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Kindred S thyroid hormone receptor is an active and constitutive silencer and a repressor for thyroid hormone and retinoic acid responses

(generalized thyroid hormone resistance/thyroid hormone receptor/v-erbA/silencing)

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ABSTRACT **Mutations in the gene encoding the human thyroid hormone receptor β (hTRβ) have been associated with generalized thyroid hormone resistance (GTHR). However, the molecular basis by which the receptor mutants cause the clinical symptoms is largely unknown. We show here that the β form of the human receptor possesses, in addition to hormone-dependent activation, the ability to repress basal-level activity of a target promoter. This silencing function is localized in the carboxyl-terminal part of the receptor and can be transferred to a heterologous DNA binding domain. This mode of silencing is therefore distinct from inhibition by competition with activator proteins on DNA. We show that two receptor mutants isolated from patients with GTHR are impaired in transcriptional activation but fully retain the silencing function, which enforces dominant negative regulation by the receptor. Interestingly, the kindred S receptor (hTRΔ332) acts as a constitutive repressor with a strong silencing ability similar to that of the v-*erbA* oncogene product. We also provide evidence for distinct transcriptional regulatory properties of both proteins. Finally, we show that both thyroid hormone- and retinoic acid-responsive genes are potentially repressed to generate the clinical manifestations of the GTHR syndrome. Our findings suggest that silencing plays an important role in the phenotypic expression of the symptoms in patients with GTHR.**

Mutations in the thyroid hormone receptor (TR) gene can lead to diverse phenotypic abnormalities. In man, generalized thyroid hormone resistance (GTHR) is a genetic disorder characterized by the resistance of peripheral and pituitary tissues to the action of thyroid hormone (1). The syndrome is manifested by elevated circulating levels of free thyroid hormones (triiodothyronine, T_3; and thyroxine, T_4) and inappropriately normal or elevated levels of thyroid-stimulating hormone (thyrotropin) (2, 3). Affected patients have in addition a variable degree of delayed bone maturation, heart abnormalities, hearing defects, and mental retardation (1–3). The syndrome segregates in an autosomal dominant manner. Genetic analysis of resistant kindreds has clearly linked this syndrome to the c-erbAβ or human TR β (hTRβ) gene (4), designated *THRB*.

The v-*erbA* oncogene represents another mutated form of TR. The oncoprotein functions by arresting erythroid differentiation (5). It is believed that erythroid transformation by v-*erbA* is caused by repression of genes that are required for development and differentiation (6, 7). Further experiments indicated that the carboxyl-terminal part of the v-*erbA* oncoprotein is critical for erythroblast transformation (10). Deletion of about 50 amino acids at the very C-terminal part abolished both neoplastic transformation (10) and silencing (11), implying that silencing plays a major role in the biological activity of v-*erbA*.

To fully understand the molecular basis of the clinical symptoms of patients with GTHR, it appeared necessary to analyze the human T_3 receptor for its transcriptional properties. The TR is a nuclear receptor, which binds DNA sequence specifically in the presence or absence of hormone. The receptor activates gene transcription in the presence of hormone, while in its absence, the receptor represses transcription of target genes. Due to the fact that TR can form stable heterodimers with other members of the TR/steroid hormone receptor superfamily, it is generally believed that repression of transcription occurs only by forming inactive heterodimers and/or by competing for DNA binding sites.

Isolation and sequencing of hTRβ genes from unrelated patients or kindreds with the GTHR syndrome revealed that the majority of the mutations analyzed so far are located in the ligand-binding domain (LBD) of the receptor. These mutations result in either failure to bind thyroid hormone (T_3) or reduced binding affinity of the hormone (18). There is a high variability in the degree of the clinical phenotype among unrelated kindreds, which is thought to be explained in part by different T_3 binding affinities of various receptors (19).

One such isolated receptor from a kindred, designated A, bears a single-base-pair substitution leading to the amino acid change Pro-448 → His (TR448H; ref. 20). This mutation leads to a 90% decrease in T_3 binding affinity (21). In contrast, the kindred S receptor fails to bind any detectable hormone. This receptor (TRΔ332) has a three-base-pair deletion that results in the loss of Thr-332 (22). Both mutant receptors show no change in DNA binding affinity (21, 22). However, the mechanism by which the receptor mutants cause thyroid hormone resistance and the clinical manifestations are largely unknown.

Here, we show that hTRβ and isolated receptor mutants from patients with GTHR possess silencing function. We have addressed the questions of whether this function plays a role in the dominant negative regulation by mutant receptors, which genes are potentially affected in patients with GTHR, whether this function contributes to the observed high variability in the phenotypes of affected kindreds, and most importantly, whether silencing plays a role in the manifestation of clinical symptoms.

MATERIALS AND METHODS

Plasmid Constructions. The full-length cDNAs coding for the mutant hTRβs hTRΔ332 (kindred S) or hTR448H (kindred

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Abbreviations: GTHR, generalized thyroid hormone resistance; RA, retinoic acid; RAR, RA receptor; TR, thyroid hormone receptor; hTRβ, human TR β; TRE, thyroid hormone response element; LBD, ligand-binding domain; DBD, DNA-binding domain; GAL DBD, yeast activator GAL4 DBD; *tk*, thymidine kinase gene; wt, wild type.

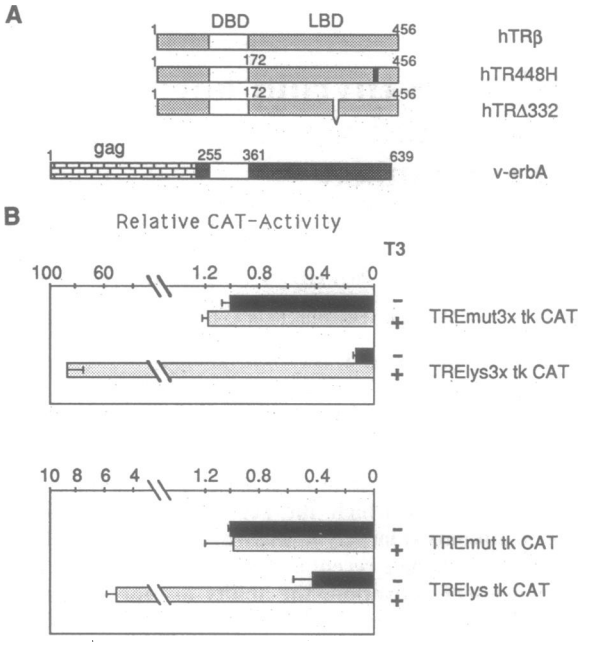
A) were isolated with *Eco*RI from pGEM3-kindred S (22) or A (20), respectively, and inserted into the filled-in Asp-718/*Bam*HI sites of pRSv-erbA (9), replacing the v-*erbA* coding sequences. Receptor fusions with the yeast activator GAL4 were constructed by insertion of the *Sfa*NI/*Hin*fI blunt-ended fragment from the coding sequences of hTRβ or the corresponding receptor mutants into the polylinker (*Sal* I/*Bam*HI filled) of the expression vector pABgal (11). The reporter 17-mer Ade-TATA CAT (chloramphenicol acetyltransferase gene) was constructed by exchanging thymidine kinase gene (*tk*)-promoter sequences with adenovirus major late gene promoter sequences from −53 to +41 and additional insertion of the 17-mer sequence *Pst* I/*Xba* I from the plasmid 17-mer tkCAT (11).

Cell Culture and Transfection. CV1 (ATCC CCL 70) and L (L/M(TK$^-$); ATCC CCL1.3) cells were plated out in Nutridoma (Boehringer Mannheim) 24 hr before transfection at a density of 10^6 or 8×10^5 cells, respectively, per 10-cm dish. Transfection was performed as described by Denner *et al.* (24). The conditions used for hormonal induction are described elsewhere (25). CAT assay was done as described (26).

RESULTS

The Kindred S Receptor (hTRΔ332) Is a Constitutive Repressor of Transcription. To analyze the transcriptional properties of the hTRβ and receptor mutants isolated from patients with GTHR, we performed cotransfection experiments in CV1 cells, which lack significant amounts of endogenous TRs (9). As reporter plasmids, we used one or three copies of either the natural lysozyme thyroid hormone response element (TRE), TRElys (F2; ref. 25), or TREpal (27) in front of the *tk* promoter–CAT gene fusion. As a control, we used a mutated TRE inserted at the same position in the reporter plasmid, TREmut-tkCAT (25). Upon cotransfection with the expression plasmid coding for hTRβ (pRShTRβ; ref. 28), the mutated TRE showed no effect on basal-level promoter activity in the presence or absence of thyroid hormone (Fig. 1*B*), and no differences in basal-level activity were seen when using one or three copies of the mutated TRE (data not shown). As expected, TRElys allowed induction of promoter activity by thyroid hormone (T_3). However, without hormone the promoter activity was repressed below the basal level. Basal-level repression was observed to a greater extent when three copies of the TRE were used (Fig. 1*B*) and was also seen when using the inverted TRE, TREpal (data not shown). Thus, repression of the basal level was dependent on a functional TRE.

The hTRβ mutants hTR448H (kindred A receptor) and hTRΔ332 (kindred S receptor) are both mutated in the hormone binding domain (Fig. 1*A*). Since it is not known whether these mutations affect only hormone binding or have in addition some influence on the repression function, we performed cotransfection experiments in CV1 cells. The coding sequences of the receptor mutants were identical in sequence and in length except for the indicated mutations. To compare wt with mutated receptors, we inserted the corresponding full-length cDNAs into the same pRS expression vector (29); consequently, we refer to these pRS expression vectors by the insertions. As a control, we used the pRS vector Δgal (see below and ref. 11). hTR448H (kindred A receptor) exhibited hormone inducibility that was 1/15th that of the wt receptor (Fig. 1*C*), which reflects its 10-fold weaker affinity for T_3 (21). However, the capability to repress the basal level was not affected by this mutation and was similar in extent to that of the wt hTRβ. Similarly, the kindred S hTRΔ332 receptor, which lacked any hormone inducibility, fully retained the repression function (Fig. 1*C*). It is noteworthy that the basal-level repression by the wt and mutant receptors was similar to that of the v-erbA oncoproteins.



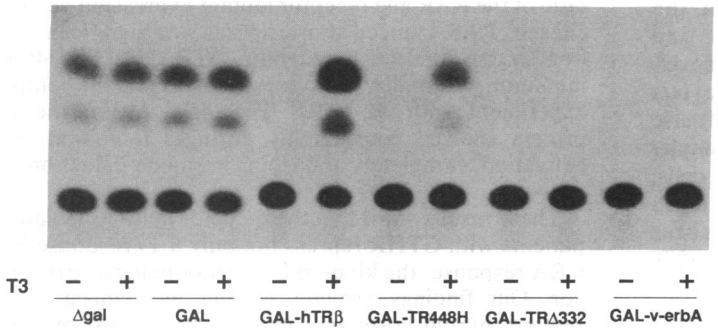
C

Expression Plasmid	T3 Hormone −	T3 Hormone +	Activation of Promoter Level	Hormone Induction	Repression of Basal Level
Control Δgal Vector	100 ±3.6	153.8 ±6.4	1.5	1.5	1.00
hTRβ	14.4 ±0.4	13900 ±364	139	965	7.1
hTR448H	12.6 ±2.9	810 ±65.4	8.1	64	7.9
hTRΔ332	15.3 ±1.4	17.9 ±3.8	0.17	1.1	6.5
v-erbA	12.4 ±0.1	13.7 ±0.1	0.12	1.1	8.0

FIG. 1. hTRβ represses basal-level promoter activity. (*A*) Schematic representation of the encoded receptor proteins. Numbers show the positions of amino acids in hTRβ and receptor mutants. DBD, DNA-binding domain; LBD, ligand-binding domain. (*B*) Cotransfection experiments with the indicated reporter constructs were done in CV1 cells. Bars represent CAT activity relative to the obtained values from the control reporter plasmid TREmut or TREmut3x-tkCAT, respectively. Black bars correspond to values without hormone, and stippled bars correspond to values with hormone (3,5,3′-triiodothyronine; T_3). Note the different scales of the two panels. (*C*) Cotransfection experiments using TRElys3x-tkCAT with wild type (wt) and various mutant receptors were done in CV1 cells. Values were normalized in percent to the activity seen with the control pRS expression vector Δgal (11). Induction by hormone was calculated by dividing the values obtained with and without hormone. Total hormone induction is composed of basal-level repression and activation above this level. Averages and standard deviations were determined from independent duplicate experiments.

Thus, the mutations in the analyzed kindred receptors did impair hormone-dependent activation but had no effect on basal-level repression. Furthermore, we show that, similar to the v-erbA oncogene product, the kindred S receptor, hTRΔ332, is a hormone-independent or constitutive repressor of transcription.

Mutated TRs Are Active Repressors (Silencers) of Gene Transcription. To prove that the mutant receptor, hTRΔ332, is an active repressor and to exclude the possibility that competition with any unknown activator proteins on authentic TR binding sites, we replaced the DBD of wt and mutant



Expression Plasmid	T3 Hormone		Hormone Induction	Repression of Basal Level
	−	+		
Δgal	100 ± 2.2	132 ± 22	1.3	1.00
GAL	151 ± 1.2	197 ± 43	1.3	0.6
GAL-hTRβ	1.3 ± 0..03	583 ± 15	448	76
GAL-TR448H	1.8 ± 0.29	89 ± 7.2	49	55
GAL-TRΔ332	1.1 ± 0.05	1.1 ± 0.13	1.0	90
GAL v-erbA	1.1 ± 0.07	1.0 ± 0.18	0.9	90

FIG. 2. Kindred S receptor is a constitutive and active repressor (silencer) of gene transcription. (*Left*) CAT assay after cotransfection of the indicated expression plasmids and the reporter 17-mer tkCAT in L cells with (+) or without (−) addition of thyroid hormone. (*Right*) Percentages from cotransfection experiments in L cells relative to the values obtained with Δgal.

receptors with the yeast activator GAL4 DBD (GAL DBD; amino acids 1–147). The GAL DBD contains several functions: it binds specifically to the upstream activation sequence or 17-mer (30), and it contains a dimerization domain and a nuclear localization signal (31). Therefore, by using the GAL DBD, any concern about receptor mutations affecting dimerization or nuclear translocation will be eliminated. The coding sequences for the carboxyl-terminal parts of the wt hTRβ and hTRΔ332 and hTR448H mutants, which include the LBD, were fused to the GAL DBD and inserted into the eukaryotic expression vector pRS (29). As a reporter we used the 17-mer tkCAT construct (25). The various GAL DBD–receptor expression plasmids were cotransfected into mouse fibroblast L cells in which the GAL DBD itself is not active (11).

The C-terminal part of hTRβ is sufficient to confer not only hormonal inducibility but also transcriptional repression of the basal level without hormone (Fig. 2). GAL-hTR448H mediated both active repression of the basal level and hormone induction, the latter being weaker by a factor of 9 than that obtained with the wt receptor (Fig. 2 *Right*). However, similar to the GAL–v-erbA fusion protein, the mutated receptor fusion, GAL–hTRΔ332, showed constitutive repression independent of hormone (Fig. 2). To quantitatively compare the silencing activity of v-erbA and hTRΔ332, a series of transfection experiments in L cells with various amounts of expression plasmids were carried out. The results confirmed that the kindred S receptor had similar strong silencing activity compared with the v-*erbA* oncogene product (data not shown). As a control, the GAL DBD itself had negligible effects on promoter activity compared with the pRS expression vector Δgal (Fig. 2) from which the coding sequences for the GAL DBD had been removed (11).

These data show that the transcriptional activities of the various GAL–receptor fusions correspond to those obtained with the full-length receptors. Furthermore, we conclude that the repression of basal-level promoter activity is not due to competition with other factors but rather is caused by direct inhibition of gene transcription. In addition, we show that the receptor mutants isolated from patients with GTHR still retain this silencing function and suggest that it might play a role in the manifestation of clinical symptoms.

Both Silencing and Hormone-Dependent Activation by hTRβ Are Effective on Different Minimal Promoters. To confirm that a variety of promoters are potential targets for silencing, we tested two different minimal promoters, the adenovirus major late promoter TATA box and the *tk* TATA box, which were fused to the CAT gene (25). A single 17-mer was inserted upstream of the TATA box of each of the promoters. Cotransfection experiments were performed with L cells. The basal-level activity of both minimal promoters did not change when the expression vectors Δgal or GAL DBD were used (Fig. 3), and the activity was also independent of T_3 hormone (data not shown). However, cotransfection with the expression plasmid coding for GAL-TRβ led clearly to the repression of basal-level activity. Upon addition of hormone this fusion protein became a strong activator, increasing transcription significantly from both minimal promoters (Fig. 3). We conclude that different promoters containing a TATA box can be silenced and activated by hTRβ dependent on the absence or presence of the hormone.

Kindred S Receptor Is an Efficient Competitor of Both T_3 and Retinoic Acid (RA) Responses. Although, both hTRΔ332 and the oncogene protein v-erbA are constitutive repressors with a similar strong silencing ability, no erythroid or myeloid leukemia was reported from patients with GTHR. Therefore, it was necessary to compare both mutant receptors for their ability to inhibit activation by the wt hTRβ. Cotransfection experiments were performed in CV1 cells in the presence of T_3 by using TRElys3x-tkCAT as reporter and constant amounts of the hTRβ expression plasmid, and increasing amounts of either hTR448H, hTRΔ332, or the v-erbA expression vectors were cotransfected (Fig. 1*A*). As a control the expression vector Δgal was used. The receptor mutant hTR448H was able to inhibit wt hTRβ activity at an equal molar ratio of the expression plasmids (Fig. 4 *A* and *B*). As expected, hTRΔ332 was a stronger inhibitor of hTRβ activation, showing nearly a 70% inhibition at a 1:1 molar ratio (Fig. 4*B*), which would probably be the situation in heterozygous patients. The v-*erbA* oncogene product, however, had the most dramatic effect. Using a 1:1 molar ratio of the expression plasmids, the hTRβ-mediated response was totally abolished. The control expression vector Δgal did not

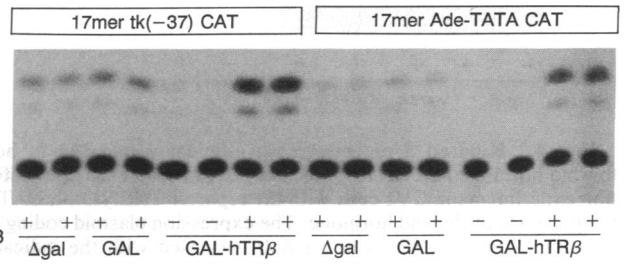


FIG. 3. Both silencing and hormone-dependent activation are effective on different minimal promoters. CAT assay showing duplicate experiments obtained by cotransfection of the indicated expression vectors with reporter plasmids containing the GAL4 DBS and either the *tk* promoter TATA box (17-mer tk(-37) CAT) or the adenovirus major late gene TATA box (17-mer Ade-TATA CAT) in L cells with (+) or without (−) addition of thyroid hormone.

have any effect on hTRβ hormonal response (only the 10:1 ratio is shown).

Recently, evidence was presented indicating that the v-erbA protein interferes also with the RA response and therefore may be oncogenic because of inhibition of development and differentiation (8). It was important to test whether the mutant receptors from patients with GTHR also have an effect on the RA pathway. We carried out a similar experiment using cotransfection experiments in CV1 cells, which lack measurable amounts of both the TR and the RAR. TREpal3x-tkCAT (25) was used as a reporter, which has been shown to be responsive to both T_3 and RA hormones (27).

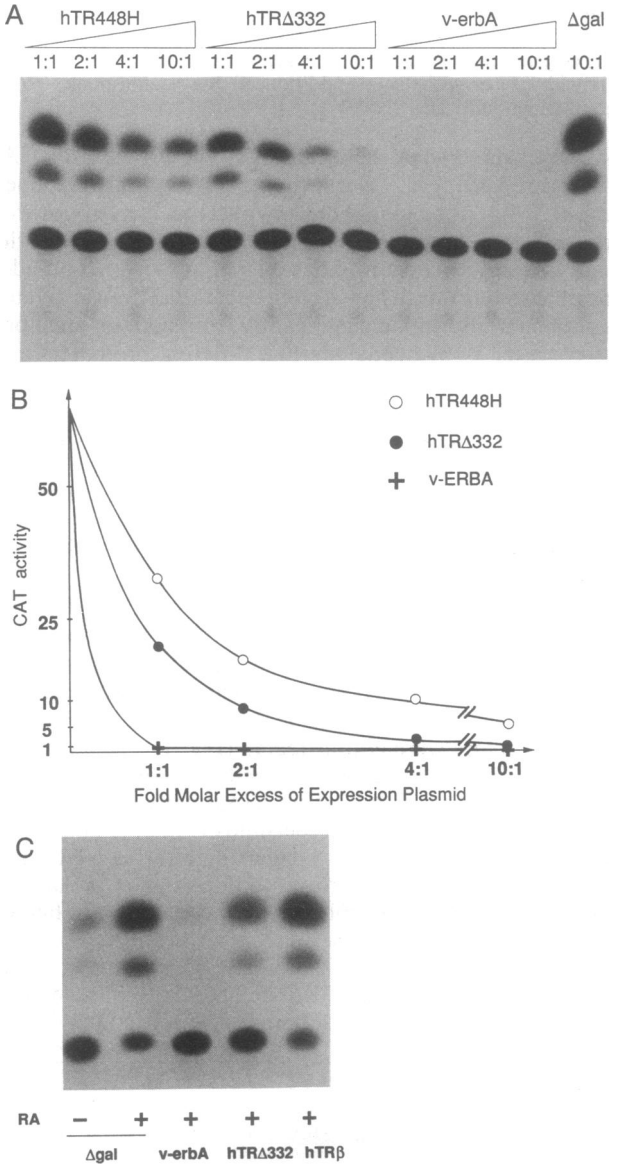


FIG. 4. Kindred S receptor is an efficient competitor of both thyroid hormone and RA response. (*A*) CAT assay from cotransfection experiments in CV1 cells with the reporter TRElys3x-tkCAT in the presence of thyroid hormone. The expression plasmid coding for full-length receptor hTRβ (0.5 pmol) was used with the indicated fold-molar excesses (from equal amounts, 1:1, up to 10-fold molar excess, 10:1) of either hTR448H, hTRΔ332, v-erbA, or the control vector Δgal. (*B*) Diagram with the quantitation of the data in *A* relative to control Δgal as 100%. (*C*) CAT assay from competition experiments in CV1 cells with the reporter TREpal3x-tkCAT, full-length human RA receptor (RAR) α (1 pmol), and the indicated expression plasmids (1 pmol), with addition of thyroid hormone and with (+) or without (−) RA.

Thyroid hormone was added to all samples, which would more closely resemble the situation in patients. A 1:1 molar ratio of the RAR and receptor mutant expression vectors was chosen. RAR-induced transcription was significantly inhibited by the kindred S receptor, hTRΔ332 (Fig. 4*C*). The inhibition was similar in extent to that seen in the competition experiment with wt hTRβ (Fig. 4*B*). Again, the v-erbA protein showed considerably stronger inhibition of RAR activation, completely abolishing hormone induction at a 1:1 ratio.

These results show that the receptor mutants isolated from patients with GTHR repress not only a T_3 response but also a RA response, the kindred S receptor being a strong repressor. Our findings provide another mechanism by which mutant thyroid hormone receptors could possibly result in human pathology.

DISCUSSION

In an attempt to fully understand the effects of mutations of hTRβ in patients with GTHR, we analyzed the transcriptional characteristics of the wt receptor and receptor mutants. The mutated receptors from kindreds with GTHR are called dominant negative because they inhibit the activity of the normal β and probably α receptors (32). From all available data, it can be concluded that exons 9 and 10 of the hTRβ gene are the predominant sites of mutation (18). These exons code for the LBD of the receptor. Therefore, we focused on analyzing in detail the C-terminal parts of the wt and various kindred receptors in determining the effects of mutation on their transcriptional properties.

Here, we studied the mutated receptor hTRΔ332, which was found in kindred S patients. This receptor does not bind hormone. Our data show that the mutated hTRΔ332 is not simply an intrinsic transcriptionally defective receptor, as stated earlier (21), but rather is a receptor that actively represses basal-level promoter activity. This type of repression is due to active silencing, which can be compared to enhancement by enhancer proteins (for reviews see refs. 33 and 34). The silencing activity is transferable to another DBD and therefore can be uncoupled from its native TRE. Thus, silencing by the full-length receptor is not due to competing for positive factors bound to a TRE. Minimal promoters containing only a TATA box are efficiently silenced, which suggests that in patients a variety of different promoters containing an authentic TRE are potential targets for transcriptional silencing. Since hTRΔ332 is an active and constitutive repressor and not an intrinsically nonfunctional receptor, obviously it is much more potent in interfering with the natural function of the hormone-bound T_3 receptor.

To understand how the clinical symptoms of patients with GTHR are manifest on a molecular level, it is necessary to know which target genes are potentially affected. Since the receptor mutants hTR448H and hTRΔ332 have DNA binding properties similar to the wt receptor (21, 22), competition at the DNA binding level would therefore occur at genes containing regulatory binding sites for TR. Due to the fact that TR and the RAR share common binding sites, the mutant receptor may repress those target genes as well. Our data confirm this possibility. It was shown that hTRβ is also able to bind to a vitamin D_3 response element (23), which suggests that the vitamin D_3 response could also be affected in patients with GTHR. This may explain the effects of mutations in TR on bone development. Taken together, this leads to the conclusion that in patients with GTHR, not only T_3-responsive genes but also some RA- and possibly vitamin D_3-responsive genes are inhibited, providing another pathway to explain the severity of specific clinical symptoms.

The mechanisms by which mutant receptors inhibit wt receptor activity and act as dominant negative regulators may

occur at different levels. One mechanism could involve the formation of nonfunctional heterodimers with the mutant receptor proteins. Such a mechanism is described for the muscle myogenic factor myoD and its inhibitor idiotype (12). A dominant negative effect would also occur if a transcriptionally defective receptor competes with the wt receptor for DNA binding. A third level would be active transcriptional repression or silencing by the mutant receptors themselves. A mutant receptor that does not bind hormone, like the kindred S receptor, is capable of interfering with the wt receptor on at least two different levels (i.e., similar DNA binding affinity and silencing) and will be a powerful inhibitor of the T_3 response.

A great variability of thyroid hormone resistance among different kindreds has been reported (18, 19). This can be explained by a variable degree of impairment of hormone binding affinity (19). We demonstrated that silencing is also localized in the C terminus of the authentic receptor and therefore is potentially an additional causative element for phenotypic differences. The many functions of the TR, such as hormone binding, dimerization, DNA binding, activation, and repression, can be affected by mutations in one way or the other and lead to potentially different clinical phenotypes. Because T_3 can autoregulate the expression of its own receptor (13, 14), even small changes in transcriptional activity might lead to very large changes in the overall transcriptional pattern.

It was known that the v-erbA protein does not bind hormone, is a dominant negative regulator of TR (9, 15, 16), and has in addition strong constitutive silencing activity (11). The question of how v-erbA causes its specific effects of differentiation arrest and changes in growth requirements in transformed cells is largely unknown. Target genes, which are thought to be repressed by the oncoprotein, are likely to contain regulatory sites for the T_3 receptor or RAR (8, 9). Because the kindred S receptor hTRΔ332 showed similar properties as the v-*erbA* oncogene product, we compared the transcriptional behavior of both proteins for active repression and competition in regard to T_3 or RA response. Interestingly, while both have similar inherent silencing potential, the v-*erbA* oncogene product was a much better inhibitor of both T_3 receptor or RAR action. This suggests that silencing may not be the sole mechanism for cellular transformation by v-erbA. Up to now, there is no report describing erythroid transformation or erythroid and myeloid leukemia in patients with GTHR, which could be explained by our findings. The reason for the more efficient inhibition is not known. Explanations for the stronger inhibition by the v-*erbA* oncogene product could lie in higher protein stability, different level of expression, different heterodimerization pattern, or higher affinity for DNA due to the mutations in the DBD. Notably, the DBD seems to have a certain influence on oncogenic transformation. Bonde *et al.* (17) reported that a back mutation of serine in the zinc finger sequence of v-erbA to that of the cellular TR, a glycine at position 61, impaired erythroid transformation; this suggests an additional important role for the DBD of the v-erbA oncoprotein. Regardless of the mechanism, neoplastic transformation by v-erbA might be due to its strongly enhanced ability to compete with TR and RA action.

The fact that the kindred S receptor is a silencer protein may help to explain the dramatic differences between the severe clinical symptoms of the homozygous Bercu patient and a homozygous patient, the Refetoff patient, who has a complete absence of functional hTRβ. The Refetoff patient has stippled epiphyses and dysmorphic features, but growth and IQ of the affected children are quite normal. In contrast, the Bercu patient, homozygous for the kindred S receptor, shows severe delay in linear growth and profound mental retardation (18, 22). This coincides with severe hypothyroidism in the bone and brain tissues in spite of the very high levels of thyroxine (T_4) and T_3 (18). Because the Bercu patient is much more affected than the Refetoff patient, it is possible that not only genes activated by hTRβ and hTRα are affected. It is likely that gene activation mediated by RA and perhaps other receptors is also severely inhibited.

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