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Article

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Novel insight from transgenic mice into thyroid hormone resistance and the regulation of thyrotropin

E. Dale Abel,¹ Helen C. Kaulbach,¹ Angel Campos-Barros,² Rexford S. Ahima,¹ Mary-Ellen Boers,¹ Koshi Hashimoto,¹ Douglas Forrest,² and Fredric E. Wondisford¹

¹Thyroid Unit and Division of Endocrinology and Metabolism, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, USA

²Department of Human Genetics, Mount Sinai School of Medicine, New York, New York 10029, USA

Address correspondence to: Fredric E. Wondisford, Thyroid Unit, Department of Medicine, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, Massachusetts 02215, USA. Phone: (617) 667-2151; Fax: (617) 667-2927; E-mail: fwondisf@bidmc.harvard.edu

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Patients with resistance to thyroid hormone (RTH) exhibit elevated thyroid hormone levels and inappropriate thyrotropin (thyroid-stimulating hormone, or TSH) production. The molecular basis of this disorder resides in the dominant inhibition of endogenous thyroid hormone receptors (TRs) by a mutant receptor. To determine the relative contributions of pituitary versus hypothalamic resistance to the dysregulated production of thyroid hormone in these patients, we developed a transgenic mouse model with pituitary-specific expression of a mutant TR ($\Delta 337T$). The equivalent mutation in humans is associated with severe generalized RTH. Transgenic mice developed profound pituitary resistance to thyroid hormone, as demonstrated by markedly elevated baseline and non-triiodothyronine (T_3)-suppressible serum TSH and pituitary TSH- β mRNA. Serum thyroxine (T_4) levels were only marginally elevated in transgenic mice and thyrotropin-releasing hormone (TRH) gene expression in the paraventricular hypothalamus was downregulated. After TRH administration, T_4 concentrations increased markedly in transgenic, but not in wild-type mice. Transgenic mice rendered hypothyroid exhibited a TSH response that was only 30% of the response observed in wild-type animals. These findings indicate that pituitary expression of this mutant TR impairs both T_3 -mediated suppression and T_3 -independent activation of TSH production *in vivo*. The discordance between basal TSH and T_4 levels and the reversal with TRH administration demonstrates that resistance at the level of both the thyrotroph and the hypothalamic TRH neurons are required to elevate thyroid hormone levels in patients with RTH.

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Introduction

Resistance to thyroid hormone (RTH) is a dominantly inherited condition with affected individuals harboring mutations in the β isoforms of the thyroid hormone receptor (TR) (1). Nearly all affected individuals are heterozygous for the mutant allele, and the molecular basis of the syndrome is presumed to be interference of wild-type TR function by the mutant TR (2, 3). Under normal circumstances, thyroid hormone concentrations are regulated by a feedback loop in which thyroid hormones inhibit the production of thyrotropin-releasing hormone (TRH) by the hypothalamus and thyrotropin (TSH) production by the thyrotrophs in the anterior pituitary (4-9). This pathway is critically dependent upon the presence of normal TRs, which bind to the promoters of TRH and TSH subunit genes and regulate their expression (10-14). In the presence of its ligand, triiodothyronine (T_3), TRs mediate ligand-dependent repression of the transcription of these genes, and in the absence of T_3 , the transcription rate is not simply returned to baseline, but ligand-independent activation is observed (15-17).

Patients with RTH have varying degrees of insensitivity to T_3 -mediated inhibition of TSH production (1). The

impact of RTH on TSH regulation in the absence of thyroid hormone is unknown. Although it has been presumed that the basis for the endocrine abnormalities is the presence of mutant TRs in the pituitary and the hypothalamic TRH neurons, the relative roles of the pituitary versus the hypothalamus in the pathogenesis of RTH remains unknown. Most of the insight into the molecular basis of RTH has been obtained from experiments using transfected mutant TRs in various cell lines. It is not always possible to generalize or apply the observations derived from these experiments to thyroid hormone-responsive cells in the *in vivo* milieu. Thus, targeted expression of mutant thyroid hormone receptors to the hypothalamus or to the thyrotrophs of transgenic mice should give valuable insight into the roles of these compartments in the genesis of the resistance phenotype.

A few *in vivo* models of RTH currently exist. Adenoviral gene transfer of a mutant TR (G345R) into mice has recapitulated some of the hepatic abnormalities observed in RTH (18). The same group also expressed this receptor transgenically in the pituitaries of mice using a fragment of the TSH- β promoter to drive transgene expression (19). Mild increases in thyroxine (T_4) concentrations with a tendency toward lower TSH concentrations were

reported in the F1 transgenic mice. Unfortunately, the elevated T₄ phenotype was lost in subsequent generations. Hence, it is difficult to know whether or not pituitary resistance truly existed in this model. Transgenic mice with ubiquitous overexpression of the frameshift mutant PV were reported to develop hyperactivity and mild increases in T₄ concentrations (20). The pituitary expression of the transgene was variable and the extent of hypothalamic expression unknown. Finally, mice with targeted ablation of the β isoform of the TR exhibit impaired T₃-mediated suppression of TSH, which underscores the important role of TR- β in the regulation of thyrotropin (21). TR- β knockout mice, however, are a model of recessive resistance and are therefore pathophysiologically distinct from the human syndrome (1, 21).

Hence, to gain further insight into the impact of mutant TR expression on the hypothalamic-pituitary-thyroid axis, we targeted a mutant TR associated with generalized RTH selectively to the pituitary in transgenic mice. Extensive characterization of the hypothalamic-pituitary-thyroid axis has been performed, and insights into the relative roles of the pituitary and the hypothalamus in the RTH phenotype have been obtained. These mice have also provided the opportunity to assess *in vivo* the impact of the mutant TR on ligand-dependent, as well as ligand-independent, functions of the endogenous receptor.

Methods

Animals. All aspects of animal care and experimentation performed in this study were approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Animals were maintained on a 12-h light/12-h dark schedule (light on at 0600) and fed laboratory chow and water *ad libitum*. Blood was obtained for total thyroxine (T₄) and thyrotropin (TSH) from the tail vein. Experimental hypothyroidism was induced by administering 150 μ Ci of iodine-131 by intraperitoneal injection to mice that had been placed on a low-iodine diet (Harlan Teklad, Madison, Wisconsin, USA) for 8 days. Experiments on hypothyroid animals were performed three weeks after the administration of iodine-131, because all mice develop undetectable T₄ concentrations by the third week. Suppression of pituitary TSH production was attempted by daily intraperitoneal injections of T₃ (1 μ g/ml) in buffered HEPES for 3 weeks at doses of 0.2 μ g/100 g mouse body weight during the first week, 0.5 μ g/100 g mouse body weight during the second week, and 1.0 μ g/100 g mouse body weight during the third week. All comparisons were made between transgenic mice and littermate controls to minimize the effect of genetic variability.

Generation of transgenic mice. The naturally occurring mutation Δ 337T (22, 23) was introduced into the human TR- β 1 cDNA by site-directed mutagenesis (CLONTECH Laboratories Inc., Palo Alto, California, USA), and the presence of the mutant was confirmed by direct sequencing. The ability of this cDNA to produce an intact protein was confirmed by *in vitro* translation and by the ability of the proteins to bind to thyroid hormone response elements (TREs) in gel mobility shift assays (data not shown). The cDNA was initially subcloned as an *Eco*RI fragment into the expression vector pGEX4T2 (Pharmacia Biotech, Piscataway, New Jersey, USA). The transgene was made by cloning a 4.6-kb promoter fragment of the mouse α -subunit gene (24) (gift of University of Colorado Health Sciences Center, Denver, Colorado, USA) into the *Kpn*I and *Hind*III sites of the expression plasmid pREP7 (Invitrogen Corp., San Diego, California, USA), which contains the SV40 polyadenylation sequence downstream of its multiple cloning site. The mutant

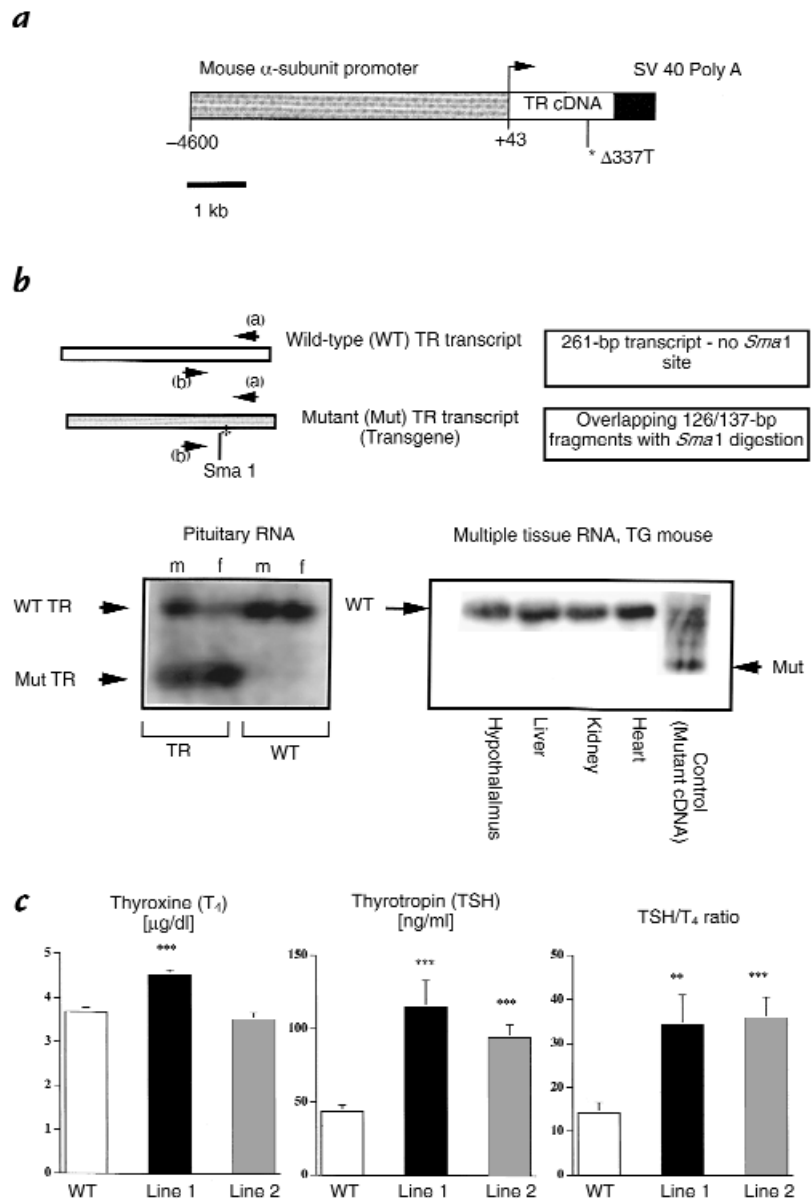
TR cDNA was removed from pGEX4T2 as a *Bam*HI-*Not*I fragment and then cloned downstream of the α -promoter fragment, immediately proximal to the SV40 polyA. The 6.2-kb transgene was released from this plasmid by *Kpn*I and *Sal*I restriction digests and gel purified by electroelution (Fig. 1a). In the Beth Israel Transgenic Facility, approximately 4 ng of the purified transgenic vector was microinjected into the male pronuclei of 1-day-old mouse zygotes of the inbred strain FVB/N and reimplanted into the uteri of pseudopregnant foster mothers. Litters were obtained after 21 days. Between the age of 14 and 18 days, preweaned mice were identified by sex and marked by earlobe punching. Then, 15 mm of tail was removed for genomic DNA extraction. Genomic DNA was extracted by overnight digestion with proteinase K and SDS. After high-salt precipitation of the SDS, phenol/chloroform extraction was performed and the DNA obtained by ethanol precipitation. Genomic DNA (10 μ g) was digested with *Bam*HI, subjected to agarose gel electrophoresis, and transferred to nylon membranes (Micron Separations Inc., Westboro, Massachusetts, USA). Southern blotting was performed using the entire human TR- β 1 cDNA as a probe. Transgenic mice were identified by the presence of a specific 1.6-kb fragment.

RNA analysis. RNA was extracted from pooled pituitaries (5–8 animals), using guanidinium thiocyanate, selective precipitation, and isopycnic centrifugation with lithium chloride and cesium trifluoroacetate (Pharmacia Biotech). Wild-type TR versus transgene expression was established by performing semiquantitative reverse transcription (RT)-PCR on DNaseI-treated samples using the following primers: 5'-GGCCAGCTGAAAAATGGGGG-3' and 5'-TGTCACCTTCATCAGGAGTTT-3', which amplify 261 bp of the COOH-terminus of TR- β . The size of the wild-type and transgenic PCR products are identical. However, the mutant human transcript contains a *Sma*I restriction site that is absent in the wild-type murine transcript. The appearance of overlapping 126- and 137-bp fragments upon *Sma*I restriction digestion of the PCR product is indicative of transgene expression. The PCR products were resolved on a 3% agarose gel, transferred to nylon membranes, and hybridized with the human and mouse PCR products. TSH- β gene expression was assayed in pituitary total RNA by Northern blotting. In hypothyroidism studies, serial dilutions of total RNA was performed, and 5, 2.5, 1.25, and 0.625 μ g of RNA were resolved on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. The membrane was hybridized to the entire cDNA of the β subunit of murine TSH under high-stringency conditions. In studies of hyperthyroid mice, 15 μ g of RNA was used.

Localization and quantification of preprothyrotropin (prepro)-TRH mRNA by *in situ* hybridization histochemistry. Male wild-type and transgenic mice ($n = 3$ per group) were anesthetized with an intraperitoneal injection of sodium pentobarbital. They were perfused transcardially with PBS prepared with diethylpyrocarbonate (DEPC)-treated water, followed by 10% neutral buffered formalin. Brains were removed, immersed in the same fixative overnight, and then cryoprotected in 20% sucrose in PBS-DEPC at 4°C. Five series of 20- μ m coronal free-floating sections were cut on a tabletop cryotome and mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, Pennsylvania, USA). The protocol for *in situ* hybridization histochemistry has been described previously (25). Prepro-TRH mRNA was localized using a sulfur-35 UTP-labeled complementary RNA probe synthesized from a plasmid containing the cDNA for the mouse prepro-TRH gene (26) (kind gift of Masatomo Mori, Gunma University, Maebashi, Japan). Control sections were hybridized with a sense cRNA probe. The slides were exposed to Kodak Biomax MR film (Eastman Kodak Co. Scientific Imaging Systems, Rochester, New York, USA) for 2 days, after which they were dipped in Kodak NTB2 photographic emulsion and developed after 4 days of exposure; then they were counterstained with cresyl violet acetate. Sections

Figure 1

Transgenic construct, pituitary expression of the transgene, and impact on basal thyroid hormone concentrations. **(a)** Schematic representation of the transgene. The vector contains 4.6 kb of the mouse glycoprotein subunit α gene cloned upstream of the human TR- β cDNA harboring the mutation Δ 337T and ligated to a 400-bp fragment containing the SV40 polyadenylation signal. **(b)** RT-PCR scheme for detecting transgene expression and expression of the mutant and wild-type TR transcripts in mouse pituitaries and other tissues. RNA was obtained from pituitary, hypothalamus, liver, kidney, and heart, and analyzed by RT-PCR using primers (denoted *a* and *b*) that are homologous both to the human and mouse TR sequences. These primers amplify 261 bp of the carboxyl-terminus of the human and mouse TR- β gene. A polymorphism in the human gene results in the presence of a *Sma*I restriction site that is not present in the mouse gene. Restriction digestion with this enzyme results in the appearance of 126/137-bp overlapping fragments indicating transgene expression. Gels shown were performed after *Sma*I digestion of the RT-PCR products. Note that the smaller transcript appears only in the pituitaries of TG mice but is absent from WT mouse pituitaries and from multiple other tissues, including the hypothalamus in TG mice. Data is shown for line 1 mice; similar results were obtained for line 2 mice, which had 50% lower pituitary transgene expression vs. line 1. **(c)** Basal total T_4 , TSH concentrations, and TSH/ T_4 ratios (means \pm SEM) in the WT and TG mice from two independent transgenic founder lines (1 and 2). The number of animals used for T_4 determinations are WT: $n = 89$; line 1: $n = 84$; and line 2: $n = 39$, respectively. The number of animals used for TSH determinations are WT: $n = 51$; line 1: $n = 40$; and line 2: $n = 46$. The number of samples on which T_4 and TSH were simultaneously measured for the purpose of calculating the TSH/ T_4 ratio are WT: $n = 24$; line 1: $n = 26$; and line 2: $n = 26$. Note the small increase in T_4 concentration in line 1 and the lack of T_4 elevation in line 2, despite large increases in TSH concentration. $**P < 0.01$ vs. WT; $***P < 0.0001$ vs. WT. RT, reverse transcription; T_4 , thyroxine; TG, transgenic; TSH, thyroid-stimulating hormone; WT, wild-type.



were analyzed with a Zeiss Axioplan light microscope using bright and dark-field optics. Dark-field photomicrographs were taken with Kodak daylight slide film and the images printed in black and white using a dye sublimation printer (Kodak 8600). Film autoradiograms of the hypothalamic paraventricular nucleus (PVN) and thalamic reticular nucleus, corresponding to bregma -0.58 mm, -0.82 mm, and -1.22 mm, respectively (Figures 36, 38, and 41 of ref. 27), from three wild-type and three transgenic mice were selected, coded, and analyzed blindly by laser densitometry (Molecular Dynamics, Sunnyvale, California, USA). Integrated densities of prepro-TRH mRNA in each half of the brain were summed, and mean densities in the PVN and reticular nucleus were determined in each animal.

Radioimmunoassay. Total T_4 levels were measured in 10- μ l serum samples in duplicate determinations by a mouse-specific RIA (ImmuChem coated tube- T_4 iodine-125 RIA Kit; ICN Pharmaceuticals, Costa Mesa, California, USA). TSH was measured in 25- μ l serum samples in triplicate determinations by a specific mouse TSH RIA using a mouse TSH/luteinizing hormone (LH) reference preparation (AFP51718mp), a mouse TSH antiserum

(AFP98991), and rat TSH antigen for radioiodination (NIDDK-rTSH-I-9). All reagents were obtained from A.F. Parlow (Harbor University of California at Los Angeles Medical Center, Torrance, California, USA). The standard curve was performed in hyperthyroid mouse serum, and the limit of sensitivity was <20 ng/ml. The inter- and intra-assay variations were less than 6%.

TRH stimulation. TRH stimulation was performed by implanting pellets impregnated with 1 mg of TRH (Innovative Research of America, Sarasota, Florida, USA) into the interscapular space. Blood was subsequently withdrawn from the tail vein for determination of total T_4 at 0, 6, 24, 48, and 72 h after pellet insertion. TSH was assayed on the 0-, 6-, and 24-h samples.

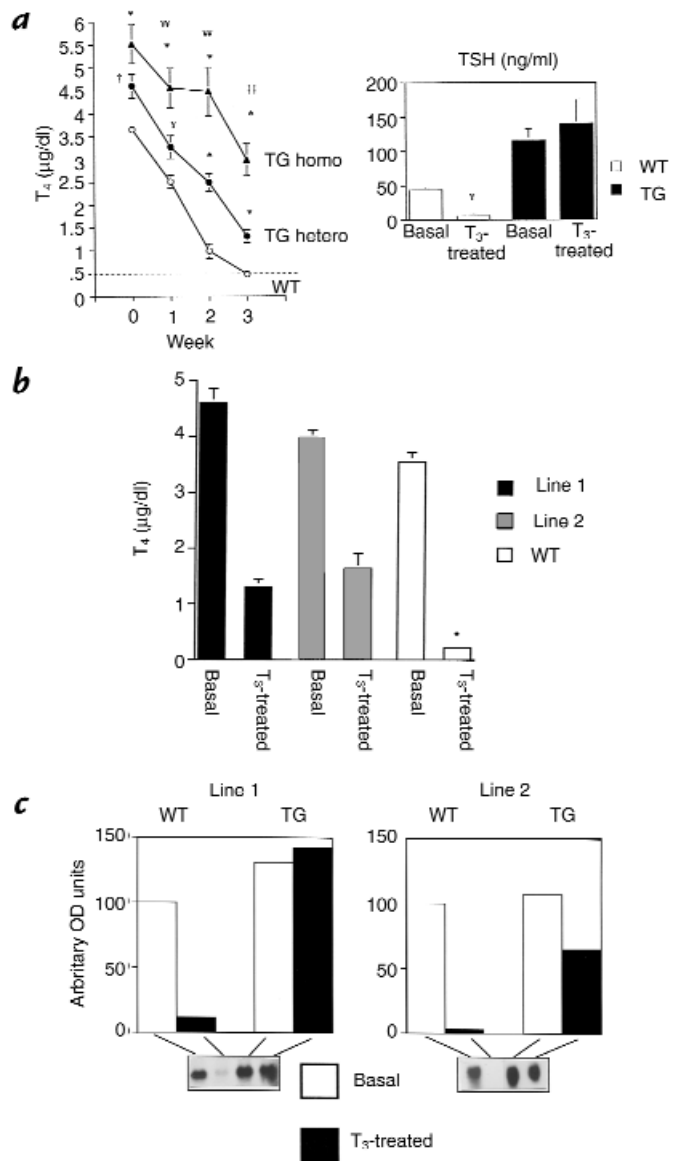
Statistical analysis. Differences were assessed by one-tailed Student's *t* test (two sample unequal variance).

Results

Generation of transgenic mice and expression level of transgene. Because of the negligible effect of the transgene on basal T_4 concentrations, founder lines were initially screened

Figure 2

Effect of T_3 administration on serum T_4 , TSH concentrations, and pituitary TSH- β mRNA. (a) Total T_4 concentrations (line 1) obtained at weekly intervals during the administration of pharmacological doses of T_3 for 3 weeks. The hatched line represents the limit of detection of the T_4 RIA. Mice with homozygous allelic expression of the transgene (TG homo; $n = 5$) were compared with TG mice harboring one transgenic allele (TG hetero; $n = 13$) and WT mice ($n = 21$). TSH concentrations were determined at baseline and at the end of 3 weeks in 11 heterozygous TG mice and 8 WT mice. Note that T_4 and TSH are undetectable in WT mice after 3 weeks. In contrast, TG mice reveal only partial suppression of T_4 and lack of suppression of TSH, indicating pituitary resistance. For the T_4 data: $^{**}P < 0.01$ and $^{\dagger\dagger}P < 0.0001$, TG homo vs. TG hetero; $^{\ddagger}P < 0.01$, $^{\dagger}P < 0.001$, $^*P < 0.0001$, TG homo and hetero vs. WT. For TSH data: $^{\ddagger}P < 0.01$, basal vs. treated WT. (b) Comparison of T_4 concentrations before and after the administration of T_3 in line 1 and line 2 TG mice. Note that despite basal thyroid hormone concentrations in line 2 that were similar to those of WT mice, T_4 concentrations in line 2 are only partially suppressed to a level that is similar to that observed in line 1. TSH concentrations in line 2 mice were also partially suppressed to levels that were equivalent to untreated WT mice (44 ng/ml) after 3 weeks of T_3 administration (data not shown). $^*P < 0.0001$ compared with T_3 treated values in lines 1 and 2. (c) TSH- β mRNA expression in pituitaries of T_3 treated mice from both lines. Total RNA from pooled pituitaries (six mice per group) was analyzed by Northern blotting. The blots were initially hybridized with the mouse TSH- β cDNA and then with cyclophilin. Densitometry was used to determine mRNA abundance and cyclophilin expression used to adjust the TSH- β message for differences in loading. TSH mRNA abundance is expressed in arbitrary OD units with WT basal expression normalized to 100. Note complete suppression of TSH- β message in WT mice, lack of suppression in line 1 mice, and partial suppression in line 2 mice. T_3 , triiodothyronine.



by measuring total T_4 levels after three weeks of T_3 suppression. Three lines exhibited incompletely suppressed T_4 levels. Two of these lines, designated 1 and 2, respectively, are analyzed in detail and reported in this paper. Transgene expression was documented by RT-PCR (Fig. 1b). Endogenous TR expression was downregulated in transgenic mice when compared with wild-type controls. The relative abundance of mutant TR to wild-type TR transcripts determined from multiple assays was approximately 1.5:1. Transgene expression was approximately twofold higher in line 1 versus line 2. Mutant TR expression was assayed by RT-PCR in the hypothalamus and in peripheral tissues (heart, liver, kidney). Expression of the transgene outside of the pituitary was not seen (Fig. 1b).

Basal T_4 and TSH concentrations. Mean total T_4 concentrations were increased by approximately 20% in mice from line 1 but were not increased in mice from line 2 (Fig. 1c). In contrast to the small effect of the transgene on T_4 concentrations, TSH concentrations were increased by greater than twofold in transgenic mice

from both lines (Fig. 1c). The disparity between TSH and T_4 concentrations suggests an impairment in the bioactivity of the TSH produced by transgenic versus wild-type mice and is evidenced by the significant increase in the TSH/ T_4 ratio observed (Fig. 1c).

Analysis of *in vivo* regulation of thyrotroph function by T_3 . Administration of T_3 suppresses endogenous TSH secretion and ultimately T_4 production by the thyroid gland. We therefore sought to determine the sensitivity of the hypothalamic-pituitary-thyroid axis in transgenic mice by measuring total T_4 concentrations at weekly intervals during three weeks of intraperitoneal administration of pharmacological doses of T_3 . TSH concentrations were measured at the end of the T_3 treatment period. In wild-type mice, T_3 administration resulted in a progressive decline in T_4 concentrations, and serum T_4 levels were not detectable by the end of three weeks. In contrast, thyroxine concentrations were only partially suppressible in transgenic mice. Compared with wild-type mice, T_4 concentrations were significantly higher in mice (line 1) har-

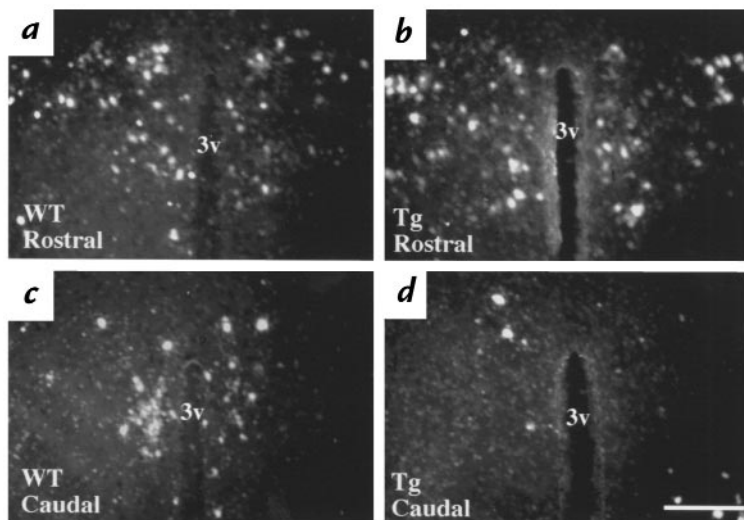


Figure 3

(a–d) Representative dark-field photomicrographs showing prepro-TRH mRNA at two rostral and caudal levels in the PVN of mice. (a and c) WT mice. (b and d) Tg mice. The rostral PVN is represented by a and b, and c and d represent the caudal PVN. Note the paucity of labeled cells in the caudal PVN of the Tg mouse. As shown in this data from line 1, a similar degree of downregulation of prepro-TRH expression in the caudal PVN was observed in line 2 mice. 3v (third ventricle): scale bar = 300 μ m. PVN, paraventricular hypothalamic nuclei; TRH, thyrotropin-releasing hormone.

boring one transgenic allele at all time points (Fig. 2a). Line 2 mice demonstrated a similar pattern of impaired T_4 suppressibility, despite the fact that they did not have any increase in baseline thyroxine concentration (Fig 2b). To assess whether increasing transgene expression resulted in increased resistance, mice from line 1 were bred to homozygosity for the transgenic allele. Homozygous animals developed more severe T_3 resistance. The increase in basal T_4 concentrations was not significantly higher than the values seen in heterozygotes. However, in response to exogenously administered T_3 , the degree of T_4 suppression was significantly blunted when compared with heterozygous and wild-type mice (Fig. 2a). To confirm that the nonsuppressibility of T_4 was due to impaired suppression of TSH, serum TSH concentrations and pituitary TSH- β mRNA were measured before and after T_3 suppression. In wild-type mice, TSH concentrations were suppressed below the detection limit of the TSH RIA after T_3 administration. This contrasts with line 1 transgenic mice in which no measurable reduction in TSH concentrations could be discerned (Fig. 2a). In line 2 mice, TSH concentrations fell by 50% but were not suppressible. TSH- β gene expression followed the pattern of serum TSH. Line 1 mice revealed complete absence of TSH- β mRNA suppression, and line 2 mice revealed a suppression of 40% (Fig 2c). In contrast, TSH- β mRNA was nearly completely obliterated by T_3 administration in wild-type mice. These data indicate that transgenic mice from both lines exhibited profound pituitary resistance to thyroid hormone.

Analysis of the role of TRH. The mutant TR in our transgenic model was targeted only to the pituitary, and given the modest effect of the transgene on T_4 concentrations, we speculated that increased TRH production may play a critical role in elevating thyroid hormone concentrations in RTH. To assess the impact of pituitary transgene expression on hypothalamic TRH gene expression, *in situ* hybridization histochemistry was performed on the brains of line 1 and line 2 transgenic mice and littermate controls. A comparison of the distribution of neurons expressing prepro-TRH mRNA in the rostral and caudal PVN of wild-type (Fig. 3, a and c, respectively) and trans-

genic mice (Fig. 3, b and d, respectively) reveal evidence of downregulated TRH gene expression in distinct subsets of TRH neurons in transgenic mice. The level of prepro-TRH mRNA in the rostral PVN of transgenic mice was similar to wild-type mice (compare Fig. 3, a and b). In contrast, prepro-TRH mRNA was markedly decreased in the caudal PVN of transgenic mice from both lines (compare Fig. 3, c and d). Densitometric analysis of film autoradiograms showed an overall decrease in PVN prepro-TRH mRNA content of 35% in transgenic mice from line 1 and 55% in transgenic mice from line 2 ($P < 0.001$). We also examined prepro-TRH mRNA content in the reticular thalamic nuclei of transgenic mice, which contain a population of TRH neurons previously demonstrated to lack T_3 regulation in rats, and found no change in TRH gene expression (data not shown).

To test the hypothesis that TRH downregulation was minimizing the phenotype in transgenic mice, TSH and T_4 responses to TRH administration were examined in transgenic and wild-type mice. Six hours after the insertion of the TRH-containing pellet, both transgenic and wild-type mice exhibited increased concentrations of T_4 and TSH (Fig. 4). This increase was transient in wild-type mice, and their concentrations returned to baseline within 24 hours. In contrast, TSH concentrations of transgenic mice were still rising at 24 hours, and this was mirrored by a sustained increase in T_4 concentrations that persisted beyond 72 hours (Fig. 4).

TSH response to hypothyroidism. Three weeks after radioactive iodine ablation, all animals studied had thyroxine levels that were at or below the level of detection of the T_4 RIA and had an associated robust increase in serum TSH concentrations. Wild-type mice increased their TSH more than 400-fold, but the response in transgenic mice was blunted, with only a 40-fold increase in TSH concentration (Fig. 5a). To determine whether these changes reflected changes at the level of TSH subunit gene expression, a Northern blot of pituitary total RNA obtained from hypothyroid animals was performed and probed with the TSH- β cDNA. In concordance with the changes in serum TSH, the TSH- β mRNA response to hypothyroidism was also blunted in transgenic mice (Fig. 5b).

Discussion

We have successfully generated a transgenic mouse model of selective pituitary resistance to thyroid hormone. The mutant TR $\Delta 337T$ does not bind thyroid hormone and is associated in humans with a severe phenotype of generalized resistance to thyroid hormone (22). The promoter fragment used to drive pituitary transgene expression was previously shown to direct expression selectively in thyrotrophs and gonadotrophs (24). The expression of the transgene in the gonadotrophs did not appear to have any effect on fertility in these mice. In two lines with independent sites of transgene integration, the phenotype observed was one of severe *in vivo* thyrotroph resistance to T_3 , but with a small effect on baseline T_4 concentration. Analysis of these mice provide novel insight into the relative contributions of the pituitary and hypothalamus to the neuroendocrine abnormalities associated with RTH.

Basal concentrations of thyroxine are determined by TSH stimulation of thyroid follicular cells. As TSH concentration rises, there is an associated increase in thyroid hormone synthesis and production (28). TSH production in the thyrotroph is regulated by feedback inhibition by circulating thyroid hormone and via stimulation by hypothalamic TRH (4). There is some evidence that TRH enhances the biologic activity of TSH by modifying its glycosylation pattern (29–31). This was recently confirmed in mice with targeted ablation of the TRH gene who exhibit T_4 levels that were 50% of those in wild-type mice, despite TSH concentrations that were elevated twofold (32). In our mice, TSH concentrations were significantly elevated, which confirms the important role of pituitary TRs in regulating basal TSH output. The TSH elevation likely reflects impairment of endogenous TR-mediated regulation of TSH gene expression by the mutant TR transgene at ambient thyroid hormone concentrations. Paradoxically, T_4 levels were not proportionately increased. The discrepancy between TSH levels and T_4 production (as evidenced by the increased TSH/ T_4 ratio in transgenic mice) could reflect a primary abnormality in the thyroids of transgenic mice. This is extremely unlikely given the robust T_4 response to TRH administration observed in transgenic mice. A more plausible explanation is that the bioactivity of the TSH produced in transgenic animals is reduced. This contrasts with patients with RTH who have been shown to produce a

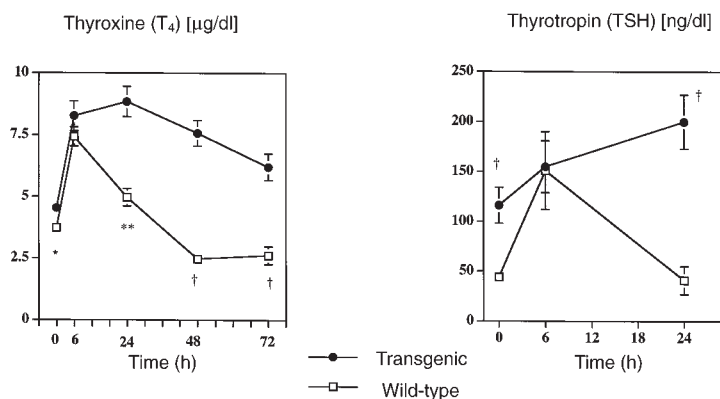
TSH with enhanced bioactivity (33). It is likely, then, that the difference between our mice and patients with RTH reside in differences in TRH production.

Since the mutant TR was selectively targeted to the pituitary, but not to the hypothalamus, basal TRH production in our transgenic mice should not be increased. In fact, when we directly assayed for hypothalamic TRH expression, we found marked downregulation of prepro-TRH mRNA in the neurons of the caudal paraventricular hypothalamus. Mapping of the distribution of T_3 -responsive TRH neurons in the PVN has been performed in the rat (7, 34, 35). In rats, the distribution of the T_3 responsive neurons are restricted to the medial parvocellular neurons of the PVN (6, 7). Mice do not possess the same discreet hypothalamic nuclear architecture as rats, and as such, the exact location of T_3 responsive neurons in the mouse are unknown. In this study, we have been able to demonstrate regional differences in TRH gene expression within the PVN of mice and show that the overall prepro-TRH mRNA content in the PVN is diminished in transgenic mice. The basis for the downregulation of TRH gene expression is likely due to the small increase in thyroxine concentrations observed in transgenic mice. This observation also suggests that the hypothalamic TRH neurons possess exquisite sensitivity to small changes in thyroid hormone concentrations, as evidenced by the downregulation of prepro-TRH gene expression in both lines of transgenic mice.

If TRH overproduction is an important component of the RTH phenotype, we hypothesized that chronic administration of TRH would result in further elevations in T_4 concentrations in transgenic mice. Persistent elevations in T_4 in response to TRH was indeed observed in transgenic mice. This suggests that resistance at the level of the hypothalamus plays an important role in sustaining the elevated thyroid hormone concentrations that are seen in RTH. There are several possible explanations for the augmented T_4 response of the transgenic mice. Because of impaired T_3 -mediated gene regulation, the thyrotroph in the transgenic mice might respond to TRH administration as would a hypothyroid pituitary with an exaggerated TSH response. In addition, TRH could be differentially changing the glycosylation pattern of TSH in a way that could prolong the half-life of TSH. Because of the small amounts of TSH that can be extracted from mouse serum,

Figure 4

Effect of TRH administration. Serum T_4 and TSH concentrations, before and after the implantation of a 1-mg sustained release TRH pellet. Nineteen TG and 18 WT mice were studied. Basal T_4 and TSH concentrations were obtained in all mice. To minimize the stress of repeated bleeding, and given the volume of serum required, TSH and T_4 concentrations were obtained in a subset of mice (12 TG and 12 WT) at 6 h and 24 h. The remaining 7 TG and 6 WT mice were bled daily starting at 24 h for T_4 only. Mice from both lines were studied, and combined data are shown. Note that TG and WT mice both show an initial T_4 and TSH response to TRH, but the response is sustained beyond 24 h in TG mice at a time when values have returned to normal in WT animals. For T_4 data: * $P < 0.05$, ** $P < 0.001$, † $P < 0.0001$, TG vs WT. For TSH data: † $P < 0.0001$, TG vs. WT.



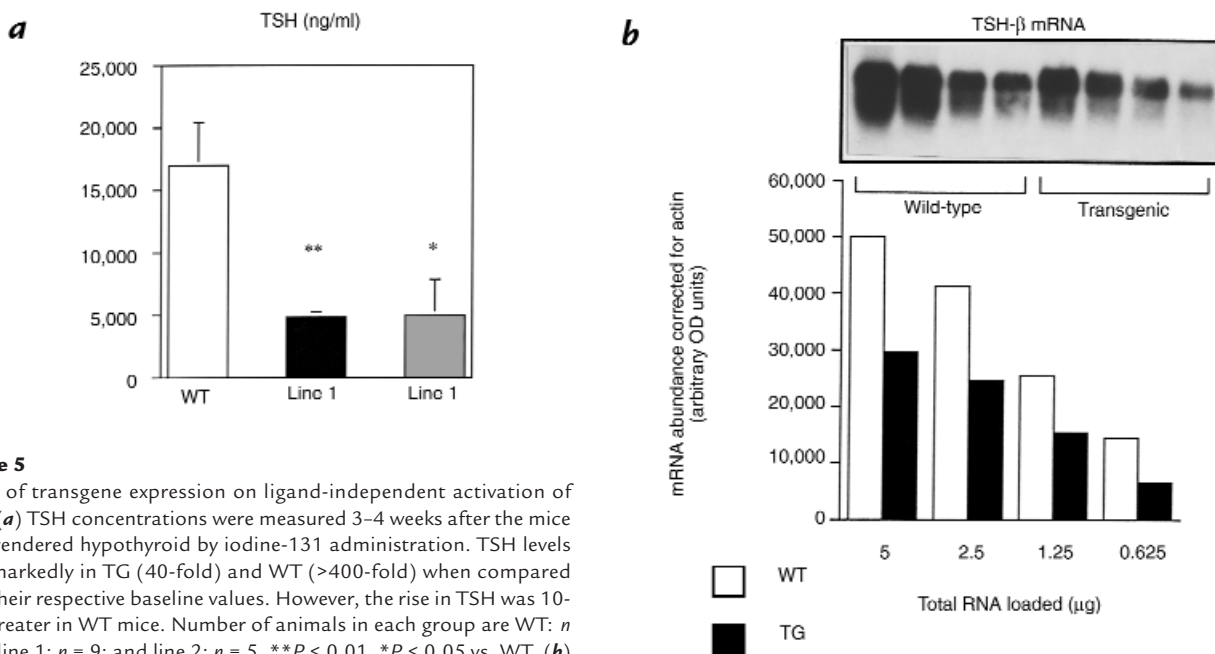


Figure 5

Effect of transgene expression on ligand-independent activation of TSH. (a) TSH concentrations were measured 3–4 weeks after the mice were rendered hypothyroid by iodine-131 administration. TSH levels rose markedly in TG (40-fold) and WT (>400-fold) when compared with their respective baseline values. However, the rise in TSH was 10-fold greater in WT mice. Number of animals in each group are WT: $n = 13$; line 1: $n = 9$; and line 2: $n = 5$. ** $P < 0.01$, * $P < 0.05$ vs. WT. (b) TSH- β mRNA in WT and TG hypothyroid mice (pooled pituitaries). A Northern blot was performed using serial dilutions of pituitary total RNA. Data from line 1 are shown. Similar data (not shown) exist for line 2 mice. The blots were initially hybridized with the mouse TSH- β cDNA, and then with actin. Densitometry determined mRNA abundance, and actin expression was used to adjust the TSH- β message for differences in loading. TSH- β mRNA abundance was 40% lower in TG vs. WT mice.

we have not directly assayed for TSH glycosylation. However, previous published data in rats and humans render this hypothesis plausible (29–31). Changes in the glycosylation of TSH could also impact its bioactivity. Hence, increased bioactivity of TSH could also be playing a role in the elevated T_4 concentrations observed.

The fall in T_4 concentration observed in wild-type mice after T_3 administration reflects, in large part, decreased thyroid gland T_4 synthesis occurring in response to TSH inhibition. The possibility exists, however, that accelerated catabolism of T_4 in response to T_3 -induced hyperthyroidism could account, in part, for its decline (36, 37). Transgenic mice displayed incomplete suppression of T_4 , lack of TSH inhibition in line 1, and partial TSH inhibition in line 2. The presence of the mutant TR in the pituitary clearly prevents the inhibition of TSH production in the face of T_3 administration. It is intriguing that the partial suppression in T_4 levels seen in transgenic mice appears greater than the corresponding change in TSH. Two possible explanations for this can be entertained. First, it is possible that T_3 administration further impairs the bioactivity of TSH through its effect on TRH neurons, which retain their sensitivity to thyroid hormone. Second, the decline observed in transgenic mice may reflect the effect of increased clearance of T_4 occurring in response to T_3 -induced hyperthyroidism; the fact that we were unable to completely suppress T_4 levels reflects the persistent effect of the nonsuppressed TSH.

The molecular mechanisms governing negative regulation of thyrotroph TSH production are unclear but are thought to involve interactions between the TR and other nuclear proteins in a ligand-dependent manner (38). In this study we observed a direct relationship between transgene expression and resistance. Line 2 mice had lower transgene

expression than line 1. This is the likely reason for the milder phenotype as evidenced by partial suppression of serum TSH and pituitary TSH- β mRNA by T_3 , which contrasts with complete lack of suppression in line 1. Furthermore, doubling of the transgene dose in homozygote line 1 mice produced a greater degree of resistance than that seen in heterozygotes. There are a number of possible explanations for the relationship between increasing transgene expression and pituitary resistance. There could simply be increasing amounts of the mutant TR bound to the promoters of the TSH subunit genes. This non- T_3 -binding mutant would then be incapable of mediating T_3 -mediated inhibition of TSH subunit gene transcription. The $\Delta 337T$ mutant TR constitutively binds to the nuclear corepressor protein NCoR, and the binding is not relieved by T_3 (39). Hence, one mechanism for resistance is that the aberrant interaction between the mutant TR and nuclear corepressors is not relieved by thyroid-hormone binding, and this prevents the recruitment of other cofactors responsible for mediating inhibition. Downregulation of endogenous TR expression was observed in heterozygous transgenic mice. Additional downregulation of the endogenous TR by the mutant TR in homozygous transgenic mice could also contribute to the dose-related increase in pituitary resistance.

Our mice also exhibited impaired ligand-independent activation of TSH- β gene expression *in vivo*. This finding is the first demonstration that ligand-independent activation of TSH production can be modulated *in vivo* and raises interesting questions about potential mechanisms. Impaired ligand-independent activation was demonstrated both at the level of measured serum TSH and at the level of TSH- β gene expression. The apparent greater differences in serum TSH, as compared with

TSH- β gene expression, likely represents differences in the kinetics and half-life of TSH protein in the serum versus that of TSH- β mRNA in the pituitary. A simple explanation for the deficient TSH response to hypothyroidism may be diminished thyrotroph reserve. Transgenic mice under basal conditions have TSH concentrations that are two- to threefold higher than wild-type mice. This chronic overproduction of TSH could render the thyrotroph incapable of mounting a vigorous TSH response to hypothyroidism. Transgenic mice are still able, however, to increase their TSH some 40-fold in the face of hypothyroidism. Although this is 10-fold less than the wild-type response, our data indicate that they retain a significant degree of responsiveness to the withdrawal of ligand. Hence, we feel that thyrotroph failure might not be the sole explanation for this phenomenon. Another contributing factor could be differential degrees of TRH induction by hypothyroidism in transgenic versus wild-type mice. We know from the TRH administration experiments that the transgenic mice exhibit an augmented response to TRH administration; thus, any deficiency in TRH rise in transgenic mice resulting from the lower baseline expression would tend to be offset by the enhanced responsiveness of the thyrotrophs of the transgenic mice to TRH.

Potential molecular mechanisms for the impaired ligand-independent activation of the TSH subunit genes observed in this study are also worthy of discussion. Recent reports have attempted to address the role of nuclear corepressors in the ligand-independent activation of the TRH and TSH subunit genes, but the results are controversial (17, 40). Hollenberg *et al.* cloned a truncated NCoR molecule (NCoRI) that retains the interacting domains but lacks the repressing domains of full-length NCoR. In transfection experiments, NCoRI acts as a dominant inhibitor of endogenous NCoR. They reported that transfection of this NCoR inhibitor results in enhanced ligand-independent activation of the TRH gene by TR- β 1 and TR- α , but not TR- β 2 (40). Their data imply that by competitively inhibiting NCoR, ligand-independent activation by the TR- β 1 and TR- α isoforms could be enhanced, and suggest that the TR- β 2 isoform may mediate ligand-independent activation via an NCoR-independent mechanism. If this is true in the pituitary, then two potential explanations for our *in vivo* observations become apparent. First, the mutant TR used in our studies was expressed as TR- β 1. If NCoR binding limits ligand-independent activation, then the degree of activation that could be mediated by this mutant that constitutively binds to NCoR would be expected to be less than that of the wild-type TR- β 2. In addition, the downregulation of the endogenous TR by the mutant transgene could further limit the degree of ligand-independent activation mediated by the wild-type TRs. It will, therefore, be important to determine whether expression of this mutation as TR- β 2 will produce a similar effect on ligand-independent activation of the TSH subunit genes.

In transfection experiments with full-length NCoR, Tagami *et al.* reported that cotransfection of NCoR augmented ligand-independent activation of the TSH- α , TSH- β , and the TRH genes by TR- β 1. In addition, a mutant TR with defective NCoR binding was defective

in ligand-independent activation (17). In contrast to the studies of Hollenberg *et al.*, they postulated that nuclear corepressor proteins may act to enhance ligand-independent TSH- β gene transcription and that TR binding to DNA may not be required. If the Tagami hypothesis is correct, we would expect that the Δ 337T mutant used in our transgenic mice should mediate enhanced ligand-independent activation, because it constitutively binds to NCoR (39). Our observation of impaired ligand-independent activation of TSH transcription does not support their hypothesis. One potential reason for this could be differences in the content of additional regulatory cofactors between the embryonic kidney cell lines in which their transfections were performed and the pituitary *in vivo*. The possibility also remains that the Δ 337T mutant TR interferes with additional pathways that govern ligand-independent activation of the TSH subunit genes by TR. Hence, it will be important to perform similar *in vivo* expression experiments using other RTH mutant TRs with different transcriptional defects than that described for Δ 337T (41–46). These studies should shed light on whether the defective ligand-independent activation of the TSH subunit genes observed with the Δ 337T mutant *in vivo* is can be generalized to other mutant TRs associated with RTH.

In summary, we have successfully demonstrated that a transgenic approach can be used to study the effect of mutant TR expression on pituitary function *in vivo*. The model can therefore be used for the *in vivo* analysis of other mutant TRs that may possess alternative transcriptional defects or are associated with varying clinical phenotypes. We have demonstrated that pituitary expression of the TR Δ 337T mutant results in impaired T₃-mediated repression, as well as impaired T₃-independent activation of TSH production. Furthermore, the discordance between basal TSH and thyroxine levels and the reversal with TRH administration, demonstrate that resistance at the level of both the pituitary and the hypothalamus is required to elevate thyroid hormone levels in patients with RTH. We suggest that central resistance, rather than pituitary resistance, may be a more appropriate term to describe the etiology of elevated thyroid hormone levels in patients with RTH.

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