

# Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor $\alpha 1$

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**Thyroid hormone, acting through several nuclear hormone receptors, plays important roles in thermogenesis, lipogenesis and maturation of the neonatal brain. The receptor specificity for mediating these effects is largely unknown, and to determine this we developed mice lacking the thyroid hormone receptor TR $\alpha 1$ . The mice have an average heart rate 20% lower than that of control animals, both under normal conditions and after thyroid hormone stimulation. Electrocardiograms show that the mice also have prolonged QRS- and QT<sub>end</sub>-durations. The mice have a body temperature 0.5°C lower than normal and exhibit a mild hypothyroidism, whereas their overall behavior and reproduction are normal. The results identify specific and important roles for TR $\alpha 1$  in regulation of tightly controlled physiological functions, such as cardiac pacemaking, ventricular repolarisation and control of body temperature.**

**Keywords:** bradycardia/hypothermia/thyroid hormone

## Introduction

Thyroid hormone (TH) is involved in the regulation of many physiological and developmental processes (Forrest 1994; Freake and Oppenheimer 1995; Silva 1995). The hormone (tri-iodothyronine, T<sub>3</sub>, and its precursor thyroxine, T<sub>4</sub>) also plays a major role in the cardiovascular system, such as regulation of heart rate, cardiac output and lipid content. Hypothyroidism in man and in animal models manifests in low heart rate and cardiac output as well as hyperlipidemia. In contrast, hyperthyroidism is associated with tachycardia that can lead to arrhythmia, elevated cardiac output and hypolipidemia (Dillman, 1996).

Thyroid hormone acts through nuclear hormone receptors (TRs), which are ligand dependent transcription factors encoded by two different genes in mammals, each one

giving rise to variant proteins (Lazar *et al.* 1988; Mitsuhashi *et al.* 1988). The proteins from the TR $\alpha$  gene have distinct properties: TR $\alpha 1$  binds TH and thereby regulates target gene expression, whereas TR $\alpha 2$  binds no known hormone. Its function is unclear, although it has been suggested that it represses TR $\alpha 1$  functions (Koenig *et al.* 1989; Katz *et al.* 1995). Both variant TR $\beta$  proteins bind TH and transactivate target genes although they differ in their N-terminal regions.

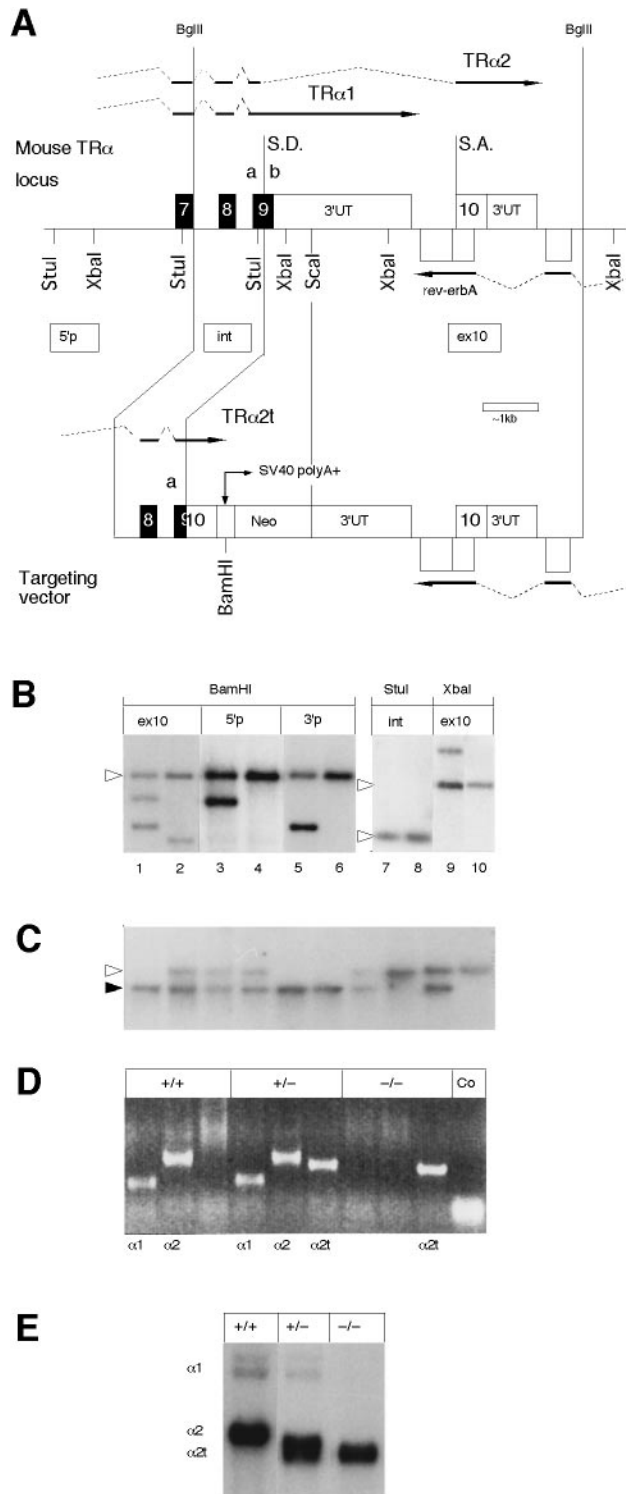
The role of TR $\beta$  in mediating the effects of TH has been elucidated in part. The syndrome of generalized resistance to thyroid hormones (GRTH) is well characterized (Refetoff *et al.* 1993; Refetoff 1994) and has given important clues to the role of TR $\beta$ . The syndrome is associated with two different genetic abnormalities, both afflicting the TR $\beta$  gene. Heterozygous patients from ~50 families with a transdominant negative version of the receptor often suffer from elevated levels of TH, growth and mental retardation, tachycardia and attention deficit/hyperactivity. They have surprisingly normal levels of thyroid stimulating hormone (TSH), resulting from the inability of the mutant receptor to downregulate the TSH genes. Moreover, patients and mice lacking the TR $\beta$  gene have a hearing loss and have elevated levels of thyroid hormones and TSH (Refetoff *et al.* 1967; Usala *et al.* 1991; Takeda *et al.* 1992; Forrest *et al.* 1996a,b). The studies suggest that TR $\beta$  regulates expression of the pituitary hormones TSH and growth hormone, and that TR $\beta$  is important for certain neuronal functions.

To clarify the difference between expression of a dominant-negative receptor to that of loss of a receptor, and to determine the roles of the individual thyroid hormone receptors in development, we generated mice with a null mutation of the TR $\alpha 1$  locus using homologous recombination. Our results define, for the first time, specific roles for TR $\alpha$  in mediating the effects of TH.

## Results

### **Generation of mice lacking TR $\alpha 1$**

To determine the roles of the individual TRs in mediating the effects of TH, we wished to develop transgenic mice from which a functional TR $\alpha 1$  gene was deleted, but which still expressed the splice variant, TR $\alpha 2$ , and the related orphan receptor, rev-erbA $\alpha$ , that is transcribed on the opposite strand (Lazar *et al.* 1988, 1989; Miyajima *et al.* 1989) (Figure 1A). For this we constructed a targeting vector that would replace the TR $\alpha 1$ -specific coding sequence with that of TR $\alpha 2$  (Figure 1A). Homologous recombination, which deleted the TR $\alpha 1$ -specific sequence in mouse embryonal stem (ES) cells was achieved in three independent cell clones. A detailed Southern blot analysis with 5', 3' and internal probes shows that the DNA in one of the ES clones contained no modifications other

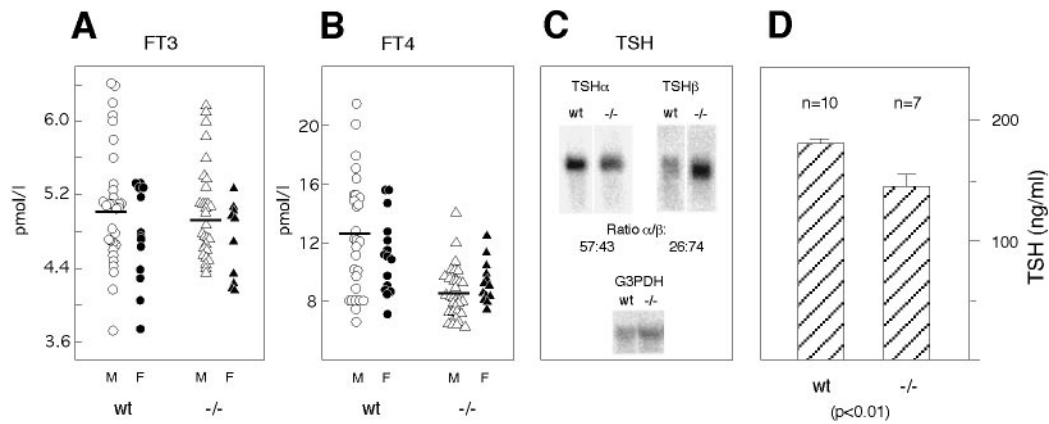


than those of the desired recombination event (Figure 1B; for details see Materials and methods).

To learn if the deletion of TR $\alpha$ 1 affected viability, heterozygous offspring from founder animals chimeric for the targeted ES cells were mated, and their progeny tested by Southern analyses (Figure 1C). The targeted TR $\alpha$ 1 gene was inherited in a Mendelian fashion: crosses between heterozygous female and male mice have given 25 +/+, 46 +/- and 25 -/- animals, with a 50:50 ratio between female and male offspring. This suggests that there is no lethality in -/- embryos. Homozygous animals are viable and survive to at least 18 months of age. Both female and male TR $\alpha$ 1 -/- animals were fertile, and the resulting litter sizes were normal. Overall, the animals appear healthy, with no overt abnormalities detected at autopsy.

Next, we wished to establish that expression of TR $\alpha$ 2 was unaffected by the TR $\alpha$ 1 targeting. RNA from the brains of adult mice of the three genotypes was subjected to RT-PCR analyses. In all three instances, the upstream 5' primer was complementary to sequences in exon 9a, common to all RNAs. The downstream primers annealed to 3' untranslated sequences specific to TR $\alpha$ 1, wild-type (wt) TR $\alpha$ 2, or to targeted TR $\alpha$ 2. The results demonstrate that products of the expected lengths were found in the respective samples (Figure 1D). Furthermore, the data confirm the absence of TR $\alpha$ 1 RNA in the -/- mice. A Northern blot analysis of polyadenylated RNAs substantiates the above results (Figure 1E) and shows that +/- animals contained ~50% of the level of TR $\alpha$ 1 RNA as compared with the control sample. The analysis also shows that all three samples contained levels of RNA comparable with a TR $\alpha$ 2 protein. The expression of the TR $\beta$  gene is unaffected in brain RNA from the TR $\alpha$ 1 -/- mice (A. Mansén and B. Vennström, data not shown), suggesting that a compensatory increase in receptor expression does not occur.

**Fig. 1.** Targeting of the TR $\alpha$  locus. (A) shows the structure of the 3'-end of the TR $\alpha$ 1/rev-erbA $\alpha$  locus with the targeting vector at bottom. Only the restriction sites relevant for the analysis of the recombination event are shown. The approximate locations for three of the probes used in Southern blotting are shown in boxes. 5'p: 5' probe; int: internal probe for the 5' half of the recombined allele; ex10: exon 10 probe. The location of the 3' probe is not shown for space reasons. Broken lines indicate splicing events. Splice donor sites are indicated by S.D., acceptor sites by S.A. (B) shows a Southern blot analysis of the recombination event in ES cells. DNA from G418 resistant clones was digested with the indicated enzymes. The filters were hybridized, stripped and rehybridized with 5', 3' and internal probes as shown in the figure. Odd-numbered lanes show DNA from targeted cells, even-numbered lanes indicate normal cells' DNA. The open arrowhead indicates the location of the normal allele, the filled arrowhead shows the mutated gene. (C) is a Southern blot analysis of BamHI digested DNA from the progeny of one representative litter from a cross between heterozygotes. The 5' probe was used for hybridization. The open arrowhead indicates the location of the normal allele, the filled arrowhead shows the mutated gene. (D) shows a RT-PCR analysis of polyadenylated brain RNA from +/-, +/- and -/- mice. The primer pairs chosen detect the RNAs for wt TR $\alpha$ 1, TR $\alpha$ 2 and targeted TR $\alpha$ 2, respectively. The RNA transcribed from the targeted allele is indicated by TR $\alpha$ 2t. The sizes of the electrophoresed products correspond to the expected sizes. (E) is a Northern blot of polyadenylated brain RNA. The cDNA probe used for hybridization contained sequences common to all forms of TR $\alpha$  RNAs. The TR $\alpha$ 2 RNA from the -/- animals migrated faster than the corresponding RNA from the control animals, due to its shorter 3' untranslated region originating from SV40 sequences in the targeted allele (see panel A).



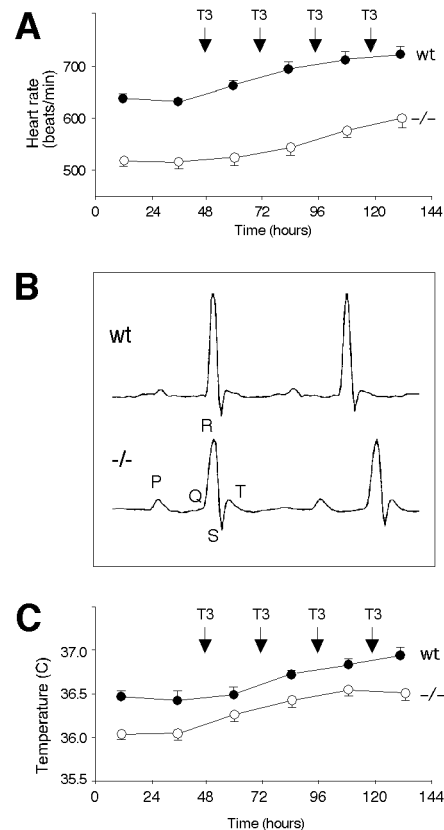
**Fig. 2.** Analysis of the pituitary–thyroid axis of thyroid hormone production. (A) and (B) show the levels of free T3 and T4 in serum. (C) is a Northern blot analysis of RNAs for TSH $\alpha$  and  $\beta$ , respectively. The serum from 2½-month-old animals was used to determine levels of free T3 and T4 using a commercially available direct competitive radioimmunoassay from Amersham. To detect TSH RNAs, pituitaries from four animals, aged 8 weeks, were pooled and polyadenylated RNA was transferred to filters as described for Figure 1. Hybridization was done with cDNA probes specific for mouse TSH $\alpha$  and  $\beta$ , respectively (Gurr *et al.*, 1984). The RNAs were quantified with a Phosphorimager, and the ratio of RNA levels between wt and TR $\alpha$ 1 $^{-/-}$  animals was normalized against the hybridization signal obtained with a probe for G3PDH. (D) shows serum levels of TSH in 5-month-old male mice, as determined by a radioimmunoassay.

### Hormonal status

TH production in the thyroid gland is induced by the pituitary thyroid stimulating hormone (TSH). Here, the ligand-bound receptor acts as a negative regulator, particularly on the TSH $\beta$  chain gene, although effects on the  $\alpha$  gene have also been observed. The role of TR $\alpha$ , if any, in the regulation of TH production via the pituitary was therefore first analyzed by determining the concentrations of free T3 and T4 in serum, an assay method that accurately reflects the availability of TH to an organism. The results (Figure 2A) show that the TR $\alpha$ -deficient male mice had lower levels of free T4 than control animals:  $8.5 \pm 1.7$  versus  $12.5 \pm 4.3$  pmol/l ( $p < 0.001$ ). In contrast, no significant differences were found between the female  $-/-$  and control mice ( $9.3 \pm 1.4$  versus  $11.1 \pm 2.6$  pmol/l;  $p = 0.04$ ). Also, the T3 levels were normal, ranging from 4.7 to 5.1 pmol/l (SD values  $\pm 0.4$ – $0.6$ ) in all mice irrespective of genotype or sex (Figure 2B). To understand this mild hypothyroidism better, the levels of RNAs for the  $\alpha$  and  $\beta$  chains of TSH in male mice was determined by Northern blot analyses and quantification. Figure 2C shows that the TSH $\alpha$  RNA level was  $\sim 30\%$  lower in the TR $\alpha$ 1 $^{-/-}$  mice than in the control mice. In contrast, the TSH $\beta$  RNA was more abundant in the  $-/-$  animals than in wt controls. We next measured the levels of serum TSH of 4-month-old male animals, to determine if the lower abundance of the TSH $\alpha$  mRNA would reflect hormone levels. The results show that the  $-/-$  mice had a concentration of 144 ng/ml (SD  $\pm 28$ ,  $n=7$ ), compared with 181 ng/ml (SD  $\pm 18$ ,  $n=9$ ) for the controls (Figure 2D). The TR $\alpha$ 1 $^{-/-}$  animals thus have slightly but significantly lower serum TSH levels ( $p > 0.01$ ). Histological analyses of the thyroid glands of the TR $\alpha$   $-/-$  mice showed no abnormalities, and no goitre has been detected in any animals during an 18 month observation period (data not shown). We conclude that the hypothyroidism in the TR $\alpha$ 1 $^{-/-}$  mice is a result of dysregulated TSH $\alpha$  production.

### Cardiac function

Hyperthyroidism is usually associated with an elevated heart rate (tachycardia). To determine if TR $\alpha$ 1 was



**Fig. 3.** Cardiac and metabolic function abnormalities. (A) is a telemetry recording of heart rate in TR $\alpha$ 1 deficient and wild-type mice. The diagram shows 24 h mean values. After 48 h of baseline registration, T3 (1 mg/kg body weight) was injected 1 p.m. for 4 consecutive days. The data are from nine wild-type and eight  $-/-$  animals. (B) shows a typical example of an averaged ECG registration from a TR $\alpha$ 1 deficient and a wild-type mouse. (C) shows the body temperature of mice as 24 h mean values, recorded with the telemetry system.

involved in regulation of heart rate, a telemetry system involving radio transmitters with sensors that record heart rate and complete electrocardiograms in freely moving animals was implanted into 2-month-old male mice (Johansson and Thorén, 1997). As shown in Figure 3A,

**Table I.** Different time intervals (ms) in the baseline ECG complex in homozygote TR $\alpha$ 1  $-/-$  mice ( $n=5$ ) compared with wild-type mice ( $n=7$ ). 24 h mean values are shown of body temperature (presented as SEM  $p < 0.05^a$  and  $p < 0.01^b$ )

Time interval	Wild-type	Homozygote
QRS	10 $\pm$ 0	12 $\pm$ 0.3 <sup>a</sup>
QT <sub>end</sub>	20 $\pm$ 1.0	28 $\pm$ 1.1 <sup>b</sup>
Basal values of body temperature	36.5 $\pm$ 0.1	36.0 $\pm$ 0.0 <sup>a</sup>

the TR $\alpha$ 1-deficient mice have a lower mean heart rate (bradycardia) than control animals of the same genetic background (mean value per 24 h is 515 beats/min, as compared with 632 beats/min for the wt controls). Since the TR $\alpha$ 1 $-/-$  mice had subnormal free T4 levels that could have resulted in the low heart rate, the mice were made hyperthyroid. Daily injections of 1 mg/kg body weight of T3 resulted in an increase in heart rate in the  $-/-$  and  $+/+$  animals (Figure 3A). The TR $\alpha$ 1  $-/-$  mice failed to reach the same heart rate as the control group, even after prolonged treatment of hormone. The concentrations of free T3 in serum were the same in both sets of animals at the termination of the experiments, ranging from 30 to 80 pmol/l when measured 1 day after the last injection. Results similar to these were also obtained after injection of one-tenth and one-quarter of the amount of T3 above (data not shown). The data suggest that ablation of TR $\alpha$ 1 expression results in a lower intrinsic heart rate, regardless of TH status.

Averaged electrocardiograms (ECG) recorded from TR $\alpha$ 1  $-/-$  and control animals were compared to provide an understanding of the bradycardia. Figure 3B shows that the TR $\alpha$ 1  $-/-$  mice have prolonged QRS- and QT<sub>end</sub>-durations, also after correction for the difference in heart rate. Table I reveals that particularly the QT<sub>end</sub> duration is markedly prolonged in the homozygotes, as compared with control mice. The prolonged QT<sub>end</sub> duration suggests a slow ventricular repolarization in the myocardium.

#### Other physiological parameters

Thyroid hormones also affect body temperature by upregulating thermogenesis in brown fat tissue and gluconeogenesis. The 24 h mean body temperature, recorded by the telemetry system, is 0.5°C lower in the TR $\alpha$ 1-deficient mice than the controls (Table I). When treating the mice with T3, a rapid parallel increase in body temperature was seen in both types of animals (Figure 3C). There was no significant difference in locomotor activity, also revealed by the telemetry, between the two groups, either before or after T3 treatment (data not shown).

## Discussion

### Targeting the TR $\alpha$ 1 gene

Since the TR $\alpha$ 1 and TR $\alpha$ 2 proteins have different properties, we designed a strategy for removing the coding sequence for TR $\alpha$ 1 while allowing continued expression of TR $\alpha$ 2. In this way, we hoped to produce mice that allowed identification of functions attributable to TR $\alpha$ 1 and interpretation of the phenotype. The results demonstrate that the production of an isoform-specific knockout was successful with our strategy. We were able to select-

ively delete sequences which encode TR $\alpha$ 1-specific RNA as shown by the absence of mRNA by Northern blotting and RT-PCR. In addition, we were able to modify the locus so that a TR $\alpha$ 2 mRNA was produced at normal levels. This demonstrates that appropriate splicing occurred between exon 8 and the modified exon 9a +10, and that the addition of an artificial polyadenylation sequence did not negatively affect the stability of the TR $\alpha$ 2 mRNA.

### Hypothyroidism

Mice and patients that lack TR $\beta$  expression have high circulating TH levels, accompanied by elevated TSH levels (Refetoff *et al.* 1967, Forrest *et al.* 1996a). Forrest *et al.* (1996a) showed that both the TSH $\beta$  (specific to thyrotropes) and, to a lesser extent, the TSH $\alpha$  (expressed in thyrotropes and gonadotropes) RNAs were upregulated in TR $\beta$  deficient mice, and therefore suggested that TR $\beta$ , highly expressed in the thyrotrophs of the pituitary, is essential for normal T3 regulation of TSH production. Our demonstration that the male TR $\alpha$ 1  $-/-$  mice have a mild hypothyroidism support and extend the results on the TR $\beta$   $-/-$  mice.

Normally, elevated TH levels suppress, and low TH levels induce, expression of pituitary TSH. However, in TR $\alpha$ 1-deficient mice the mild hypothyroidism does not result in any increase in TSH $\alpha$  RNA levels but, on the contrary, it is associated with a decrease in the RNA levels. These findings run counter to expectations based on traditional experiments using hormone manipulation, and highlight the necessity of investigating the individual functions of each TR variant. These data suggest that the induction of TSH in hypothyroidism is not merely a passive mechanism of de-repression in the absence of TH, and that TR $\alpha$ 1 serves an important role in this process.

Furthermore, whereas TSH $\alpha$  RNA is reduced, there is the opposite effect on the TSH $\beta$  subunit gene, which displays a slightly increased RNA level in TR $\alpha$ 1 $-/-$  mice. Taken together with the findings in TR $\beta$   $-/-$  mice (Forrest *et al.*, 1996a), a dynamic picture emerges in which individual TR variants are likely to mediate distinct aspects of positive and negative regulation of TSH expression, possibly involving differential regulation of the two TSH subunit genes in the case of the TR $\alpha$ 1 isoform.

Although in the absence of TR $\alpha$ 1 there may be complex underlying mechanisms of regulation, the net outcome is that serum TSH levels are only slightly reduced below normal. The fact that the elevated expression of TSH $\beta$  RNA fails to result in elevation of serum TSH suggests that there is no corresponding increase in secreted protein or that elevation of serum TSH levels cannot occur without a concomitant increase in TSH $\alpha$  subunit expression. The modest reduction of serum TSH is consistent with the mild hypothyroidism observed. This is further supported by our analyses of the thyroid glands from the TR $\alpha$   $-/-$  mice, which revealed no abnormalities indicative of gross aberrations of the TSH response.

### Cardiac function

The bradycardia of the TR $\alpha$ 1  $-/-$  mice could theoretically be interpreted as being due to genes specific to the 129 strain of mice used for generation of the ES cells (Gerlai, 1996). When located in the vicinity of the targeted TR $\alpha$

locus, such genes would be inherited along with the mutated gene in the  $-/-$  mice, but not the control mice that had been bred from TR $\alpha$ 1  $+/-$  mice (Gerlai, 1996). However, our experiments rule out this possibility, since mice of the 129 strain as well as mice deficient for expression of the rev-erbA $\alpha$  gene do not exhibit bradycardia (C.Johansson and P.Chomez, data not shown), thus indicating that traits specific to the 129 strain cause the low heart rate. The observation that the heart rate increased in the TR $\alpha$ 1  $-/-$  mice upon T3 treatment indicates that TR $\beta$  can also affect heart rate via indirect mechanisms, by affecting pacemaking functions other than those of TR $\alpha$ 1 or by substituting for TR $\alpha$ 1 in certain aspects. The latter views are supported by our finding that both receptor isoforms are expressed in the mouse heart (A.Mansén and B.Vennström, unpublished).

The reason for the bradycardia in the TR $\alpha$ 1  $-/-$  mice is at present unclear. The intrinsic heart rate is set by the cardiac sinoatrial node, and positive or negative stimulation is mediated by the sympathetic and vagal nervous systems respectively. A close examination of the functions of the above tissues will be required, including the expression of  $\beta$ -adrenergic and muscarinic receptors that mediate effects of the sympathetic and parasympathetic nervous systems (Dillmann, 1996). It is also possible that the lack of TR $\alpha$ 1 has perturbed the expression of ion channels and pumps. The pacemaker function is dependent on an unstable resting membrane potential, which generates the action potential necessary for depolarization across the atria. TH is known to induce the cardiac Na-K ATPase, and to concomitantly downregulate the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (Magyar *et al.*, 1995; Dillmann, 1996). The role of these two activities in the pacemaker function is unclear, although it is known that the sympathetic stimulus affects heart rate by causing an inward current of Na<sup>+</sup> and Ca<sup>2+</sup> ions (DiFrancesco, 1993). A further understanding of how TR $\alpha$ 1 causes bradycardia will thus require localization of TR expression in the different regions of the heart.

The electrocardiograms in the TR $\alpha$ 1  $-/-$  mice revealed prolonged QT<sub>end</sub> and QRS durations, a phenomenon similar to that seen in hypothyroid rat myocytes (Shimoni and Severson, 1993) and hypothyroid patients (Dillmann, 1996). The QT<sub>end</sub> duration is indicative of a slower ventricular repolarization, a phenomenon not normally associated with bradycardia in patients. The TR $\alpha$ 1  $-/-$  mice had been treated with T3, and our data therefore indicate that TR $\alpha$ 1 is involved in regulating the ion currents mediating the ventricular repolarization. A multitude of Na<sup>+</sup>, Ca<sup>2+</sup> and K<sup>+</sup> ion channels and transporters are involved in regulating heart activity (Warmke and Ganetsky, 1994; Shimoni and Severson, 1995). Our unpublished data have, however, revealed that none of the well known TR target genes in the heart (sarcoplasmic Ca ATPase, Na-K ATPase,  $\beta$ -adrenergic receptors) is dysregulated in the TR $\alpha$ 1  $-/-$  mice (A.Mansén, C.Johansson and B.Vennström). Identification of novel cardiac target genes for TRs, and detailed electrophysiological studies on isolated cardiac myocytes from different TR-deficient mice, will therefore be instrumental for dissecting the mechanism(s) by which thyroid hormones affect heart function.

### Other physiological parameters

Our results also show that TR $\alpha$ 1 deficiency leads to a reduction in body temperature of 0.5°C. Extreme hypothyroidism caused by thyroidectomy of rats causes a major reduction in body temperature, usually of several degrees (Guernsey and Edelman, 1983). It is therefore possible that TR $\beta$  in addition to TR $\alpha$  plays a role in thermogenesis in brown fat or in other metabolic processes. The mice have normal amounts of brown adipose tissue, suggesting that the reduced body temperature is due to perturbed metabolic processes rather than aberrant tissue development. Moreover, since the TR $\alpha$ 1  $-/-$  mice were as active as the control mice, the data suggest that the hypothermia is due to the absence of TR $\alpha$ 1 and not to differences in locomotor activity.

### TR $\alpha$ 1 and disease

Important clues to the function of TR $\beta$  have been provided by the >50 families with GRTH. Patients with a lack of TR $\beta$  expression display high TH and TSH levels and a loss of hearing (Refetoff *et al.*, 1967; Forrest *et al.*, 1996a,b). GRTH patients with the transdominant-negative version of the receptor often have elevated levels of TH, growth and mental retardation and tachycardia. Their TSH levels are surprisingly normal, resulting from the inability of the mutant receptor to downregulate the TSH genes. Taken together, these results suggest that TR $\beta$  has important roles in cells of neuronal and neuroendocrine origin.

Only one indication of a specific role for TR $\alpha$ 1 has emerged from the studies of GRTH patients. The combination of tachycardia and elevated TH levels in patients carrying a transdominant-negative version of TR $\beta$  is compatible with the absence of the mutant TR $\beta$  but the presence of TR $\alpha$  expression in the cardiac tissue that regulates heart rate. Our study identifies specific functions for TR $\alpha$ 1, namely in regulation of the pacemaker function and in ventricular repolarization. The TR $\alpha$ 1  $-/-$  mice thus provide a unique model for identification of TH regulation of ion currents in isolated cardiac pacemaker and contractile cells. The data also suggest that searches for TH agonists or antagonists for use in treating cardiac dysfunction should be targeted towards TR $\alpha$ 1.

The fact that no patients with mutant TR $\alpha$  genes have been found had resulted in the hypothesis that TR $\alpha$ 1 was indispensable. However, our data demonstrate the contrary: the mice are fertile, do not exhibit any gross deficiencies and appear to be only mildly hypothyroid. At first hand, it is puzzling that no patients with defective TR $\alpha$  genes have been found. However, our data raise the possibility that patients with defective TR $\alpha$ 1 genes could have subnormal levels of TH and TSH which would contrast with the hallmarks of GRTH. The analysis of the TR $\alpha$ 1  $-/-$  mice potentially offers important clues to the identification of patients with mutant TR $\alpha$  genes.

## Materials and methods

### Targeting vector

We designed a targeting strategy that would ablate only TR $\alpha$ 1 expression and leave that of TR $\alpha$ 2 intact. The two proteins arise from alternative splicing which generates proteins with different carboxy termini (Figure 1A). TR $\alpha$ 1 is encoded by exons 1-9, whereas TR $\alpha$ 2 results from alternative splicing from a donor site present 128 bp after the start of exon 9 (here referred to as exon 9a) to an acceptor site for exon 10.

This alternative splicing event replaces the 40 C-terminal amino acids encoded by TR $\alpha$ 1 with 120 amino acids specific for TR $\alpha$ 2. A targeted disruption of any of the exons 1–9a would consequently ablate both receptor variants, whereas deletion of nucleotides in exon 9, which are after the splice site (here referred to as exon 9b), would selectively disrupt TR $\alpha$ 1. However, the orphan hormone receptor rev-erbA $\alpha$  is encoded by the opposite strand of the TR $\alpha$  locus and its last coding exon overlaps exon 10 of the TR $\alpha$  locus (Figure 1A). Therefore, the targeting construct was designed as shown in Figure 1A so that (i) the coding sequence specific to TR $\alpha$ 1 was removed, (ii) the production of a TR $\alpha$ 2 mRNA with a normal coding sequence was possible, and (iii) the region of the TR $\alpha$  exon 10 which overlaps the rev-erbA $\alpha$  locus was undisturbed.

The targeting vector was made from two independent phage  $\lambda$  clones which contained a total of 19 kb of 3' TR $\alpha$  locus from the FVB mouse strain. A 6.6 kb *Bgl*II fragment, including the region of TR $\alpha$  from intron 7 to exon 10, was isolated and subcloned into the SP70 vector (Promega). A *Sca*I site in the 3' UT region of exon 9 was converted into a *Sal*I site. To change exon 9a+b to an exon encoding 9a + exon 10, RT-PCR was performed on poly(A) selected RNA from mouse brain using polyd(T) as the reverse primer. The PCR primers had sequences encoding a *Sal*I site followed by a sequence corresponding to either the beginning of exon 9 (5'SAL) (5'-GGAGTCGACCGAGAAGAGTCAGGAG-3') or the 3' UT region of exon 10, 47 nucleotides after the stop codon (3'SAL) (5'-GCTACTCTTCTCCCGTCGACCAAC-3'). The resulting PCR product (9a+10) was purified and cloned into pGEM 3Zf(+) vector at a *Sal*I site (pGEM 9a+10) and sequenced. The 1100 bp sequence just upstream of the splice donor site in exon 9 to part of the 3' UT region after exon 9 was excised from the pSP70*Bgl*II vector containing the genomic fragment by cleavage with *Sna*I and *Sal*I, and replaced with the *Sna*I–*Sal*I fragment of pGEM 9a+10, containing exon 9a downstream of the *Sna*I site and exon 10, 47 bp after the stop codon. This yielded the clone pmTR $\alpha$ ex9a+10. To allow for selection and proper processing, the SV40 poly(A) signal was cloned into the *Bam*HI site of pMCneo/poly(A) (Stratagene). *Sal*I/*Xho*I digestion released the fused SV40-neomycin selectable marker, which was cloned into the *Sal*I site of pmTR $\alpha$ ex9a+10 to give a targeting vector with the neomycin transcriptional unit in the opposite orientation. All molecular manipulations were done according to standard procedures (Bensen-Chanda, 1995).

### Probes

The 5' probe (5'p) was isolated by restriction digestion using the *Bam*HI site in the plasmid intermediate and a *Kpn*I site in the clone. The 3' probe (3'p) corresponds to a *Bam*HI–*Hind*III fragment distal to exon 10. The exon 10 probe (ex10) was generated by PCR using a 5' primer with the sequence GCGGAATTCAAGCTTTGGGGAAGACGACAGCA-GTG (the underlined portion represents 1377–1401 of mTR $\alpha$ 2 cDNA) and the 3'SAL primer described above to generate the PCR fragment used in the targeting construct. The 568 bp internal probe (int) was generated by PCR using a 5' primer specific to exon 8 (nucleotides 961–980 in the mouse cDNA) and a 3' primer corresponding to the beginning of exon 9 (nucleotides 1002–1024 in the mouse cDNA).

### ES cells

E14 ES cells were grown on mitomycin C-treated primary mouse embryo fibroblasts from neomycin-resistant mice and grown in standard ES media (Muller *et al.*, 1991) supplemented with 1000 U/ml leukemia inhibitory factor. The targeting vector was electroporated (BioRad Gene Pulser at 250 V, 960 mF) into ES cells after cleavage with *Bgl*II. Neomycin-resistant (375  $\mu$ g/ml G418) clones were screened for homologous recombination events by Southern blotting, after digestion with *Bam*HI, and hybridized with the 5' probe. Three clones that had undergone homologous recombination were identified. The accuracy of the recombination was assessed by Southern blot analyses (Figure 1B). DNA was digested with *Bam*HI which generated a 23 kb fragment from the normal locus. In contrast, the recombined locus has two *Bam*HI sites 226 bp apart which divides the 23 kb fragment into a 13 kb 5' fragment and a 9.9 kb 3' fragment. The filter was hybridized consecutively with four different probes: (i) an internal probe (denoted ex10 in Figure 1A) specific for exon 10 sequences, (ii) a 5' probe (5'p) which recognizes sequences 5' to the targeting vector, (iii) an internal probe (int) detecting the 5' half of the recombined locus and (iv) a 3' probe (3'p) recognizing sequences 3' to the targeting vector. As shown in Figure 1B, the ex10 probe detects, as expected, one 23 kb band (indicated by an open arrowhead) in DNA from control cells (even numbered lanes), whereas the targeted ES cell DNA (odd numbered lanes) gave three fragments

corresponding to the 13 and 9.9 kb fragments from the recombined gene, in addition to the 23 kb normal allele. Moreover, the ex10 probe gave no extra bands, indicating that no additional, non-homologous integration had occurred. Hybridization with the 5' and 3' probes detected the 23 kb normal allele and the 13 kb and 9.9 kb recombined alleles, respectively. To verify that the 5' and 3' recombination sites remained intact, the DNA was digested with *Stu*I or *Xba*I and probed with the internal probe for analysis of the 5' event and with the ex10 probe for the 3'. The appropriate recombination gave one band with the 5' probe and two bands with the 3' probe, as expected (Figure 1B). We conclude that an accurate homologous recombination had occurred.

### Generation of mice

Blastocysts from C57Bl females were collected to generate the transgenic mice, injected with ES cells and implanted into pseudopregnant F1(B<sub>6</sub>CBA) females. Male offspring with coat color chimerism were bred with *Balb*c females, and germline transmission was scored by coat color. TR $\alpha$ 1 +/- mice were identified by PCR and were then interbred to obtain homozygotes. DNA was prepared from tail clips as described (Laird *et al.*, 1991), and analyzed by PCR using the 5'SAL primers described above or by Southern blotting using the 5'p probe. The 3' primers were different for each paired reaction. The 3' primer (3'rvTR $\alpha$ 1) corresponds to nucleotides 1349–1362 of mTR $\alpha$ 1 cDNA and the 3' primer (3'SV40) corresponds to the sequence 5'-ACCACAACCTCG-AATGCAGTG-3' of the SV40 poly(A) tail. All three ES lines that had undergone homologous recombination yielded viable mice homozygous for deletion of TR $\alpha$ 1.

### Analysis of RNA

RT-PCR was done using polyd(T) as a primer for cDNA synthesis. The 5' primer, 5'SAL, and 3' primers, 3' rvTR $\alpha$ 1 and 3'SV40 are described above. In addition, a primer specific for the most 3' end of exon 10, called 3'ex.10 (5'-GAACCAAGTAAGCACAGACGAC-3') was used to identify wild-type TR $\alpha$ 2. Polyadenylated RNA (5  $\mu$ g/lane) was used for Northern blotting. Filters were hybridized with a full length mouse TR $\alpha$ 1 cDNA probe which recognizes both TR $\alpha$  isoforms. cDNA clones for detecting the TSH $\alpha$  and - $\beta$  RNAs were prepared from the respective cDNA clones (Gurr and Kourides, 1984).

### Hormone assays

The serum from 2- to 4-month-old males was used to determine levels of free T3 and T4 using a commercially available, direct competitive radioimmunoassay from Amersham (Amerlex-MAB FT3 and FT4 Kit). Serum from 4-month-old males was used for the determination of TSH levels with a specific mouse TSH radioimmunoassay using a mouse TSH/LH reference preparation (AFP51718mp), a mouse TSH antiserum (AFP98991), and rat TSH antigen for radio-iodination (NIDDK-rTSH-I-9). All reagents were obtained from Dr A.F.Parlow (Harbor University of California at Los Angeles Medical Center, Torrance, CA). 25  $\mu$ l serum in triplicate determinations were used for the radioimmunoassay. The limit of sensitivity was 0.6 ng and the intra-assay variation <6%. Full details will be published elsewhere.

### Telemetry

The telemetric device and its sensors for ECG (Data Sciences) were implanted as described previously (Johansson and Thorén, 1997). Monitoring of physiological parameters was initiated 7 days after the operation. The recordings were done continuously for up to 14 days in unstressed, freely moving mice kept in a separate animal room. All TH injections were subcutaneous. QRS and QT<sub>end</sub> durations were calculated from computer-averaged ECG recordings by manual placing of cursors. The QT<sub>end</sub> was defined as the timepoint at which the T-wave reached the isoelectric line.

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