TRβ1.

Transcriptional Capacity of h-TRβ1 Mutants

Transient transfection studies were then performed using h-TRβ1 mutants in HeLa and NIH3T3 cells, in order to characterize the behavior of these mutants on natural TREs. In HeLa cells, ED and OK displayed a dose-dependent effect of T_3, achieving full transcriptional capacity as compared with WT h-TRβ1. As expected, mutant PV, which does not bind T_3, was unable to transactivate even at high T_3 concentrations. On the Lys-TRE, OK and ED differed most in their response with 10 nM T_3; at higher concentrations, both approached the WT h-TRβ1 response, although at 100 nM T_3 they were still clearly less transactivated than WT receptor. A representative graph is shown in Figure 2, where transactivation of WT and mutant h-TRβ1 on the Lys-TRE in HeLa cells was studied. These results are similar to those of Meier et al. (20) where the artificial TRE MTV-Pal-CAT was used, except that the mutant OK was not completely transactivated on the Pal-TRE at 1000 nM T_3.

TABLE 2. Sequences of natural TREs

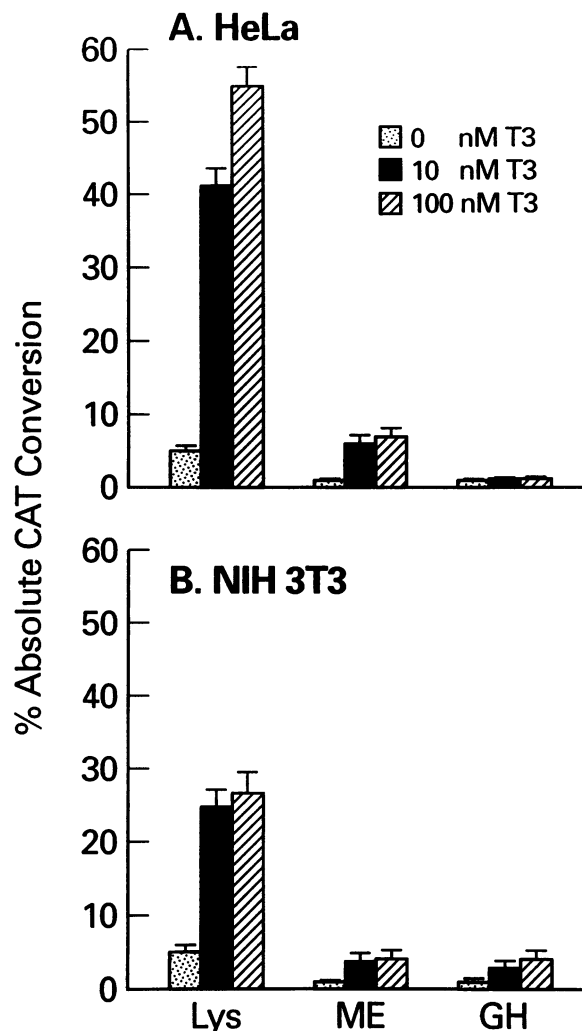| TRE	Sequences
Rat Malic Enzyme (ME-TRE)	−289 **AGGACGTTGGGGTT**AGGGG**AGGACA**GTG-260
Rat Growth Hormone (GH-TRE)	−190 **AAGGTAA**GATC**AGGGACGTGACC**TC -166
Chicken Lysozyme Silencer (Lys-TRE)	−2352 **ATTGACCCC**AGCTG**AGGTCA**AGTTACG -2326

FIG. 1. Transactivation activities of WT h-TRβ1 on the Lys-, ME- and GH-TREs in (A) HeLa cells and (B) NIH3T3 cells

Transfections were performed with 1 µg of pSV2 WT h-TRβ1 and 5 µg of TK-TRE-CAT reporter plasmid/plate, followed 24 hr later by treatment of the cells with the appropriate T₃ concentrations. The results are expressed as percentage of absolute CAT conversion at 0, 10, and 100 nM T₃. The y-axis was drawn to the same scale to emphasize the differences in transactivation between the two cell types. Results of two experiments, each with duplicate plates per receptor/T₃ concentration, were expressed as means ± SEM. Basal values at 0 nM T₃ in 3T3 cells were normalized to that obtained in HeLa cells, to allow comparison between two cell types.

Dominant Negative Effect of h-TRβ1 Mutants

The ability of a mutant h-TRβ1 to inhibit the function of the normal h-TRβ1 and α1 is termed the dominant negative effect, and this property
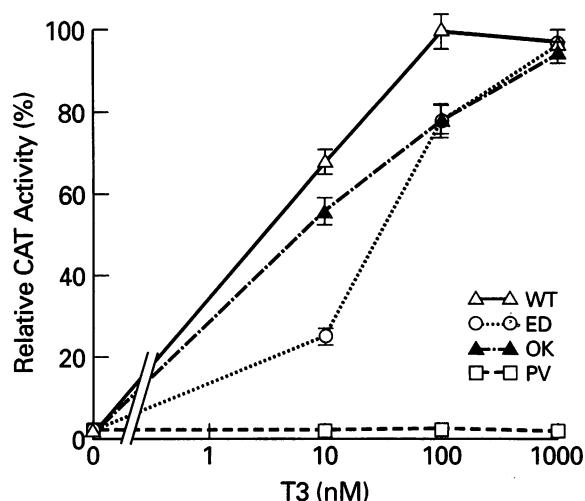

FIG. 2. Transactivation activities of WT and mutant h-TRβ1, ED, OK, and PV on Lys-TRE in HeLa cells

HeLa cells were transfected with the respective h-TRβ1 and 5 µg of TK-TRE-CAT plasmids and the cells treated with the appropriate T₃ concentrations 24 hr later. CAT activities were normalized to that obtained by WT h-TRβ1 at 1000 nM T₃. Results of two experiments, each with duplicate plates per receptor/T₃ concentration were expressed as relative CAT activity (%) ± SEM.

of the mutant receptor is thought to determine the clinical manifestations of RTH. An understanding of how h-TRβ1 mutants behave in various cell types may help explain why there is variable organ resistance in kindreds with RTH. Thus, we used two cell types, HeLa and NIH3T3 cells, and cotransfected WT and mutant h-TRβ1 at different ratios to study potential differences in dominant negative activities. Figure 3 compares the dominant negative effect of ED on the three TREs studied in HeLa and NIH3T3 cells at 10 nM T₃. On the Lys-TRE, a significant difference (p < 0.05) in dominant negative potencies between the two cell types occurred at a 1:2 ratio of wild-type:mutant receptor (WT:M). On the ME-TRE, there were differences in dominant negative potencies at 1:1, 1:2, and 1:5 WT:M ratios between cell types. The GH-TRE was not transactivated by T₃ in HeLa cells and thus this represents a clear difference between the cell types. In Figure 4, where mutant OK was used on the Lys-TRE, the dominant negative potency was only observed at a 1:5 WT:M ratio in HeLa cells. This contrasted
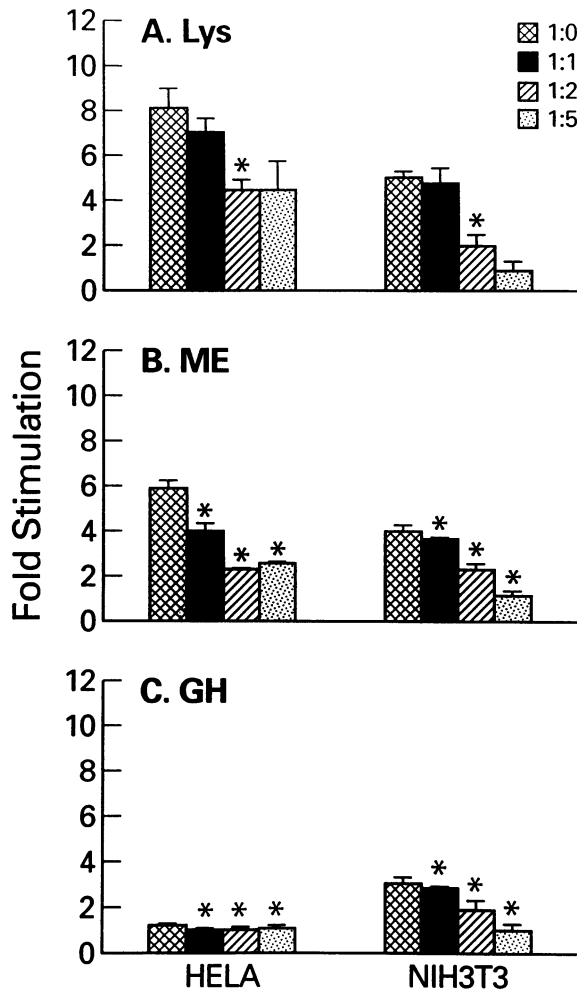
FIG. 3. The dominant negative effect of mutant ED

Three natural TREs (A) Lys, (B) ME, and (C) GH, were examined in HeLa and NIH3T3 cells at 10 nM T_3. Cells were cotransfected with various WT: M ratios (i.e., 1:0, 1:1, 1:2, and 1:5) and treated with 10 nM T_3 24 hr later. Experiments were performed at least twice, with duplicate plates within each experiment and results expressed as fold stimulation by 10 nM T_3 over 0 nM T_3 at each of the WT:M ratios ± SEM. The differences in fold stimulation between 1:0 and the other WT:M ratios were calculated for each cell type, and a significance value was obtained by Student's paired t test. *The difference in dominant negative potencies between the cell types at the designated WT:M ratio is significant ($p < 0.05$).

FIG. 4. The dominant negative effect of mutant OK

Three natural TREs, (A) Lys, (B) ME, and (C) GH, were examined in HeLa and NIH3T3 cells at 10 nM T_3. Cells were cotransfected with various WT:M ratios (i.e., 1:0, 1:1, 1:2, and 1:5) and treated with 10 nM T_3 24 hr later. Experiments were performed at least twice, with duplicate plates within each experiment and results expressed as fold stimulation by 10 nM T_3 over 0 nM T_3 at each of the WT:M ratios ± SEM. The differences in fold stimulation between 1:0 and the other WT:M ratios were calculated for each cell type, and a significance value was obtained by Student's paired t test. *The difference in dominant negative potencies between the cell types at the designated WT:M ratio is significant ($p < 0.05$).

sharply with the effect seen in NIH3T3 cells, where the dominant negative effect was also observed at 1:1 and 1:2 WT:M ratios. On the ME-TRE, differences were seen between cell types at all wild-type:mutant receptor ratios. The situation on the GH-TRE was similar to that observed with mutant ED in that the GH-TRE was not
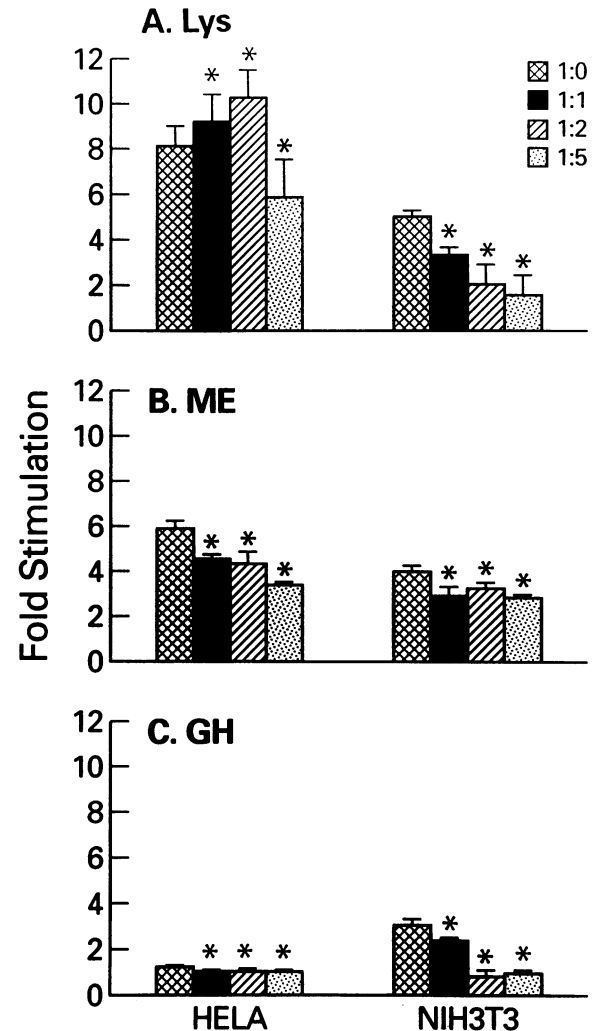
transactivated by T_3 in HeLa cells but in 3T3 cells a dominant negative effect occurred, again representing a significant difference between the cell types. Figure 5 shows the dominant negative effect of mutant PV in the two cell types. On the
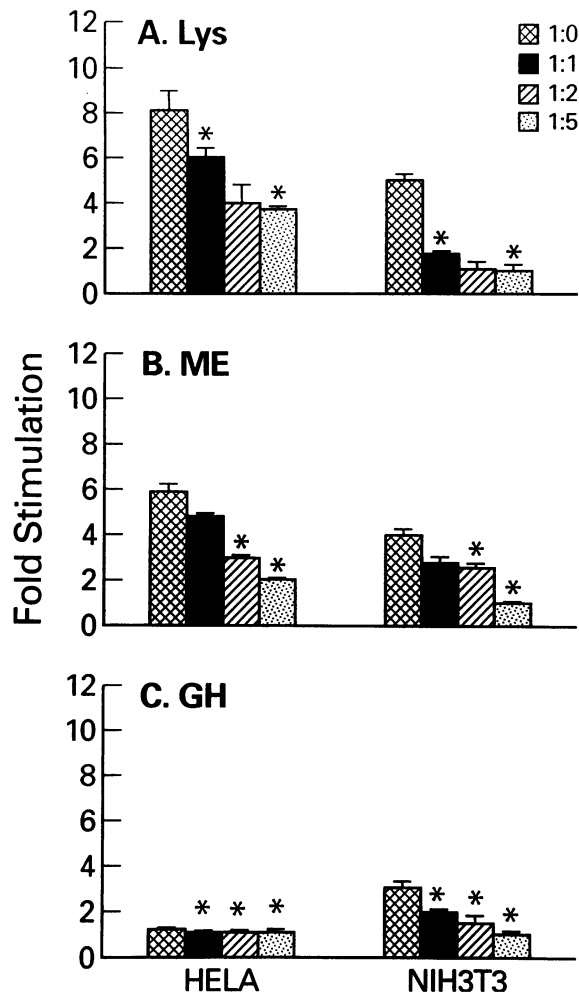
FIG. 5.   The dominant negative effect of mutant PV

Three natural TREs, (A) Lys, (B) ME, and (C) GH, were examined in HeLa and NIH3T3 cells at 10 nM T_3. Cells were cotransfected with various WT:M ratio (i.e., 1:0, 1:1, 1:2, and 1:5) and treated with 10 nM T_3 24 hr later. Experiments were performed at least twice, with duplicate plates within each experiment and results expressed as fold stimulation by 10 nM T_3 over 0 nM T_3 at each of the WT:M ratios ± SEM. The differences in fold stimulation between 1:0 and the other WT:M ratios were calculated for each cell type, and a significance value was obtained by Student's paired t test. *The difference in dominant negative potencies between the cell types at the designated WT:M ratio is significant ($p < 0.05$).

Lys-TRE, a significant difference occurred particularly at a 1:1 WT:M ratio. Significant differences in dominant negative potencies also occurred on the ME-TRE at 1:2 and 1:5 WT:M ratios. The situation on the GH-TRE was similar to that observed with mutants ED and OK.

Relative Abundance of Hetero- and Homodimers

In order to explain differences in the dominant negative activities seen in the two cell types above, and since it is known that TRAPs are present in different amounts in different cells (32) and are thought to enhance the action of TRs (25,27), we performed gel-shift analyses using nuclear extracts of HeLa and 3T3 cells. Figure 6A shows interesting differences between HeLa and NIH3T3 cells using Lys-TRE. The HeLa nuclear extract and WT h-TRβ1 formed two bands (Lane 5), an upper one which migrated with the same electrophoretic mobility as the h-TRβ1/RXRβ heterodimer band (Lane 2) and a lower, faster-migrating one (open arrowhead), which most likely represents a second heterodimer band. Addition of specific competitor resulted in loss of these heterodimer bands, indicating the specific binding of TR/RXR heterodimers to Lys (Lane 6). In contrast, under identical experimental conditions, NIH3T3 nuclear extract interacted with h-TRβ1 only weakly as the upper, slower-migrating heterodimer but more interestingly, also formed a band with the same electrophoretic mobility as the homodimer (Lane 9). Specific competitor abolished these two bands (Lane 10). These findings are in agreement with those of Sugawara et al. (32), who characterized the nature of TRAPs in several cell lines and rodent tissues, and found that in HeLa and NIH3T3 cells two heterodimer bands formed: a slower migrating one composed mostly of RXRβ and a faster migrating one composed mostly of RXRα. Furthermore, these bands were twice as intense in HeLa cells as in NIH3T3 cells, as was the case in our experiments. The absence of the lower band in our NIH3T3 cells could be due to a lower abundance of RXRα in our cell line. Since it has been postulated that transcription of TRs occurs via the stable heterodimer (25,27), these gel-shift data potentially explain the different transcriptional capacities of h-TRβ1 on the Lys-TRE in the two cell types (Fig. 1). While there was a 10-fold stimulation of the Lys-TRE in HeLa cells, there was only a 5-fold stimulation in 3T3 cells, in keeping with the finding of strong heterodimer bands seen with the HeLa nuclear extract and much weaker ones with the 3T3 nuclear extract. The background signal observed with the HeLa nuclear extract is due to the nonspecific interaction of probe with other proteins.

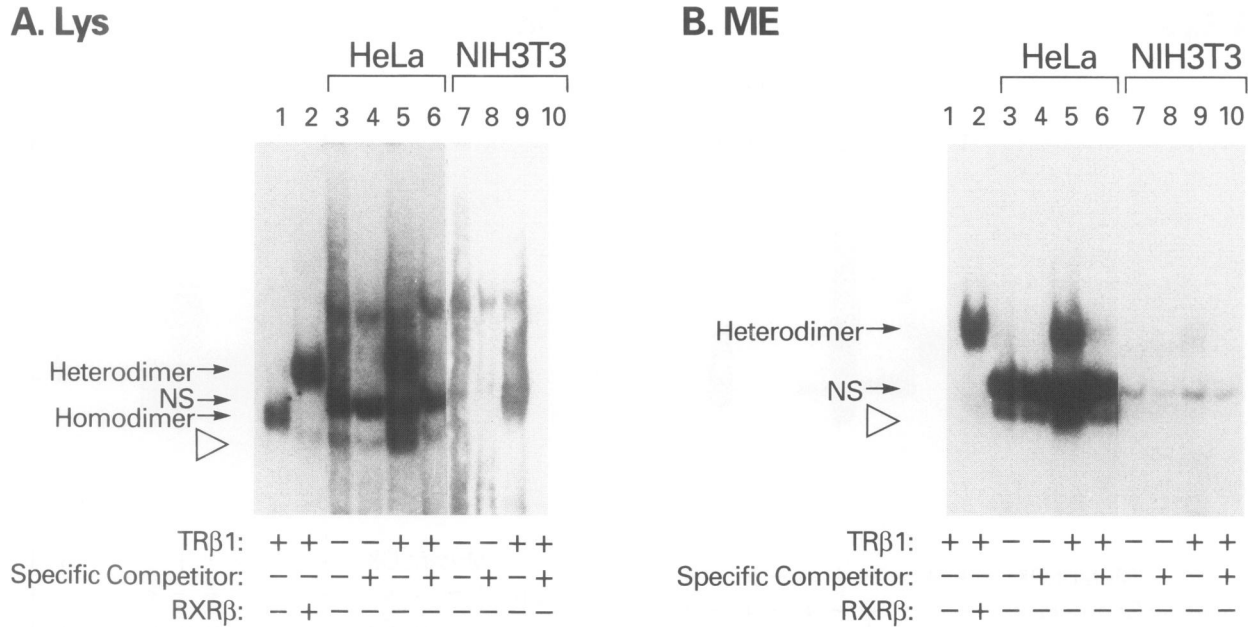In contrast, WT h-TRβ1 did not bind to the ME-TRE probe (Fig. 6B, Lane 1). Strong het-

A. Lys

HeLa   NIH3T3

1 2 3 4 5 6 7 8 9 10

Heterodimer→
NS→
Homodimer→
▷

TRβ1:   + + − − + + − − + +
Specific Competitor: − − − + − + − + − +
RXRβ: − + − − − − − − − −

B. ME

HeLa   NIH3T3

1 2 3 4 5 6 7 8 9 10

Heterodimer→
NS→
▷

TRβ1:   + + − − + + − − + +
Specific Competitor: − − − + − + − + − +
RXRβ: − + − − − − − − − −

FIG. 6. Gel mobility shift analyses using nuclear extracts from HeLa cells and NIH3T3 cells with WT h-TRβ1 on the (A) Lys- and (B) ME- TREs

An equal amount of in vitro translated receptor was incubated with labeled Lys-TRE and 5.0 μg of HeLa or 3T3 nuclear extract and electrophoresed. Exposure of the dried gels was kept constant between experiments at 14 hr. The 3T3 section in Panel A was exposed for 2.5 days in order to bring out the heterodimer and homodimer bands. Note that a nonspecific band (NS) runs just above the homodimer band in the panel with HeLa nuclear extract and is not competed away by specific competitor. (▷) The lower band in Lane 5 likely represents a faster migrating heterodimer band competed away by specific competitor (Lane 6), and overlaps a nonspecific band. Note that in Panels A and B, all lanes represent each of two separate gels, respectively.

erodimer bands, as with Lys-TRE, formed with the HeLa nuclear extract (Lane 5). A similar band of increased electrophoretic mobility formed, which was partially competed away by specific competitor. The heterodimer bands formed with the 3T3 nuclear extract (Lane 9) were much weaker than with HeLa nuclear extract, indicating that the quantity of RXRβ/α or RXR-like TRAPs may be lower in 3T3 cells.

To understand the interaction of TRAPs with mutant receptors, we have carried out similar studies with the mutant OK. Figure 7A and B shows binding of the OK mutant to the Lys- and ME-TREs, respectively. The pattern of heterodimerization on the Lys-TRE in HeLa cells is identical to that for WT h-TRβ1 (Fig. 6A). There was specific competition of both heterodimer bands by the unlabeled probe (Lane 6). In the case of the 3T3 nuclear extract, however, prominent homodimer bands were seen, together with a much weaker upper heterodimer band (Lane 9). Both bands represent specific interactions of the mutant h-TRβ1 with factors in the nuclear extract, which disappear upon addition of unlabeled probe in lane 10.

Using the ME-TRE in HeLa cells, prominent heterodimers formed when either the WT h-TRβ1 or mutant receptors were used (Fig. 6B, Lane 5 versus Fig. 7B, Lane 5). No homodimers were seen using either HeLa or 3T3 nuclear extract on the ME-TRE. In 3T3 cells, upper heterodimers of weaker intensities formed when either WT or mutant h-TRβ1 were used (Fig. 6B, Lane 9 versus Fig. 7B, Lane 9). Thus, depending on the cell type and the TRE, both mutant hetero- and homodimers may contribute to the dominant negative activities of mutant receptors. Our findings also suggested that on the identical TRE, the same mutation could mediate varying strengths of dominant negative inhibition depending on whether homodimers or heterodimers form. It could further be postulated that, since transactivation is thought to occur mostly via the stable heterodimer, the greater the ability to form heterodimers, the less potent the dominant negative effect of a mutant receptor will be. Since h-TRβ1 was only able to form heterodimers on the ME-TRE, the dominant negative potencies of mutant receptors on this element could be predicted to be less than on the
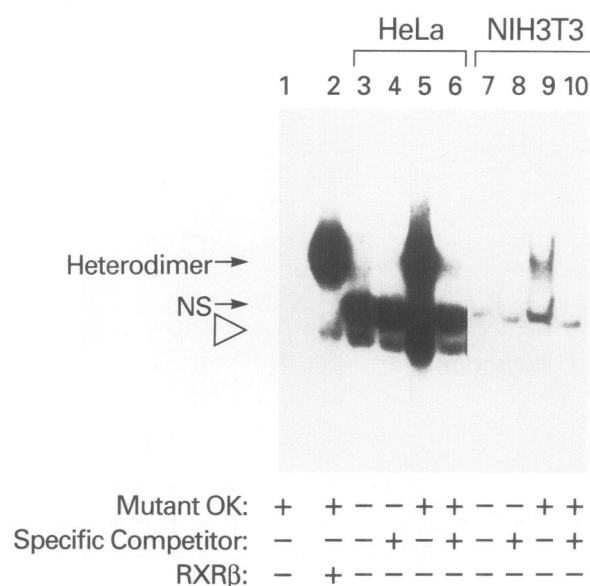
A. Lys
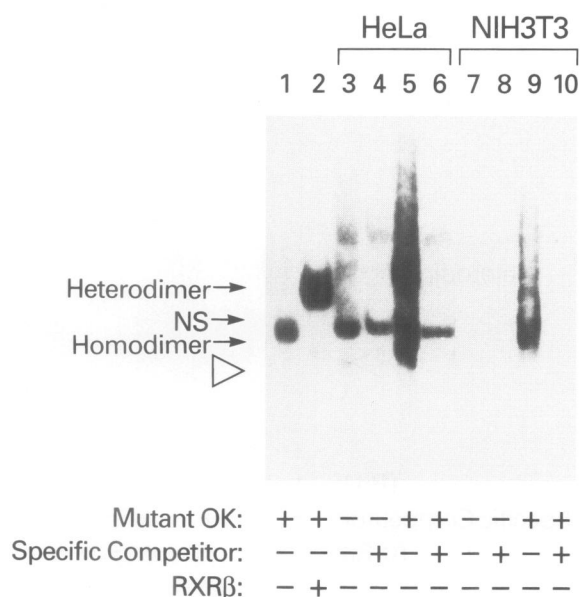
B. ME

FIG. 7. Gel mobility analyses using nuclear extracts from HeLa cells and NIH3T3 cells with mutant receptor OK on the (A) Lys- and (B) ME-TREs

Experimental conditions were identical to those for Fig. 6. In this case, however, both gels were exposed for 14 hr. There is a nonspecific band (NS) that runs just a little above the homodimer band in the panel with HeLa nuclear extract and again, as in Fig. 6A, is not competed away by specific competitor. (▷) The lower band in Lane 5 likely represents a faster migrating heterodimer band competed away by specific competitor (Lane 6) and overlaps a nonspecific band. As in Fig. 6, the Panels A and B each represent a gel, respectively.

Lys-TRE, where both heterodimers and homodimers formed. This approach of using nuclear extract to identify potential hetero- and/or homodimerization complexes could be useful in further studies to assess the relative roles of these complexes in the dominant negative activities of mutant receptors.

Expression of TRβ1 Protein

In order to exclude the possibility that the expression of the transfected pSV2-h-TRβ1 could be widely discrepant between the two cell types, immunocytochemistry, utilizing a monoclonal antibody directed at the amino terminus of WT h-TRβ1, was performed. Figure 8 shows representative immunofluorescence and phase-contrast fields from HeLa and NIH3T3 cells and confirms that there is approximately equivalent receptor expression in both cell types. Furthermore, as demonstrated by Meier et al. (19), the levels of nuclear h-TRβ1 protein increased in parallel with the amount of WT or mutant pSV2-h-TRβ1 transfected in HeLa cells, when using the

same expression plasmid. It is thus reasonable to assume that this will also be the case for NIH3T3 cells.

DISCUSSION

The molecular mechanisms underlying the variable organ resistance within an individual and between kindreds with RTH, sharing identical mutations, have remained unresolved. We characterized positive TREs to investigate the role of cell type in modulating the dominant negative effect of mutant h-TRβ1. Three different naturally occurring mutations from kindreds with RTH, which had previously been shown to have three distinct types of functional impairment on an idealized TRE (19,20,31), were used. Mutant ED has a 5-fold reduction in T_3-binding affinity, a correspondingly shifted T_3-dose response curve in transient transfection assays and, as expected, high levels of T_3 restored its transcriptional capacity to wild-type levels and obliterated its dominant negative effect. Despite having a sim-
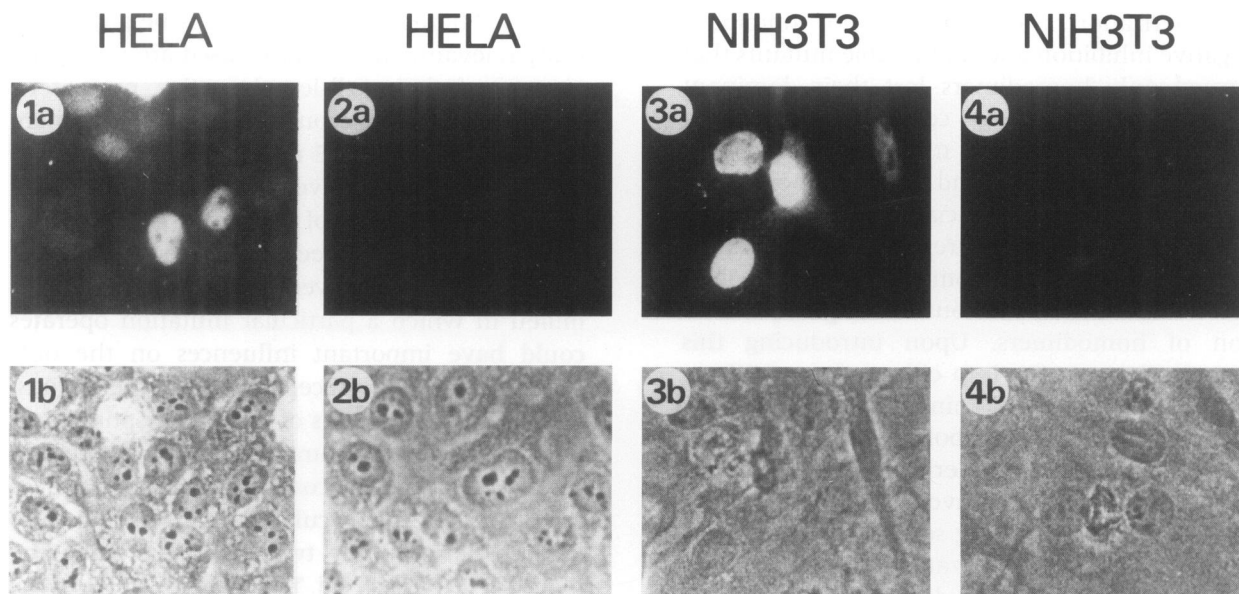
FIG. 8.   Expression of WT h-TRβ1 in HeLa (1 a and b, 2 a and b) and NIH3T3 (3 a and b, 4 a and b) cells
Immunocytochemistry with a monoclonal antibody recognizing the amino terminus of the WT h-TRβ1 was performed after transfecting cells. Plates were transfected with either 2.0 μg of pSV2-WT h-TRβ1 (1 a and b, 3 a and b) or 2.0 μg of empty pSV2 vector (2 a and b, 4 a and b). 1a–4a, Fluorescence micrographs; 1b–4b, the corresponding phase contrast micrographs (magnification: 763×).

ilar 5-fold reduction in T₃-binding affinity, mutant OK was only partially transactivated at high T₃ levels, and its dominant negative potency was likewise not completely reversed. It was speculated that this point mutation not only affected T₃-binding but also the transactivation domain (20). PV has a frame shifted carboxy terminus, which abolished T₃-binding, predictably failed to transactivate with T₃, and exhibited the most potent dominant negative effect on the Lap-TRE.

In this study, we systematically compared two different cell types and found that, in addition to the TRE motif and the nature of the mutation in h-TRβ1 (20,33), the dominant negative potencies of mutant receptors were modulated by the cellular environment. This may be the result of whether heterodimers or homodimers can form in the particular cell type in question.

Sugawara et al. (32) have recently demonstrated a heterogeneity of TRAPs in different rat tissues and cell lines that were differentially distributed and which were able to heterodimerize with TRs. Petty (34) also demonstrated, by Far-Western Blotting, multiple bands of different molecular sizes, many of which differed from that of the RXRs, that formed between TRβ1 and nuclear extracts of rat brain, kidney, liver, GH₃

cells, and HeLa cells. Suen and Chin (35) also showed that distinct, cell-specific TR-DNA complexes formed when GH₃ and HeLa nuclear extracts were used in cell-free in vitro transcription assays. In this paper, we showed that the interactions of WT h-TRβ1 and mutant OK on the Lys- and ME-TREs differed in HeLa and 3T3 cells, as seen by gel-mobility shift assays. Heterodimers between WT h-TRβ1 and RXRβ/α could mediate a 10-fold transcription from the Lys-TRE observed in HeLa cells, whereas, in 3T3 cells, there was a weaker 5-fold transcriptional response, together with weaker heterodimer bands and the presence of homodimers. Thus, the strength of the transcriptional capacity of wild-type and mutant receptors may be dependent on the degree of heterodimerization, which, in turn, is dependent on the amount of TRAPs present in different tissues.

Almost all of the mutations that cause RTH cluster in two "hot spot" regions on either side of the dimerization domain (5). This domain consists of nine heptad repeats, which are thought to be important for both dominant negative activity and transcription (36,37); furthermore, a dimerization domain encompassing codons 281–300 has been defined by in vitro mutagenesis (38–41). Nagaya et al. (6) showed that receptor het-

erodimerization was important for dominant negative inhibition and that double mutants that formed only homodimers lost their dominant negative capacity in JEG3 cells. They studied two naturally occurring RTH mutant receptors that formed homodimers and heterodimers with RXRα, as well as an artificial mutation in one of the hydrophobic heptad repeats of the putative receptor dimerization domain, which impaired heterodimerization without altering the formation of homodimers. Upon introducing this dimerization mutant into either of the RTH receptor mutants, the dominant negative activity of the RTH mutant was abolished, suggesting the importance of heterodimerization for the dominant negative effect. Conversely, Hao et al. (22) were able to show, using several artificial TREs, preferential homodimerization with the S-mutant receptor, a powerful dominant negative receptor (42,19), and predominant heterodimerization with the GH receptor, which has compromised dominant negative function and was found in both normal individuals and in a patient with severe pituitary resistance to thyroid hormone (42,43). The latter finding with the GH receptor also supports one of our contentions that the greater the degree of heterodimerization versus homodimerization, the lesser the dominant negative potency of the receptor. The findings of Hao et al. (22) are also in contrast to those of Au-Fleigner et al. (23), where mutational analysis of the ninth heptad repeat of the c-erbAα1 receptor demonstrated that this region was required for heterodimerization but not homodimerization and, furthermore, that the chick c-erbAα1 mutant with intact homodimerization lacked dominant negative activity. Our present study, however, suggests the possibility that both hetero- and homodimers in different amounts are capable of mediating the dominant negative effect depending on the contributions of cell type, mutation, and the TRE motif. Using h-TRβ1 mutants in NIH3T3 cells on the Lys-TRE, homodimers were the predominant species; however, in HeLa cells, only heterodimers formed on the Lys- and ME-TREs, and this species may thus be responsible for dominant negative action in this cell.

All the studies to date have mostly focused on the TR-TRE relationships within one particular cell type. We present the first systematic study of mutant h-TRβ1 interactions on natural TREs using two different cell types in order to assess the contribution of cell type to dominant negative potencies of mutant receptors. Although the cells used here may not necessarily be physiologically relevant, and the TREs used are not in the context of their full-length native promoters, certain preliminary conclusions can be drawn. Not only does the TRE motif and the isoform of the endogenously active receptor determine the degree of inhibition of a specific gene in RTH individuals as has been suggested by others (19,20), but we showed here that the cellular milieu in which a particular mutation operates could have important influences on the outcomes of these receptor-DNA interactions. Firstly, the magnitudes of the transcriptional activities on various natural TREs were different in the two cell types. Secondly, the dominant negative effect of a particular mutant receptor also differed between the two cell types when the identical TRE was used. Thirdly, the nature of the protein-protein complexes formed as assessed by gel-shift assays differed depending on the TRE and cell used. Fourthly, we have established that h-TRβ1 expression was equal in the two cells studied here, confirming that the differences in functional activities of the receptors observed herein were truly cell type specific. Thus, the cell type in which a mutant receptor is found could determine the relative amounts of hetero- and homodimers, and this, together with the nature of the mutation and the TRE motif, could influence the strength of the dominant negative action of mutant receptors and potentially contribute to the degree of organ resistance. Studies are underway to extend these observations to characterize the behavior of mutant h-TRβ1 on various natural TREs in the respective cell types in which they normally function. It is clear that more physiological approaches are required to further elucidate the mechanisms by which the dominant negative effect of mutant h-TRβ1 correlates with the clinical phenotypes, and, to this end, we have developed a transgenic mouse model of RTH using the PV mutant receptor (44).

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