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The T3Rα gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production

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The diverse functions of thyroid hormones are thought to be mediated by two nuclear receptors, T3Rα1 and T3Rβ, encoded by the genes T3Rα and T3Rβ respectively. The T3Rα gene also produces a non-ligand-binding protein T3Rα2. The *in vivo* functions of these receptors are still unclear. We describe here the homozygous inactivation of the T3Rα gene which abrogates the production of both T3Rα1 and T3Rα2 isoforms and that leads to death in mice within 5 weeks after birth. After 2 weeks of life, the homozygous mice become progressively hypothyroidic and exhibit a growth arrest. Small intestine and bones showed a strongly delayed maturation. In contrast to the negative regulatory function of the T3Rβ gene on thyroid hormone production, our data show that the T3Rα gene products are involved in up-regulation of thyroid hormone production at weaning time. Thus, thyroid hormone production might be balanced through a positive T3Rα and a negative T3Rβ pathway. The abnormal phenotypes observed on the homozygous mutant mice strongly suggest that the T3Rα gene is essential for the transformation of a mother-dependent pup to an 'adult' mouse. These data define crucial *in vivo* functions for thyroid hormones through a T3Rα pathway during post-natal development.

Keywords: knock-out mice/nuclear receptors/post-natal development/thyroid hormones/T3Rα

Introduction

Thyroid hormone (T3) functions are mediated by three nuclear receptors, T3Rα1, T3Rβ1 and T3Rβ2, encoded by two genes, T3Rα and T3Rβ, also called c-erbAα and c-erbAβ, respectively (Sap *et al.*, 1986; Weinberger *et al.*, 1986). These receptors belong to a family of nuclear receptors (Laudet *et al.*, 1992) that includes retinoic acid receptors (RAR), 9-*cis*-retinoic acid receptors (RXR), vitamin D3 receptors (VDR) and peroxisome proliferator-activated receptors (PPAR). All these receptors contain a DNA-binding domain and a ligand-binding domain and mediate ligand-dependent transcriptional control of target genes (Mangelsdorf *et al.*, 1995). Differential splicing of the T3Rα primary transcript produces two transcripts, T3Rα1 and T3Rα2 mRNAs, the latter being expressed at higher levels in all tissues (Mitsuhashi *et al.*, 1988). The T3Rα2 protein lacks the T3-binding and AF-2 transactivator domains but retains the DNA-binding domain and can exert dominant-negative activity over the T3Rα1 receptor (Koenig *et al.*, 1989). Two short mRNAs are also transcribed from an internal promoter located in intron 7 of the T3Rα gene. These short mRNAs exhibit specific patterns of expression in mouse tissues. They encode truncated products T3RΔα1 and T3RΔα2, which retain the C-terminal part of T3Rα1 and T3Rα2 respectively. These truncated proteins exert transdominant functional inhibition over T3Rα1 and RARα (Chassande *et al.*, 1997). These data suggest a complex pattern of regulation of the cellular response to T3, since most of these isoforms exhibit potent regulatory activities toward T3Rs.

Although it has been clearly established that thyroid hormones are essential for normal post-natal development, the precise functions of thyroid hormones and of their receptors remain to be elucidated. Thyroid hormones are known to induce metamorphosis in amphibians by mediating changes in specific tissues and organs, for example in the development of the nervous system and in the repatterning of the gastrointestinal tract (for review, see Tata, 1993; Kaltenbach, 1996). In mammals, hypothyroidism conditions in humans or experimental hypothyroidism in rats demonstrates that thyroid hormones are involved in numerous developmental processes such as growth and neurogenesis (Legrand, 1986). The existence of two thyroid hormone receptor genes, T3Rα and T3Rβ, suggests that distinct thyroid hormone receptors may mediate distinct thyroid hormone functions. Whereas the T3Rα gene is widely expressed from early developmental stages, the expression of the T3Rβ gene is highly restricted and starts later in embryogenesis (Forrest *et al.*, 1990; Strait *et al.*, 1990; our unpublished data). Moreover, mice with targeted disruption of the T3Rβ gene exhibit overproduction of thyroid hormones and impairment of auditory functions, but no developmental defects are detected (Forrest *et al.*, 1996a,b). These data suggest that thyroid hormone actions during embryogenesis and post-natal life could be mediated by the T3Rα gene.

To investigate further the *in vivo* functions of the T3Rα gene products, we have generated mice with a targeted disruption of the T3Rα gene which abrogates the production of both T3Rα1 and T3Rα2 isoforms. After birth, the homozygous mutant mice showed a strongly reduced production of both T4 and T3 associated with a growth arrest and a delayed development of bones and small intestine. Although homozygous mutants usually die within 5 weeks, they can be rescued by injections of T3

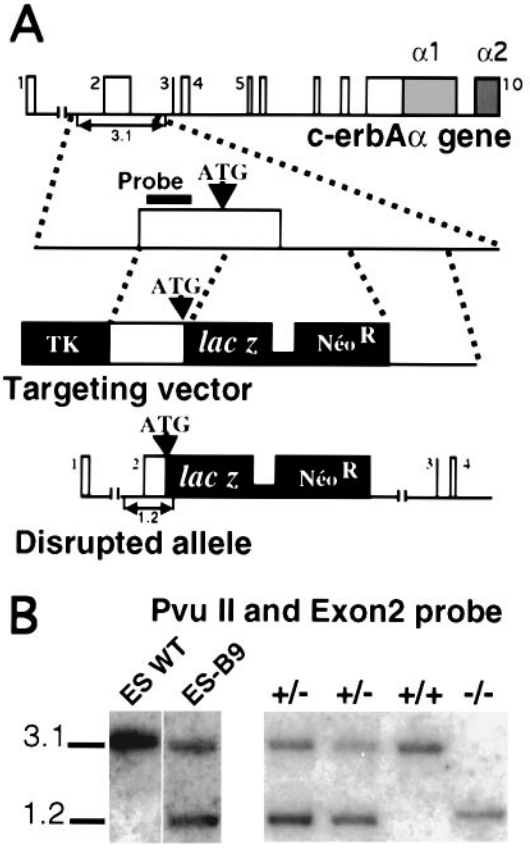

Fig. 1. Disruption of the T3Rα gene by homologous recombination. (**A**) Diagram of the T3Rα gene. α1 and α2 represent the part of exons 9 and 10 which encode the specific regions of T3Rα1 and T3Rα2 isoforms. The targeting construct contained a 0.25 kb deletion. The structure of the mutated allele is shown on the bottom line. The exon 2 probe used for Southern blot analysis and the sizes of the fragment detected after digestion with *Pvu*II are indicated. (**B**) Genotypes of wild-type ES cells, B9 clone and progenies of T3Rα$^{+/-}$ intercrosses. DNA samples digested with *Pvu*II were analysed by Southern blotting with the exon 2 probe.

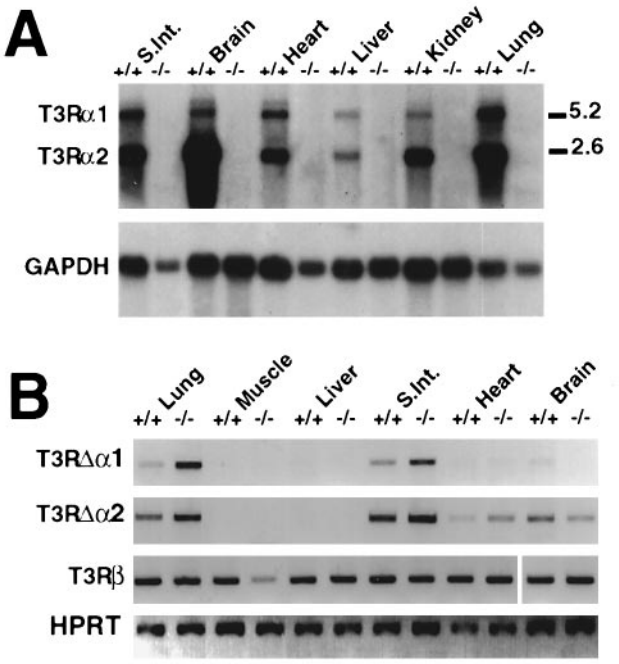

Fig. 2. Expression patterns of the T3Rα and T3Rβ genes. (**A**) Northern blot analysis of T3Rα1 and T3Rα2 mRNAs in different tissues of T3Rα$^{+/+}$ and T3Rα$^{-/-}$ mice. The sizes of the mRNAs are indicated in kb. S. Int.: small intestine. (**B**) RT–PCR analyses of T3Rβ, T3RΔα1 and T3RΔα2 mRNAs in different tissues of T3Rα$^{+/+}$ and T3Rα$^{-/-}$ mice.

for 1 week. The phenotype of the T3Rα$^{-/-}$ mice is then quite different from that of the T3Rβ$^{-/-}$ mice. These data demonstrate that the products of the T3Rα gene are involved in the positive control of thyroid hormone production and are necessary for post-natal development.

Results

Production of mice with inactivated T3Rα gene

To inactivate the T3Rα gene, a recombination cassette containing both a *LacZ* coding frame and a *NeoR* gene driven by the β-actin promoter was introduced in the first coding exon (exon 2), immediately downstream of the T3Rα initiation codon (Figure 1A). The presence of two polyadenylation sites downstream of the *LacZ* and *NeoR* genes, respectively, was designed to prevent further transcription of both T3Rα1 and T3Rα2 mRNAs. This construction does not affect the internal promoter located in intron 7 of the T3Rα gene. The mutated allele was introduced into the T3Rα locus by homologous recombination in embryonic stem (ES) cells. Two independent recombinant cell clones, B9 (Figure 1B) and A7, were obtained and injected into host blastocysts. Heterozygous mice were derived in an outbred OF1 genetic background for B9 and an inbred C57Bl6 background for A7. The growth rate of heterozygous mice was slightly slower than the growth rate of wild-type mice and, after 4 weeks, their body weight was reproducibly 15–25% lower than that of wild-type (see below, Figure 3A). Later on, these heterozygous mice reached the regular size of wild-type animals and were fertile. They were intercrossed to produce homozygous mutants. For the B9 clone, among 1354 born pups, 365 (27%), 660 (48.7%) and 329 (24.3%) were respectively wild-type, heterozygous and homozygous progenies. For the A7 clone, among 237 born pups, 62 (26.2%), 116 (48.9%) and 59 (24.9%) were respectively wild-type, heterozygous and homozygous progenies. These data show that homozygous disruption of the T3Rα gene is not deleterious to embryonic development.

Expression of T3Rα and T3Rβ genes

Northern blots hybridized with antisense oligonucleotides from exons 8 and 9 showed that T3Rα1 and T3Rα2 mRNAs could be detected in the tissues of wild-type but not of homozygous mutant mice (Figure 2A). RT–PCR experiments using several combinations of oligonucleotide primers located downstream of the recombination cassette demonstrated the absence of abnormal mRNAs produced within the T3Rα locus and that would encode truncated T3Rα proteins (data not shown) in homozygous mutant mice. As expected, short transcripts initiated at the internal promoter in intron 7 were still expressed (Figure 2B). In small intestine and lung, the expression level of these short transcripts was 2–4 times greater than in wild-type. The pattern of expression of the T3Rβ gene was similar in homozygous mutants and wild-type mice in all organs

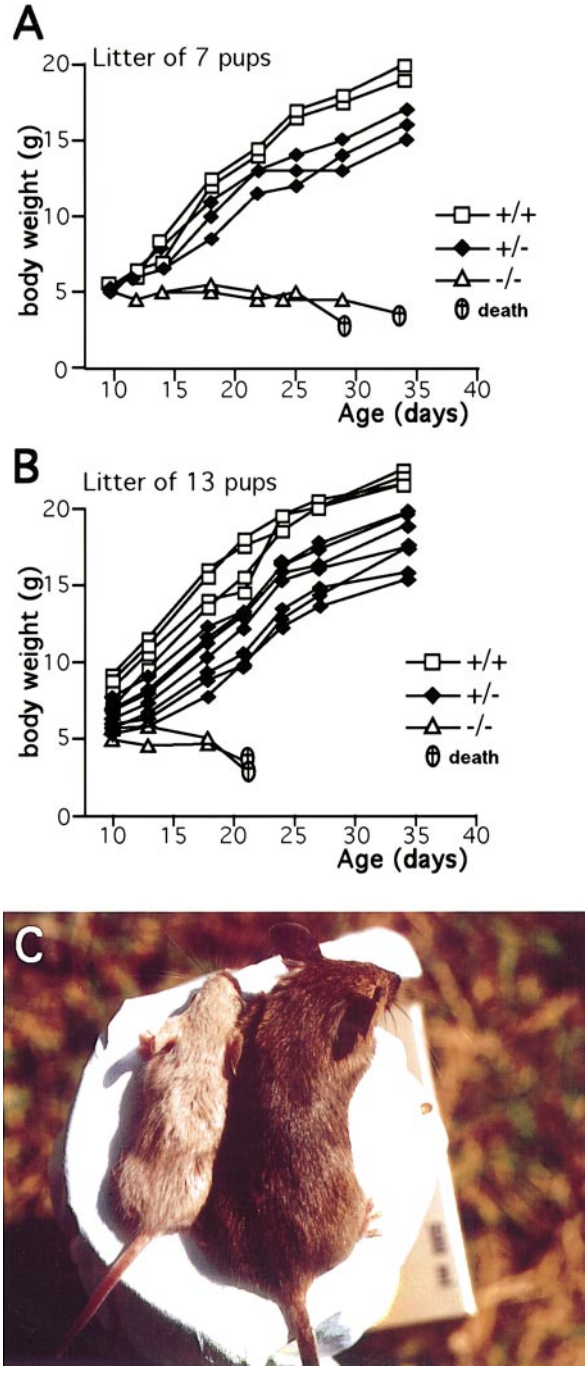

Fig. 3. Growth curves of progeny resulting from T3Rα$^{+/-}$ mice intercrosses. Growth curves of a seven pup litter (**A**) and of a 13 pup litter (**B**) are shown. (**C**) Photograph of a T3Rα$^{-/-}$ (left) and a T3Rα$^{+/+}$ (right) 4-week-old mouse.

examined, with the exception of skeletal muscle which showed a 2- to 4-fold decrease in expression. These observations show that the homologous recombination has totally abrogated the production of T3Rα1 and T3Rα2 mRNAs and that it has not significantly altered the overall expression pattern of the T3Rα and T3Rβ genes.

Post-natal phenotype of the T3Rα$^{-/-}$ mice

Homozygous mutant mice derived from the B9 and A7 clones did not exhibit any obvious abnormal phenotypes up until the first two post-natal weeks. Subsequently the growth of these mutant mice stopped completely at a body weight of 5–6 g, whereas the weight of wild-type animals increased up to 15–20 g within 2 weeks (Figure 3A). From the fourth week on, homozygous mutant animals lost 30–50% of their weight and then died. Thus, the homozygous mutant mice cannot survive beyond the time of weaning. This phenotype was observed for almost all homozygous mutant animals, whereas 99.8% of heterozygous and wild-type mice were still healthy 2 months after birth. These observations demonstrate that the phenotype strictly correlates with the T3Rα gene disruption and that there was no interference with the genetic background. However, two out of 151 B9 homozygous mutant mice (1.3%) and 0 out of 39 A7 homozygous mutant mice spontaneously survived for up to 3–7 months (see below). Adoption of pups by a wild-type mother did not modify the abnormal phenotype of homozygous mutants, showing that the growth defect did not result from the inability of heterozygous mothers to properly nurse their progeny. In bigger litters, the death of homozygous mutant mice occurred during the fourth week (compare Figure 3A and 3B). Alternatively, after experimental reduction of the litter size to four pups, death occurred only during the sixth week, suggesting that the phenotype was attenuated by maternal factors. Detailed analyses were carried out on 14- 21-day-old B9 and A7 homozygous mutant mice (from hereon referred to as 3-week-old mice). At that age, the growth retardation was clearly established but the animals were still healthy and seemed to exhibit normal behaviour. To characterize the phenotype of the T3Rα$^{-/-}$ mice further, histological analyses were performed on various organs. The liver, kidney, heart, lung, spleen, smooth and skeletal muscle of homozygous mutant mice did not display overt abnormalities (data not shown). Although the size of the brain appeared marginally reduced in homozygous mutants, we failed to detect any obvious cellular and morphological differences between homozygous mutant and wild-type brains. Cerebral cortex of homozygous mutant mice showed normal laminar organization and cytoarchitectonics as revealed by Nissl stain. Acetylcholine esterase and cytochrome oxidase histochemistry did not show any abnormality in cortical parcellation nor in the organization of subcortical nuclei. Detailed analyses of the small intestine, bones and thyroid hormone production have been carried out.

Delayed maturation of the small intestine

Dissection of T3Rα$^{-/-}$ mice did not reveal overall alterations in the morphology of the stomach, the duodenum or the colon. In contrast, the small intestine appeared much smaller, softer and more fragile than in wild-type mice. Histological analyses revealed impaired structural features of the small intestine. In T3Rα$^{-/-}$ mice, the diameters of the jejunum and of the ileum were respectively 2- and 3-fold smaller than in wild-type mice (Figure 4A). The number and size of the villi were decreased. The overall mucosal connective tissue and the overlying epithelium appeared almost normal, as assessed by the typical expression of specific markers (cytokeratin, sucrase–isomaltase and lactase, data not shown). The total number of epithelial cells per crypt–villus unit was strongly reduced (almost 65% reduction in the ileum), probably reflecting a decrease in enterocytic absorptive cell number since enterocytes

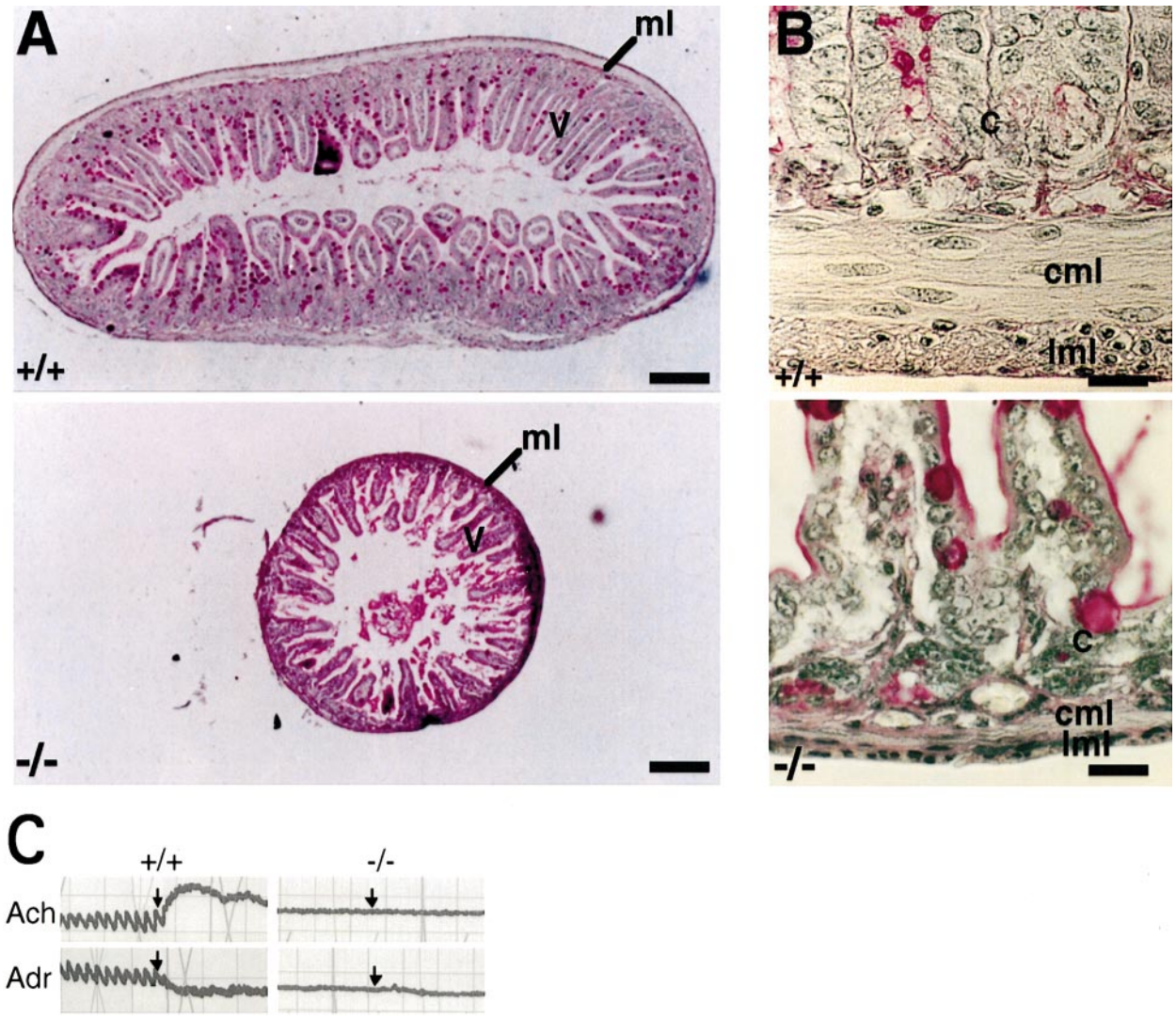

Fig. 4. Small intestinal phenotypic maturation is impaired in T3Rα$^{-/-}$ mice. (**A**) PAS staining of transverse section of T3Rα$^{+/+}$ (top left) and T3Rα$^{-/-}$ (bottom left) ileum (same magnification). Red spots correspond to mucus-containing goblet cells. ml: muscle layers. v: villi. Bar = 193 μm. (**B**) Higher magnification of the same transverse section of T3Rα$^{+/+}$ (top right) and T3Rα$^{-/-}$ (bottom right) focusing on the outer muscle layers. cml: circular muscle layer. lml: longitudinal muscle layer. c: crypt. Bar = 15 μm. (**C**) Recording of ileum spontaneous muscle activity or in response to acetylcholine (Ach) or adrenaline (Adr). Both drugs were used at a final concentration of 2 μg/ml. Ileum was allowed to equilibrate for at least 15 min before drugs were added (vertical arrow).

represent almost 90% of total epithelial cells in wild-type mice (Kedinger, 1994). Among the other epithelial cell types, the frequency of the goblet cells was reduced by 2.5-fold in the ileum, whereas Paneth cell frequency was not affected. The circular and longitudinal smooth muscle layers were strongly reduced (Figure 4B). Functional analyses were also performed on the small intestine. They showed that morphological alterations of the muscular layers correlated with an altered motility. Wild-type ileum showed a high level of spontaneous contractions, immediate tetanization in response to acethylcholine and relaxation in response to adrenaline (Figure 4C). In contrast, the T3Rα$^{-/-}$ ileum exhibited neither spontaneous contraction nor responses to acethylcholine or adrenaline even when a 3-fold increase in neuromediator concentration was used. Interestingly, these results could be correlated with a stronger expression level of the T3Rα gene in muscle layers than in epithelium and connective tissue (as detected by LacZ staining, data not shown). Enzyme activities such as dipeptidases (*N*-aminopeptidase and dipeptidyl-dipeptidase IV) and disaccharidases (sucrase and lactase) showed a 2-fold reduction in homozygous mutant mice. However, absorption of peptides and disaccharides appeared almost normal, as assessed by absorption assays using radiolabelled leucine and sucrose (data not shown). These characteristics are very similar to those of the small intestine of younger mice (Henning *et al.*, 1994). All these histological and functional data show that in T3Rα$^{-/-}$ mice the overall structure of the small intestine was properly developed but exhibited a strongly retarded maturation.

Delayed bone development

Although the skeleton was not affected in its general morphology in T3Rα$^{-/-}$ mice, long bones appeared to be softer than in wild-type, suggesting an impaired ossification. To confirm this observation, histomorphometry was performed on undecalcified tibia sections (Figure 5A1 and

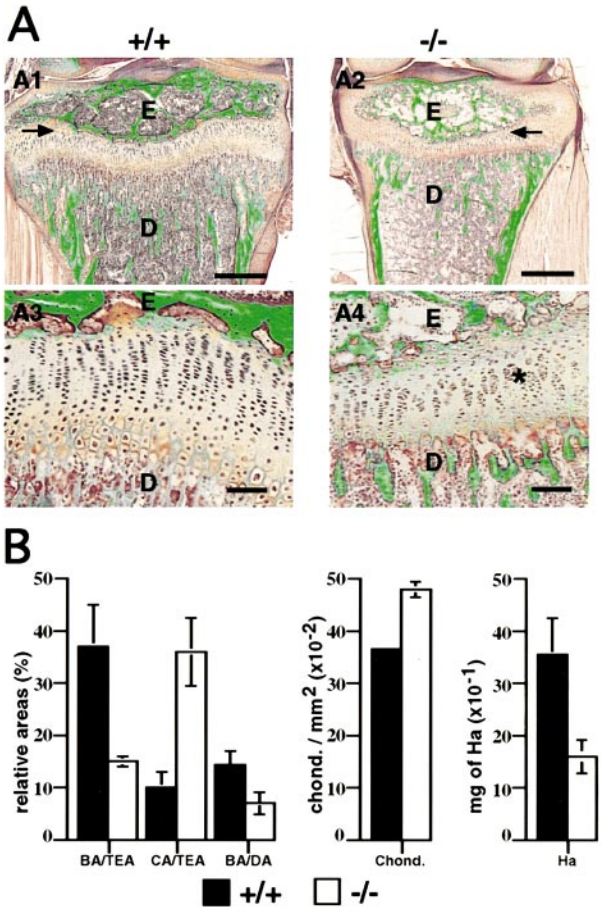

Fig. 5. Bone development is impaired in T3Rα$^{-/-}$ mice. (**A**) Longitudinal medial bone sections (7 μm) of T3Rα$^{+/+}$ (A1) and T3Rα$^{-/-}$ (A2) mice. Bar = 500 μm. E: epiphysis, D: diaphysis. The horizontal arrows indicate the growth plate. Higher magnification of A1 and A2, focusing on the growth cartilage, are shown in A3 and A4 respectively. The star indicates disorganized chondrocyte columns. Bar = 50 μm. (**B**) Histomorphometry analysis was performed on these sections for T3Rα$^{+/+}$ (black column) and T3Rα$^{-/-}$ (white column) mice. The bone area (BA) and the cartilage area (CA) were determined on the total epiphysis area (TEA) and on the diaphysis area (DA) and are presented as a percentage of this TEA (BA/TEA and CA/TEA) or DA (BA/DA). The density of chondrocytes (Chond.) was calculated in the growth plate. The total amount of hydroxyapatite (Ha) was measured by dual X-ray absorptiometry.

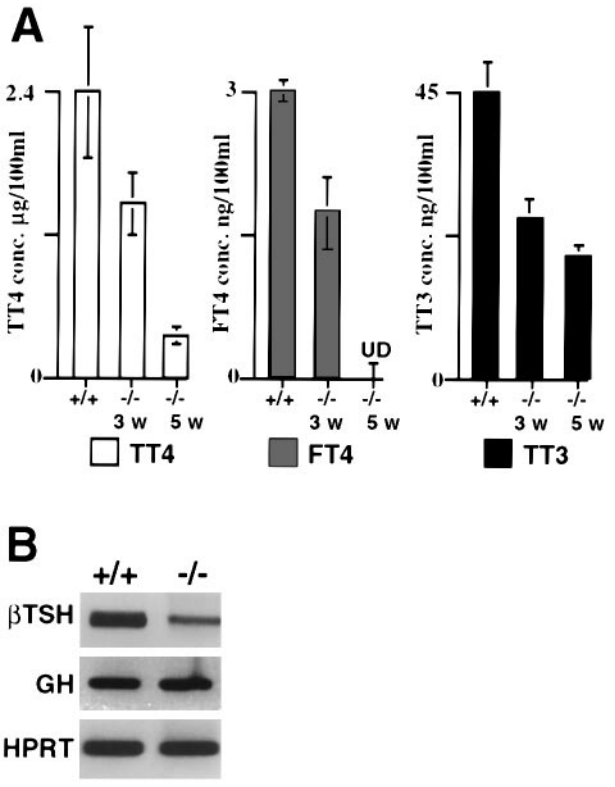

Fig. 6. Analysis of thyroid hormones, β thyroid-stimulating hormone (βTSH) and growth hormone (GH). (**A**) Thyroid hormone concentrations (means ± SEM) in serum from homozygous mutant mice at 3 and 5 weeks of age. Values were normalized to those obtained for 3- to 5-week-old wild-type mice (TT4 = 2.4 ± 0.3 μg/100 ml and TT3 = 44.7 ± 2.1 ng/100 ml). Thyroid hormone concentrations were not different in 3- and 5-week-old wild-type mice. The numbers of T3Rα$^{+/+}$, 3-week-old T3Rα$^{-/-}$ and 5-week-old T3Rα$^{-/-}$ animals were respectively n = 6, 3 and 7 for total T3 (TT3) and for total T4 (T4) and n = 5, 6 and 6 for free T4 (FT4). Concentrations of FT4 in 5-week-old T3Rα$^{-/-}$ mice were under the detection limit (UD) and corresponded to a level at least 10 times lower than in the wild-type mice. (**B**) RT–PCR analysis of βTSH and GH mRNAs in pituitary gland total RNA extracts.

A2). A reduction of the bone area (mean ± SEM, BA = 15 ± 1.1%, n = 3 versus 37 ± 7.9%, n = 3) paralleled by an excessive development of the cartilage area (CA = 36 ± 6.6%, n = 3 versus 10 ± 2.8%, n = 3) was observed in epiphysis as compared with wild-type animals (Figure 5B). These data demonstrated that epiphyseal bone centres were not properly developed. The growth cartilage of homozygous mutant mice was thinner, contained a higher density of chondrocytes (4793 ± 135, n = 3 versus 3652 ± 58, n = 3 chondrocytes/mm^2) and showed a reduction in the number of hypertrophic chondrocytes (Figure 5A3 and A4). In addition, chondrocyte columns were sometimes disrupted and chondrocytes were arranged more randomly. The bone area within the diaphysis was also strongly reduced in homozygous mutant mice (7.4 ± 1.6%, n = 3) as compared with wild-type animals (13.9 ± 2.0%, n = 3). The calcium content of homozygous mutant mice skeletons, as assessed by measurement of hydroxyapatite by X-ray absorptiometry, was at least 2- to 3-fold lower than in wild-type skeletons (Figure 5B). Moreover, bone marrow seemed to be highly hypoplasic in T3Rα$^{-/-}$ mice (Figure 5A1 and A2). In conclusion, bone ossification in the epiphysis and in the diaphysis, growth cartilage and mineralization seemed to be strongly delayed in mutant mice.

Impaired production of thyroid hormones

To explore the possibility that the abnormal phenotypes of the T3Rα$^{-/-}$ mice were due to abnormal thyroid or pituitary hormone concentrations, hormone levels were determined. Data presented in Figure 6A clearly show that the concentrations of total T3 (TT3), total T4 (TT4) and free T4 (FT4) in 3-week-old homozygous mutants were reduced to ~60% of the concentrations found in wild-type animals (P <0.05). In 5-week-old animals, the concentrations of TT3, TT4 and FT4 were reduced respectively to 40, 10 and <10% of the levels measured in wild-type animals. The stronger decrease of TT4 than TT3 suggests that the hypothyroidism resulted from an

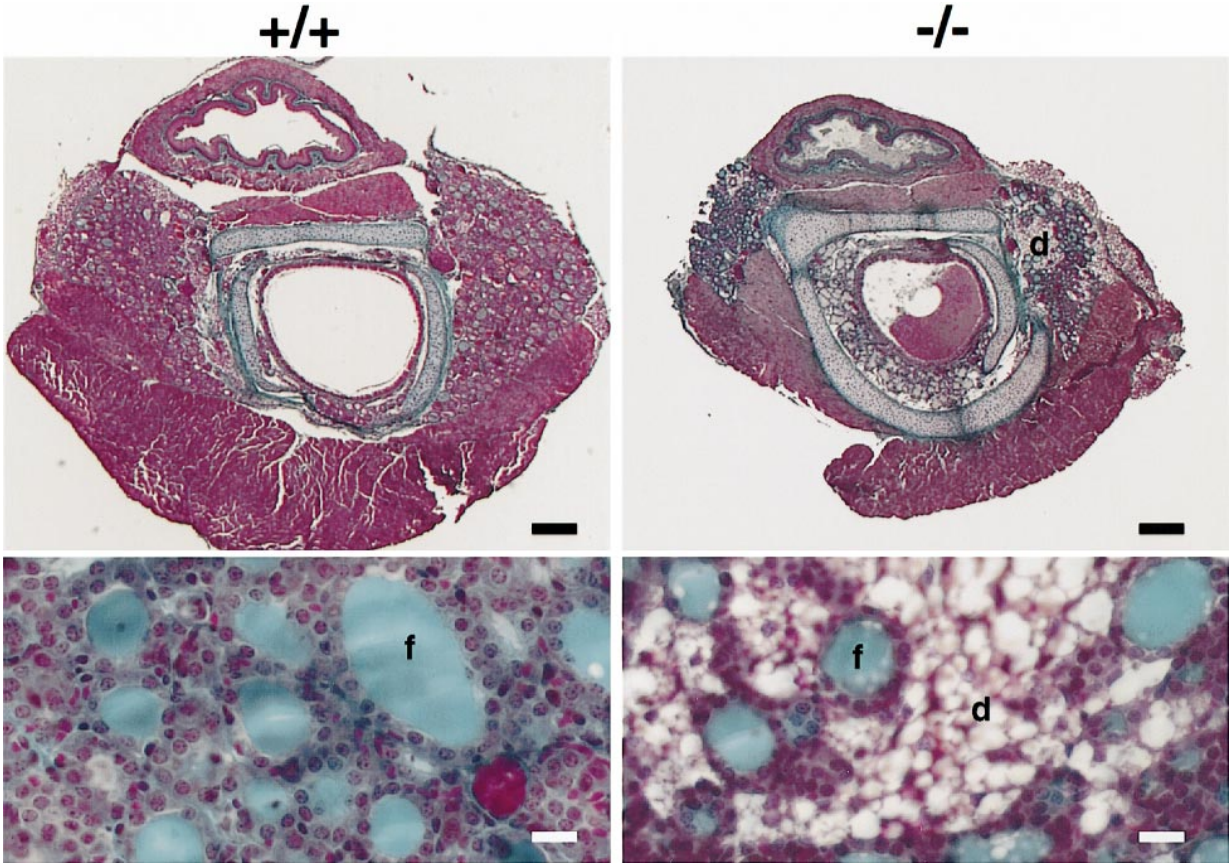

Fig. 7. Histology of the thyroid gland in wild-type mice and homozygous mutant mice. Transverse section through the trachea–esophagus block of T3Rα$^{+/+}$ (upper left) and T3Rα$^{-/-}$ (upper right) mice. Bar = 200 μm. Higher magnification of the thyroid gland of T3Rα$^{+/+}$ mice (bottom left) and T3Rα$^{-/-}$ mice (bottom right). Bar = 20 μm. f: follicles. d: area of vacuolar degeneration.

impaired T4 production by the pituitary–thyroid axis rather than from peripheral dysfunctions.

Histological examination of thyroid glands were performed on 3-week-old mutant mice exhibiting a small weight reduction (only 30–35%) and compared with those of wild-type mice. In these conditions, we could establish more clearly if the size of the thyroid gland would be affected much more than that of the overall body. Quantitative image analyses indicate that thyroid areas were reduced in overall size in 3-week-old homozygous mutant mice (mean ± SEM, 0.32 ± 0.06 mm^2, n = 5, P <0.05) as compared with wild-type animals (0.71 ± 0.17 mm^2, n = 5). The length of the thyroid gland was also reduced (860 ± 120 μm, n = 5) as compared with wild-type (1450 ± 100 μm, n = 5). Thus, the thyroid gland exhibited a clear hypoplasia. Observation at higher magnification showed a vacuolar degeneration of some thyroid follicular cells (see d on Figure 7).

β thyroid-stimulating hormone (TSH) production by the pituitary gland was assessed by RT–PCR on total mRNA (Figure 6B). βTSH mRNA was reduced at least 3-fold in T3Rα$^{-/-}$ mice as compared with wild-type mice. In contrast, growth hormone (GH) mRNA and protein levels in the pituitary gland and the serum concentration of prolactin were not significantly affected in homozygous mutant mice (Figure 6B and data not shown). Taken together, these data show that thyroid hormone production is impaired progressively in the T3Rα$^{-/-}$ mice, resulting in severe hypothyroidism at weaning time.

T3Rα$^{-/-}$ mice can be rescued by T3 injections for 1 week

Since the homozygous mutant mice suffered from deep hypothyroidism, we anticipated that these animals might be cured by exogenous supply of T3. Three-week-old homozygous mutants were injected s.c. with 1 μg of T3 daily for 7 days. Out of 15 treated animals, six survived for 3–6 months. This survival rate was much higher than the 1.3% spontaneous survival rate. Preliminary data showed that both males and females seemed to be sterile. These rescued mice exhibited a shorter life-span than wild-type and heterozygous animals. T3 injections restored the body growth but did not allow the rescued mice to reach a normal weight before death (Figure 8A). Preliminary data showed that small intestine and bones seemed to be normal (data not shown), suggesting that the delayed maturation observed in 3-week-old animals had been fully recovered. Moreover, the serum concentrations of thyroid hormone of 2.5-month-old mice were normal, showing that the pituitary–thyroid axis was fully functional (Figure 8B). In conclusion, injections of T3 for 1 week rescued mice from death and allowed complete maturation of most organs as well as efficient production of thyroid hormones.

Discussion

Impaired post-natal development results from hypothyroidism

The purpose of this work was to investigate the role of the T3Rα gene in embryonic and post-natal development

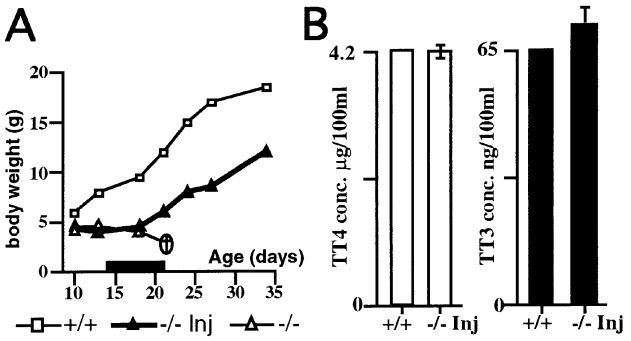

Fig. 8. Analysis of rescued T3Rα$^{-/-}$ animals. (**A**) Growth rate of T3-injected animals (▲, –/– Inj) and one non-injected (△, –/–) T3Rα$^{-/-}$ animal in comparison with one wild-type animal (□). Injections were made during the third week of life (black horizontal line). (**B**) Two months after T3 injections, thyroid hormone concentrations were determined in three rescued homozygous mutant mice and compared with those obtained in three T3Rα$^{+/+}$ mice of the same age (TT4 = 4.2 ± 0.1 µg/100 ml and TT3 = 65 ± 0.8 ng/100 ml, $P < 0.05$).

in the mouse. LacZ staining of T3Rα$^{+/-}$ mice showed that expression of the T3Rα gene is detectable at 9.5 days post-coitum (d.p.c.) in the heart, at 10.5 d.p.c. in neural tube and then in almost all tissues of the embryo from the tenth day of gestation onwards (unpublished data). Moreover, thyroid hormones are detected in mouse embryos, and hypothyroidism of the mother and of the fetus leads to abnormal embryonic development (Morreale de Escobar *et al.*, 1985; de Zegher *et al.*, 1995). These data suggest that thyroid hormones could be involved in embryonic development. Surprisingly, T3Rα$^{-/-}$ mice do not exhibit any embryonic phenotype. We assume that functional redundancies with other nuclear receptors of the same family take place, as has been previously shown between T3Rα1 and RARs in chicken erythropoietic progenitors (Schroeder *et al.*, 1992; Gandrillon *et al.*, 1994).

An overt abnormal phenotype has been observed during post-natal life in the T3Rα$^{-/-}$ mice. The mutants exhibit growth arrest, incomplete maturation of the small intestine (in particular of the muscle layers), impaired bone mineralization, delayed ossification of epiphysis and diaphysis, bone marrow hypoplasia, hypothyroidism and early lethality. Most of these abnormal phenotypes are reminiscent of syndromes described in experimental hypothyroidism in rodents and in hypothyroidism conditions in human. In rats surgically thyroidectomized or treated with propylthioluracil at birth, ossification is delayed, growth ceases after the first post-natal month and sexual development is markedly affected (Legrand, 1986). In human, congenital thyroid deficiency leads to the absence or dysgenesis of the bone epiphyses, abnormal intestine motility, growth retardation and hypothermia (Legrand, 1986; Baran, 1995; Vassilopoulou-Sellin and Sellin, 1995). These data suggest that hypothyroidism might be responsible for most of the abnormal phenotypes of the T3Rα$^{-/-}$ mice. Moreover, severe hypothyroidism might functionally inactivate T3Rβ and even turn it into a transdominant repressor for the transcriptional regulation of target genes. Such a transdominant negative activity has been proposed in human GRTH syndrome (generalized resistance to thyroid hormones), where mutations in T3Rβ abrogate binding of T3 (Refetoff *et al.*, 1993). Mental functions, growth and bone maturation are affected in these patients. This dominant-negative activity could be reinforced in our mutant animals by the absence of the T3Rα proteins. We thus assume that severe hypothyroidism leads to the death of the homozygous mutant mice. This hypothesis is demonstrated further by T3 injections, which were shown to rescue 40% of the mutant mice from death and allowed a progressive recovery of most of the abnormal phenotype. We assume that this rescue occurred through a T3Rβ-dependent pathway. Nevertheless, the recovery of the injected animals was slow, suggesting that inactivation of the T3Rα gene impaired an efficient response to T3. Moreover, growth and life-span were not totally rescued, showing that these physiological functions required a functional T3Rα gene. Because we deleted both isoforms T3Rα1 and T3Rα2 produced by the T3Rα gene, we are not able to discriminate between their respective functions. By knocking-out the T3Rα1 or the T3Rα2 isoforms independently, we would be able to answer this question and demonstrate if any functional compensation could take place between these two proteins.

T3Rα products positively control the production of thyroid hormones

The hypothyroidism of the T3Rα$^{-/-}$ mice clearly indicates that T3Rα gene products are involved in up-regulating production of thyroid hormones. The decrease in the βTSH mRNA level in the T3Rα$^{-/-}$ mice suggests that T3Rα gene products activate, directly or indirectly, the βTSH gene. The efficient production of GH and prolactin by the pituitary gland suggests that hypothyroidism does not result simply from a non-functional pituitary gland. The molecular target(s) of the T3Rα gene products is still unknown. We assume that this target should be located upstream of or at the βTSH gene level. The assumption that the products of the T3Rα gene stimulate production of the βTSH gene is reinforced by recent data with transgenic mice overexpressing the v-*erbA* oncogene (Barlow *et al.*, 1994). v-*erbA* transgenic mice exhibit hypothyroidism, but no increase in the plasma concentrations of TSH and thyroid-releasing hormone (TRH) have been detected, as might have been expected. This last observation suggests that the v-erbA protein antagonizes a putative activator of the TSH and/or TRH genes. As the v-*erbA* oncogene is known to be an efficient transdominant inactivator of T3Rs and RARs (Damm *et al.*, 1989; Sap *et al.*, 1989; Sharif and Privalsky, 1991; Chen and Privalsky, 1993), we assume that the target of the v-*erbA* oncogene might be a T3Rα product. As opposed to this positive activity of the T3Rα gene products, it has been clearly shown that the T3Rβ gene products act as inhibitors of thyroid hormone production (Forrest *et al.*, 1996b). Therefore, thyroid hormone production might be balanced through a positive T3Rα-dependent and a negative T3Rβ-dependent pathway.

The decrease of thyroid hormone concentrations in T3Rα$^{-/-}$ mice suggests that the T3Rα-dependent thyroid hormone production takes place progressively during weaning. We assume that in newborn as well as in young homozygous mutant mice, thyroid hormones are provided by the mother through milk (as suggested by attenuated phenotypes obtained in smaller litters and by Oberkotter,

1988), and/or by the thyroid gland of the young animal but through a T3Rα-independent pathway. Our data clearly demonstrate that the T3Rα gene products play a key role in switching on thyroid hormone production by weaning time. Two hypotheses can be proposed in order to explain the rescue of homozygous mutant mice and the long term restoration of thyroid hormone production by transient T3 treatment. Either injected T3 can activate the T3Rβ-dependent pathway that compensates for the deficient T3Rα pathway, or the injected T3 transiently cures animals before the onset of a delayed T3Rα-independent production of thyroid hormone. In both cases, the T3Rα gene appears to be responsible for turning on an 'adult-type' production of thyroid hormones during weaning.

Functions of the T3Rα gene during weaning

During weaning, totally mother-dependent pups become self-autonomous mice. Most of the physiological functions are modified, correlating with important organ maturation. The increase in intestine size is characterized by an extensive development of the villi and of the muscular layers. Bone ossification and calcification are enhanced. Autonomous thermoregulation and immunoprotection take place. Moreover, young mice have to find their food and to respond to external stimuli. It is striking that the phenotypes of the 3-week-old homozygous mutant mice are similar to that of younger wild-type animals, suggesting that T3Rα$^{-/-}$ mice have not switched on the 'adult' phenotype. These data are highly reminiscent of T3-controlled metamorphosis in amphibians (Tata, 1993) and strongly suggest that thyroid hormones are important during weaning in mammals to trigger the switch from a 'newborn' to an 'adult' phenotype. As no such developmental alterations occur in T3Rβ$^{-/-}$ mice, we propose that these thyroid hormone-dependent developmental functions are mediated mainly through a T3Rα pathway.

In conclusion, these data show that the T3Rα gene products are essential for post-natal development and that they are involved in a positive regulatory mechanism controlling the production of thyroid hormones.

Materials and methods

ES cell selection and generation of mutant mice

R1 ES cells (Nagy *et al.*, 1993) were electroporated with the targeting vector, then selected with G418 (Gibcco) and Gancyclovir and, after 10 days, clones were picked for screening as described (Chassande *et al.*, 1997). Two independent clones, B9 and A7, were identified, karyotyped and then injected into $C_{57}Bl_6$ blastocysts. Male chimeras were crossed with OF1 and $C_{57}Bl_6$ females to generate heterozygous mice (IFFA-CREDO, France). All animal experiments were performed according to approved protocols. ES cell and mouse tail DNA were extracted by SDS/proteinase K solubilization, purified and then digested with *Pvu*II. Southern blot was performed using the alkaline transfer procedure on Hybond N$^+$ membranes, according to the manufacturer's instructions. A 250 bp PCR fragment containing the 5′ end of exon 2 from T3Rα was used as a probe.

RNA analysis

Northern blots. RNA was extracted using the guanidium thiocyanate/acidic phenol method (Chomczinski and Sacchi, 1987). Poly(A)$^+$ mRNA was purified using the PolyAT tract System (Promega). Northern blots were carried out using the glyoxal denaturation technique and alkaline transfer onto Hybond N$^+$. Hybridization buffer (4× SSC, 0.25% low-fat milk, 1% SDS, 10 mM sodium phosphate pH 6.8 and 0.1 mg/ml denatured herring sperm DNA) was supplemented with 1% diethyl pyrocarbonate and incubated at 56°C overnight. Membranes were pre-incubated for 1 h in this solution and hybridization was performed overnight at 54°C. To detect the T3Rα transcripts, the membrane was probed using 5 ng/ml of an equimolar mixture of 9A, 15A (exon 9) and 3A (exon 8) end-labelled oligonucleotides. RNA quantification was carried out with a Phosphorimager (Molecular Dynamics, USA).

RT–PCR analysis. Oligonucleotides were from Genset (France). Reverse transcription was performed as follows, unless specified otherwise: 1 μg of total RNAs and 0.5 μg of random primers (Promega) were mixed in 10 μl, 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 (DTT), 6 mM $MgCl_2$, 0.5 mM each dNTP] were added and the mixture was incubated at 37°C for 1 h. The reaction was stopped by heating at 68°C for 5 min. PCR was performed using Goldstar thermostable polymerase (Eurogentech, Belgium) on a Perkin-Elmer 9600 thermocycler, with 1 μl of reverse transcription mix. The reaction was performed in Goldstar reaction buffer containing 2 mM $MgCl_2$ and using 100 ng of each oligonucleotide and 0.3 U of enzyme in a final volume of 50 μl. The thermocycler was pre-heated to 95°C before the introduction of the reaction mix. To allow the detection of the T3Rβ transcripts (T3Rβ1 and T3Rβ2), oligonucleotides β (GGCTTCTTTA-GAAGAACCATTCAG) and βA (CAGGAATTTCCGCTTCTGCTT) were used as primers. Thirty five cycles were performed with an annealing temperature of 56°C. HPRT and T3RΔα transcripts were detected as described previously (Chassande *et al.*, 1997).

Intestine and bone analysis

Intestine analysis. Jejunum and ileum, the proximal and distal portions, respectively, of the small intestine were dissected, 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 periodic acid–Schiff (PAS) protocol (Segal and Petras, 1992). Muscle contraction of the ileum was recorded with a force displacement transducer and an ink-writing recorder (Physiograph® MK III from Narco Biosystem, Houston, USA) as described previously (Isozaki *et al.*, 1995). Adrenaline (Lab. Aguettant, Lyon, France) and acetylcholine (ref. A66 25, Sigma) were used at a final concentration of 2 μg/ml.

Bone analysis. Leg tibias were dissected out, fixed in 70% alcohol, embedded, cut (7 μm) and stained according to the standard protocol (Meunier, 1983). Longitudinal medial sections were analysed with a true colour image-processing workstation Visiolab 1000 (Biocom®, France). The total amount of hydroxyapatite was measured by dual energy X-ray absorptiometry (pDEXA from Norland, USA; Tysarczyk-Niemeyer *et al.*, 1995).

Hormone assays and thyroid gland histology

Mice were anaesthetized with ketamine (Panpharma, France) + Hypnovel (Roche, France) and bled intracardiacally. Sampling for all experiments was performed at 2–4 p.m., to avoid day–night cycle variations. Serum concentrations of TT3 and TT4 were determined by specific radio-immunoassay, using sodium anilino-naphthalene sulfonate (ANS) and acidic buffer, as binding inhibitors of serum proteins (Rousset *et al.*, 1984). FT4 was measured using an enzyme-linked fluorescent assay kit (Vidas-FT4, Biomerieux, France). Rescued animals were obtained by daily s.c. injection of 1 μg of T3 (stock solution in 10^{-3} M NaOH) for 7 days. Tracheal blocks of mice were dissected out, immediately fixed in Bouin Holland's reagent, embedded in paraffin and cut into 5 μm thick sections as described previously (Trouillas *et al.*, 1996). Sections were stained according to the standard Masson's Trichrome protocol. Thyroid areas were determined on the largest sections by computer imaging (workstation Visiolab 1000, Biocom®, France).

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