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Selective Pituitary Resistance to Thyroid Hormone Produced by Expression of a Mutant Thyroid Hormone Receptor β Gene in the Pituitary Gland of Transgenic Mice

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Resistance to thyroid hormone (RTH) has been subdivided into generalized resistance (GRTH) and pituitary resistance (PRTH) based on the clinical impression of absence or presence of thyrotoxicosis. However, due to lack of objective clinical and genetic criteria, the existence of PRTH as a distinct entity became controversial. To determine what the phenotype would be if RTH was confined to the pituitary, a transgenic mouse was developed in which expression of the mutant thyroid hormone receptor (TR) β (G345R) was targeted to the pituitary thyrotrophs by placing it downstream of the mouse thyrotropin β promoter. This construct exhibited an antagonistic effect on the thyroid hormone-dependent transactivation, mediated through the wild-type TRβ1, only when cotransfected with the thyrotroph embryonic factor in a heterologous cell line. As expected the transgene was transcribed predominantly in the pituitary gland but not in liver. These mice showed a significant, though modest, increase in serum T_4 concentration. A decrease in the serum cholesterol was observed in keeping with the selective tissue hyposensitivity to thyroid hormone.

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Abbreviations used: CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GH, growth hormone; GRTH, generalized resistance to thyroid hormone; PRTH, pituitary resistance to thyroid hormone; RT, reverse transcriptase; RTH, resistance to thyroid hormone; T_3, 3,3′,5-triiodothyronine; T_4, 3,3′,5,5′-tetraiodothyronine or thyroxine; TEF, thyrotroph embryonic factor; TR, thyroid hormone receptor; TSH, thyrotropin or thyroid stimulating hormone; WT, wild-type.

Resistance to thyroid hormone (RTH) is a syndrome of reduced responsiveness to thyroid hormone. In most cases it is inherited as a dominant trait due to mutations in the thyroid hormone receptor (TR) β gene producing mutant TRβ molecules that interfere with the function of the wild-type (WT) TRβ expressed by the normal allele (1). Originally defined as a condition of inappropriate secretion of TSH, RTH was subdivided into generalized resistance to thyroid hormone (GRTH) and pituitary resistance to thyroid hormone (PRTH). This classification as GRTH and PRTH was based principally on the clinical impression of absence or presence, respectively, of symptoms and signs suggestive of thyrotoxicosis (2).

The recognition that RTH is associated with TRβ defects led to the identification of TRβ gene mutations in more than 100 families (3). In many instances different individuals with an identical mutation were categorized as having GRTH and PRTH, sometimes within the same family (4–7). Furthermore, a detailed clinical analysis of 312 patients with GRTH and 72 with PRTH showed no significant differences in the clinical features and laboratory findings between the two groups of patients, pointing to the subjective basis of the subclassification of RTH (8). Thus, the diagnosis of PRTH has become controversial (9).

To determine whether PRTH exists as a distinct entity, it is necessary to sort out the manifestations that are due to pituitary from peripheral tissue RTH. To this purpose we set out to produce a transgenic mouse that fulfilled the criteria of PRTH, by targeting the expression of a mutant TRβ to the pituitary thyrotrophs. To insure dominant negative interaction with the WT TRs, we selected the natural mutant TRβ G345R that causes severe RTH in the affected individuals (10). This mutant TRβ has been used successfully to produce RTH in the liver of mice by somatic gene transfer (11). We reasoned that the resulting pheno-

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type in the transgenic mouse could be then compared to that observed in man.

The targeted transgene consisted of the human TRβ1(G345R) under the control of the mouse TSHβ promoter. The resulting transgenic mouse manifested the expected findings of PRTH, namely, increased serum concentration of T_4 but normal TSH and reduced serum cholesterol. The former reflects the resistance of the thyrotrophs to thyroid hormone and the latter is a manifestation of the normal response of the liver to the increased circulating level of T_4. The severity of the PRTH was, however, mild. This did not seem to be due to faulty targeting or reduced expression of the transgene as shown by analysis of the mouse and human TRβ mRNAs in pituitary and liver of the animals. Species and isoform specific action of the TRβ protein needs to be considered in future work aimed to determine the PRTH phenotype.

MATERIALS AND METHODS

Preparation of DNA for microinjection: The mouse (m) TSHβ promoter fused to a mutant human (h) TRβ1 cDNA. A 1039 bp fragment (−1031 to +8) of the mTSHβ promoter (12,13) was amplified by PCR using the oligonucleotide primers 5′-AAAGCTTTGGGCATGTTTAGTTACAGTGTGG-3′ and 5′-AGGATCCTTACTGCTGTGATGACCCACTCTCCGT-3′ (sense and antisense, respectively, and the adapters containing Hind III and Bam HI restriction sites are underlined). The sequence of this DNA fragment was verified after cloning into a plasmid (pGEM-mTSHβpro). The mutant G345R hTRβ1-cDNA was excised from pcDNAI/Amp-hTRβ1(G345R) (14). The polyadenylation signal of the bovine growth hormone (GH) gene was obtained from the plasmid pRc/CMV (Invitrogen Corp., San Diego CA). These three DNA fragments were fused in the proper order and direction and subcloned into pGEM3Z (Promega, Madison, WI) to construct the plasmid pGEM-mTSHβpro-hTRβ1(G345R), as outlined in Fig. 1.

Cell culture, transfection, and luciferase assay. The human hepatoblastoma cell line HepG2 was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2, 95% air and 100% humidity. To test the tissue specific expression and level of dominant negative activity of the plasmid pGEM-mTSHβ-hTRβ1(G345R), cells were transfected with 0.5 μg of this plasmid along with 0.05 μg of the wild-type hTRβ1 plasmid driven by the human cytomegalovirus (CMV) enhancer/promoter [pcDNAI/Amp-hTRβ1(WT)], 1 μg of the thyroid hormone responsive-luciferase reporter plasmid, F2×3-Luc (14) and 0.2 μg of either the sense or antisense expression vectors of the mouse thyrotroph embryonic factor (pCMV-mTEF) (15), kindly provided by Dr. D.W.Drolet. The CMV driven expression vector, pcDNAI/Amp-hTRβ1(G345R) (14), was also used as a positive control for the determination of dominant negative effect. After 16-20 h exposure to DNA-calcium phosphate precipitate, the medium was replaced with DMEM containing 10% T_3-deficient FBS or the same medium containing 100 nM T_3. Luciferase activity was measured as described (14) and values are expressed as mean ±SD fold induction by the addition of T_3.

Production of transgenic mice. The expression cassette containing the mTSHβ promoter, the hTRβ1(G345R)cDNA and the bovine GH poly adenylation signal, was excised from the plasmid pGEM-mTSHβpro-hTRβ1(G345R) and purified through sucrose-gradient ultracentrifugation. The purified fragment was microinjected into 1-cell stage embryos of CD-1 mice. Twenty four hours later the embryos were implanted into the oviducts of pseudopregnant mice as described (16).

Presence of the transgene in the founders and offspring was tested by PCR of genomic DNA. The founders were also tested by dot blotting and by Southern blotting. For the latter, DNA samples were digested with Eco RI and Bam HI and hybridized with a ^{32}P labeled full length hTRβ cDNA probe. The sense primer complementary to the mTSHβ promoter sequence (5′-GATGCATGCTATAATAATGTCAGAG-3′) and an antisense primer complementary to the hTRβ1 sequence (5′-GCCTCTAGATGTCATCCAGCACCAAATCTGT-3′) were used to amplify the fusion construct of the transgene and the sense primer 5′-GCACAGGGAGGAAGTAGGCTGTTCT-3′ and antisense primer 5′-CCCTGGAGGCCAAAGGTCATCAATG-3′, complementary to intronic sequences that flank exon 3 of the mTRβ2 gene (17), were used in the same reaction to amplify the mouse TRβ gene as positive PCR control. DNA was obtained from 2–5 mm of tail biopsies by lysis for 1 h at 56°C in 20 μl of 50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, 0.2% SDS and 100 μg/ml proteinase K. Samples were diluted with 200 μl of water, boiled for 5 min and the debris were removed by centrifugation. Two μl with 35 pmol of each primer were used in a 35 cycle PCR at 56°C annealing temperature. For Southern and dot blotting, DNA was extracted from the lysed tail biopsies by phenol/chloroform.

Detection of transgene expression. Total RNA was purified from pituitaries and liver of the transgenic and non transgenic (wild-type) mice by the acid guanidinium isothiocyanate technique (18). Complementary DNA was synthesized by reverse transcription using oligo dT primer. The reverse transcript corresponding to 1 μg of total RNA was amplified by two consecutive PCR reactions (RT-PCR). The first using exonic oligonucleotide primers located across two introns and the second, using nested primers that amplify a region containing species specific sequence differences creating distinct restriction sites; Nhe I for mTRβ and Sma I for hTRβ. The conditions of the first PCR were 25 cycles consisting of 10 s at 96°C, 1 min at 58°C, and 1 min at 68°C with the sense primer 5′-CAAAAATCATCACACCAGCAAT-3′ and the antisense primer 5′-AGGAAGCGGCTGGCATGGCA-3′. The second PCR used as a template 0.1 μl of the first PCR product under the same conditions except for the sense primer 5′-CCAGCAATTACCAGAGTGGTGGAT-3′ and the antisense primer 5′-CGGTAATTGATATAGTGTTCAAAGG-3′. All primers match human and mouse TRβ1 exonic sequences. The final PCR products were treated with Sma I which digests the human but not mouse cDNA fragment, and with Nhe I which digests the mouse but not human cDNA. The digested PCR products were analyzed on 3% Nusieve 1% agarose gel.

Handling of mice. Mice were weaned on the fourth week after birth and were fed Purina Rodent Chow ad libitum and given tap water. They were housed, 4-5 mice per cage, in an environment of controlled 19°C temperature and 12 h alternating cycles of artificial light and darkness. All animal experiments were performed according to approved protocols at the University of Chicago. Approximately 300 μl of blood was obtained from the tail vein under light methoxyflurane (Pitman Moor, Mundelein, IL) anesthesia at 28, 38–40, 56–60 and 70 days of age. Animals were killed at 4 to 7 months of age by exsanguination through eye vein puncture under anesthesia. Tissues were immediately removed, frozen on dry ice and stored at −85°C until extraction of RNA. The blood was allowed to clot at room temperature, the serum was separated by centrifugation and stored at −20°C until analyzed in the same assay.

Tests of thyroid function. Serum thyroxine (T_4) concentration was measured by double antibody precipitation RIA (Diagnostic Products, Los Angeles, CA) modified to measure T_4 in 15 μl of serum with a sensitivity of 0.5 μg/dl (6.4 nmol/liter). Serum TSH level was determined by a heterologous, disequilibrium, double antibody precipitation RIA developed in our laboratory (19). Using 50 μl of serum, the sensitivity of this TSH assay is 0.002-0.004 ng/ml. Cholesterol was measured in 10 μl of serum by an automated clinical laboratory assay system requiring an additional 40 μl of serum for priming. Values are reported as mean±SD and P values were calculated using the unpaired Student's t test.

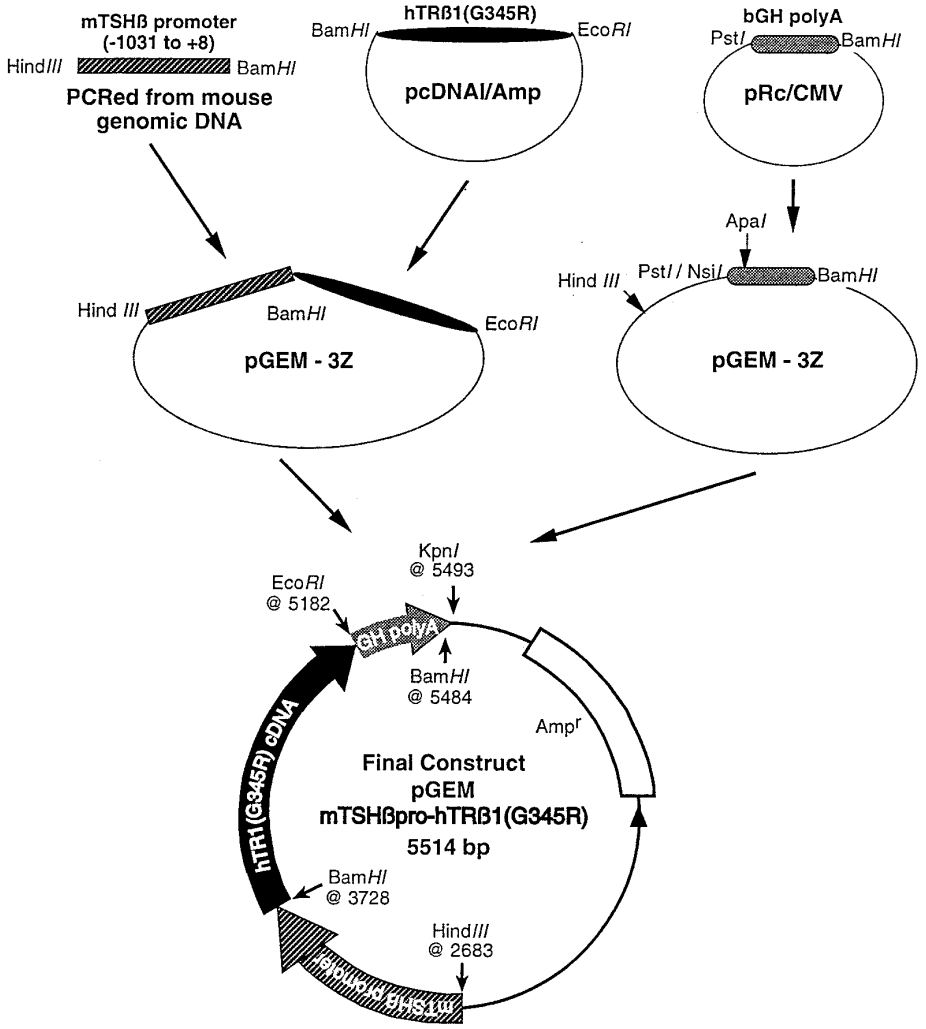

FIG. 1. Construction of the chimeric gene used as transgene that consists of dominant negative mutant hTRβ1, G345R under the control of the mTSHβ promoter. The mTSHβ promoter, generated by PCR, was inserted upstream of the hTRβ1(G345R), excised from pcDNAI/Amp, and cloned in pGEM-3Z. To add a polyadenylation (polyA) signal, this fused gene construct was digested with Eco RI, polished, and the insert was released with Hind III. The bGH polyA signal was first excised from pRc/CMV by digestion with Nsi I and Bam HI and ligated into the Pst I - Bam HI digested pGEM-3Z. It was then digested with Apa I, polished and digested again with Hind III, ready to accept the released fused gene. The final construct, mTSHβpro-hTRβ1(G345R), containing the polyA signal is shown schematically.

RESULTS

The mTSHβ promoter contains cell type specific enhancer elements which are active in thyrotroph-derived TtT97 cell but not in cells of other tissue origin, such as mouse L-cell fibroblasts (12). Furthermore, the -1031 to $+8$ region of the mTSHβ promoter has three binding sites for the TEF (13,20). To determine if the chimeric construct, mTSHβpro-hTRβ1(G345R), would be targeted to the thyrotrophs of transgenic mice, we tested whether the mutant hTRβ1 is expressed in a TEF-dependent manner. For this purpose we examined the dominant negative potency of this plasmid on the T_3-dependent transactivation of the CMV-driven hTRβ1(WT) in HepG2 cells. As shown in Fig. 2, mTSHβ-hTRβ1(G345R) inhibited hTRβ1(WT) function only when the expression vector for TEF was co-transfected. This result suggested that the construct was suitable for thyrotroph targeting.

Three founder mice carrying at least 3 copies of the mTSHpro-hTRβ1(G346R) transgene were identified, as determined by Southern blot analysis (data not shown). Two were male and one was a female. Each produced from 28-33 offspring by mating with three non transgenic (wild-type) mice. All three were able to transmit the transgene to their offspring but only those from one male exhibited a significant increase in serum T_4 concentrations and form the basis for this report.

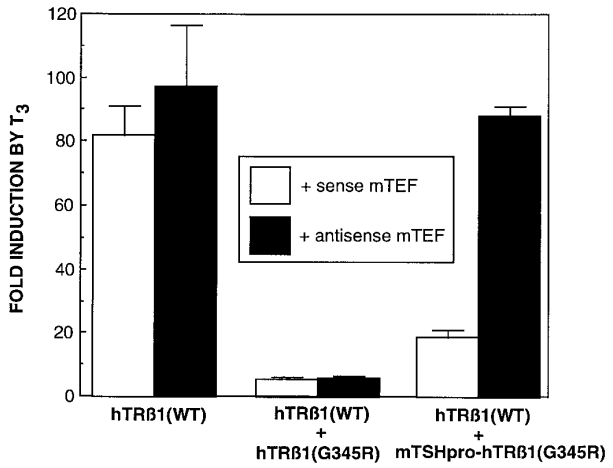

FIG. 2. Tissue specific expression of the construct, mTSHβpro-hTRβ1(G345R), prepared for the production of transgenic mice. This chimeric construct, that expresses a mutant human TRβ1 under the control of the mouse TSHβ promoter, was transfected into HepG2 cells together with the WT hTRβ1 and a luciferase reporter gene, that responds to T_3-induced transactivation mediated by the hTRβ1(WT), in the presence either the sense or antisense of the mouse thyrotroph specific factor expression vector, pCMV-mTEF. The hTRβ1(WT) and hTRβ1(G345R) driven by the CMV promoter were used as control in transfections carried out simultaneously. The T_3-induced increase in luciferase activity is expressed as fold induction of the basal activity in the absence of T_3. Note that the mutant hTRβ1, (G345R), effectively inhibits the ability of the hTRβ1(WT) to respond to T_3 by induction of luciferase activity (middle two bars). Note that the chimeric construct expressing the same mutant hTRβ1 but under the control of the mouse TSHβ promoter, inhibited the hTRβ1(WT) mediated transactivation by T_3 only if the mTEF was coexpressed (two bars on the right). Concentrations of vectors are indicated in Methods.

No significant differences were observed between the non transgenic (wild-type) and transgenic mice in terms of number of mice per litter (8 to 15), survival and body weight from birth to 6 months of age. Significantly higher serum T_4 concentrations were first observed in transgenic female mice at the age of 28 and 38 days and persisted throughout the 7 month observation. Significantly higher serum T_4 concentrations were first observed in transgenic male mice at 56 days of age and persisted at throughout the 7 month observation.

Data from 6 F1 litters (71 mice), offspring of the male founder and wild-type female mice and 4 F2 litters (40 mice) offspring from mating between transgenic F1 mice, selected for serum T_4 levels above the average, are shown in Table 1. Approximately 1/2 of the F1 and 3/4 of the F2 mice carried the transgene, suggesting that the transgenes in the founder were incorporated into a single site. Males had higher serum T_4 concentrations in both wild-type and transgenic groups of mice. In addition, transgenic F1 mice had significantly higher T_4 levels than their litter mates. The serum T_4 concentration did not increase further in F2 mice, despite being the progeny of transgenic F1 parents with above average serum T_4 levels. The mean T_4 difference between transgenic and wild-type littermates of F2 failed to achieve significant difference due to the low number of wild-type mice.

Sufficient serum from 70 day old mice was available to measure serum T_4, TSH and cholesterol concentrations in four groups of 10 mice (wild-type males and females and transgenic males and females) and results are shown in Fig. 3. Wild-type and transgenic males had higher concentrations of T_4 and cholesterol but not TSH than the corresponding groups of female mice. Mean serum T_4 levels were significantly higher in transgenic mice of both sexes but serum TSH concentrations were not significantly different. Mean T_4 values $\pm$SD for transgenic and wild-type mice, respectively, were 6.07 ± 0.87 and 4.99 ± 1.30 μg/dl for male mice; 5.06 ± 0.74 and 3.91 ± 0.62 μg/dl for female mice and 5.62 ± 0.95 and 4.45 ± 1.14 μg/dl ($P < 0.001$) for both sexes combined. The corresponding mean TSH values were: 0.060 ± 0.033 and 0.084 ± 0.061 ng/ml for male mice; 0.051 ± 0.038 and 0.070 ± 0.042 ng/ml for female mice and 0.055 ± 0.035 and 0.077 ± 0.054 ($P = 0.140$) for both sexes combined. Compatible with the predicted selective thyrotroph resistance to TSH, the mean cholesterol concentration in transgenic mice of both sexes was lower and the combined means were significantly different. Mean values $\pm$SD for transgenic and wild-type mice, respectively, were 127 ± 11 and 137 ± 33 mg/dl for male mice; 95 ± 8 and 121 ± 32 mg/dl for female mice and 112 ± 18 and 130 ± 32 mg/dl ($P < 0.05$) for both sexes combined.

To confirm the postulated mechanism for the selective resistance to thyroid hormone at the thyrotroph level, expression of the transgene in the pituitary gland and in the liver was tested by RT-PCR. As shown in Fig. 4, the PCR product from pituitary gland RNA of the wild-type littermate was completely digested by Nhe I but not by Sma I, the enzymes that digests specifically DNA fragment amplified from mouse and human TRβcDNA, respectively. Similar results were obtained using cDNA generated from liver RNA of the wild-type mouse and liver RNA from the two transgenic mice. In the latter, less than 10% failed to completely digest with Nhe I or was digested by Sma I, that can be attributed to human TRβcDNA. This was likely due to contamination of the RNA preparation with genomic DNA containing the chimeric construct, although expression of a very small amount of transgenic cannot be excluded. However, in sharp contrast, most of the PCR product generated from pituitary gland RNA of the transgenic mice was digested by Sma I and not by Nhe I, indicating the predominate expression of the hTRβ1(G345R) transgene. Although this RT-PCR

TABLE 1

Number of Transgenic Mice and T_4 Concentrations in F1 Offspring of the Founder Male and in F2 Offspring of F1 Transgenic Mice at 56 and 60 Days, Respectively

	Male			Female			Both sexes		
Generation	WT	Transgenic	*P* value	WT	Transgenic	*P* value	WT	Transgenic	*P* value
Number of mice									
F1	15	20		23	17		38	37	
F2	7	17		3	13		10	30	
T_4 (μg/dl)									
F1	5.44 ± 1.12	6.29 ± 1.00	0.025	4.73 ± 0.68	5.59 ± 1.03	0.003	5.01 ± 0.93	5.96 ± 1.06	<0.001
F2	4.96 ± 1.05	5.75 ± 1.08	0.114	4.43 ± 0.12	5.53 ± 1.29	0.172	4.80 ± 0.90	5.59 ± 1.22	0.040
P (F1 vs F2)	0.399	0.123		0.469	0.882		0.531	0.184	

Note. 6 litters are included in F1 and 4 litters in F2. T_4 concentrations are expressed as means ± SD. *P* values for T_4 differences among sexes were 0.019 for wild type, and 0.046 for transgenic of F1; 0.425 for wild type and 0.620 for transgenic of F2.

provides only semi-quantitative information, it can be concluded from these results that the transgene was expressed efficiently in the pituitary gland and to a far lesser extent, if at all, in liver.

DISCUSSION

Mice deficient in TRα and TRβ have been developed by homologous recombination in embryonic cells (21,22). Although they have proven to be invaluable in the understanding of TRα and TRβ function, respectively, they are not models for the most common form of dominantly inherited RTH in which both mutant and WT receptor genes are expressed. The ideal animal model for RTH, a knock-in (23), has not yet been generated and transgenic mice bearing a mutant TRβ1 linked to the β-actin promoter, in order to promote ubiquitous expression of the transgene, produced minimal changes in serum T_4 concentration (24).

To date the only animal model of RTH in which dominant negative effect of the transgene was documented at the tissue level, is that produced by somatic gene transfer of a mutant hTRβ1 (11). Delivery of the mutant gene to liver was achieved by means of the replica-

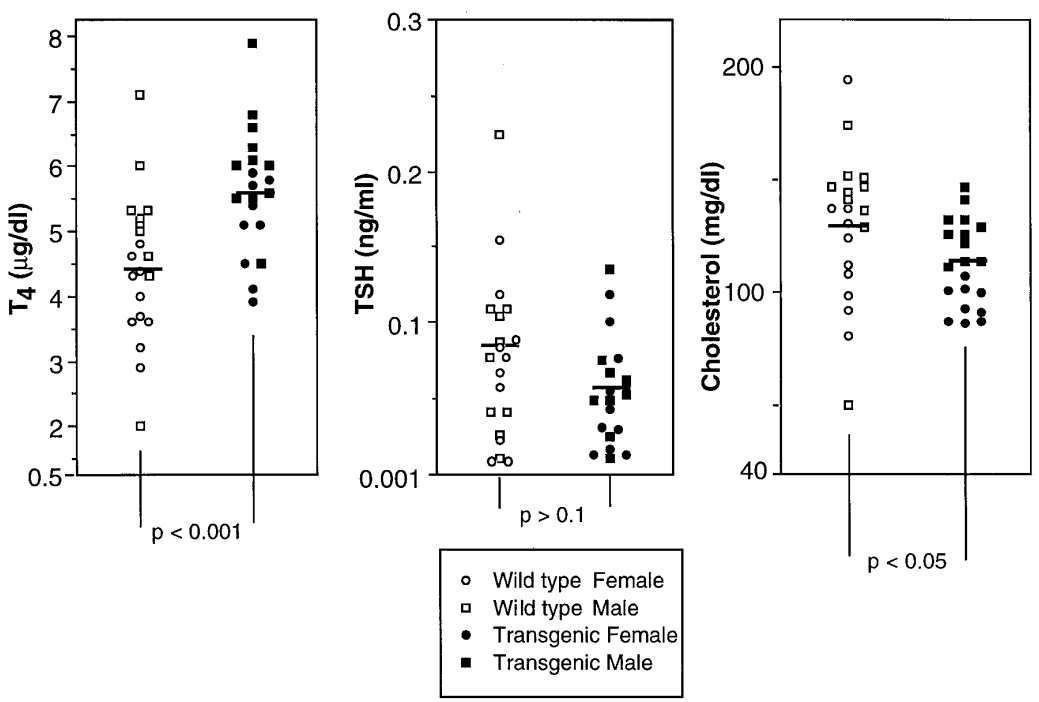

FIG 3. Serum T_4 TSH and cholesterol concentrations in F1 transgenic mice expressing a the mutant hTRβ1(G345R) under the control of the mTSHβ promoter and their wild-type litter mates.

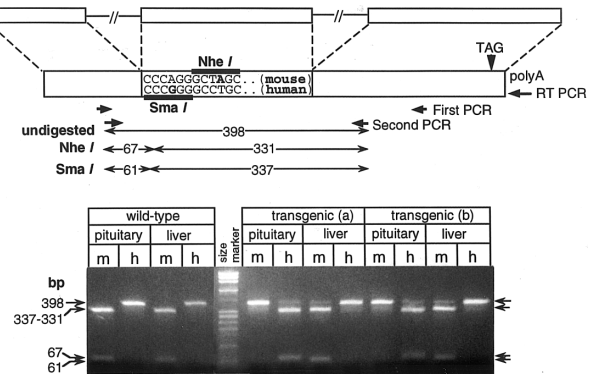

FIG. 4. Detection of transgene expression in pituitary and liver tissue. cDNA synthesized by reverse transcription of RNA, extracted from pituitary and liver of a non-transgenic (wild-type) and two transgenic mice, was submitted to two rounds of PCR using primers complimentary to absolutely identical sequences of human and mouse TRβ1 and TRβ2. The resulting fragment of 398 bp was digested with Nhe I, specific for the mouse (m) TRβ, and Sma I, specific for the human (h) TRβ, as shown schematically on top of the figure. Note that RNA derived from the wild-type mouse had only mouse specific TRβ mRNA in both pituitary and liver. In contrast, the two transgenic mice, (a) and (b), express predominantly human TRβ mRNA in the pituitary (the transgene), while the liver had virtually only mouse TRβ mRNA.

tion-defective adenovirus. In these animals, administration of T_3 produced blunted responses of serum cholesterol and the mRNAs of several thyroid hormone-dependent liver proteins. Unfortunately, gene delivery of the mutant hTRβ to the pituitary gland by means of adenovirus administered intravenously is very poor and is insufficient to produce a measurable effect.

The purpose of this work was to develop a mouse with RTH confined to the thyrotrophs in order to determine the phenotype of PRTH. We expected that from the observations of this mouse model, the corresponding phenotype in man could be deduced to help clear the controversy regarding the existence of PRTH in man. In vitro testing of the chimeric construct, developed for this purpose, showed that expression of the transgene was dependent on the presence of the thyrotroph specific factor, TEF. Thus, it is not surprising that the mutant hTRβ1 transgene was expressed preferentially and possibly exclusively in the thyrotrophs. The small amount of hTRβ1 in liver was likely amplified from contaminant transgene in DNA rather than from hTRβ1 mRNA.

We were, however, surprised to find that the strong signal of the transgene, manifested by the predominant accumulation of hTRβ1 mRNA in the pituitary gland, produced a weak phenotype of PRTH. Indeed, while about 90% of the TRβ mRNA in pituitaries of transgenic animals were derived from mutant hTRβ1, the increase in serum T_4 was on the average only 19%, and produced a 13% decline in the mean serum cholesterol. Several hypotheses can be advanced to explain this discrepancy. First, the hTRβ1 may not be efficiently translated or may be degraded more rapidly. This could not be verified because of the unavailability of mutation specific (one amino acid) or species specific antibody. Second, the dominant negative effect of the mutant hTRβ1 may be reduced because of species or isoform differences in competing with the endogenous mTRβ2. Such effect has not been shown in vitro, though indirect preliminary data suggest that it may exist (25).

From this first successful attempt to produce a mouse with PRTH, it appears that in order to study the putative human phenotype, the transgenic mouse model would require the targeted expression of a species and isoform specific transgene. Such a transgene could be the mouse equivalent of a mutant TRβ naturally occurring in man. It should be placed on a backbone of a mouse TRβ2 sequence. Though the efficiency of targeting may be debatable in view of the finding of Camper, et al and Yusta et al (26,27) that showed a stronger enhancer/promoter activity of the pituitary glycoprotein α-subunit, our results suggest that this mTSHβ promoter directed efficiently transgene targeting.

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