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Different functions for the thyroid hormone receptors TRα and TRβ in the control of thyroid hormone production and post-natal development

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The biological activities of thyroid hormones are thought to be mediated by receptors generated by the *TR*α and *TR*β loci. The existence of several receptor isoforms suggests that different functions are mediated by specific isoforms and raises the possibility of functional redundancies. We have inactivated both *TR*α and *TR*β genes by homologous recombination in the mouse and compared the phenotypes of wild-type, and single and double mutant mice. We show by this method that the TRβ receptors are the most potent regulators of the production of thyroid stimulating hormone (TSH). However, in the absence of *TR*β, the products of the *TR*α gene can fulfill this function as, in the absence of any receptors, TSH and thyroid hormone concentrations reach very high levels. We also show that TRβ, in contrast to TRα, is dispensable for the normal development of bone and intestine. In bone, the disruption of both *TR*α and *TR*β genes does not modify the maturation delay observed in *TR*α$^{-/-}$ mice. In the ileum, the absence of any receptor results in a much more severe impairment than that observed in *TR*α$^{-/-}$ animals. We conclude that each of the two families of proteins mediate specific functions of triiodothyronin (T3), and that redundancy is only partial and concerns a limited number of functions.
Keywords: bone/intestine/knockout/thyroid hormone receptor/TSH

Introduction

Thyroid hormones are known to induce metamorphosis in amphibians by mediating remodelling of specific tissues and organs (for review see Tata, 1993; Kaltenbach, 1996). Triiodothyronin (T3) is essential for post-natal development of mammals, as hypothyroidism leads to growth retardation and impaired neurogenesis (Legrand, 1986). T3 is also involved in many aspects of adult life. In humans, hypothyroidism affects the function of many organs and results in alterations of thermogenesis and behaviour (Legrand, 1986).

The functions of T3 are mediated by three nuclear thyroid receptors, TRα1, TRβ1 and TRβ2, encoded by two genes, *TR*α and *TR*β, respectively (Sap *et al*., 1986; Weinberger *et al*., 1986). The *TR*β locus generates the β1 and β2 receptors by using two different promoters and alternative splicing. The *TR*α locus generates the α1 receptor and three non-T3 binding proteins: TRα2, which results from alternative splicing of the TRα primary transcript (Koenig *et al*., 1989; Lazar *et al*., 1989); and TRΔα1 and TRΔα2 transcripts, which are generated from an internal promoter located in intron 7 of the *TR*α locus (Chassande *et al*., 1997). The thyroid hormone receptors belong to the family of nuclear receptors which includes retinoic acid receptors, 9-*cis* retinoic acid receptors, vitamin D3 receptors and peroxisome proliferator-activated receptors (Laudet *et al*., 1992). All these receptors contain a DNA-binding domain and a ligand-binding domain; they mediate ligand-dependent transcriptional control of target genes (Mangelsdorf *et al*., 1995). The β1 and β2 receptors only differ in their N-terminal region. The α and β receptors display remarkably conserved sequences in their DNA-binding, ligand-binding and ligand-dependent transactivation domains, but their sequences differ completely in the N-terminal region. The consequences of these structural homologies is that all three receptors bind the same ligand and the same motifs on DNA. Despite these similarities, recent *in vivo* investigations of the respective functions of these receptors have suggested that each of them is likely to mediate a limited number of the physiological activities of T3.

The TRs not only mediate the action of T3 but also play a role in maintaining the concentration of ligand at a constant level. This is achieved by feedback regulation involving pituitary thyroid stimulating hormone (TSH). T3 negatively controls the synthesis and secretion of TSH and thyrotropin releasing hormone (TRH) at the level of pituitary and hypothalamus, respectively, through a negative feedback loop (Lezoualc'h *et al*., 1992; Scanlon and Toft, 1996). The disruption of the *TR*β gene leads to hyperthyroxinemia (Forrest *et al*., 1996; Weiss *et al*., 1998). In contrast, mice with targeted disruption of the *TRα1* and *TRΔα1* genes (TRα1$^{-/-}$) exhibit a moderate hypothyroxinemia and mild hypothyroidism (Wikstrom *et al*., 1998), and mice lacking both *TRα1* and *TRα2* display hypothyroxinemia which increases upon aging and severe hypothyroidism (Fraichard *et al*., 1997). These data suggest that the products of both the α and β genes are involved, although in different ways, in the control of TSH production.

Other physiological functions are differentially affected by the deletion of either of the receptors. The inactivation of the *TR*β gene results in impairment of the auditory

function, but no alteration in development, metabolism or neurological functions has been described in these animals (Forrest *et al*., 1996). *TRα1*$^{-/-}$ mice show an abnormal heart rate and lower body temperature (Wikstrom *et al*., 1998). Mice which lack both the *TRα1* and the *TRα2* isoforms (TRα$^{-/-}$) exhibit growth retardation, a lower body temperature, a delayed maturation of bone and intestine and they die shortly after weaning (Fraichard *et al*., 1997). From these studies, it is clear that α and β receptors are differentially involved in the control of developmental, endocrine and metabolic processes.

The difference in the pattern of expression of the three receptors may partly account for these functional differences. The pleiotropic effect of the mutation in *TR*α is consistent with the wide distribution of TRα transcripts, whereas the more limited alterations generated by the ablation of the *TR*β gene are in agreement with the more restricted pattern of expression observed for TRβ transcripts. A few organisms and tissues such as early chick embryos (Forrest *et al*., 1991; Flamant and Samarut, 1998) and rat cerebellum (Bradley *et al*., 1989) express only α isoforms but most tissues express several isoforms with varying ratios. Redundancy may occur in tissues where both α and β receptors are expressed.

To investigate the respective roles of the *TR*α and *TR*β genes, we have generated mice with a targeted disruption of the *TR*β gene and, using the previously described *TR*α$^{-/-}$ mice, produced double mutant mice. We have compared the phenotypes of wild-type, *TR*α$^{-/-}$, *TR*β$^{-/-}$ and *TR*α$^{-/-}$*TR*β$^{-/-}$ mice, and investigated the pituitary and thyroid functions of these mice in order to analyse the respective parts played by the α and β receptors in the control of the production of thyroid hormones. We have also compared the effects of the single and double mutations on the maturation of bone and intestine, in order to analyse potential functional redundancies between α and β receptors in these organs.

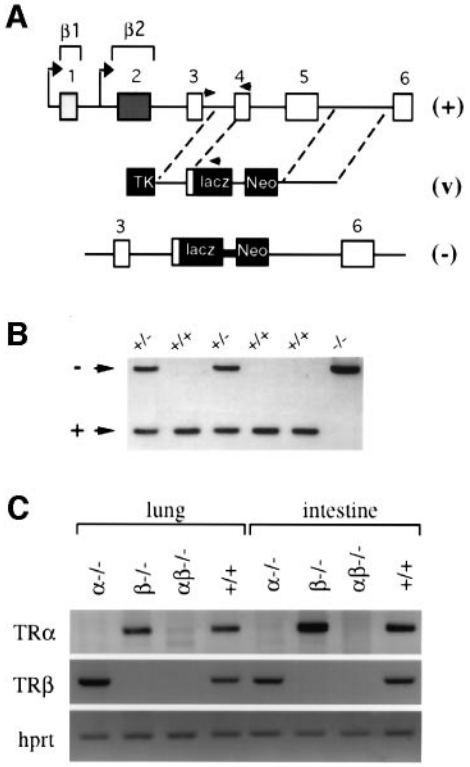

Fig. 1. Disruption of the *TR*β gene by homologous recombination. (**A**) Diagram of the *TR*β gene. Large arrows represent the two alternative transcriptional initiation sites used to generate the β1 and β2 transcripts. Arrowheads represent the position of the oligonucleotides used to identify the wild-type and mutant alleles by PCR. (**B**) Genotypes of the progenies of the *TR*β$^{+/-}$ intercrosses. The upper band ('–') is the mutant 1.2 kb fragment amplified with oligonucleotides βi3 and laczAS. The lower band ('+') is the wild-type 210 bp fragment amplified using the βe5 and βe5A primers. (**C**) Expression of the TRα and TRβ transcripts in wild-type and mutant mice. The TRα and TRβ transcripts (lines) were amplified in lung and intestine from *TR*α$^{-/-}$, *TR*β$^{-/-}$, double mutant and wild-type mice (columns).

Results

Production of mice with inactivated TRβ

To inactivate the *TR*β gene, a recombination cassette containing both a *lacZ* coding sequence and a *Neo*^{R} gene driven by the *β-actin* promoter was introduced downstream of exon 3 (Figure 1A). The presence of two polyadenylation sites downstream of the *lacZ* and *Neo*^{R} genes, respectively, was designed to prevent further transcription of both TRβ1 and TRβ2 mRNAs. Moreover, the region of the gene which encodes the DNA-binding domain of the receptors was removed in the replacement vector, to prevent the production of a chimeric protein that could potentially compete with other receptors for the binding to DNA. Two independent clones of recombinant embryonic stem (ES) cell clones were isolated and injected into blastocysts. Heterozygous mice were derived in an inbred 129SV background. Homozygous animals were obtained by intercrossing heterozygous animals (Figure 1B). As previously described (Forrest *et al*., 1996), homozygous animals were fertile and displayed hyperthyroxinemia.

Production of mice with both inactivated TRα and TRβ

*TR*α$^{+/+}$*TR*β$^{-/-}$ mice were first crossed with *TR*α$^{+/-}$*TR*β$^{+/+}$ mice to generate double heterozygous animals, which were fertile and intercrossed to yield viable and fertile *TR*α$^{+/-}$*TR*β$^{-/-}$ mice. These latter mice were further intercrossed to generate double homozygous animals. *TR*α$^{-/-}$*TR*β$^{-/-}$ mice were born alive with no obvious morphological or physiological alterations. Among 148 *TR*β$^{-/-}$ pups, 45 (30.4%), 69 (46.6%) and 34 (23%) were *TR*α$^{+/+}$, *TR*α$^{+/-}$ and *TR*α$^{-/-}$, respectively, The newborn mice were able to move and suckle normally, compared with *TR*β$^{-/-}$ or wild-type mice. These data show that the absence of both TRα and TRβ receptors does not impair the embryonic development of the mouse.

RT–PCR experiments were designed to monitor the expression of the transcripts produced from the *TR*α and *TR*β loci. Figure 1C shows that no transcript containing sequences downstream of the *lacZNeo*^{R} cassette was detected in intestine or lung from *TR*β$^{-/-}$ or *TR*α$^{-/-}$*TR*β$^{-/-}$ mice.

Altered growth rate and post-natal death in TRα$^{-/-}$ TRβ$^{-/-}$ mice

There was no obvious difference in the growth rates of wild-type and *TR*β$^{-/-}$ animals, respectively (data not shown), in agreement with previous observations (Forrest *et al*., 1996). As previously described for the *TR*α$^{-/-}$ mice,

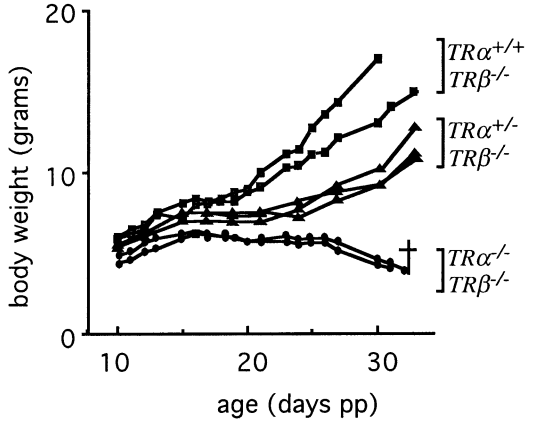

Fig. 2. Growth curves of the seven pups' progeny resulting from a $TR\alpha^{+/-}TR\beta^{-/-}$ mice intercross. Squares, $TR\beta^{-/-}$ animals; triangles, $TR\beta^{-/-}TR\alpha^{+/-}$ animals; circles, $TR\beta^{-/-}TR\alpha^{-/-}$ animals.

the newborn double mutant animals exhibited an altered growth rate. Until the age of 15 days, the difference in body weight between $TR\beta^{-/-}$ and double mutant mice was moderate (Figure 2). From this age on, the growth of double mutants was stopped and after 3 weeks a decrease in body weight was observed, leading to death by the age of 5 weeks. Growth of the $TR\alpha^{+/-}TR\beta^{-/-}$ mice was slower than that of $TR\beta^{-/-}$ mice.

As for the $TR\alpha^{-/-}$ mice, morphological examination of skeleton, liver, heart, lung and kidneys of 3-week-old $TR\alpha^{-/-}TR\beta^{-/-}$ mice showed that these organs did not reveal overt abnormalities despite a significant reduction in size compared with wild-type or $TR\beta^{-/-}$ animals (data not shown).

Hyperproduction of TSH and thyroid hormones in $TR\alpha^{-/-}TR\beta^{-/-}$ mice

We showed previously that from the age of 3 weeks $TR\alpha^{-/-}$ mice were progressively becoming hypothyroid. By the age of 5 weeks, plasma thyroid hormone concentrations reached values representing ~30% of hormone levels in normal mice (Fraichard *et al*., 1997). On the contrary, elevated plasma concentrations of TSH, T3 and thyroxin (T4) have been reported for $TR\beta^{-/-}$ mice (Forrest *et al*., 1996). We measured the plasma concentrations of these hormones in 3-week-old wild-type, $TR\alpha^{-/-}$, $TR\beta^{-/-}$ and $TR\alpha^{-/-}$ $TR\beta^{-/-}$ mice (Figure 3). In these experiments, comparison of the concentrations of TSH and thyroid hormones in $TR\alpha^{-/-}$ mice versus wild-type mice did not have any statistical significance, as assessed by an unpaired *t*-test ($p > 0.37$ for the three parameters). This was due to the large variance and the low number of animals tested. Comparisons of all parameters in $TR\beta^{-/-}$ and $TR\alpha^{-/-}TR\beta^{-/-}$ versus wild-type on the one hand, $TR\alpha^{-/-}$ and $TR\beta^{-/-}$ versus $TR\alpha^{-/-}TR\beta^{-/-}$ on the other, were meaningful as assessed by p values <0.001 (see Figure 3). Comparison of $TR\alpha^{-/-}$ versus $TR\beta^{-/-}$ was valid for T3 and T4 but not for TSH. In agreement with the observations from others (Forrest *et al*., 1996; Weiss *et al*., 1998), TSH, T4 and T3 concentrations in $TR\beta^{-/-}$ mice were increased 3- to 6-fold compared with normal controls (Figure 3). In double mutant mice, both TSH and thyroid hormone concentration were surprisingly high. Plasma T4 and T3 levels were ~10-fold higher than those of normal mice, whereas

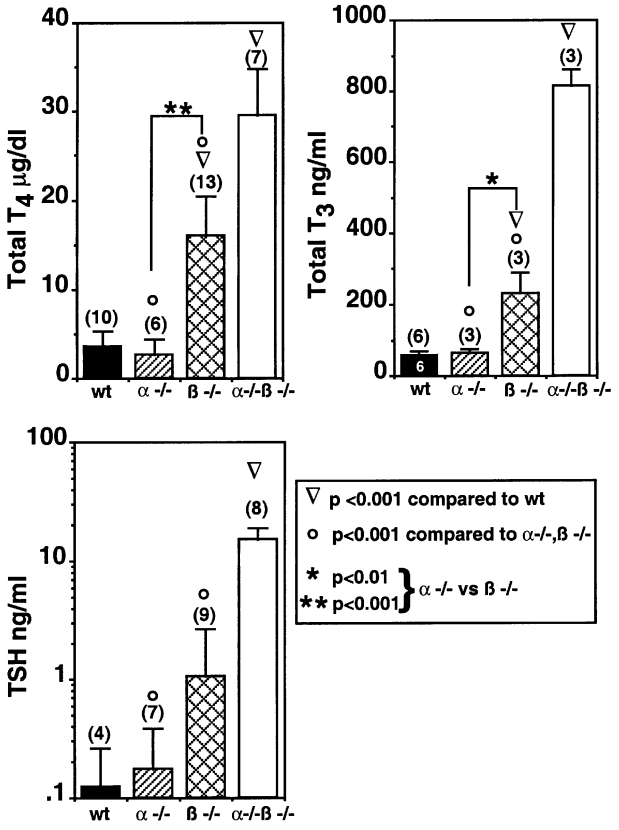

Fig. 3. T3, T4 and TSH are markedly increased in mice lacking TRβ receptors. Thyroid hormones (total T4, total T3) and TSH concentrations in serum from 3-week-old wild-type (wt; black bars), $TR\alpha^{-/-}$ (hatched bars), $TR\beta^{-/-}$ (cross-hatched bars) or double mutant mice (white bars). The number of animals used for each measurement is indicated in parentheses on the top of each bar. Statistical analysis was performed using an unpaired *t*-test to assess the significance of the differences observed between the values obtained in the different genotypes.

plasma TSH concentration was increased by >100-fold (Figure 3).

Histological features of the thyroid in $TR\alpha^{-/-}$, $TR\beta^{-/-}$ and double mutants

At the autopsy of 3-week-old mice, gross examination revealed an enlargement of the thyroid gland in $TR\beta^{-/-}$ and $TR\alpha^{-/-}TR\beta^{-/-}$ mice compared with wild-type mice, whereas in $TR\alpha^{-/-}$ mice the thyroid gland was much smaller and could not be distinguished from the muscular and the adipose surrounding tissues. Histological comparison of the largest sections of the tracheal block confirmed the diffuse hyperplasia of the thyroid gland in the $TR\beta^{-/-}$ (Figure 4, compare C1 with A1) and in the $TR\alpha^{-/-}TR\beta^{-/-}$ mice (compare D1 with A1 in Figure 4), in contrast to the hypoplasia observed in the thyroid gland of the $TR\alpha^{-/-}$ mice (Figure 4, compare B1 with A1). On the high magnification, the follicles in $TR\alpha^{-/-}$ mice were small and disorganized (Figure 4, compare B2 with A2). $TR\beta^{-/-}$ and double mutants exhibited an increase in the number of follicles but the thyroid glands of these animals differed in size and content of the follicles, aspects of colloid and development of vascularization (Figure 4, compare C2 and D2 with A2). In agreement with the observations of Forrest *et al*. (1996), Figure 4C2 clearly shows an increase in size of the follicles in $TR\beta^{-/-}$ compared with wild-type

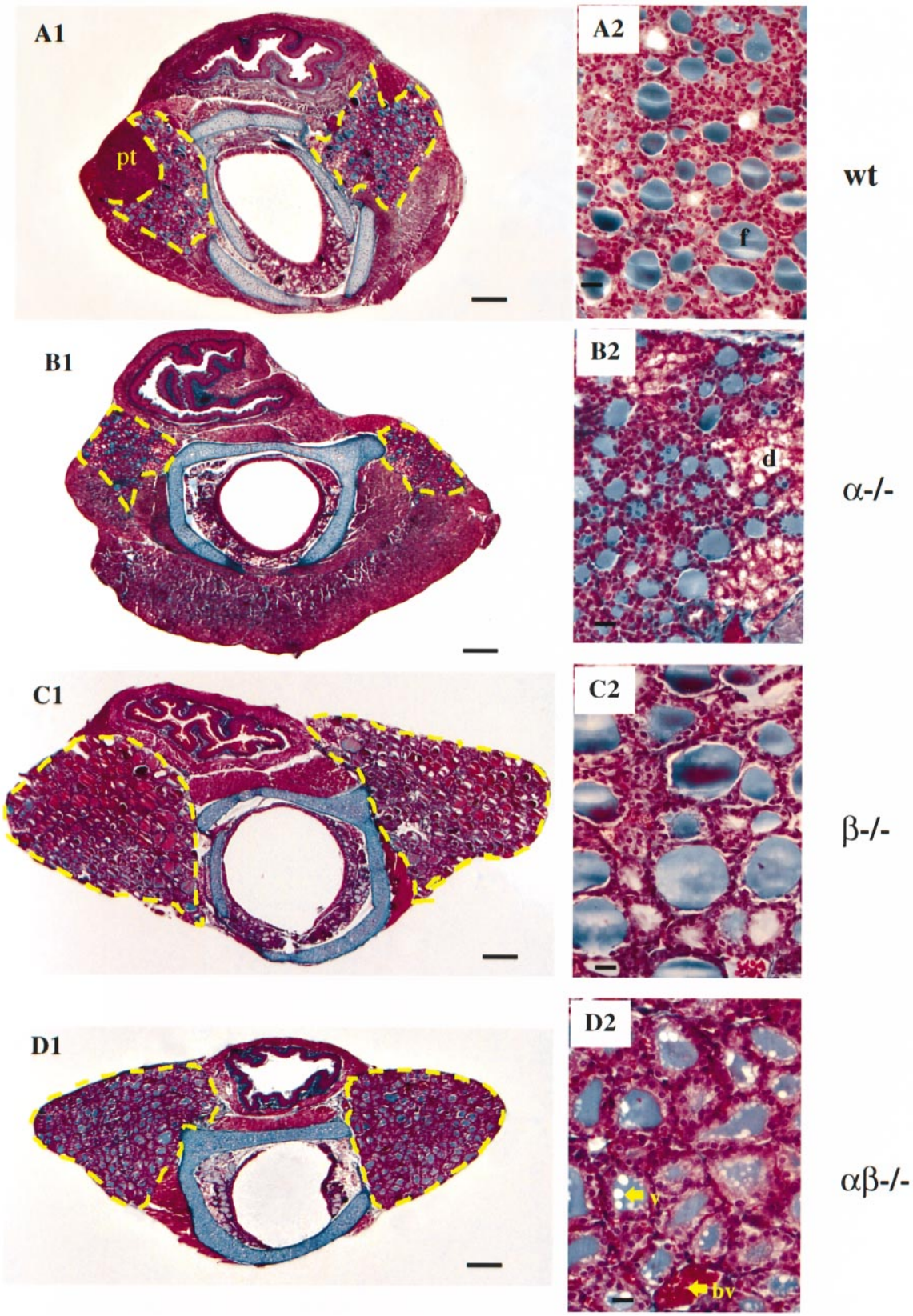

Fig. 4. Histology of the thyroid gland in TR mutants. Transverse sections through the thyroid trachea block of a 3-week-old control mouse (**A**), and *TR*α$^{-/-}$ (**B**), *TR*β$^{-/-}$ (**C**) and *TR*α$^{-/-}$*TR*β$^{-/-}$ (**D**) mutant mice. The *TR*β$^{-/-}$ (C1) and *TR*α$^{-/-}$*TR*β$^{-/-}$ (D1) exhibited a bilateral thyroid enlargement and the *TR*α$^{-/-}$ (B1) exhibited a moderate but clear hypoplasia, compared with controls (A1). Scale bars in A1–D1, 200 μm. pt: parathyroid gland. Higher magnification showed that enlargement was due to increased number and size of follicles (f) in *TR*β$^{-/-}$ mice (C2), compared with controls (A2). In *TR*α$^{-/-}$*TR*β$^{-/-}$ mutants (D2), small follicles exhibited signs of hyperactivity: colloid vacuoles (v), cuboid epithelium and hypervascularization (bv). Scale bars in A2–D2, 20 μm.

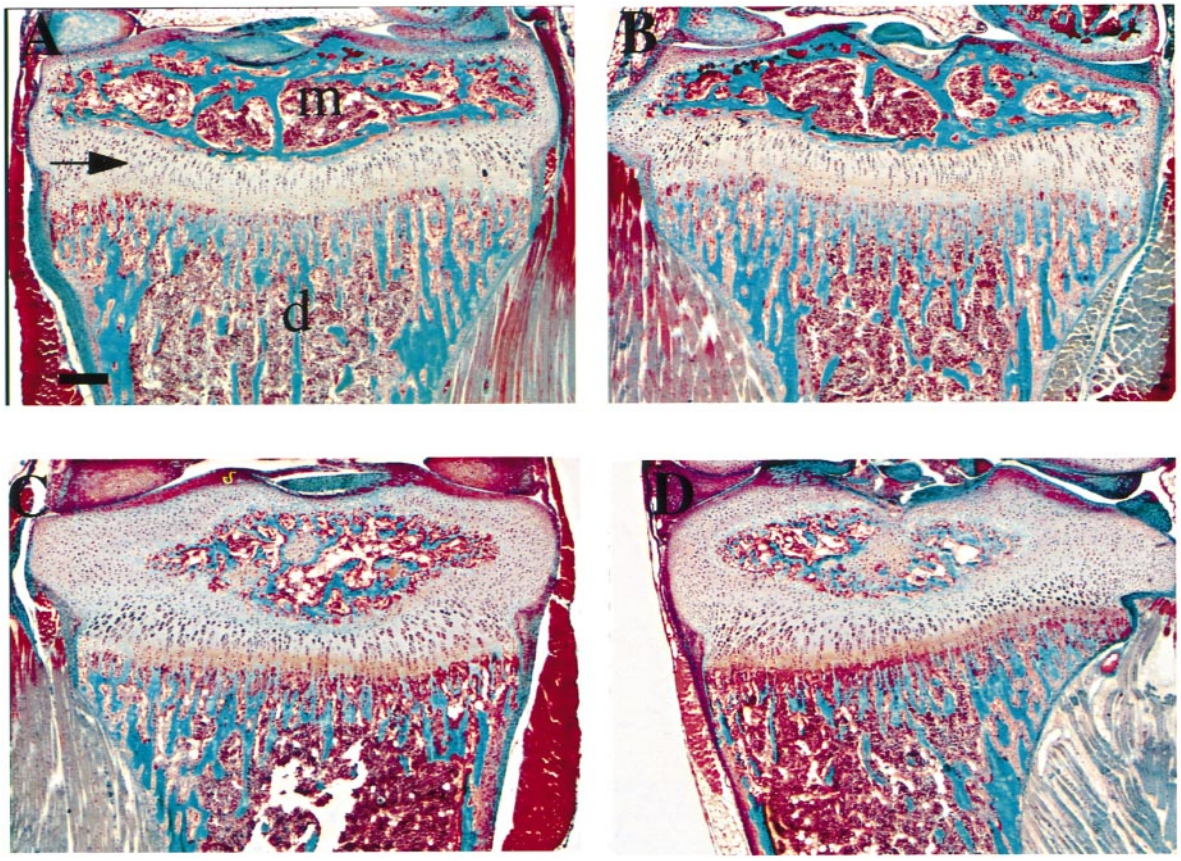

Fig. 5. Bone development is impaired in double mutant as in *TR*α$^{-/-}$ mice. Longitudinal bone sections (7 μm) of wild-type (**A**), *TR*β$^{-/-}$ (**B**), *TR*α$^{-/-}$ (**C**) and *TR*α$^{-/-}$*TR*β$^{-/-}$ (**D**) mice. Bar, 200 μm. m, metaphysis; d, diaphysis. The horizontal arrow indicates the growth plate.

mice (Figure 4A2). In *TR*α$^{-/-}$*TR*β$^{-/-}$ mice (Figure 4D2), the follicles were smaller and contained a less abundant colloid with vacuoles near the epithelium. The capillaries filled with red cells seemed more numerous in *TR*α$^{-/-}$ *TR*β$^{-/-}$ than in *TR*β$^{-/-}$ mice. Active colloid resorption and increased vascularity observed in the thyroid gland of double mutant animals are consistent with hyperactivity.

Delayed maturation of bone and intestine in TRα$^{-/-}$TRβ$^{-/-}$ mice

The overall morphology of the skeleton was not disturbed in *TR*β$^{-/-}$ or in double mutant animals. Histological examination of the tibia of *TR*β$^{-/-}$ mice did not reveal any difference compared with the wild-type animals (Figure 5A and B). In contrast, the tibia of *TR*α$^{-/-}$*TR*β$^{-/-}$ mice exhibited impaired development of epiphyseal bone centres characterized by a hypertrophied cartilage associated with a low ossification (Figure 5D). This phenotype is identical to the one observed in *TR*α$^{-/-}$ mice (Figure 5C; Fraichard *et al*., 1997). This study was carried out with 2-week-old animals. At this age, *TR*α$^{-/-}$ and wild-type animals have similar thyroid hormone plasma concentrations (data not shown), avoiding any interference of hypothyroidy with the phenotype.

Morphological appearance of the small intestine in the four groups of 2-week-old animals was also studied. As previously described, *TR*α$^{-/-}$ animals showed reduced length of the intestine as well as a greater fragility compared with the wild-types. This was also observed in the double mutant mice. On the contrary, we did not observe any difference between wild-type and *TR*β$^{-/-}$ animals. Histological analysis was then conducted at the proximal and distal levels of the small intestine and in the proximal colon. In accordance with previous observations, the colon was not affected by the receptor deficiency. Here we show histological staining at the distal ileum level (Figure 6). The intestinal size and the length of the crypt-villus unit was reduced in the *TR*α$^{-/-}$ compared with wild-type mice (Figure 6, C and D versus A and B). On the contrary, *TR*β$^{-/-}$ animals did not show such intestinal alterations (Figure 6E and F). The intestines of the double knockout mice displayed a histological impairment similar to *TR*α$^{-/-}$ mice at the proximal jejunum level (data not shown; Fraichard *et al*., 1997). Strikingly, a much more dramatic alteration was observed in the distal ileum of double mutant mice compared with *TR*α$^{-/-}$ mice (Figure 6G–I). This severe phenotype was characterized by very few and short villi-lined, flat epithelial cells (Figure 6I). The thickness of the external muscle coats in double mutants was similar to *TR*α$^{-/-}$ DI (Figure 6H versus D).

Discussion

Unliganded TRβ is not responsible for the phenotype resulting from the disruption of the TRα gene

We have previously described a severe phenotype in mice lacking the TRα1 and TRα2 proteins (Fraichard *et al*.,

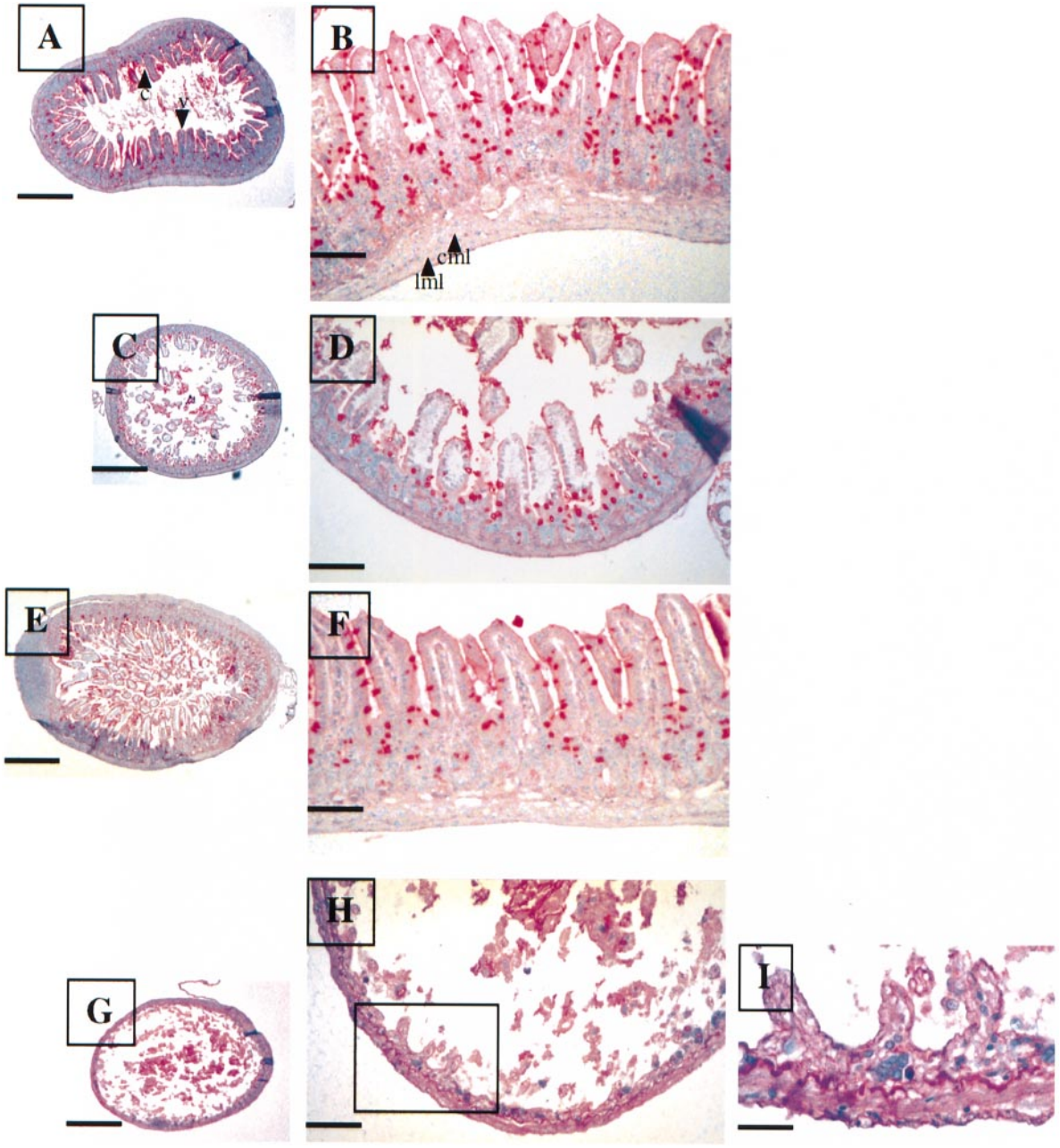

Fig. 6. Small intestinal phenotypic maturation is more severely impaired in double mutant than in *TR*α$^{-/-}$ mice. Periodic acid–Schiff (PAS) staining of transverse section of wild-type (**A** and **B**), *TR*α$^{-/-}$ (**C** and **D**), *TR*β$^{-/-}$ (**E** and **F**) and *TR*α$^{-/-}$*TR*β$^{-/-}$ (**G** and **H**) ileum. At low magnification (A, C, E and G), bar = 250 μm. v, villi; c, crypt. At higher magnification (B, D, F and H), bar = 100 μm. cml, circular muscle layer; lml, longitudinal muscle layer. At very high magnification (I), bar = 40 μm.

1997). *TR*α$^{-/-}$ animals displayed progressive hypothyroidism together with multiple disorders leading to death shortly after the weaning period. We postulated that this phenotype could be the result of hypothyroidism reinforced by the dominant negative activity of unliganded TRβ in these mice. The ablation of the *TR*β gene does not correct the severe phenotype observed in *TR*α$^{-/-}$ animals, showing that the phenotype generated by the disruption of *TR*α is not the result of a dominant-negative activity of the TRβ aporeceptor.

TRα and TRβ cooperate to repress TSH production

The hyperthyroxinemia observed in *TR*β$^{-/-}$ animals (our data; Forrest *et al*., 1996) indicates that, in normal mice, liganded TRβ receptors repress TSH synthesis, and this very probably mainly results from direct interaction of TRβ with negative response elements present in the *TSH*β gene promoter (Wondisford *et al*., 1989; Wood *et al*., 1989), although TRH disregulation may also occur (Feng *et al*., 1994; Yamada *et al*., 1997). These data suggest that TRα alone is not able to fully control TSHβ expression. However, the comparison between *TR*β$^{-/-}$ and *TR*β$^{-/-}$ *TR*α$^{-/-}$ mice presenting extremely high circulating TSH levels, shows that TRα is also capable of repressing TSHβ expression. This confirms recent observations suggesting that TRα1 could regulate the production of TSH and thyroid hormones in the absence of TRβ receptors (Weiss *et al*., 1997). We propose that in normal mice, TRβ and

TRα cooperate to repress TSHβ transcription, TRβ being a more potent repressor.

In apparent contradiction with this model, we have previously shown that the absence of TRα receptors in wild-type mice leads to hypothyroxinemia (Fraichard *et al*., 1997), and others have described limited hypothyroxinemia in *TRα1*$^{-/-}$ mice (Wikstrom *et al*., 1998). These data suggested that the TRα proteins might balance the action of TRβ receptors and positively control the production of thyroid hormones. This contradiction is easily resolved if we assume that the absence of the weak repressor, TRα, eliminates the competition between the two types of receptors and enables the stronger repressor, TRβ, to fully exert its repression potential. This assumption is supported by data showing that TRβ proteins are more efficient than TRα1 in binding the negative thyroid hormone response element of the TSH promoter and repressing its activity (McCabe *et al*., 1998).

The TRα and TRβ proteins have redundant functions in some but not all tissues

The extended pattern of expression of the *TR*α gene may account for the pleiotropic phenotype of *TR*α$^{-/-}$ mice. In contrast, *TR*β$^{-/-}$ mice display a mild and tissue-restricted phenotype (Forrest *et al*., 1996; our data), consistent with their more restricted pattern of expression. In some tissues, however, the *TR*α and *TR*β genes are coexpressed (Bradley *et al*., 1989, 1992; Forrest *et al*., 1990, 1991; Macchia *et al*., 1990; Strait *et al*., 1990, 1991). This raises the possibility of a functional redundancy between TRα and TRβ proteins. Our data show that this assumption is verified in some, but not all tissues.

As bones from young *TR*α$^{-/-}$ and *TR*α$^{-/-}$*TR*β$^{-/-}$ mice look identical, and are normal in *TR*β$^{-/-}$ animals, it seems unlikely that *TR*β plays any function in long bone formation although it is expressed in chondrocytes (Abu *et al*., 1997). We conclude that TRα, but not TRβ, is essential for the normal postnatal maturation of bone, and that TRβ cannot substitute for TRα in this process.

In contrast, histological analysis reveals clear differences between *TR*α$^{-/-}$*TR*β$^{-/-}$ and *TR*α$^{-/-}$ in the distal ileum, but not in the jejunum or in the colon. In the ileum, both *TR*α and *TR*β are coexpressed in the epithelium (Plateroti,M., Chassande,O., Fraichard,A., Gauthier,K., Freund,J.-N., Samarut,J. and Kedinger,M., submitted). Our data suggest that these genes are redundant in distal ileum, although *TR*β is not able to fully compensate for the absence of *TR*α. They also suggest that TRβ is responsible for the partial recovery of the intestinal phenotype after treatment of *TR*α$^{-/-}$ mice by T3 injections (Plateroti,M., Chassande,O., Fraichard,A., Gauthier,K., Freund,J.-N., Samarut,J. and Kedinger,M., submitted). We assume that redundancy occurs for other functions and enables the rescue of *TR*α$^{-/-}$ mice by T3 (Fraichard *et al*., 1997).

The thyrocytes express TRβ and TRα isoforms (Selmi-Ruby and Rousset, 1996), but the functions of these proteins in the thyroid gland remain poorly understood. TSH exerts a tight control on the development and metabolism of the thyroid, as an increase in TSH concentration triggers hypertrophy and hyperplasia of the thyroid gland and results in enhanced secretion of thyroid hormones. Therefore, it is likely that the morphological and histological features of the thyroid gland in the *TR*β$^{-/-}$ mice is the consequence of the increase of TSH concentration. Further increase in TSH concentration would be expected to result in enhanced hypertrophy, as it is observed in pathological or experimental hypothyroidism or in *TR*β$^{-/-}$*TRα1*$^{-/-}$ mice (D.Forrest, personal communication). However, in our double mutant animals, which display a very high concentration of TSH, the hypertrophy of the gland is blunted. Since the disruption of the *TR*α gene in *TR*α$^{-/-}$ animals leads to hypotrophy of the thyroid gland, it is possible that their absence in double mutant mice prevents a larger hypertrophy in response to TSH. From these data we assume that *TR*α plays an important role in the development of the thyroid gland. In conclusion, this work shows that the currently known T3 receptors are dispensable for the embryonic development of the mouse and that the products of the *TR*α and *TR*β genes display tissue-specific functions with limited redundancy.

Further investigations using targeted disruption of the *TR*α and *TR*β genes should unravel the respective contribution of each receptor isoform to peripheric functions, and the molecular basis of the tissue-specific functions.

Materials and methods

ES cell selection and generation of mutant mice

The TRβ targeting vector was constructed using pGNAβ as starting plasmid (gift from Dr P.Brulet). The 3′ arm homologous to *TR*β was cloned from a λEMBL4 library (gift from Dr J.P.Magaud). The 5′ arm was amplified by PCR from mouse 129 DNA. The thymidine kinase gene was inserted as previously described (Fraichard *et al.*, 1997). The targeting vector was digested with *Spe*I. 'ENS' ES cells were established from 129sv blastocysts in our laboratory by B.Pain and D.Aubert according to the technique described previously (Robertson, 1987). These cells were karyotyped as male and were proven to contribute with a very high efficiency to the germline when injected into blastocysts. We routinely maintain 'ENS' cells on MEF feeders in Glasgow minimal essential medium supplemented with 10% fetal bovine serum and 1000 U/ml LIF. 'ENS' ES cells were electroporated with 40 μg linearized plasmid, then selected with G418 (Gibco-BRL) and Gancyclovir and, after 10 days, clones were picked for screening. Positive clones were subsequently tested for correct karyotype and absence of mycoplasma and subsequently injected into blastocysts from C57Bl6 recipient mice. The genotype of the progenies of the mice carrying a *TR*β mutation was analysed using a PCR-based screening procedure. DNA (20–100 ng) extracted from tails was subjected to amplification using a mixture of four oligonucleotides: βi3 (GGAGTCCTCACTAGAGTCACC) and lacZAS (CCTCTTCGCTATTACGCCAGCTGG) allowed the amplification of the mutant allele. βe5 (TGGTGCTGGATGACAGCAAG) and βe5A (CAGGAATTTCCGCTTCTGCTT) allowed the amplification of the fragment from the wild-type allele. The amplification was carried out on a thermocycler (Perkin Elmer) using the following procedure: 94°C, 2 min, then (94°C, 15 s, 70°C, 15 s, 72°C, 60 s) for 10 cycles then (94°C, 15 s, 60°C, 15 s, 72°C, 60 s) for 30 cycles.

RNA analysis by RT–PCR

Oligonucleotides were from MWG ScienceTech (France). Reverse transcription was performed as follows: 1 μg of total RNA and 0.5 μg of random primers were mixed in 10 μl of water, heated to 68°C for 5 min and cooled to 37°C. Then, 10 μl of polymerization mix [100 mM Tris–HCl pH 8.3, 150 mM KCl, 20 mM dithiothreitol, 6 mM $MgCl_2$, 0.5 mM each dNTP, 200 U reverse transcriptase (Promega)] were added and the mixture was incubated at 37°C for 30 min. PCR was performed using 0.8 units Eurobiotaq thermostable DNA polymerase (Eurobio, France) in a 50 μl reaction mix. One microlitre of reverse transcription mix was used in each reaction. The detection of TRβ transcripts was carried out using oligonucleotides E6 (GGTGCTGGATGACAGCAAGA) and E7A (GCATTCACGATGGGTGCTTGT) were used as primers. Oligonucleotides VIS (GGAGATGATTCGCTCACTGCAG) and 15A (CAGCCTGCAGCAGAGCCACTTCCGT) were used as primers to detect TRα transcripts. The amplification was performed using the following parameters: 94°C, 2 min, (94°C, 15 s, 58°C, 15 s, 72°C, 60 s) for 35 cycles.

Intestine and bone analysis

Intestine analysis. Jejunum and ileum were dissected from 2-week-old pups, fixed in Bouin Holland's reagent and embedded in paraffin for serial sections and morphological observations. Sections of 5 μm were stained according to the standard PAS protocol (Segal and Petras, 1992).

Bone analysis. Leg tibia were dissected out from 2-week-old pups, fixed in 70% alcohol and embedded. Undecalcified sections (7 μm) were obtained and stained with Goldner's trichrome, according to the bone histomorphometric protocol (Meunier, 1983). Longitudinal medial sections were analysed with a true colour image-processing workstation Visiolab 1000 (Biocom, France).

Hormone assays and thyroid gland histology

Three-week-old mice were anaesthetized with ketamine (Panpharma, France) + Hypnovel (Roche, France) and bled intracardiacally. Sampling for all experiments was performed from 12 a.m. to 3 p.m. to minimize day–night variations. Serum concentrations of total T3 and total T4 were determined by specific radioimmunoassay, using sodium anilino-naphtalene sulfonate and acidic buffer, as binding inhibitors of serum proteins (Rousset *et al.*, 1984). TSH was measured by specific radioimmunoassay, as described previously (Weiss *et al.*, 1997). Tracheal blocks of mice were dissected out, immediately fixed in Bouin Holland, embedded in paraffin and cut in totality by serial sections of 5 μm. Sections were stained according to the standard Masson's Trichrome protocol. Thyroid areas were compared on the largest sections.

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