

# A targeted dominant negative mutation of the thyroid hormone $\alpha$ 1 receptor causes increased mortality, infertility, and dwarfism in mice

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Mutations in the thyroid hormone receptor  $\beta$  (TR $\beta$ ) gene result in resistance to thyroid hormone. However, it is unknown whether mutations in the TR $\alpha$  gene could lead to a similar disease. To address this question, we prepared mutant mice by targeting mutant thyroid hormone receptor kindred PV (PV) mutation to the TR $\alpha$  gene locus by means of homologous recombination (TR $\alpha$ 1PV mice). The PV mutation was derived from a patient with severe resistance to thyroid hormone that has a frameshift of the C-terminal 14 aa of TR $\beta$ 1. We knocked in the same PV mutation to the corresponding TR $\alpha$  gene locus to compare the phenotypes of TR $\alpha$ 1<sup>PV/+</sup> mice with those of TR $\beta$ 1<sup>PV/+</sup> mice. TR $\alpha$ 1<sup>PV/+</sup> mice were viable, indicating that the mutation of the TR $\alpha$  gene is not embryonic lethal. In drastic contrast to the TR $\beta$ 1<sup>PV/+</sup> mice, which do not exhibit a growth abnormality, TR $\alpha$ 1<sup>PV/+</sup> mice were dwarfs. These dwarfs exhibited increased mortality and reduced fertility. In contrast to TR $\beta$ 1<sup>PV/+</sup> mice, which have a hyperactive thyroid, TR $\alpha$ 1<sup>PV/+</sup> mice exhibited mild thyroid failure. The *in vivo* pattern of abnormal regulation of T3 target genes in TR $\alpha$ 1<sup>PV/+</sup> mice was unique from those of TR $\beta$ 1<sup>PV/+</sup> mice. The distinct phenotypes exhibited by TR $\alpha$ 1<sup>PV/+</sup> and TR $\beta$ 1<sup>PV/+</sup> mice indicate that the *in vivo* functions of TR mutants are isoform-dependent. The TR $\alpha$ 1<sup>PV/+</sup> mice may be used as a tool to uncover human diseases associated with mutations in the TR $\alpha$  gene and, furthermore, to understand the molecular mechanisms by which TR isoforms exert their biological activities.

The thyroid hormone, T3, has profound effects on growth, development, and homeostasis. These biological activities are mediated mainly by thyroid hormone receptors (TRs) that are ligand-dependent transcription factors (1). Three ligand-binding TR isoforms have been identified, TR $\alpha$ 1, TR $\beta$ 1, and TR $\beta$ 2, which are derived from the TR $\alpha$  and TR $\beta$  genes, by alternative splicing of the primary transcripts. Each TR isoform has a unique developmental and tissue-specific expression (1, 2). Studies using a gene-inactivation approach indicate that these TR isoforms have distinct and common functions *in vivo* (3). The action of TR depends not only on the types of DNA elements on the T3 target genes but also on a host of corepressor and coactivator proteins (1, 2).

Resistance to thyroid hormone (RTH) is a syndrome characterized by reduction in the sensitivity of tissues to the action of thyroid hormones. Mutations in the TR $\beta$  gene result in TR $\beta$  mutants, which mediate the clinical phenotype by interfering with transcription of T3-regulated genes by means of a dominant negative effect. This disease is manifested by elevated levels of circulating thyroid hormones associated with normal or high levels of serum thyroid-stimulating hormone (TSH) (4). The other clinical features include short stature, decreased weight, tachycardia, cardiac disease and hearing loss, attention-deficit hyperactivity disorder, decreased IQ, and dyslexia (4). Based on the extensive sequence homology in the functional domains of  $\alpha$  and  $\beta$  TR and their similar *in vitro* functional characteristics, it is intriguing that no TR $\alpha$  mutations have ever been found in RTH patients.

It has been postulated that mutations of the TR $\alpha$  gene could be embryonic lethal, inconsequential, or not associated with abnormalities of RTH. To test these possibilities and to understand further the functions of TR $\alpha$  mutants *in vivo*, we prepared mutant mice by targeting the PV mutation to the TR $\alpha$  gene locus by means of homologous recombination (TR $\alpha$ 1PV mice). PV has a mutation in exon 10 of the TR $\beta$  gene, a C insertion at codon 448, which produces a frameshift of the carboxyl-terminal 14 aa of TR $\beta$ 1 (5). We knocked in the same PV mutation to the corresponding TR $\alpha$  gene locus and compared the phenotypes of mice with a similar mutation at the TR $\beta$  locus (TR $\beta$ 1PV mice; ref. 6). TR $\alpha$ 1<sup>PV/+</sup> mice show significant mortality, and TR $\alpha$ 1<sup>PV/PV</sup> mice were rarely obtained and died shortly after birth. TR $\alpha$ 1<sup>PV/+</sup> mice were dwarfs and exhibited increased mortality, reduced fertility, and mild thyroid failure. These different phenotypes in the pituitary–thyroid axis of TR $\alpha$ 1<sup>PV/+</sup> and TR $\beta$ 1<sup>PV/+</sup> mice are consistent with the fact that no TR $\alpha$  mutations could be identified in RTH patients. The abnormal regulation patterns of T3 target genes differed in the tissues of TR $\alpha$ 1<sup>PV/+</sup> and TR $\beta$ 1<sup>PV/+</sup> mice. The distinct phenotypes exhibited by TR $\alpha$ 1<sup>PV/+</sup> mice indicate that the *in vivo* signaling pathways of TR mutants are isoform-dependent.

## Materials and Methods

**Preparation of TR $\alpha$ 1PV Mutant Mice.** The targeting vector, pmTR $\alpha$ -1, was modified as described by Wikstrom *et al.* (7). A 522-bp fragment containing exons 9 and 10 of the TR $\alpha$  gene was released by digesting the plasmid with *Stu*I and *Sal*I. In its place, a 209-bp fragment containing the mutated TR $\alpha$  sequence (TR $\alpha$ 1PV) was ligated to give an intermediate plasmid (pmTR $\alpha$ 1PV-Intermediate). This 209-bp fragment was obtained by PCR by using the mouse TR $\alpha$ 1 cDNA as a template and the following two primers: 5' primer, 5'-AGTCAGGAGGCCTACTGCTGGCGTTTGAGCACTAC; 3' primer, 5'-TGAGTCGTCGACAGATCTTCAGTCTAATCCTCGAACGGATCCAAGAACAAGGGGGGAAGAGTTCTGTGGGGGCACTCGACTTTCATGTGGAG.

The 3' primer contained a C insertion at the mouse TR $\alpha$ 1 cDNA nucleotide position 1173 followed by a human PV sequence, and a *Bam*HI site was placed downstream of the stop codon (see Fig. 2A). The resulting DNA fragment contained a *Stu*I and *Sal*I at the 5' and 3' ends, respectively. The sequence of this insert was confirmed by DNA sequencing.

Abbreviations: T3, 3,3',5'-triiodo-L-thyronine; T4, L-thyroxine; TR, thyroid hormone receptor; GH, growth hormone; RTH, resistance to thyroid hormone; PV, mutant thyroid hormone receptor kindred PV; TSH, thyroid-stimulating hormone; MBP, myelin basic protein; ME, malic enzyme;  $\alpha$ -SU,  $\alpha$ -glycoprotein subunit; D1, type 1 deiodinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSV-TK, herpes simplex virus thymidine kinase.

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To enhance the selection efficiency, a 1.854-kb herpes simplex virus thymidine kinase (HSV-TK) cDNA was placed 1.2 kb upstream of the pmTR $\alpha$ 1PV-Intermediate. The HSV-TK cDNA was restricted from pPyfEnh TK (8) by *Xho*I and *Hind*III and blunted by using Klenow fragment. pmTR $\alpha$ 1PV-Intermediate was restricted with *Eco*RV, and both ends were blunted by treating with Klenow. The blunt-ended HSV-TK cDNA was ligated to the blunt-ended pmTR $\alpha$ 1PV-Intermediate to give the final targeting vector that we designated as pTR $\alpha$ 1PV (see Fig. 2A). The inserted HSV-TK cDNA was confirmed by sequencing.

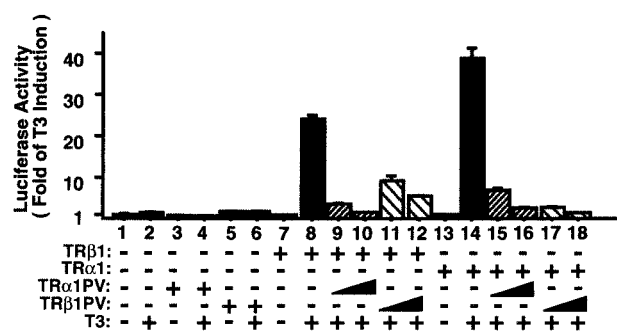
The targeting vector was linearized by *Xba*I digestion and transfected (25  $\mu$ g) into the TC-1 embryonic stem cells. The selection of the recombinant clones was performed as described (6). From six transfection experiments, six positive recombinant clones were identified by Southern blotting analysis. The recombinant clones were microinjected into C57BL/6J blastocysts to produce chimeras that were crossed with NIH Black Swiss mice to establish germ-line transmission. Mice harboring the targeted PV mutation were designated as TR $\alpha$ 1PV mice (see Fig. 2C).

F-2 mice were genotyped by PCR by using two sets of primers. For the identification of the wild-type allele, the sequence of the 5' primer (W5) is 5'-TCTTGTCTCCTCGGGCCTCATGCC, and the 3' primer (W3) is 5'-CTCTGGCCGCTGAGGCTTTAG. For the identification of the mutant allele, the sequence of the 5' primer (M5) is 5'-CTGTGCGTGGACAAGATCGAGA, and the 3' primer (M3) is 5'-CTGACCGCTTCCTCGTGCTTTACG. PCRs were carried out for 35 cycles (94°C, 30 s; 63°C, 30 s; 74°C, 30 s for the determination of the wild-type allele) (94°C, 30 s; 63°C, 30 s; 74°C, 30 s for the mutant allele) in a buffer containing MgCl<sub>2</sub> by using TaKaRa EX-*Taq* polymerase (Intergen, Purchase, NY).

**Northern Blot Analyses.** Total RNA (5–10  $\mu$ g) was used for Northern blot analysis. After electrophoresis, RNA was transferred onto membranes (Hybond N+, Amersham Pharmacia), which were hybridized with appropriate probes. cDNA probes for the mouse wild-type TR $\alpha$ 1, glycoprotein  $\alpha$  common subunit ( $\alpha$ -SU), TSH $\beta$ , growth hormone (GH), malic enzyme (ME), type 1 deiodinase (D1), myelin basic protein (MBP), and Pcp2 genes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by using a random primer hexamer protocol. For quantification, the intensities of the mRNA bands were normalized against the intensities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Thus, the blots were stripped and rehybridized with <sup>32</sup>P-labeled cDNA for GAPDH. The quantification of the bands was performed by using a Molecular Dynamics PhosphorImager.

**Transient Transfection Assay.** Transient transfection experiments were carried out by using CV1 cells as described by Zhu *et al.* (9). Briefly, cells were transfected with plasmids containing the reporter pdoublePal-TK-Luc (0.4  $\mu$ g; a generous gift of J. L. Jameson, Northwestern University School of Medicine, Chicago) and the expression plasmid for TR $\beta$ 1 (pCLC51; 0.2  $\mu$ g), or for TR $\alpha$ 1 (pCLC61; 0.2  $\mu$ g) in the absence or presence of the expression plasmid for TR $\alpha$ 1PV mutant (pCDNA3.1 TR $\alpha$ 1PV; 0.2 or 1  $\mu$ g) or for TR $\beta$ 1PV (pCLC51PV; 0.2 or 1  $\mu$ g). Five hours after transfection, cells were incubated in T3-deficient medium (Td medium). Twenty hours after transfection, T3 (100 nM) was added and incubated for an additional 24 h. Cells were lysed, and the luciferase activity was determined. The values were normalized against the protein concentrations that were determined by the BCA protein assay kit (Pierce).

**Hormone Assays.** The serum levels of total T4 (TT4) and T3 (TT3) were determined by using a Gamma Coat T4 and T3 assay RIA kit, respectively, and free T4 (fT4) was determined by using Clinical Assays Gamma Coat Free T4 (direct one-step) (Dia-Solin, Stillwater, MN) according to the manufacturer's instructions. TSH levels in serum were measured as described (10).



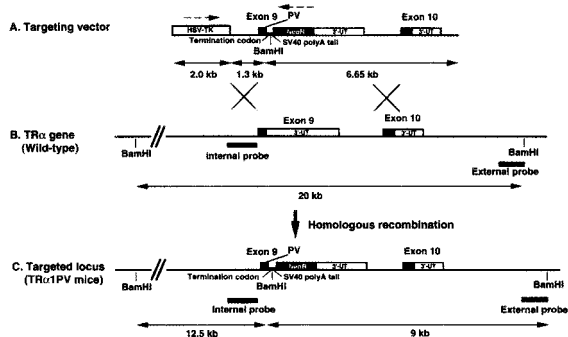
**Fig. 1.** TR $\alpha$ 1PV and TR $\beta$ 1PV repress the transactivation activity of w-TR $\alpha$ 1 and w-TR $\beta$ 1. CV1 cells ( $1.25 \times 10^5$  cells/well) were cotransfected with pdoublePal-TK-Luc (0.4  $\mu$ g) and the expression plasmid for w-TR $\alpha$ 1 (pCLC61; 0.2  $\mu$ g) or w-TR $\beta$ 1 (pCLC51; 0.2  $\mu$ g) in the absence or presence of TR $\alpha$ 1PV (pCDNA3.1 TR $\alpha$ 1PV; 0.2 or 1  $\mu$ g) or TR $\beta$ 1PV (pCLC51PV; 0.2 or 1  $\mu$ g). The luciferase activity was determined as described in *Materials and Methods*. The data are expressed as mean  $\pm$  SD ( $n = 3$ ).

**Data Analysis.** All data are expressed as mean  $\pm$  SE. Statistical analyses used the Student's *t* test, and  $P < 0.05$  was considered significant. Group analyses were also conducted by using single-factor ANOVA.

## Results

**The TR $\alpha$ 1PV Interferes with the Transactivation Activity of the Wild-Type TR $\alpha$ 1 and TR $\beta$ 1 *in Vitro*.** Because naturally occurring TR $\alpha$ 1 mutants have never been identified in RTH patients, we first evaluated whether an identical mutation in the corresponding TR $\alpha$  gene resulted in functional impairment of the wild-type TRs. An expression vector containing both the cytomegalovirus and T7 promoters was constructed by a C insertion at the nucleotide position 1180 of the mouse TR $\alpha$ 1 cDNA followed by the human TR $\beta$ 1PV mutant sequence (5). This C insertion led to a frameshift mutation to give the TR $\alpha$ 1PV protein sequence. TR $\alpha$ 1PV protein had a total of 409 aa, 1 aa shorter than the w-TR $\alpha$ 1. We prepared TR $\alpha$ 1PV protein by *in vitro* transcription/translation and evaluated its T3 and transactivation activities. Similar to that found for TR $\beta$ 1PV (11), TR $\alpha$ 1PV had totally lost the T3 binding activity (data not shown). Fig. 1 shows that, consistent with the loss of T3 binding activity and similar to TR $\beta$ 1PV (bar 6 vs. 2), TR $\alpha$ 1PV had also lost its T3-dependent transactivation activity (bar 4 vs. 2). Comparison of bars 9 and 10 with bar 8 shows that the T3-dependent transactivation activity mediated by w-TR $\beta$ 1 was repressed 85% and 92% by the cotransfection of the TR $\alpha$ 1PV expression plasmid at the TR $\alpha$ 1PV/w-TR $\beta$ 1 ratios of 1 and 5, respectively. A similar extent of repression on the w-TR $\alpha$ 1 transactivation activity by TR $\alpha$ 1PV (TR $\alpha$ 1PV/w-TR $\alpha$ 1 plasmid ratios of 1 and 5 for bars 15 and 16, respectively) was also detected (80% and 92% repression shown in bars 15 and 16 vs. bar 14, respectively). These results indicate that TR $\alpha$ 1PV was a potent dominant negative mutant receptor that interferes with the transcriptional activity of the w-TR $\beta$ 1 and w-TR $\alpha$ 1. Interesting, we found that TR $\beta$ 1PV exerted less dominant negative action than TR $\alpha$ 1PV on w-TR $\beta$ 1 transactivation activity (65% and 80% repression at TR $\beta$ 1PV/w-TR $\beta$ 1 plasmid ratios of 1 and 5 for bars 11 and 12, respectively) but exerted a slightly more potent dominant negative action than TR $\alpha$ 1PV on w-TR $\alpha$ 1 transcriptional activity (92% and 97% repression at TR $\beta$ 1PV/w-TR $\alpha$ 1 plasmid ratios of 1 and 5 for bars 17 and 18, respectively).

**Generation of Mice with Targeted TR $\alpha$ 1 Mutant PV.** Fig. 2A shows the targeting vector that contained the PV mutation in exon 9 (see *Materials and Methods*). Other features of the 10-kb targeting vector included a *Bam*HI site, which was placed immediately after the stop codon for screening purposes (Fig. 2A). Positive



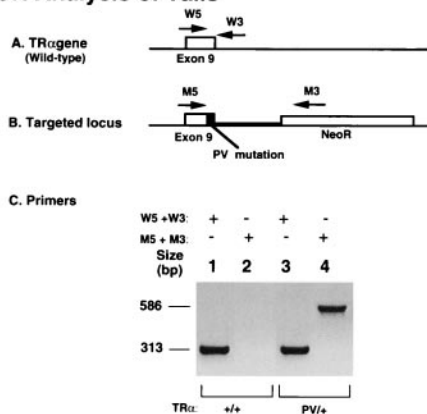
**Fig. 2.** Targeting of the PV mutation onto the TR $\alpha$  gene locus by homologous recombination. (A) Schematic representation of the TR $\alpha$ 1PV targeting vector; the 10-kb targeting vector contains the PV mutation in exon 9. The locations of the NeoR and the HSV-TK genes are indicated. (B) Restriction map of the wild-type TR $\alpha$  gene locus; the expected sizes of the fragments digested by BamHI, which were detected by internal and external probes, respectively, are indicated. (C) Restriction map of the targeted TR $\alpha$ 1PV gene locus. The expected sizes of the fragments digested by BamHI, which were detected by the internal and external probes, are indicated. SV40, simian virus 40.

embryonic stem cells were identified by using Southern blot analysis by both internal and external probes (Fig. 2A). BamHI digests of the genomic DNA revealed 20- and 12.5-kb fragments when hybridized with the internal probe encompassing the PV mutation site in exon 9 (Fig. 2C; data not shown), whereas the wild-type clones showed the expected 20-kb band (Fig. 2B). When the same BamHI digests were hybridized with the external probe (Fig. 2C), a 9-kb fragment was detected together with the 20-kb fragment derived from the wild-type allele (Fig. 2C; data not shown). These results indicate that the PV mutation was correctly targeted onto the TR $\alpha$  gene locus.

The mice carrying the TR $\alpha$ 1PV gene were identified by PCR analysis by using genomic DNA prepared from mouse tails (Fig. 3). Using the primers pairs specifically for detecting the wild-type (W5 and W3; Fig. 3A) and mutant genes (M5 and M3; Fig. 3B), the PCR products with sizes of 586 bp (lane 4 of Fig. 3C) and 313 bp (lanes 1 and 3 of Fig. 3C) were detected for the mutant mice and wild-type mice, respectively. The genotypes of the mutant mice were further confirmed by Southern blot analysis (data not shown).

**Fertility and Survival.** TR $\alpha$ 1<sup>PV/+</sup> mice were fertile; however, the fertility of TR $\alpha$ 1<sup>PV/+</sup> mice was reduced as shown by decreases in the

### PCR Analysis of Tails



**Fig. 3.** Genotyping of TR $\alpha$ 1<sup>PV/+</sup> mice by PCR. Using the primer pairs of M5 and M3 shown in B, a 586-bp fragment was obtained from TR $\alpha$ 1<sup>PV/+</sup> mice (C, lane 4). Using the primer pairs W5 and W3 as indicated in A, a 313-bp fragment was obtained from the genomic DNA of the same mice (C, lane 3). When the 586-bp fragment was absent, the mice were wild type (C, lane 2).

**Table 1. Reduced fertility in TR $\alpha$ 1<sup>PV/+</sup> mice**

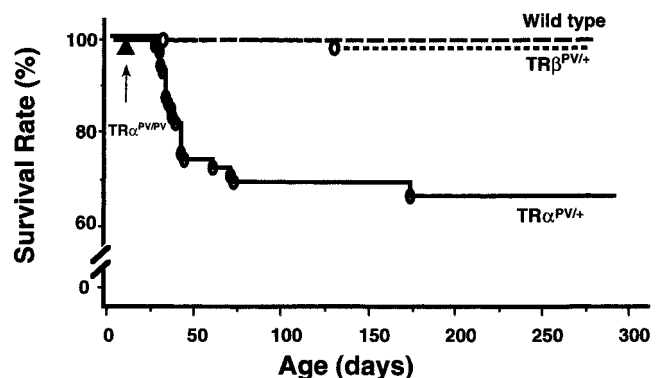
Mice	Pregnancy/mating, %	Litter size, number of pups
Wild-type $\times$ wild-type	>99 (n = 10)	10–12
F-TR $\alpha$ 1 <sup>PV/+</sup> $\times$ M-wild-type	62.5 (n = 8)	3.2
M-TR $\alpha$ 1 <sup>PV/+</sup> $\times$ F-wild-type	62.5 (n = 8)	5.1
M-TR $\alpha$ 1 <sup>PV/+</sup> $\times$ F-TR $\alpha$ 1 <sup>PV/+</sup>	21.4 (n = 14)	3.0

frequencies of pregnancy and the litter size (Table 1). Compared with the wild-type mice, the success rate in mating was reduced to 62.5% for both female and male TR $\alpha$ 1<sup>PV/+</sup> mice (n = 8). The litter size was reduced from 10–12 to 3–5 per litter. When both female and male TR $\alpha$ 1<sup>PV/+</sup> mice were used in the mating, the frequency of pregnancy was further reduced to 21.4%, and the average litter size was 3.0 (n = 14). These results indicate that both the male and female TR $\alpha$ 1<sup>PV/+</sup> mice were less fertile than wild-type mice. As a result of the reduction in the fertility in both male and female TR $\alpha$ 1<sup>PV/+</sup> mice, we had no success in obtaining homozygous offspring despite numerous repeated attempts for 18 months. There was only one homozygous neonate that died shortly after birth with unknown cause.

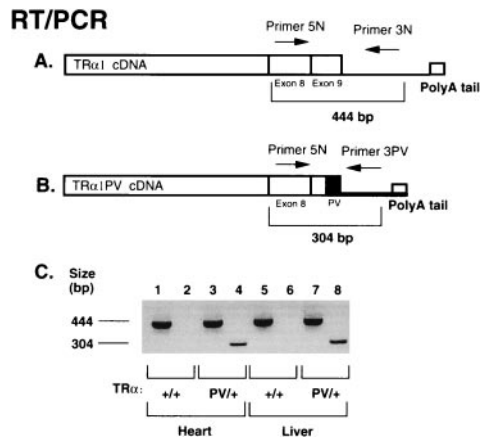
In addition, even though both male and female TR $\alpha$ 1<sup>PV/+</sup> mice were viable, the mortality rate was high, in that 30% of mice died (24 of 79). This finding is in contrast to the absence of death for the wild-type siblings (0 of 99) and tow deaths for the TR $\beta$ <sup>PV/+</sup> mice (2 of 49) during the same observation period (Fig. 4). Death occurred frequently before reaching adulthood, in that 83% (20 of 24) and 96% (23 of 24) of deaths occurred by 6 weeks and 11 weeks of age, respectively. No obvious cause of death could be found. These results suggest that mutation of the TR $\alpha$  gene seemed to compromise the ability of TR $\alpha$ 1<sup>PV/+</sup> mice to survive, especially during early life. Because it was not possible to obtain homozygous mice, the characterization of the phenotypes was focused on the TR $\alpha$ 1<sup>PV/+</sup> mice.

### The TR $\alpha$ 1PV Gene Is Expressed in All Expected T3 Target Tissues Examined.

Two sets of primers flanking the mutated exon 9 were used to assess the expressed RNA in different tissues. Using the primer pairs of 5N and 3N (Fig. 5A) or 5N and 3PV (Fig. 5B), cDNA fragments with sizes of 444 bp or 304 bp were expected for the expression of wild-type and mutant alleles, respectively. Representative results from the expression of mutant PV in the heart and liver are shown in Fig. 5C. For the TR $\alpha$ 1<sup>PV/+</sup> mice, both 444 and 304 bp were detected (lanes 3 and 4 for the heart and 7 and 8 for the liver; Fig. 5C). Using this analysis, we found that the TR $\alpha$ 1PV allele was expressed in the cerebrum, cere-



**Fig. 4.** Comparison of survival rates of TR $\alpha$ 1PV and TR $\beta$ <sup>PV/+</sup> mice. The survival rates of TR $\alpha$ 1<sup>PV/+</sup> (n = 79), TR $\beta$ <sup>PV/+</sup> (n = 49), and wild-type sibling (n = 99) mice were determined within 300 days.



**Fig. 5.** Expression of TR $\alpha$ 1PV mRNA in the tissues of TR $\alpha$ 1PV mice by reverse transcription–PCR (RT-PCR) analysis. Total RNA was isolated from the heart (C, lanes 1–4) and liver (C, lanes 5–8). RT-PCR was carried out by using the primer pairs of 5N and 3N as shown in A and 5N and 3PV shown in B for the w-TR $\alpha$ 1 cDNA and TR $\alpha$ 1PV cDNA, respectively. The 444-bp and 304-bp fragments represent the expression of the wild-type and mutant alleles, respectively, as shown in C. The genotypes are marked.

bellum, pituitary, muscle, white and brown adipocytes, lung, spleen, and kidney (data not shown), indicating that the PV mutant allele was detected in the expected T3 target tissues.

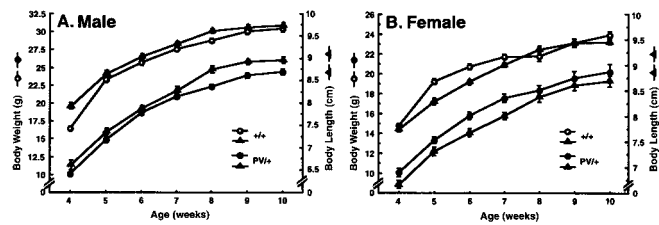
The expression of the TR $\alpha$ 1PV allele was further confirmed by Northern blot analysis by using cDNA encoding TR $\alpha$ 1. Wild-type TR $\alpha$ 1 mRNA was expressed with a size of 5.0 kb (12) and the TR $\alpha$ 1PV mRNA as a size of 1.8 kb of TR $\alpha$ 1<sup>PV/+</sup> mice. In TR $\alpha$ 1<sup>PV/+</sup> mice, the expression of TR $\alpha$ 1 mRNA was weaker than that in wild-type mice as expected (data not shown).

**TR $\alpha$ 1<sup>PV/+</sup> Mice Are Dwarfs.** TR $\alpha$ 1<sup>PV/+</sup> mice exhibited severe growth impairment evident shortly after birth. TR $\alpha$ 1<sup>PV/+</sup> mice were dwarfs, and by 4 weeks of age, the mean weight of TR $\alpha$ 1<sup>PV/+</sup> male pups was  $10.07 \pm 2.9$  g ( $n = 10$ ), which was  $\approx 40\%$  less than that of their wild-type siblings  $16.39 \pm 1.39$  g ( $n = 23$ ;  $P < 0.0001$ ; Fig. 6A). The lengths of male TR $\alpha$ 1<sup>PV/+</sup> were also shorter. By 4 weeks of age, the mean length of TR $\alpha$ 1<sup>PV/+</sup> male pups was  $6.6 \pm 0.3$  cm ( $n = 10$ ), which was 17% shorter than that of their wild-type siblings at  $8.0 \pm 0.3$  cm ( $n = 23$ ;  $P < 0.0001$ ; Fig. 6A). The weight and length differences persisted into adulthood.

Similar growth impairment was also detected in female TR $\alpha$ 1<sup>PV/+</sup> mice. At the age of 4 weeks, the mean weight of female TR $\alpha$ 1<sup>PV/+</sup> pups was  $10.08 \pm 1.12$  g ( $n = 7$ ), which was 30% less than that of their wild-type siblings ( $14.78 \pm 1.2$  g;  $n = 23$ ), and the mean length was  $6.6 \pm 0.2$  cm ( $n = 7$ ), which was 15% shorter than that of their wild-type siblings ( $7.7 \pm 0.2$  cm;  $n = 23$ ;  $P < 0.0001$ ; Fig. 6B).

**Mild Thyroid Failure in TR $\alpha$ 1<sup>PV/+</sup> Mice.** To evaluate whether the mutation of the TR $\alpha$  gene leads to functional impairment in the pituitary–thyroid axis, we determined the serum levels of TT4, fT4, TT3, and TSH. As shown in Fig. 7A, the mean TT4 concentration was  $4.77 \pm 1.07$   $\mu$ g/dl ( $n = 21$ ) in TR $\alpha$ 1<sup>PV/+</sup> mice, which was not significantly different from that in the wild-type mice ( $4.48 \pm 1.13$   $\mu$ g/dl;  $n = 27$ ). This was further confirmed by fT4 (Fig. 7B) in that the mean concentration was  $49.79 \pm 7.72$  pmol/liter ( $n = 13$ ) and  $44.68 \pm 8.65$  pmol/liter ( $n = 19$ ) for the TR $\alpha$ 1<sup>PV/+</sup> and wild-type mice, respectively ( $P = 0.1$ , no significant differences).

However, as shown in Fig. 7C, a significant 15% increase was found in the mean TT3 concentration for TR $\alpha$ 1<sup>PV/+</sup> mice ( $1.67 \pm 0.23$  ng/ml;  $n = 21$ ) as compared with their wild-type siblings [ $1.43 \pm 0.31$  ng/ml;  $n = 26$ ;  $P < 0.005$  by Student's *t* test;  $F(1,47) =$

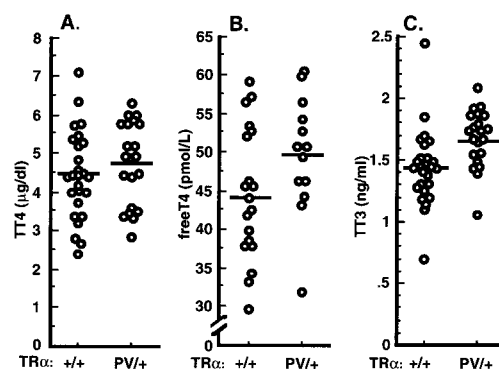


**Fig. 6.** Impairment in growth of male (A) and female (B) TR $\alpha$ 1<sup>PV/+</sup> mice. The weights and lengths of wild-type and TR $\alpha$ 1<sup>PV/+</sup> mice were measured over the first 10 postnatal weeks. Significant differences in weights and lengths were detected in both male and female TR $\alpha$ 1<sup>PV/+</sup> mice ( $P < 0.0001$ ).

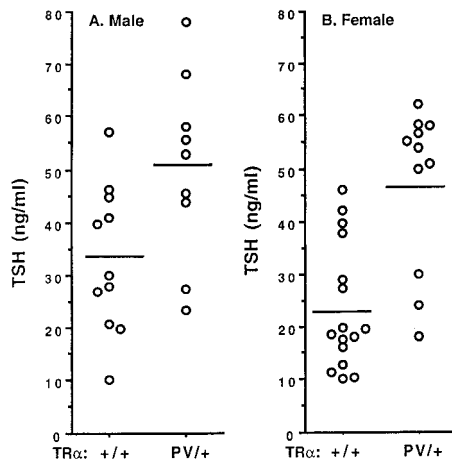
8.502,  $P = 0.005$  by ANOVA]. Furthermore, histological assessment indicates that neither abnormal follicular morphology nor lymphoid infiltration was detected in the thyroid glands of TR $\alpha$ 1<sup>PV/+</sup> mice (data not shown). These findings are in great contrast to TR $\beta$ <sup>PV/+</sup> mice, which have a 2- to 4-fold higher levels of circulating thyroid hormone and enlarged thyroid follicles (6).

Intriguingly, TR $\alpha$ 1<sup>PV/+</sup> mice had a significantly higher TSH level (Fig. 8). The mean TSH concentration in TR $\alpha$ 1<sup>PV/+</sup> mice was  $47.58 \pm 16.16$  ng/ml ( $n = 21$ ), which was 1.7-fold higher than that in their wild-type siblings [ $27.45 \pm 13.48$  ng/ml;  $n = 27$ ;  $P < 0.0001$  by Student's *t* test;  $F(1,48) = 21.005$ ,  $P = 0.00004$  by ANOVA]. The lack of lymphoid infiltration in the thyroid glands of TR $\alpha$ 1<sup>PV/+</sup> mice makes autoimmune thyroid disease less likely to account for the results of thyroid function tests. The elevated TSH, together with lower T4/T3 ratios (2.85 for TR $\alpha$ 1<sup>PV/+</sup> mice vs. 3.1 for their wild-type siblings), suggested mild thyroid failure in TR $\alpha$ 1<sup>PV/+</sup> mice. The causes of the mild thyroid failure are not clear at present.

**Abnormal Expression Patterns of T3-Target Genes in Tissues of TR $\alpha$ 1<sup>PV/+</sup> Mice.** As a first step to understanding the molecular action of TR $\alpha$ 1PV *in vivo*, we evaluated the expression of T3 target genes in the tissues of TR $\alpha$ 1<sup>PV/+</sup> mice. Fig. 9A shows the expression of TSH and GH mRNA in the pituitary gland. TSH consists of two polypeptides, the TSH-specific  $\beta$  subunit and the common  $\alpha$ -SU, whose expression is suppressed by T3. There was no difference in the expression levels of TSH $\beta$  between wild-type and TR $\alpha$ 1<sup>PV/+</sup> mice (Fig. 9Ab). It is not clear why there is a lack of concordance between the circulating TSH level and the TSH $\beta$  mRNA expression. One possibility could be that the circulating TSH in TR $\alpha$ 1<sup>PV/+</sup> mice is more stable than that in the wild-type mice. However, a 2.3-fold increase in the expression of  $\alpha$ -SU was detected in TR $\alpha$ 1<sup>PV/+</sup> mice (Fig. 9Aa), indicating a selective activation of the  $\alpha$ -SU gene in the pituitary by thyroid hormone.



**Fig. 7.** Concentrations of total T4 (A), free T4 (B), and total T3 (C) in wild-type and TR $\alpha$ 1<sup>PV/+</sup> mice. No significant differences in TT4 (A) and fT4 (B) between wild-type and TR $\alpha$ 1<sup>PV/+</sup> mice were detected. TT3 was 15% higher in TR $\alpha$ 1<sup>PV/+</sup> mice than in wild-type mice ( $P < 0.005$  by Student's *t* test).



**Fig. 8.** Elevated serum TSH levels in male (A) and female (B)  $TR\alpha^{PV/+}$  mice. The mean TSH concentrations in male (A) and female (B)  $TR\alpha^{PV/+}$  mice were significantly higher than those of wild-type mice ( $P < 0.02$  and  $P < 0.0003$ , respectively).

In  $TR\beta^{PV/+}$  mice, which have 2- to 4-fold elevated thyroid hormone levels, a 1.7-fold activation of  $\alpha$ -SU gene was observed (ref. 6 and Table 2). However, no effect on the expression of the GH gene was observed (Fig. 9A), which was similar to that seen in  $TR\beta^{PV/+}$  mice (ref. 6 and Table 2).

A different profile of abnormal regulation of T3 target genes was detected in the liver. ME and D1 are T3-positively regulated genes and were activated 2.2-fold (Fig. 9Ba) and 9.2-fold (Fig. 9Bc) in  $TR\alpha^{PV/+}$  mice, respectively, suggesting a hypersensitivity of these two genes to thyroid hormone. Previously, an increased sensitivity of target genes to thyroid hormone in mice with complete deficiency of  $TR\alpha$  ( $TR\alpha^{-/-}$   $TR\alpha 2^{-/-}$ ) was also reported (13). This finding is in contrast to the abnormal expression patterns of these two genes observed in  $TR\beta^{PV/+}$  mice in which a repression was detected (Table 2; ref. 6). The increased expression of D1 in the liver is consistent with the increased circulating levels of TT3 in  $TR\alpha^{PV/+}$  mice.

More differences in the abnormal expression patterns of T3 target genes were also found in the cerebellum. Fig. 9 C and D

**Table 2. Comparison of the expression of T3-target genes in the tissues of  $TR\alpha^{PV/+}$  and  $TR\beta^{PV/+}$  mice**

Target gene	mRNA Fold of mRNA in wild-type mice	
	$TR\alpha^{PV/+}$	$TR\beta^{PV/+}$
$\alpha$ -SU*	2.3	1.7
TSH $\beta$ *	1.0	1.0
GH $\dagger$	1.0	1.0
ME $\dagger$	2.2	0.6
D1 $\dagger$	9.2	0.8
MBP $\dagger$	1.4	1.0
Pcp2 $\dagger$	1.2	1.0

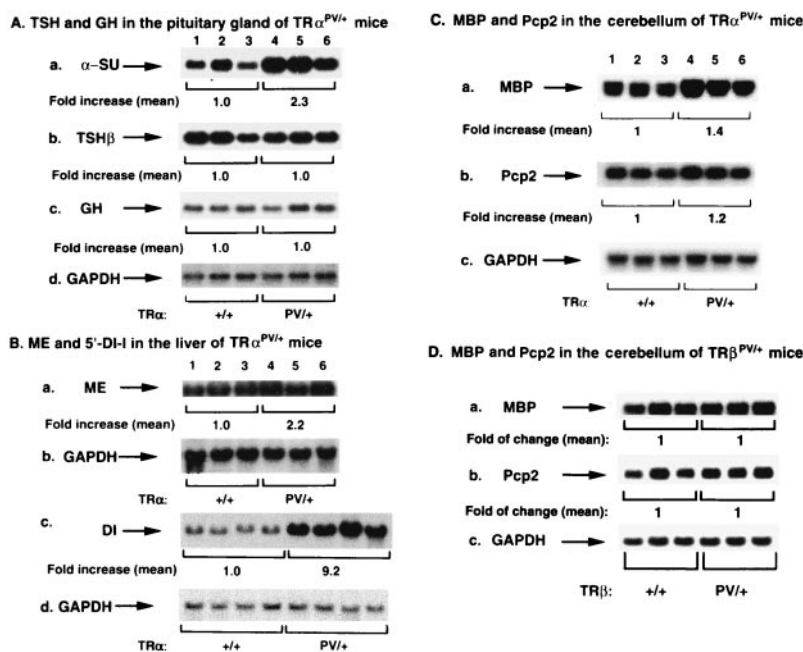
\*T3 negatively regulated genes.

$\dagger$ T3 positively regulated genes.

compares the expression of MBP and Pcp2 in the cerebellum of  $TR\alpha^{PV/+}$  and  $TR\beta^{PV/+}$  mice, respectively. No changes in the expression of MBP and Pcp2 were found in  $TR\beta^{PV/+}$  mice, whereas an activation of 1.4-fold and 1.2-fold was detected for the expression of MBP and Pcp2 genes, respectively, in  $TR\alpha^{PV/+}$  mice (Fig. 9 C and D and Table 2).

## Discussion

The generation of the  $TR\alpha$ 1PV knock-in mice has provided evidence that the mutation of the  $TR\alpha$  gene is not inconsequential. The mild thyroid failure in the  $TR\alpha^{PV/+}$  mice, which is clearly distinct from the hyperactive thyroid exhibited by the  $TR\beta$ PV and  $TR\beta\Delta 337T$  knock-in mice (6, 14), is consistent with the fact that no  $TR\alpha$  mutations have ever been identified in RTH patients. RTH patients are diagnosed based on high circulating levels of thyroid hormones associated with inappropriately normal or elevated TSH (4). The findings that  $TR\alpha^{PV/+}$  mice were viable and the majority of them could survive into adulthood suggested that the mutation of one of the  $TR\alpha$  gene alleles is not embryonic lethal. Therefore, it is reasonable to postulate that mutations of the  $TR\alpha$  gene could occur in humans, but the phenotypes would be expected to be distinct from those of RTH. The present mouse model could be used as a tool to uncover human diseases associated with the mutations of the  $TR\alpha$  gene.



**Fig. 9.** Abnormal expression patterns of T3-target genes in the tissues of  $TR\alpha^{PV/+}$  and  $TR\beta^{PV/+}$  mice. Total RNA was isolated from the pituitary (A), liver (B), and cerebellum (C) of  $TR\alpha^{PV/+}$  mice or the cerebellum of  $TR\beta^{PV/+}$  mice (D). Northern blot analyses were carried out as described in *Materials and Methods*. The levels of the expression of the T3-target genes were normalized by using GAPDH mRNA. Quantification was performed by using Molecular Dynamics PhosphorImager. The mean fold of changes is indicated ( $n = 3-4$  for each group of 12-week-old male mice).

**Table 3. Distinct phenotypes manifested by TR $\alpha$ <sup>PV/+</sup> and TR $\beta$ <sup>PV/+</sup> mice**

Phenotype	TR $\alpha$ <sup>PV/+</sup> mice	TR $\beta$ <sup>PV/+</sup> mice
Dysfunction of the pituitary–thyroid axis	Mild thyroid failure	Hyperactive thyroid
Impaired growth	Dwarfs	No
Reduced survival	Yes	No
High mortality	Yes	No
Reduced fertility	Yes	No
Expression of ME and D1 mRNA	Activation	Repression
Expression of MBP and Pcp2 mRNA	Activation	No change

Mice deficient in TR $\alpha$ 1 (TR $\alpha$ 1 knockouts) exhibit mild hypothyroidism with lower levels of thyroid hormones and TSH, normal reproduction ability, and mild abnormal heart rate and body temperature (15, 16). The mild phenotype exhibited by mice deficient in TR $\alpha$ 1 suggests that TR $\beta$  could compensate for the overlapping functions (3). Although it is not known whether TR $\alpha$ 1<sup>PV/+</sup> mice also have the phenotypes of abnormality in heart function and body temperature, the known phenotypes of TR $\alpha$ 1<sup>PV/+</sup> mice characterized so far are clearly distinct from mice deficient in TR $\alpha$ 1. It is remarkable that the mutation of one of the TR $\alpha$  alleles led to more severe phenotypes than the inactivation of both TR $\alpha$ 1 alleles.

Mice deficient in both TR $\alpha$ 1 and TR $\alpha$ 2 (TR $\alpha$ 1<sup>-/-</sup> TR $\alpha$ 2<sup>-/-</sup> mice) exhibited severe hypothyroidism, growth arrest, reduced postnatal survival, and intestine abnormality (17). The reduced postnatal survival could be rescued by injection of T3 in TR $\alpha$ 1<sup>-/-</sup> TR $\alpha$ 2<sup>-/-</sup> mice (17). The known phenotypes of TR $\alpha$ 1<sup>PV/+</sup> mice clearly are different from those of TR $\alpha$ 1<sup>-/-</sup> TR $\alpha$ 2<sup>-/-</sup> mice in that TT4 and TT3 were nearly normal such that the reduced postnatal survival is most likely not because of thyroid hormone deficiency. Moreover, the known phenotypes of TR $\alpha$ 1<sup>PV/+</sup> mice are also distinct from TR $\alpha$ 1<sup>-/-</sup> TR $\beta$ <sup>-/-</sup> mice (3, 18), which manifest more severe phenotypes, notably in the differences of the impairment of the pituitary–thyroid axis. The latter exhibit hyperactive thyroid glands with very high thyroid hormone levels together with extraordinarily high TSH (3, 18). These observations suggest that TR $\alpha$ 1PV could act by means of a gain of function, rather than simply as a result of interfering with the functions of both TR $\alpha$ 1 and TR $\beta$  functions. This notion is further supported by the transgenic mice expressing v-erbA, which is a non-T3 binding homolog of TR $\alpha$ 1 (19). The phenotypes of the transgenic mice expressing v-erbA overlap in many aspects to TR $\alpha$ 1<sup>PV/+</sup> mice in that increased mortality, reduced fertility, and reduction in weight were observed (19).

As summarized in Table 3, the phenotypes exhibited by the

TR $\alpha$ 1<sup>PV/+</sup> mice are clearly different from those of TR $\beta$ <sup>PV/+</sup> mice. Therefore, the *in vivo* signaling pathways of  $\alpha$  and  $\beta$  TR mutants apparently are distinct. At present, the molecular mechanisms by which these two TR mutant isoforms exert their distinct phenotypes are not clear. Based on the abnormal expression patterns of T3 target genes in the pituitary, liver, and cerebellum of TR $\beta$ <sup>PV/+</sup> mice (Table 2), TR $\beta$ PV could interfere with the functions of wild-type TRs by means of a dominant negative effect (6). TSH $\beta$  and  $\alpha$ -SU are T3-negatively regulated genes. Instead of being repressed by the high thyroid hormone levels in TR $\beta$ <sup>PV/+</sup> mice, no repression and further activation was observed for TSH $\beta$  and  $\alpha$ -SU, respectively (Table 2). GH, ME, and D1 are T3-positively regulated genes. However, no activation was seen in the expression of the GH gene, and repression was detected in the expression of ME and D1 genes in TR $\beta$ <sup>PV/+</sup> mice (Table 2). However, we could not exclude the possibility that TR $\beta$ PV could also act by means of a T3-independent pathway.

In contrast to TR $\beta$ PV, the abnormal expression patterns of T3 target genes *in vivo* shown in Table 2 do not support the notion that TR $\alpha$ 1PV acted by means of a dominant negative effect. In contrast to TR $\beta$ <sup>PV/+</sup> mice, the expression of  $\alpha$ -SU in the pituitary, ME and D1 in the liver, and MBP and Pcp2 in the cerebellum was activated. This observation is not because of the possibility that TR $\alpha$ 1PV cannot interfere with the transcriptional activity of the wild-type TRs. *In vitro* studies have shown that TR $\alpha$ 1PV did not bind T3, lacked *in vitro* transactivation activity, and acted to repress strongly the transactivation activity of both w-TR $\beta$ 1 and w-TR $\alpha$ 1. Therefore, the dominant negative effect of TR $\alpha$ 1PV could be masked *in vivo* by coregulatory proteins that act in a TR mutant isoform-dependent way (20). Alternatively, it is possible that TR $\alpha$ 1PV structurally and functionally is intrinsically different from TR $\beta$ 1PV; therefore, it could act by means of a T3-independent pathway differently from TR $\beta$ 1PV. This notion is supported by the recent x-ray crystallographic studies of the ligand binding domains of the wild-type TR $\alpha$  and TR $\beta$  isoforms (21). The two wild-type TR subtypes differ in the loop between helices 1 and 3, which could affect both ligand recognition and interaction with coactivators and corepressors (21). In line with these findings, it is reasonable to postulate that the differences in the structures of the TR $\alpha$ 1PV and TR $\beta$ 1PV isoforms could interact and recruit corepressors differently, leading to distinct functional consequences. The important *in vivo* role of corepressors in the action of mutant TR $\beta$  is exemplified by the manifestation of specific and deleterious action in mutant mice harboring TR $\beta$ ( $\Delta$ 337T) mutant (14). However, the validation of these possibilities needs further study.

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