



**HAL**  
open science

## **Genetic Analysis Reveals Different Functions for the Products of the Thyroid Hormone Receptor a Locus**

Jacques Samarut, Karine Gauthier, Michelina Plateroti, Clare B. Harvey, Graham R. Williams, Roy E. Weiss, Samuel Refetoff, Ames F. Willott, Victoria Sundin, Jean-Paul Roux, et al.

► **To cite this version:**

Jacques Samarut, Karine Gauthier, Michelina Plateroti, Clare B. Harvey, Graham R. Williams, et al.. Genetic Analysis Reveals Different Functions for the Products of the Thyroid Hormone Receptor a Locus. *Molecular and Cellular Biology*, 2001, 21 (14), pp.4748-4760. ensl-00000006

**HAL Id: ensl-00000006**

**<https://ens-lyon.hal.science/ensl-00000006>**

Submitted on 7 Apr 2006

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Genetic Analysis Reveals Different Functions for the Products of the Thyroid Hormone Receptor $\alpha$ Locus

KARINE GAUTHIER,<sup>1</sup> MICHELINA PLATEROTI,<sup>1</sup> CLARE B. HARVEY,<sup>2</sup> GRAHAM R. WILLIAMS,<sup>2</sup>  
ROY E. WEISS,<sup>3</sup> SAMUEL REFETOFF,<sup>3,4</sup> JAMES F. WILLOTT,<sup>5</sup> VICTORIA SUNDIN,<sup>6</sup>  
JEAN-PAUL ROUX,<sup>7</sup> LUC MALAVAL,<sup>7</sup> MASAHIRO HARA,<sup>1</sup>  
JACQUES SAMARUT,<sup>1\*</sup> AND OLIVIER CHASSANDE<sup>1</sup>

*Laboratoire de Biologie Moléculaire et Cellulaire de l'Ecole Normale Supérieure, UMR 5665 CNRS, LA 913 INRA, 69364 Lyon cedex 07,<sup>1</sup> and INSERM U369, Faculté de médecine, RTH Laennec, Lyon,<sup>7</sup> France; ICSM Molecular Endocrinology Group, Division of Medicine and MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, London, United Kingdom<sup>2</sup>; Departments of Medicine<sup>3</sup> and Pediatrics,<sup>4</sup> University of Chicago, Chicago, Illinois 60637; Department of Psychology, University of South Florida, Tampa, Florida 33620<sup>5</sup>; and Department of Psychology, Northern Illinois University, Dekalb, Illinois 60115<sup>6</sup>*

Received 27 November 2000/Returned for modification 13 February 2001/Accepted 15 April 2001

**Thyroid hormone receptors are encoded by the  $TR\alpha$  (NR1A1) and  $TR\beta$  (NR1A2) loci. These genes are transcribed into multiple variants whose functions are unclear. Analysis by gene inactivation in mice has provided new insights into the functional complexity of these products. Different strategies designed to modify the  $TR\alpha$  locus have led to strikingly different phenotypes. In order to analyze the molecular basis for these alterations, we generated mice devoid of all known isoforms produced from the  $TR\alpha$  locus ( $TR\alpha^{0/0}$ ). These mice are viable and exhibit reduced linear growth, bone maturation delay, moderate hypothermia, and reduced thickness of the intestinal mucosa. Compounding  $TR\alpha^0$  and  $TR\beta^-$  mutations produces viable  $TR\alpha^{0/0}\beta^{-/-}$  mice, which display a more severe linear growth reduction and a more profound hypothermia as well as impaired hearing. A striking phenotypic difference is observed between  $TR\alpha^{0/0}$  and the previously described  $TR\alpha^{-/-}$  mice, which retain truncated  $TR\Delta\alpha$  isoforms arising from a newly described promoter in intron 7. The lethality and severe impairment of the intestinal maturation in  $TR\alpha^{-/-}$  mice are rescued in  $TR\alpha^{0/0}$  animals. We demonstrate that the  $TR\Delta\alpha$  protein isoforms, which are natural products of the  $TR\alpha$  locus, are the key determinants of these phenotypical differences. These data reveal the functional importance of the non-T3-binding variants encoded by the  $TR\alpha$  locus in vertebrate postnatal development and homeostasis.**

Thyroid hormone receptors (TRs), encoded by the  $TR\alpha$  and  $TR\beta$  loci, mediate the action of triiodothyronine (T3) in all vertebrates. These nuclear proteins activate or repress the transcription of target genes by recruiting several protein complexes which modify the structure of chromatin (15). In mammals, T3 exerts homeostatic and developmental functions. Thyroid hormone (TH) deprivation leads to developmental (growth retardation, impaired neurogenesis) (8, 23, 36) or metabolic (reduced oxygen consumption, bradycardia, weakness, fatigue) disorders (13). Point mutations in the  $TR\beta$  gene result in syndromes known as resistance to thyroid hormone (30). Recently, the introduction of mutations in the genes encoding these receptors in mice has allowed further understanding of the mechanisms mediating the physiological actions of T3 (17, 19). The  $TR\beta$  locus encodes the  $TR\beta 1$  and  $TR\beta 2$  receptors. A new receptor,  $TR\beta 3$ , and its non-DNA-binding  $TR\Delta\beta 3$  variant have recently been described. The latter isoforms are proposed to arise from a novel promoter located upstream of the exons encoding the DNA binding domain (41). Genetic invalidation of the  $TR\beta 2$  gene leads to pituitary resistance to TH with elevated thyrotropin (TSH) and thyroxine (T4) levels in serum

(1). Knocking out all  $TR\beta 1$ ,  $TR\beta 2$ , and  $TR\beta 3/\Delta\beta 3$  genes reproduces this phenotype (10, 14) but also induces the loss of auditory function, due to delayed expression of a potassium channel in the cochlea (11), and results in impaired transcriptional response to T3 in liver (38). The  $TR\alpha$  locus encodes the ubiquitous  $TR\alpha 1$  receptor and a splice variant,  $TR\alpha 2$ , which cannot bind T3 and behaves as a weak inhibitor of TRs in transfection assays (21, 22). We have previously shown that, in a limited number of tissues, the  $TR\alpha$  locus also encodes two transcripts, previously characterized by Northern blotting, RNase protection, and reverse transcription (RT)-PCR analysis in embryonic stem (ES) cells, driven by a promoter located in intron 7, which generate  $TR\Delta\alpha 1$  and  $TR\Delta\alpha 2$  proteins. These proteins do not bind DNA or T3; hence, they are inhibitors of the activities of TRs and retinoic acid receptors in transfection assays (6). In vivo genetic modifications of the  $TR\alpha$  locus result in different phenotypes, according to the precise location of the mutation. In  $TR\alpha^{-/-}$  mice, the expression of  $TR\alpha 1$  and  $TR\alpha 2$  transcripts is abolished, but the  $TR\Delta\alpha$  transcripts are still expressed (12). These mice suffer from hypothyroidism and display several developmental defects, including growth retardation, altered intestinal development (28), impaired T- and B-lymphocyte maturation (3), expansion of cartilage regions in long bones, and finally growth arrest and death shortly after weaning (12). Mice which lack the  $TR\alpha 1$  and  $TR\Delta\alpha 1$  products ( $TR\alpha 1^{-/-}$ ) have been reported to exhibit

\* Corresponding author. Mailing address: Laboratoire de Biologie Moléculaire et Cellulaire, CNRS UMR 5665 ENS LA 913 INRA, Ecole Normale Supérieure, 46 allée d'Italie, 69364 Lyon Cedex 07, France. Phone: 33 4 72 72 81 71. Fax: 33 4 72 72 85 36. E-mail: Jacques.Samarut@ens-lyon.fr.

mild hypothyroxinemia, bradycardia, and lower body temperature, but they display normal growth and life span (39). The lethal phenotype of TR $\alpha^{-/-}$  mice compared to the viability of TR $\alpha 1^{-/-}$  mice might be attributed to the deeper hypothyroxinemia of the former. The involvement of TH as the cause of this phenotypic discrepancy can be ruled out. Indeed, although TR $\alpha^{-/-}\beta^{-/-}$  and TR $\alpha 1^{-/-}\beta^{-/-}$  mice both lack the known TRs and have very high serum levels of TH, TR $\alpha^{-/-}\beta^{-/-}$  mice die upon weaning (14), whereas TR $\alpha 1^{-/-}\beta^{-/-}$  mice are viable (16). Hence, the phenotypic differences must be ascribed to structural differences between the TR $\alpha$  mutant alleles. They could be attributed either to the TR $\alpha 2$  isoform, which is expressed in TR $\alpha 1^{-/-}$  but not in TR $\alpha^{-/-}$  mice, or to the balance between the TR $\alpha$  and TR $\Delta\alpha$  isoforms. In an attempt to determine the role of the non-T3-binding products, we have generated a new mouse strain harboring a targeted deletion designed to prevent the expression of all transcripts from the TR $\alpha$  locus. In this paper we describe the phenotype of these TR $\alpha^{0/0}$  mutant mice and point out similarities and striking differences with previously described TR $\alpha 1^{-/-}$  and TR $\alpha^{-/-}$  mice. This comparative analysis brings a new piece in the genetic analysis of the complex TR $\alpha$  locus and sheds new light on the respective functions of the TR $\alpha$  products in body growth, bone maturation, thermogenesis, audition, and intestinal maturation.

#### MATERIALS AND METHODS

**Targeted disruption of TR $\alpha$  gene.** The TR $\alpha^0$ -targeting vector was constructed using pGNA $\beta$  as starting plasmid (gift from P. Brulet, Institut Pasteur, Paris, France). The 3' arm homologous to TR $\alpha$  was amplified by PCR from ENS ES cell DNA (14), using the Expand High Fi system (Roche). The 5' arm was cloned from a  $\lambda$ EMBL4 library (gift from J. P. Magaud). The thymidine kinase gene was inserted downstream of the 3' arm.

ENS ES cells were electroporated with 40  $\mu$ g of linearized targeting vector and then selected with 250  $\mu$ g of G418 (Gibco-BRL) per ml and 0.2  $\mu$ M ganciclovir. Targeted clones were confirmed by Southern blot analysis using *PvuII* enzyme and a 900-bp PCR fragment extending from the 3' end of exon 3 to the 5' end of exon 4 of TR $\alpha$  as a probe. Two positive clones were injected into blastocysts of C57BL/6 recipient mice. TR $\alpha^{0/+}$  animals were derived in an inbred 129sv background. TR $\alpha^{0/0}$  mice were obtained by intercrossing heterozygous animals. Mouse breeding and handling were carried out in a certified animal facility, according to procedures approved by the local animal care and use committee.

**Purification of RNA, Northern blotting RNase protection assay, and RT-PCR analysis.** Total RNA from distal small intestine or pituitary was isolated by the improved acid-guanidine-phenol-chloroform method. For Northern blot analysis, single-stranded DNA probes were generated by a 30-cycle reaction using *Taq* DNA polymerase from Eurobio using 10 ng of TR $\alpha$  (exon 8), growth hormone (GH), or hypoxanthine phosphoribosyltransferase (HPRT) PCR fragments as a template, and 20 ng of a specific antisense primer: oligonucleotides  $\alpha 15A$  (CA GCCTGCAGCAGGCCACTTCCG), GH $\alpha$  (GTCAAACCTGTTCATAGGTTTG), and HPRT $\alpha$  (CACAGGACTAGAACACCTGC) were used as primers to generate the TR $\alpha$ , GH, and HPRT probes, respectively.

RNase protection was performed using reagents from Ambion (Austin, Tex.) according to the manufacturer's instructions. HPRT and TR $\alpha$  cDNA fragments were generated by RT-PCR and inserted into a pGEMt easy vector (Promega). Oligonucleotides HPRTs (GCTGGTGAAGGACCTCT) and HPRT $\alpha$  (see above) were used to generate the HPRT fragment, and TR $\alpha$ Cs (CTCTGTGATCCTGCTGTCCACAG) and TR $\alpha 1\alpha$  (CGACTTTCATGTGGAGGAAG) were used to generate the TR $\alpha$  cDNA.

RT was performed as described in reference 12. cDNA (0.05  $\mu$ g) was used for each PCR with Eurobio *Taq*. To allow the detection of TR $\alpha$  gene products (TR $\alpha 1$ , TR $\alpha 2$ , TR $\Delta\alpha 1$ , and TR $\Delta\alpha 2$ ) by RT-PCR, we used a sense primer within exon 8 ( $\alpha 2'$ , CTGCCTTGCGAAGACCAGATC) and an antisense primer within exon 9 ( $\alpha 3$ , GCGGTGGTTGACGTAGTGCTC). All the oligonucleotides were purchased from MWG (Ebersberg, Germany).

**Morphological analysis of long bones and growth plate cartilage.** Skin and muscle were dissected from forelimbs and hindlimbs of 3-week-old wild-type and TR $\alpha^{0/0}$  null mice. For anatomical analysis of long bones, one upper and one

lower limb from each animal were fixed in 80% ethanol for 1 to 4 days and transferred to 95% ethanol for a further 1 to 8 days, followed by acetone for 1 to 2 days at 4°C. Fixed limbs were stained with alizarin red and alcian blue 8GX (0.3% alcian blue in 70% ethanol [1 ml]–0.1% alizarin red in 95% ethanol [1 ml]–glacial acetic acid [1 ml]–70% ethanol [17 ml]) for 2 days at 37°C and transferred sequentially for 2- to 4-day periods through solutions of 1% KOH, 1% KOH–20% glycerol, 1% KOH–40% glycerol, 1% KOH–60% glycerol, and 1% KOH–80% glycerol at 4°C until, finally, destained limbs were stored in 100% glycerol at 4°C prior to analysis and photography under a dissecting microscope. In these preparations, mineralized bone is stained by alizarin red and cartilage is stained by alcian blue 8GX. For mineralization analysis, tibias were dissected out, fixed in 70% alcohol, embedded in paraffin, and cut (7  $\mu$ m), and undecalcified sections were stained according to the standard protocol (26). For histological analyses, the paired limbs were fixed in 10% neutral buffered formalin for 4 to 6 days and subsequently decalcified for 2 days in 10% formic acid–10% neutral buffered formalin at 20°C. Limbs were embedded in paraffin and 3- $\mu$ m sections were cut, deparaffinized in xylene, and rehydrated. Sections were stained with hematoxylin and eosin or alcian blue 8GX and van Gieson to visualize cartilage matrix mucopolysaccharides in blue and collagen-containing bone osteoid in red, according to standard histological protocols.

**Body temperature.** The system we used, ELAMS (Electronic Laboratory Animal Monitoring System by BioMedic Data Systems, Inc., Seaford, Del.), consisted of implantable programmable temperature transponders (IPTT-100) and a portable data scanner (DAS-5007). The transponders were implanted subcutaneously in the backs of three 8- to 12-week-old males from each genotype. Animals were maintained under anesthesia with midazolam. All animals were kept in a climate-controlled room with 12-h alternating light and dark cycles. Temperature measurements were initiated 3 days after the implantation and were recorded in duplicate at 10 a.m., while the animals were moving freely in their cage with the lid removed, and the scanner was placed within 5 cm of the transponder. Body temperature monitoring continued for up to 7 days.

Temperature was measured rectally at the same time in TR $\alpha^{0/0}\beta^{-/-}$  by means of a digital recording with a thermistor system. Values were not significantly different from those recorded by the transponder (data not shown).

**Growth measurements.** Male mice anesthetized with methoxyflurane were weighed and measured (nose to tail base) weekly from 2 to 9 weeks of age. Mice were housed five per cage, and food and water were provided ad libitum. The numbers of mice used from each genotype were as follows: wild type, 19 to 29; TR $\beta^{-/-}$ , 6 to 17; and TR $\alpha^{0/0}$ , 10 to 22.

**Hormone measurements.** Serum T4 and TSH were measured by radioimmunoassays as previously described (29).

**Hearing tests.** The auditory brainstem response (ABR) was used to assess the auditory sensitivity by a procedure that has been described in detail elsewhere (35). Tucker-Davis Technologies (Gainesville, Fla.) hardware and software (AeP and Sigen packages) were employed. Briefly, mice were anesthetized with sodium pentobarbital (Nembutal, 1.2  $\mu$ l per g of body weight intraperitoneally [i.p.]) and chlorprothixene (Taractan, 0.5  $\mu$ l/g i.p.) and kept warm with a heating pad. The average response (waveform) per 100 presentations of tone bursts (1 ms rise and fall; 3 ms duration; rate, 21 Hz; frequencies of 4, 8, 12, 16, and 24 kHz) was recorded using a subdermal needle electrode placed in the scalp. Each tone frequency was presented in descending intensity steps beginning at 80-dB sound pressure level and ending when a visually discernible ABR waveform could no longer be detected. Thresholds were then determined to within 5 dB for each frequency (35). The numbers of mice (60 to 90 days old) tested from each genotype were as follows: wild type, 7; TR $\beta^{-/-}$ , 9; TR $\alpha^{0/0}$ , 7; and combined TR $\beta^{-/-}$ –TR $\alpha^{0/0}$ , 4.

**Morphological staining and immunohistochemistry.** Three-week-old mice were killed by cervical dislocation and the small intestines were immediately removed. Proximal jejunums and distal ilea were collected separately and fixed in Carson's solution overnight at 4°C. They were then embedded in paraffin and 5- $\mu$ m sections were applied on polylysine-coated slides. For morphological observations, after dewaxing and rehydration, the slides were stained with hematoxylin and eosin.

Immunocytochemistry experiments were performed with a monoclonal antibody (Novocastra Laboratories) for Ki-67 detection. Polyclonal antibody PAI-211 (Affinity Bioreagents, Inc.) for TR $\alpha 1$  and TR $\Delta\alpha 1$  protein localization and another recognizing the N-terminal part of both TR $\alpha 1$  and TR $\alpha 2$  proteins (generous gift of D. Baas [4]) were used in combination with secondary biotinylated antibody and streptavidin-peroxidase detection system (Histomouse; Zymed). The tissue was counterstained lightly with hematoxylin. Before incubation with the primary antibody, the slides were immersed in 0.01 M citrate buffer, pH 6, and microwaved for 15 min.

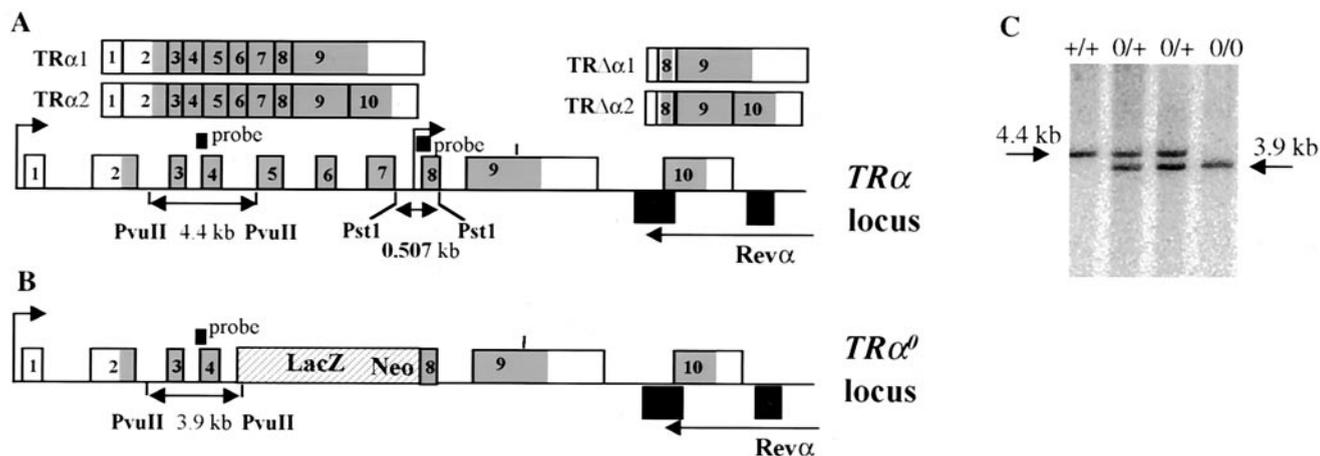


FIG. 1. Targeted disruption of the *TRα* gene by homologous recombination. (A) Structure of the *TRα* gene and of the various isoforms encoded by this gene. The two transcription start sites are indicated by upper arrows. The differential splice site in exon 9 is indicated by a vertical bar. Exons are numbered starting downstream to the distal promoter. Grey, coding regions. The structure of the transcripts is shown. (B) The targeted *TRα<sup>0</sup>* allele contains a deletion extending from the middle part of intron 4 to the beginning of exon 8. The probe used for Southern blot analysis and the size of the fragment detected after digestion with *PvuII* are indicated. (C) Detection of wild-type (+/+), heterozygous (0/+), and homozygous (0/0) littermates by Southern blot analysis.

**Cells, plasmids, transfections, and Western blotting.** HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The plasmids containing the cDNAs of human *TRα1* (pSG5h*TRα1*), rat *TRβ1* (pSG5r*TRβ1*), mouse *TRΔα1* (pSG5m*TRΔα1*), Flag*TRΔα1* (CMV-Fm*TRΔα1*), and mouse *TRΔα2* (pSG5m*TRΔα2*) have been described previously (6). HeLa cells were transfected using a calcium phosphate procedure as previously described (7) and harvested after 48 h. Lactacystin was a gift from Satoshi Omura (Tokyo, Japan). Western blotting was performed as previously described (6). We used the no. 21 polyclonal antibody (gift of J. Ghysdael) as described in reference 5 to detect *TRα1* and *TRΔα1* proteins and the M2 anti-Flag antibody (IBI) to detect the Flag-*TRΔα1* protein.

## RESULTS

**The *TRα<sup>0/0</sup>* mutation abolishes the production of all products from the *TRα* locus.** To generate mice deprived of any *TRα* gene product (*TRα1* and *TRα2* and their truncated counterparts *TRΔα1* and *TRΔα2*), a *lacZ neo<sup>r</sup>* cassette (12) was introduced by homologous recombination between exon 4 and exon 8 of the *TRα* gene (Fig. 1A) to generate the *TRα<sup>0</sup>* allele (Fig. 1B). This removed the DNA binding region of *TRα1* and *TRα2*, preventing the production of a chimeric protein that could act in a dominant-negative manner upon *TRβ*. It also removed the internal promoter in intron 7 which has been shown to drive the transcription of *TRΔα1* and *TRΔα2* (6). The *TRα<sup>0</sup>* mutated allele was introduced into ES cells by homologous recombination. The structure of the modified allele was checked by Southern blotting (Fig. 1C). Two independent positive clones were injected into host blastocysts. Heterozygous mice were derived from an inbred 129Sv genetic background. They were then intercrossed to obtain *TRα<sup>0/0</sup>* mice.

We checked for the expression of *TRα* transcripts in wild-type, *TRα<sup>0/0</sup>*, and previously described *TRα<sup>-/-</sup>* mice (12). This analysis was performed on intestine RNA where the four *TRα* isoforms have been shown to be expressed. Northern blot analysis (Fig. 2A) showed that all four transcripts could be identified in the distal ileum of wild-type mice. In *TRα<sup>-/-</sup>* mice, *TRα1* and *TRα2* RNAs were undetectable, whereas the *TRΔα1* and *TRΔα2* transcripts were still detected. In *TRα<sup>0/0</sup>*

mice, neither *TRα* nor *TRΔα* transcripts were detected. In agreement with these data, RT-PCR did not allow the detection of transcripts containing exons 8 and 9 in the intestines of *TRα<sup>0/0</sup>* mice (Fig. 2B). RNase protection analysis (Fig. 2C) revealed that the *TRα1* and *TRα2* isoforms, which are highly expressed in wild-type samples, are almost undetectable in *TRα<sup>-/-</sup>* and *TRα<sup>0/0</sup>* samples. Occasionally, the 448- and 386-nucleotide fragments were detected in the mutant mice, corresponding to transcription reading through the stop sites within the *lacZ neo<sup>r</sup>* cassette. It is noteworthy that RNase protection showed that wild-type ileum contained similar amounts of *TRα1* and *TRα2* transcripts. Underrepresentation of the large *TRα1* transcript in Northern blot experiments may have been caused by poor transfer onto nylon membranes. Two protected fragments, whose sizes correspond to the *TRΔα1* and *TRΔα2* isoforms, were detected in wild-type and *TRα<sup>-/-</sup>* but not in *TRα<sup>0/0</sup>* samples. It is thus concluded that in the distal ileum of wild-type mice, four *TRα* isoforms are expressed, including *TRΔα1* and *TRΔα2*, which are expressed at much lower levels than *TRα1* and *TRα2*. The *TRα<sup>0</sup>* mutation completely abolishes the transcription from the *TRα* locus, in contrast with the *TRα* mutation which still allows the production of *TRΔα* transcripts, as previously described (Fig. 2D).

***TRα<sup>0/0</sup>* and *TRα<sup>0/0</sup>β<sup>-/-</sup>* mice display normal embryonic development and are viable.** It has previously been shown that *TRα<sup>-/-</sup>* and *TRα<sup>-/-</sup>β<sup>-/-</sup>* mice were born in Mendelian proportions but died after weaning (12, 14). Lethality was found in 129SV, BALB/c, and C57BL/6 genetic backgrounds after eight backcrosses. When *TRα<sup>0/+</sup>* mice were intercrossed, among 346 born pups, 98 (28.3%), 170 (49%), and 78 (22.5%) were, respectively, wild-type, heterozygous, and homozygous mutant progenies. Unlike the *TRα<sup>-/-</sup>* mice, all *TRα<sup>0/0</sup>* mice survived over the weaning period and the mortality in the adult population was not different from that observed in the wild type. Male and female *TRα<sup>0/0</sup>* mice were fertile. Viability and fertility were observed in C57BL/6 and 129SV backgrounds. *TRα<sup>0/0</sup>* mice were crossed with *TRβ<sup>-/-</sup>* mice to obtain *TRα<sup>0/+</sup>*

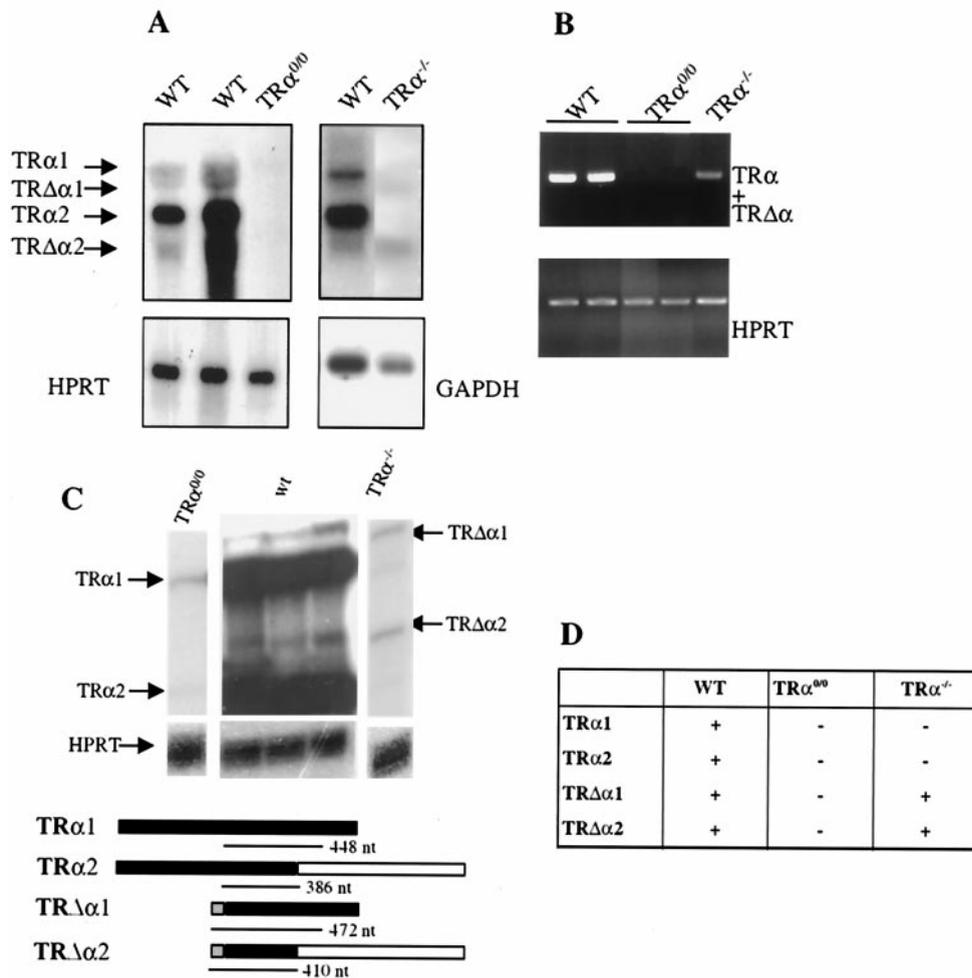


FIG. 2. Expression of the different transcripts encoded by the *TR $\alpha$*  locus in the distal ilea of the different mutants. (A) A Northern blot assay was performed using 20  $\mu$ g of total RNA isolated from the distal ilea of animals carrying the indicated genotypes. The radiolabeled probe hybridized to both TR $\alpha$  and TR $\Delta\alpha$  transcripts. A glyceraldehyde-3-phosphate dehydrogenase or a hypoxanthine phosphoribosyl transferase probe was used as an internal control. (B) Representative semiquantitative RT-PCR analysis of mRNAs encoded by the *TR $\alpha$*  locus in wild-type (WT), TR $\alpha^{-/-}$ , and TR $\alpha^{0/0}$  mice. The primers used allowed the amplification of both TR $\alpha$  and TR $\Delta\alpha$  transcripts. HPRT was used as an internal control. (C) RNase protection assay. Twenty micrograms of total distal ileum RNA from either wild-type, TR $\alpha^{0/0}$ , or TR $\alpha^{-/-}$  mice was mixed with 75 fmol of HPRT and 75 fmol of TR $\alpha$  RNA probes. The sizes of the protected TR $\alpha$  fragments and the positions of the TR $\alpha$  transcript isoforms are indicated. (D) Summary of the TR $\alpha$  and TR $\Delta\alpha$  mRNA expression in the distal ileum of each genotype.

$\beta^{-/-}$  progenies. These mice were then intercrossed and their progenies contained TR $\alpha^{0/0}\beta^{-/+}$  and TR $\alpha^{0/+}\beta^{-/-}$  animals that were all viable. Because of the low fertility displayed by TR $\alpha^{0/+}\beta^{-/-}$  animals, only TR $\alpha^{0/0}\beta^{-/+}$  mice were used to further generate double-homozygous TR mutants (TR $\alpha^{0/0}\beta^{-/-}$ ). The proportion of TR $\alpha^{0/0}\beta^{-/-}$  progenies was 19.5%. This slight deviation from Mendelian ratios may reflect marginal embryonic lethality or occasional improper maternal care for TR $\alpha^{0/0}\beta^{-/-}$  newborn mice. However, this result indicates that TRs are not absolutely required for embryonic viability. TR $\alpha^{0/0}\beta^{-/-}$  mice survived beyond weaning time and were viable for at least six months. Only occasionally were progenies obtained from TR $\alpha^{0/0}\beta^{-/-}$  intercrossings or from TR $\alpha^{0/0}\beta^{-/-}$  males or females mated with wild-type partners, suggesting that these animals have a very low fertility.

**Deregulation of the pituitary-thyroid axis in TR $\alpha^{0/0}\beta^{-/-}$  mice.** The serum levels of T3 and T4 are strictly controlled by

the direct hormonal feedback on TSH-releasing hormone (TRH) and TSH secretion, in the hypothalamus and the pituitary, respectively (20, 24, 29). We and others have previously demonstrated that TR $\beta$  plays a key role in this feedback loop since TR $\beta^{-/-}$  mice display high T3, T4, and TSH levels (11, 14, 1). The role of TR $\alpha$  in this regulation has not been clearly established. TR $\alpha 1^{-/-}$  mice demonstrated only a slight decrease of T4 and TSH concentrations in males (39), whereas TR $\alpha^{-/-}$  mice presented a progressive but severe reduction of circulating T4 and T3 between the third and the fourth week after birth (12). In TR $\alpha^{0/0}$  adult males, basal concentration of T4, but not T3 or TSH, is slightly but significantly reduced compared to wild-type mice. Furthermore, TSH and T4 suppression by increasing doses of T3 is more profound in TR $\alpha^{0/0}$  than in wild-type mice (25). In adult TR $\alpha^{0/0}\beta^{-/-}$  mice, the concentrations of serum T4, T3, and TSH were increased 14-fold, 13-fold, and more than 200-fold respectively, compared to

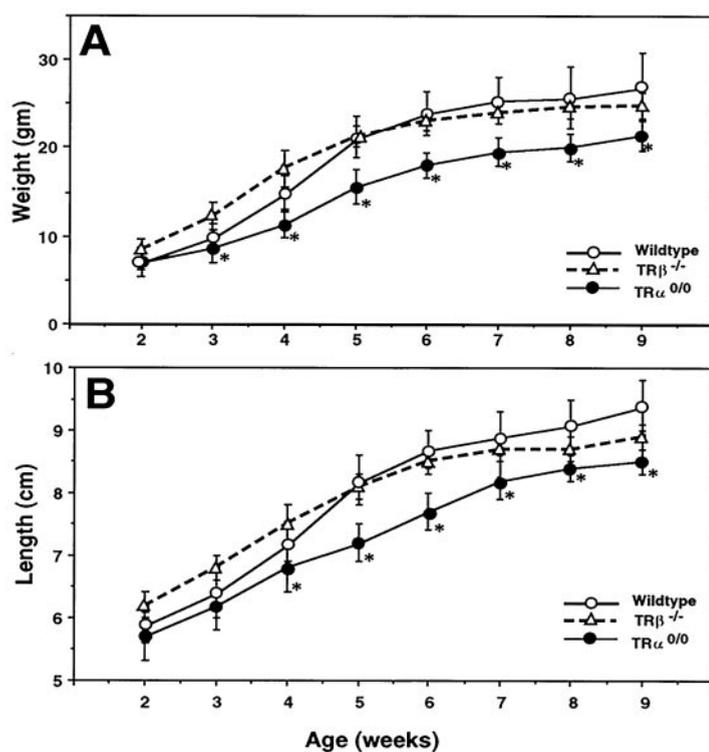


FIG. 3. Comparison of growth in wild-type and TR $\alpha$ - and TR $\beta$ -deficient mice. Weight (A) and length (B) were measured weekly from 2 to 9 weeks and plotted as means  $\pm$  standard deviations. The numbers of animals are given in Materials and Methods. \*,  $P < 0.01$  for wild-type and TR $\alpha^{0/0}$  mice. (C) Body weights of wild-type, TR $\alpha^{0/0}$ , and TR $\alpha^{0/0}\beta^{-/-}$  mice (in grams). (D) Northern blot analysis of pituitary GH mRNA from wild-type, TR $\alpha^{0/0}$ , and TR $\alpha^{0/0}\beta^{-/-}$  mice. One to two micrograms of total RNA from individual pituitaries was loaded. Single-stranded DNA probes for HPRT and GH were used.

those of wild-type mice. These levels are comparable with those previously reported for TR $\alpha 1^{-/-}\beta^{-/-}$  animals (11-, 30-, and 60- to 160-fold, respectively) (16) or 3-week-old TR $\alpha^{-/-}\beta^{-/-}$  mice (7-, 20-, and more than 100-fold, respectively) (14).

**Reduced growth rate and delayed bone maturation in TR $\alpha^{0/0}$  and TR $\alpha^{0/0}\beta^{-/-}$  mice.** The growth of TR $\alpha^{0/0}$  males and their wild-type littermates was monitored over a two-month period. TR $\alpha^{0/0}$  mice showed a reduced increase of body weight compared to wild-type animals, at all ages analyzed, in both 129Sv (not shown) and C57BL/6 genetic backgrounds (Fig. 3A). The time course of linear growth displayed similar differences (Fig. 3B), showing that alteration of body growth fully accounts for the weight differences and that the TR $\alpha^0$  mutation does not result in an abnormal weight-to-length ratio. TR $\beta^{-/-}$  mice displayed no difference in weight or length growth compared to that of the wild type (Fig. 3A and B).

The growth of TR $\alpha^{0/0}\beta^{-/-}$  mice was further reduced compared to the TR $\alpha^{0/0}$  mutants, though no difference could be observed at birth. Weight reductions of 50 and 30% were observed in mice at weaning time and in adult mice, respectively, compared to wild-type mice (Fig. 3C).

The synthesis of GH has been shown to be regulated by TH and to be decreased in TR $\alpha 1^{-/-}\beta^{-/-}$  mice (16), which display marked growth retardation. We compared the expressions of the GH mRNA in the pituitary glands of wild-type, TR $\alpha^{0/0}$ , and TR $\alpha^{0/0}\beta^{-/-}$  mice. As shown in Fig. 3D, the amount of GH RNA was not altered in TR $\alpha^{0/0}$  mice but was markedly re-

duced in double-knockout animals. These data suggest that the reduced growth observed in TR $\alpha^{0/0}$  mice may not be due to a reduced production of GH.

Since linear growth was affected in TR $\alpha^{0/0}$  mice, we studied endochondral ossification in their long bones. For this purpose, anatomical and histological analyses were performed. In 3-week-old TR $\alpha^{0/0}$  animals at weaning, the ends of metatarsals and phalanges of the foot, the condyles and growth plate of the femur, and the growth plates of the tibia and fibula stained positively for cartilage with alcian blue (Fig. 4A). In wild-type mice, alcian blue staining was markedly diminished and replaced by alizarin red staining in these areas, indicating that bone formation and mineralization were more advanced than in TR $\alpha^{0/0}$  animals. Furthermore, ossification of the patella was delayed in TR $\alpha^{0/0}$  mice relative to that in wild-type mice (Fig. 4A). Similar evidence of delayed bone formation and mineralization was seen in the upper limbs of 3-week-old TR $\alpha^{0/0}$  mice (data not shown). Goldner staining of undecalcified longitudinal sections of tibias revealed that the ossification of the epiphyses was delayed in TR $\alpha^{0/0}$  mice, and the density of trabecular bone in the metaphyses was markedly lower than in control mice (Fig. 4B). Histological analyses demonstrated that tibial growth plates from TR $\alpha^{0/0}$  mice were grossly disorganized compared to those of wild-type animals (Fig. 4B, C, and D), and similar findings were observed in other growth plates throughout the upper and lower limbs of TR $\alpha^{0/0}$  mice (data not shown). In particular, proliferating growth plate

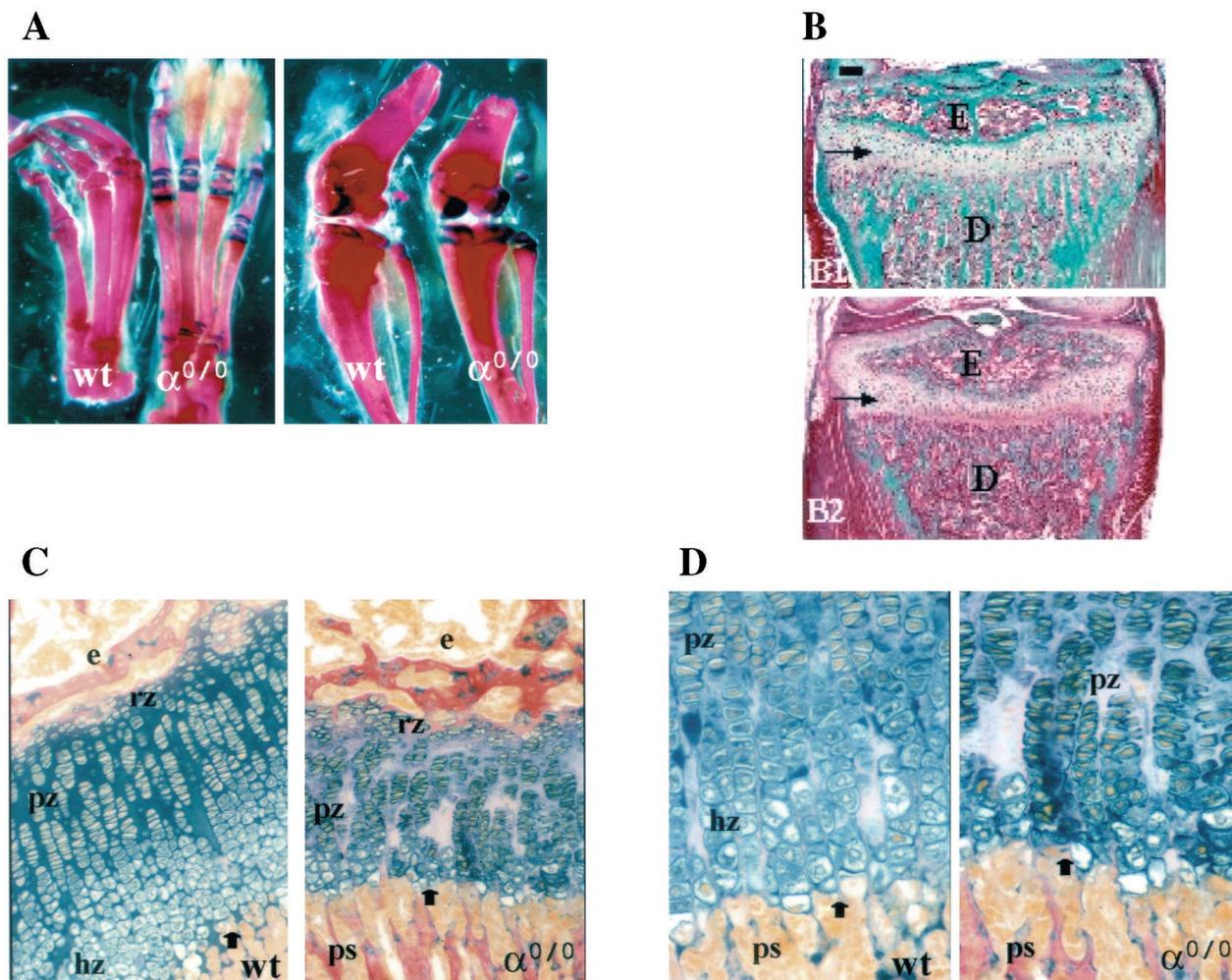


FIG. 4. Morphological appearance of lower limbs and tibial growth plates in 3-week-old wild-type (wt) and TR $\alpha$  gene knockout ( $\alpha^{0/0}$ ) animals. (A) Lower-limb skeletal mounts stained with alizarin red and alcian blue 8GX showing feet (left) and knee joint including lower femur and upper tibia with fibula (right); magnification,  $\times 8$ . Red staining shows mineralized bone and blue shows cartilage growth plates; the patella is shown to the right of each knee joint and is stained red in the wt, indicating that mineralization is complete, but blue in TR $\alpha^{0/0}$ , demonstrating the persistence of cartilage. (B) Longitudinal medial bone sections stained with modified Goldner of TR $\alpha^{+/+}$  (wt) and TR $\alpha^{0/0}$  mice. Mineralized matrix is stained in green. E, epiphysis; D, diaphysis; horizontal arrows, growth plate. (C and D) alcian blue 8GX and van Gieson staining of proximal tibial growth plates from wt and TR $\alpha^{0/0}$  mice; a section through the whole growth plate (magnification,  $\times 200$ ) is shown in panel C and a section containing the distal half of the growth plate (magnification,  $\times 400$ ) is shown in panel D. Black arrows, resorption front which separates the growth plate from underlying primary spongiosum; e, epiphysis; ps, primary spongiosum; rz, reserve zone chondrocytes; pz, proliferative zone; hz, hypertrophic zone.

chondrocytes failed to form discrete columns and the hypertrophic zone was markedly diminished and morphologically indistinct in TR $\alpha^{0/0}$  mice compared to wild-type mice, suggesting that hypertrophic chondrocyte differentiation failed to progress normally in TR $\alpha^{0/0}$  mice. Furthermore, the degree of alcian blue staining of the growth plate cartilage matrix in TR $\alpha^{0/0}$  mice was diminished and patchy compared to that in wild-type animals, supporting the view that epiphyseal growth plate architecture is disorganized and endochondral ossification is disrupted in TR $\alpha^{0/0}$  mice (Fig. 4C and D). In TR $\alpha^{0/0}$   $\beta^{-/-}$  mice, a similar phenotype was observed (data not shown). These alterations were similar to those described previously for TR $\alpha^{-/-}$  and TR $\alpha^{-/-}\beta^{-/-}$  mice (12, 14) and are reminiscent of abnormalities in epiphyseal growth plates of hypothyroid rats (34).

**TR $\alpha^{0/0}\beta^{-/-}$  mice display severe hypothermia.** TH plays a crucial role in the control of thermogenesis, and hypothyroidism results in decreased oxygen consumption and heat production (13). We examined the temperatures of adult wild-type and mutant mice, using both a rectal probe and a temperature-sensitive probe implanted under the skin. The two techniques provided similar data. The mean body temperature of TR $\beta^{-/-}$  mice was similar to that of wild-type mice, but it was 0.4°C lower in TR $\alpha^{0/0}$  mice (Fig. 5). Interestingly, the mean body temperature of TR $\alpha^{0/0}\beta^{-/-}$  animals was dramatically decreased (-4°C) relative to that of the wild type (Fig. 5). These data suggest functional redundancy between the TR $\beta$  and the TR $\alpha$  loci in the control of basal body temperature.

**Decrease of auditory sensitivity in TR $\alpha^{0/0}$  mice and deafness in TR $\alpha^{0/0}\beta^{-/-}$  mice.** Several authors have described deafness

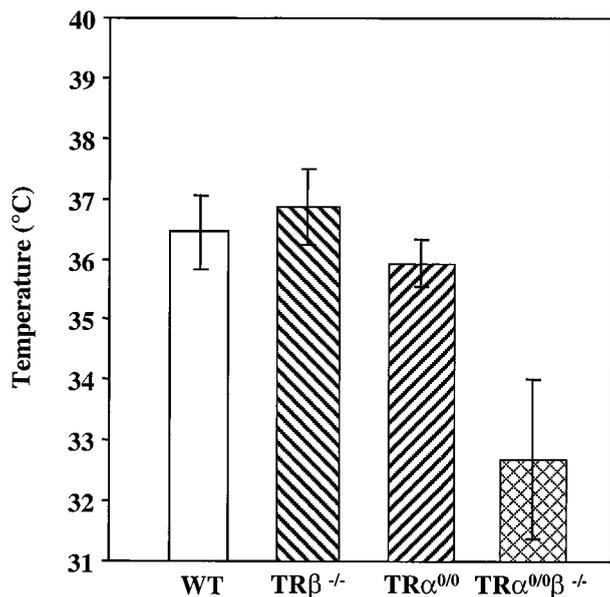


FIG. 5. Body temperature was measured at 10 a.m. with implantable transponders for 7 days in at least 3 male mice aged 8 to 12 weeks for each of the wild-type (WT) TRβ<sup>-/-</sup>, TRα<sup>0/0</sup>, and TRα<sup>0/0</sup>β<sup>-/-</sup> genotypes. Each bar represents a mean value ( $\pm$  standard deviation). Body temperatures of TRα<sup>0/0</sup> and TRα<sup>0/0</sup>β<sup>-/-</sup> animals were statistically different from those of wild-type control animals ( $P < 0.001$  and  $P < 0.001$ , respectively, by the Student  $t$  test).

in mice lacking both TRβ1 and TRβ2, but not in mice lacking TRβ2 only or TRα1 (1, 11), suggesting that TRβ1 is the only mediator of this T3-dependent function. However, the role of TRα2 and the effect of the double knockout on the integrity of hearing have not been documented. We therefore measured the ABR threshold in TRα<sup>0/0</sup> and TRα<sup>0/0</sup>β<sup>-/-</sup> mice. Whereas at low or middle frequencies TRα<sup>0/0</sup> mice had a normal hearing threshold, they exhibited a marked loss of sensitivity at high tone frequencies (Fig. 6). In TRα<sup>0/0</sup>β<sup>-/-</sup> mice, the loss of

hearing threshold was significantly more severe than that observed in TRβ<sup>-/-</sup> animals. These data suggest that, although the TRβ1 receptor is the major mediator of T3 action, in addition the integrity of the TRα gene is required to achieve full function of the auditory apparatus.

**The intestinal phenotype is mildly altered in TRα<sup>0/0</sup> mice compared to that of TRα<sup>-/-</sup> mice.** In mammals, the small intestine is lined by a continuously renewable population of epithelial cells. The pluripotent crypt stem cells give rise to immature cells that migrate and differentiate in the villus compartment and die after exfoliation at the villus tip. It was previously demonstrated that TRα<sup>-/-</sup> mice displayed a strong impairment of postnatal intestinal development (14, 28). It is noteworthy that the intestine is one of the few organs where the TRΔα and the TRα isoforms are coexpressed. In order to differentiate functions of the TRα1/α2 and TRΔα1/Δα2 isoforms in the intestine epithelium, we carried out a comparative investigation of morphofunctional parameters in TRα<sup>0/0</sup>, TRα<sup>-/-</sup>, and wild-type mice.

The size of the small intestine mucosa was reduced in TRα<sup>0/0</sup> mutants compared to that of wild-type animals. This was mainly due to the reduced lengths of the villi, resulting from a decreased number of epithelial cells per crypt-villus axis (Fig. 7A and B). These features were more strongly affected in the TRα<sup>-/-</sup> mice (Fig. 7C). The proliferation and differentiation parameters of the epithelial cells also indicated a stronger impairment in TRα<sup>-/-</sup> than in TRα<sup>0/0</sup> mice (27a). In contrast to the TRα<sup>-/-</sup> mutant animals that displayed a thinning of the smooth muscular layers (12, 28), TRα<sup>0/0</sup> mutants appeared normal in this respect. Taken together, these data strongly suggest that the severity of the intestinal phenotype correlates mostly with the persistence of the TRΔα transcripts and not with the absence of TRα1 and TRα2 isoforms.

**The intestinal phenotype is more severe in TRα<sup>-/-</sup>β<sup>-/-</sup> than in TRα<sup>0/0</sup>β<sup>-/-</sup> mice.** It was previously shown that combining the TRα<sup>-</sup> and TRβ<sup>-</sup> mutations in TRα<sup>-/-</sup>β<sup>-/-</sup> mice results in a more dramatic alteration of the structure of the

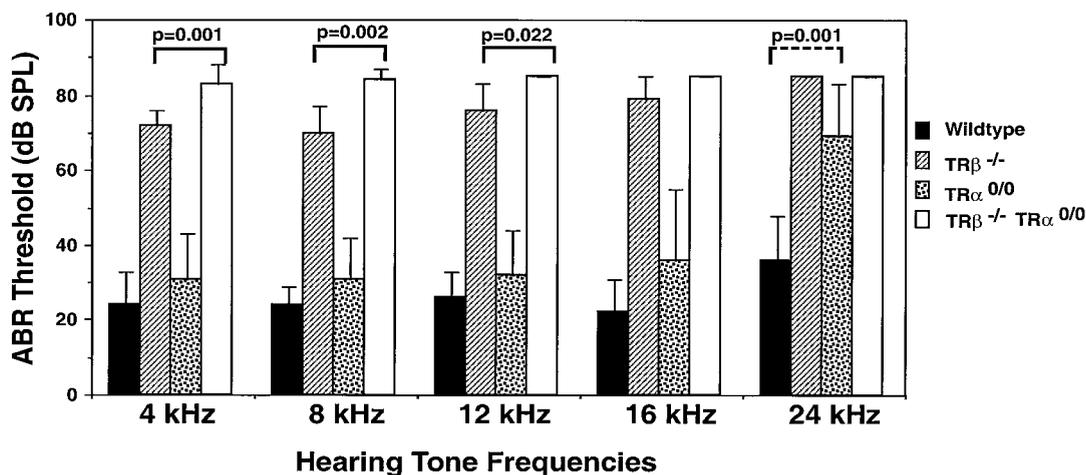


FIG. 6. ABR threshold in wild-type and TR gene knockout mice. The average response per 100 presentations of tone bursts (1 ms rise and fall; 3 ms duration; rate, 21 Hz; frequencies of 4, 8, 12, 16, and 24 kHz) were recorded. The numbers of animals are given in Materials and Methods. At all frequencies the difference between wild-type and TRβ<sup>-/-</sup> and TRα<sup>0/0</sup>β<sup>-/-</sup> mice was  $P < 0.001$ . Solid lines,  $P$  values for differences between TRβ<sup>-/-</sup> and TRα<sup>0/0</sup>β<sup>-/-</sup>; dashed line,  $P$  values for differences between the wild type and TRα<sup>0/0</sup>.

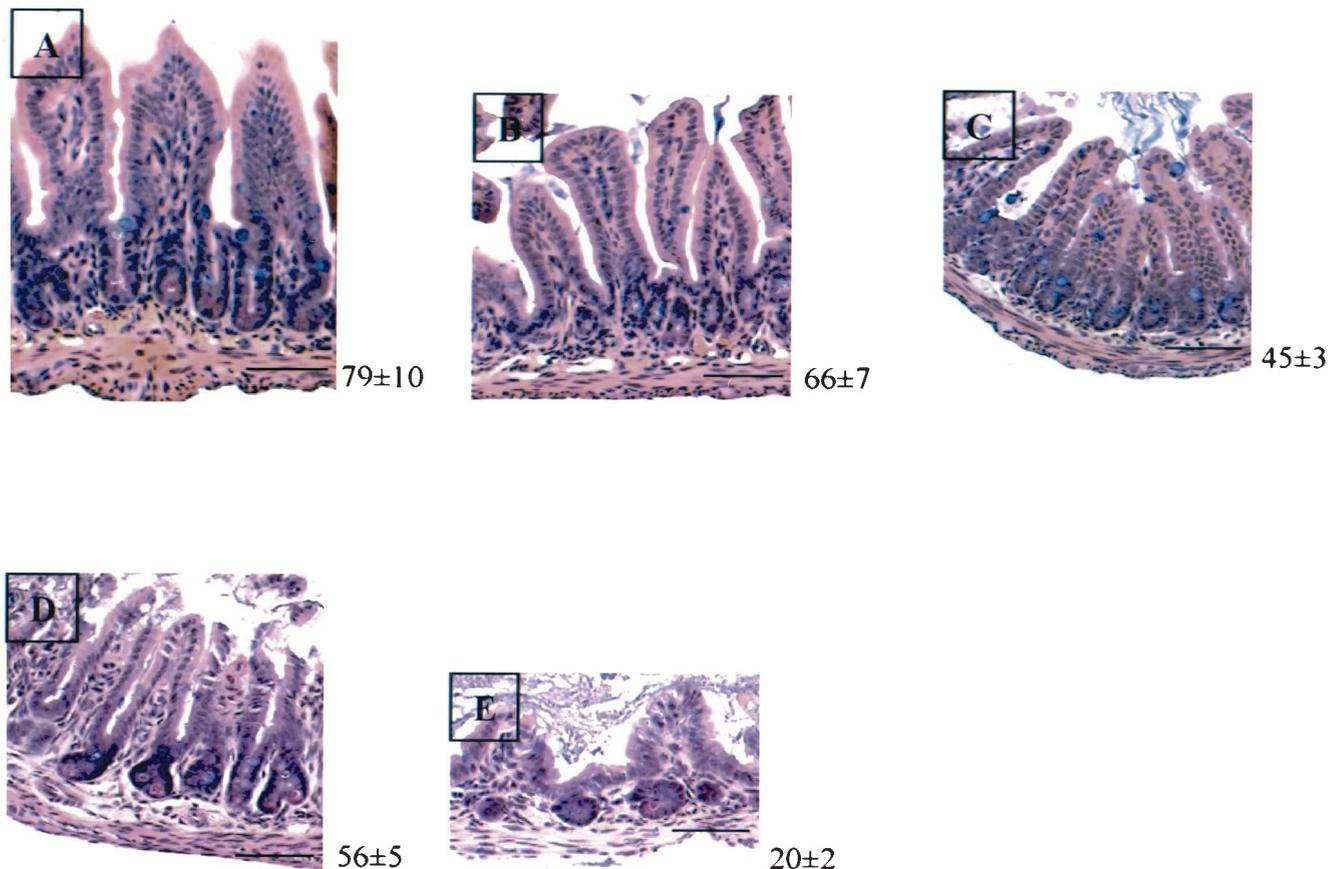


FIG. 7. Morphological appearance of distal small intestine in 3-week-old animals. Sections (5  $\mu$ m) from wild-type (A), TR $\alpha^{0/0}$  (B), TR $\alpha^{-/-}$  (C), TR $\alpha^{0/0}\beta^{-/-}$  (D), and TR $\alpha^{-/-}\beta^{-/-}$  (E) mice were stained with hematoxylin and eosin. The numbers at the right of each picture indicate the number of epithelial cells per crypto-villus axis. Statistical analysis indicated that the number of the epithelial cells in TR $\alpha^{-/-}$ , TR $\alpha^{0/0}\beta^{-/-}$ , and TR $\alpha^{-/-}\beta^{-/-}$  animals was significantly decreased compared to that in wild-type mice ( $P < 0.001$ ,  $P < 0.05$ , and  $P < 0.0001$ , respectively, by the Student  $t$  test;  $n = 5$ ). In addition, the number of epithelial cells was also significantly decreased in TR $\alpha^{-/-}\beta^{-/-}$  compared to TR $\alpha^{-/-}$  mice ( $P < 0.001$  by the Student  $t$  test). Bar, 30  $\mu$ m.

distal ileum than that observed in TR $\alpha^{-/-}$  mice (14, 28). When we analyzed the distal ilea of TR $\alpha^{0/0}\beta^{-/-}$  versus those of TR $\alpha^{0/0}$  mice, no obvious morphological difference was observed (Fig. 7B and D) and no statistically significant difference was found in the number of epithelial cells per crypt-villus axis. In contrast, in the ilea of TR $\alpha^{-/-}\beta^{-/-}$  mice, the number of epithelial cells was further decreased compared with that of TR $\alpha^{-/-}$  mice (Fig. 7C and E) and the morphology was dramatically altered (Fig. 7E), in agreement with previous observations. The number of proliferating cells in the crypts of TR $\alpha^{0/0}\beta^{-/-}$  mice ( $8 \pm 1$ ) was decreased to the same extent as in TR $\alpha^{0/0}$  animals ( $7 \pm 1$ ). In contrast, this number was more reduced in TR $\alpha^{-/-}\beta^{-/-}$  ( $2 \pm 1$ ) than in TR $\alpha^{-/-}$  animals ( $5 \pm 1$ ). The analysis of epithelial cell differentiation markers also demonstrated similar features in TR $\alpha^{0/0}$  and TR $\alpha^{0/0}\beta^{-/-}$  animals but showed a stronger impairment in TR $\alpha^{-/-}\beta^{-/-}$  than in TR $\alpha^{-/-}$  mice. In summary, the knockout of the TR $\beta$  gene does not worsen the phenotype observed in TR $\alpha^{0/0}$  mice, but clearly enhances the alterations observed in TR $\alpha^{-/-}$  mice. These data rule out functional redundancy between TRs in the control of postnatal development of the intestine. They suggest instead that the expression of the TR $\Delta\alpha$  isoforms, which is

deleterious in the absence of TR $\alpha 1$  and TR $\alpha 2$ , has even more toxic effects in the absence of all TRs.

**Identification of the TR $\Delta\alpha$  proteins in the distal ileum by immunohistochemistry.** The intestinal epithelium is composed of proliferating and undifferentiated cells which form the crypt compartment and of mature cells located in the villi. In order to analyze the presence of the TR $\Delta\alpha$  proteins in the intestinal mucosa and their pattern of expression along the crypt-villus axis, we performed immunocytochemical analysis. Since TR $\Delta\alpha$  proteins do not contain any peptide sequence which would differentiate them from their complete TR $\alpha$  counterpart, we had to compare the patterns in distal ilea from wild-type, TR $\alpha^{-/-}$ , and TR $\alpha^{0/0}$  mice, using the following antisera: anti-C $\alpha 1$  raised against the common C-terminal part of TR $\alpha 1$  and TR $\Delta\alpha 1$ , anti-C $\alpha 2$  raised against the common C-terminal part of TR $\alpha 2$  and TR $\Delta\alpha 2$  but different from TR $\alpha 1$ , and anti-N $\alpha$  raised against the N-terminal part common to TR $\alpha 1$  and TR $\alpha 2$ . No staining was obtained with the anti-C $\alpha 2$  antibody in any genotypes (data not shown), whereas the expression of TR $\alpha 2$  and TR $\Delta\alpha 2$  mRNAs has been demonstrated by semi-quantitative RT-PCR (28), Northern blotting, and RNase protection (this study) in wild-type mice. As this antiserum was

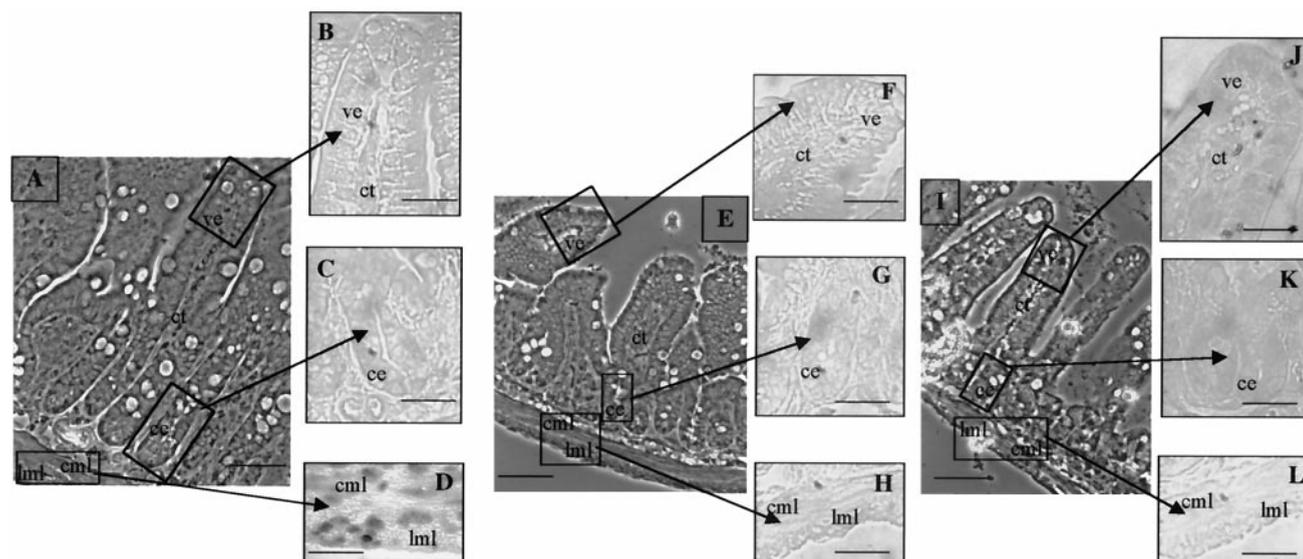


FIG. 8. Immunolocalization of TR $\alpha$ 1/TR $\alpha$ 2 proteins. The pattern of expression of TR $\alpha$  proteins of distal ileum was analyzed in paraffin-embedded sections (5  $\mu$ m) in wild-type (A to D), TR $\alpha^{0/0}$  (E to H), and TR $\alpha^{-/-}$  (I to L) 3-week-old mice. The antibody used recognizes the N-terminal part common to TR $\alpha$ 1 and TR $\alpha$ 2 (anti-N $\alpha$ ) on wild-type sections (B to D), TR $\alpha^{0/0}$  (F to H), and TR $\alpha^{-/-}$  (J to L). Technical controls included the use of phosphate-buffered saline or nonimmune serum instead of the primary antibody. (A, E, and I) Phase contrast low magnification; bar, 30  $\mu$ m; (B to D, F to H, and J to L) bright field high magnification; bar, 70  $\mu$ m. ce, crypt epithelium; ve, villus epithelium; ct, connective tissue; cml, circular muscle layer; lml, longitudinal muscle layer.

used successfully in immunocytochemical detection of TR $\Delta\alpha$ 2 and TR $\alpha$ 2 proteins in transfected cells (data not shown), the failure to detect these proteins in the small intestine probably results from their very low concentration in this tissue.

Using the anti-N $\alpha$  antiserum, we observed a nuclear labeling in muscle layers of wild-type mice (Fig. 8A to D), but no staining was observed in the epithelium or mesenchyme (Fig. 8B and C). No positive cells were detected in TR $\alpha^{0/0}$  or TR $\alpha^{-/-}$  mice (Fig. 8E to H and I to L). By semiquantitative RT-PCR, it was previously shown that in smooth muscle layers only the TR $\alpha$ 1 RNA was detected (28). Consequently, the signal detected with the anti-N $\alpha$  antibody in this tissue can be attributed to TR $\alpha$ 1. In the same experiment, the TR $\alpha$ 1 mRNA was found in the epithelium and connective tissues, whereas the TR $\alpha$ 2 mRNA was found only in the epithelium (28); therefore, we assume that the corresponding proteins are expressed in these tissue regions but at levels undetectable by this technique.

Using the anti-C $\alpha$ 1 antibody, we detected a strong nuclear signal in the differentiated epithelial cells of the villi in wild-type mice (Fig. 9A and B). Only a faint signal could be detected in the undifferentiated epithelial cells of crypts, in the lamina propria, and in smooth muscle cells (Fig. 9C and D). However, unlike the signal detected with the anti-N $\alpha$  reagent, this staining persisted in TR $\alpha^{-/-}$  mice (Fig. 9I to L), which retain the expression of TR $\Delta\alpha$  transcripts, and disappeared in TR $\alpha^{0/0}$  mice (Fig. 9E to H). These observations demonstrate that the protein labeled by the anti-C $\alpha$ 1 reagent is TR $\Delta\alpha$ 1. They also suggest that, unlike the anti-N $\alpha$  antibody, the anti-C $\alpha$ 1 antibody is unable to recognize the TR $\alpha$ 1 protein, presumably due to the small amount of this protein in the distal ileum, and to the reduced accessibility of the target epitope in the tight conformation of the ligand binding domain. Alto-

gether, these data indicate that in wild-type mice, the TR $\alpha$ 1 protein is mainly located in the nuclei of smooth muscle cells, whereas the TR $\Delta\alpha$ 1 protein is mainly present in the nuclei of differentiated epithelial cells. It is worth noting that in TR $\alpha^{-/-}$  undifferentiated and differentiated epithelial cells have similar nuclear labeling.

**The activity of TR $\Delta\alpha$  proteins is down-regulated by TR $\alpha$ 1 via the proteasome pathway.** Our data show that the deleterious activity of the TR $\Delta\alpha$  isoforms is triggered by the inactivation of the TR $\alpha$ 1 and TR $\alpha$ 2 genes. To clarify the molecular mechanisms which could account for this gene inactivation-dependent activity, we tested the possibility that TR $\alpha$ 1 and TR $\alpha$ 2 could interfere with the synthesis or degradation of the TR $\Delta\alpha$  isoforms, thereby modulating their activity. For this purpose, we measured the expression of the TR $\Delta\alpha$ 1 protein in HeLa cells after cotransfection of a TR $\Delta\alpha$ 1 expression vector together with either an empty vector or a vector expressing either TR $\alpha$ 1 or TR $\alpha$ 2. Figure 10 shows that, while the amount of TR $\Delta\alpha$ 1 transcripts was unaffected by the coexpression of the TR $\alpha$ 1 protein (Fig. 10C), the amount of TR $\Delta\alpha$ 1 protein was reduced by increasing amounts of TR $\alpha$ 1 (Fig. 10A). This effect was independent of the presence of T3 (data not shown). In contrast to these data, the expression of small amounts of TR $\alpha$ 2 did not produce any decrease in the amount of TR $\Delta\alpha$ 1 protein (Fig. 10B). It is noteworthy that expression of TR $\beta$ 1 did not change the amount of TR $\Delta\alpha$ 1 (data not shown). In order to show that degradation, and not the rate of protein synthesis, accounted for the reduction in the amount of the TR $\Delta\alpha$ 1 protein, we tested the abilities of different permeant protease inhibitors to prevent this effect. When cotransfected cells were treated with lactacystin, a potent inhibitor of the proteasome pathway (9), the amount of TR $\alpha$ 1 protein was not changed, but the amount of TR $\Delta\alpha$ 1 protein was considerably

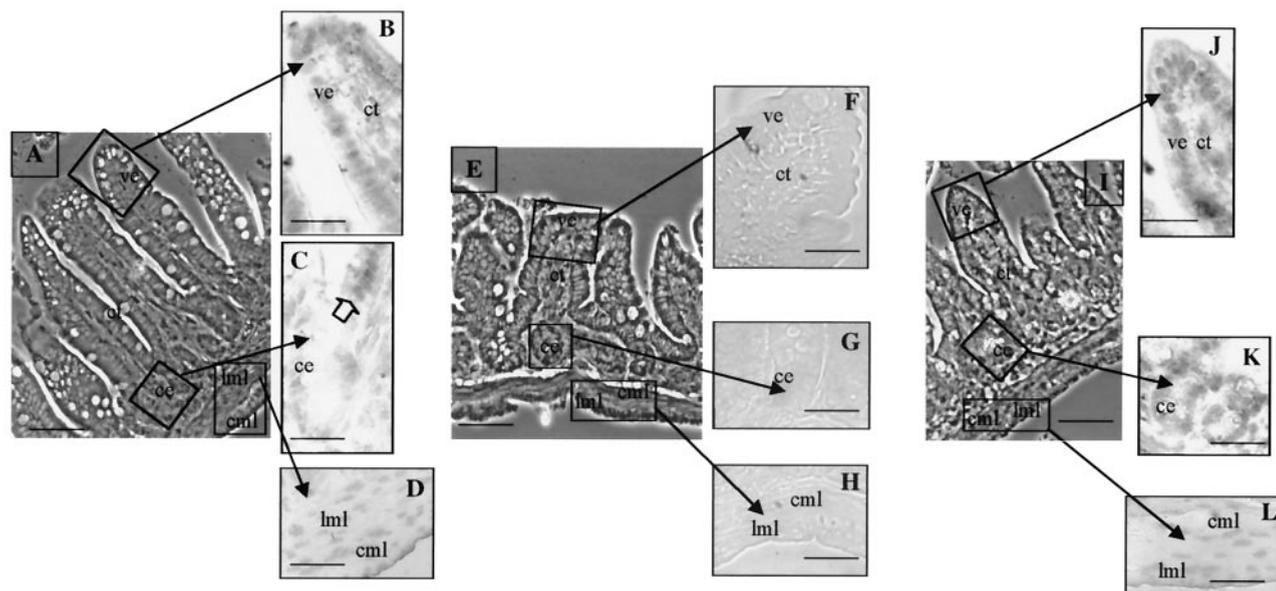


FIG. 9. Immunolocalization of TR $\Delta\alpha 1$  protein. Its pattern of expression in distal ileum was analyzed in paraffin-embedded sections (5  $\mu\text{m}$ ) in wild-type (WT) (A to D), TR $\alpha^{0/0}$  (E to H), and TR $\alpha^{-/-}$  (I to L) 3-week-old mice. The antibody recognized the C-terminal sequence common to TR $\alpha 1$  and TR $\Delta\alpha 1$  (anti-C $\alpha 1$ ) on WT (B to D), TR $\alpha^{0/0}$  (F to H) and TR $\alpha^{-/-}$  (J to L). Technical controls included the use of phosphate-buffered saline or the antibody preincubated with an excess of the recognized peptide. (A, E, and I) Phase contrast low magnification; bar, 30  $\mu\text{m}$ ; (B to D, F to H, and J to L) bright field high magnification; bar, 70  $\mu\text{m}$ . ce, crypt epithelium; ve, villus epithelium; ct, connective tissue; cml, circular muscle layer; lml, longitudinal muscle layer. The open arrow in C indicates the stronger nuclear staining of cells outside the crypts.

increased, reaching levels higher than those observed in the absence of TR $\alpha 1$  (Fig. 10D). These data suggest that TR $\alpha 1$  triggers the degradation of the TR $\Delta\alpha 1$  protein through a proteasome-dependent pathway.

## DISCUSSION

The TR $\alpha$  locus encodes the TR $\alpha 1$  receptor, whose targeted inactivation results in bradycardia and a slight decrease in body temperature (39). This locus also generates the TR $\alpha 2$  variant, which cannot bind T3, poorly heterodimerizes with RXRs, and does not contain any transactivation domain. Transfection studies have shown that the TR $\alpha 2$  protein can inhibit the transcriptional activity of TRs. The TR $\alpha 2$  transcript is present in all mammalian tissues and is generally more abundant than the TR $\alpha 1$  transcript, at least at the level of whole organ analysis (22). Despite these many data, the *in vivo* function of the TR $\alpha 2$  protein remains unknown. In addition to TR $\alpha 1$  and TR $\alpha 2$ , we have identified the TR $\Delta\alpha 1$  and TR $\Delta\alpha 2$  transcripts, which are expressed in murine embryonic stem cells and in intestine, lung, and brain tissues of adult mice. The proteins encoded by these transcripts do not bind T3 or DNA but inhibit the transcriptional activity of several nuclear receptors when they are associated with RXR partners (6). Their *in vivo* function is unknown. Several genetic modifications of the TR $\alpha$  locus which have been generated in a mouse now provide tools with which to obtain insights into the functions of these different products. The goal of this discussion is to draw some conclusions from the comparison of the phenotypes observed in the different TR $\alpha$  gene knockout mice. For the sake of clarity, we have indicated in Fig. 2C the TR $\alpha$  isoforms which are expressed or abolished in the different knockout strains.

**TR $\alpha 1$  and TR $\beta$ s cooperate to regulate TSH production.** Abolishing a TR $\beta 1$ , TR $\beta 2$ , TR $\beta 3$  and TR $\Delta\beta 3$ , or TR $\beta 2$  only has been shown to result in altered TH-dependent feedback regulation of TSH production by T3 (1, 37). In contrast, abolishing the TR $\alpha 1/\Delta\alpha 1$  isoforms in TR $\alpha 1^{-/-}$  mice (R. E. Weiss, O. Chassande, P. E. Macchia, K. Cua, J. Samarut, and S. E. Refetoff, submitted for publication), TR $\alpha 1/\alpha 2$  in TR $\alpha^{-/-}$  mice (12), or TR $\alpha 1/\alpha 2/\Delta\alpha 1/\Delta\alpha 2$  in TR $\alpha^{0/0}$  mice (25) results in mild alterations in the serum concentrations of TH and TSH, indicating that the TR $\alpha$  isoforms may play minor roles in this feedback control. However, TR $\alpha 1^{-/-}$  mice display decreased T4 and TSH levels, suggestive of secondary hypothyroidism, while TR $\alpha^{0/0}$  mice have decreased T4 but normal TSH levels, indicating increased sensitivity of the pituitary to TH. Moreover, careful examination of central responses to varying doses of T3 in TR $\alpha^{0/0}$  mice reveals increased sensitivity to TH (25). Altogether, these data suggest that the TR $\alpha 2$  isoform weakly antagonizes the activity of TR $\beta$ s in thyrotroph cells. In the absence of any true TRs, in TR $\alpha 1^{-/-}\beta^{-/-}$  (16), TR $\alpha^{-/-}\beta^{-/-}$  (14), and TR $\alpha^{0/0}\beta^{-/-}$  mice, TSH reaches very high levels despite large amounts of TH, independently of the presence of non-T3-binding TR isoforms. This shows that in thyrotroph cells, TR $\alpha 2$  could modulate TSH production only in the presence of a TR $\beta$ -mediated T3 response.

**TR $\alpha 1$ , TR $\alpha 2$ , and TR $\beta$ s cooperate to maintain basal body temperature.** Mice lacking the TR $\alpha 1/\Delta\alpha 1$  isoforms (TR $\alpha 1^{-/-}$ ) have a body temperature 0.5°C lower than that of wild-type mice (18). We show here that TR $\alpha^{0/0}$  mice display a similar deficit, suggesting that abolishing TR $\alpha 2$  does not alter thermogenesis, at least under basal conditions. TR $\beta^{-/-}$  mice have normal body temperatures suggesting that the contribution of the TR $\beta$  isoforms in the T3-dependent basal thermogenesis is

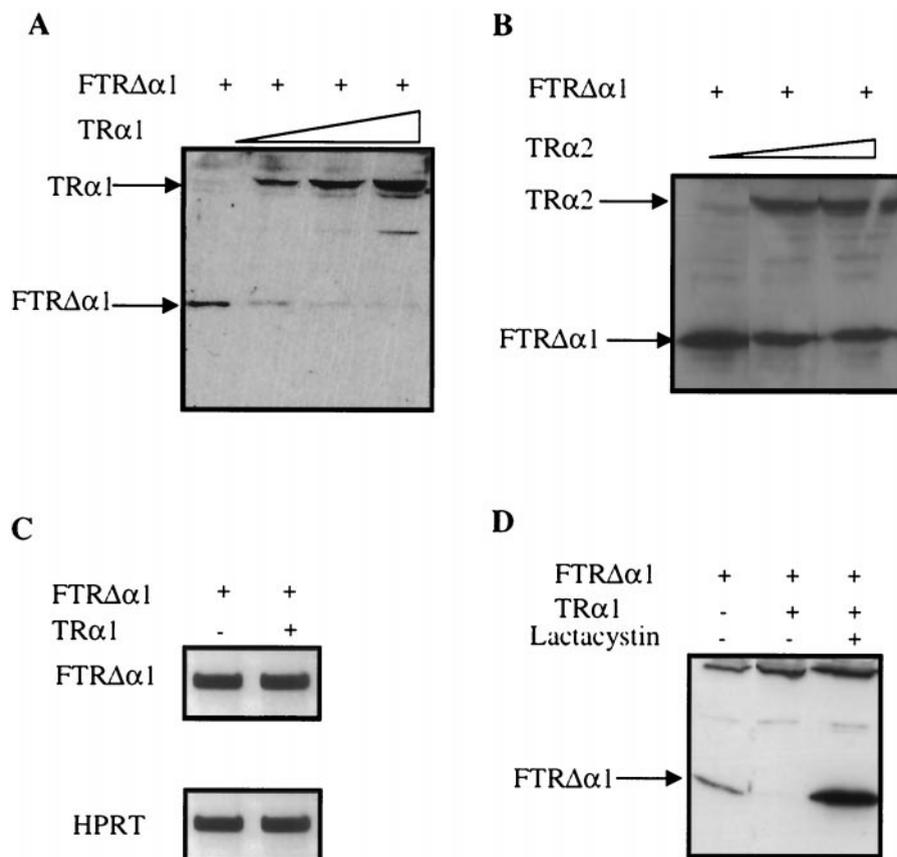


FIG. 10. Regulation of the stability of TR $\Delta\alpha$ 1 protein in HeLa cells. (A) Effects of increasing amounts of the expression vector pSG5hTR $\alpha$ 1 (0, 1.5, 5, and 15  $\mu$ g) on the TR $\Delta\alpha$ 1 protein (plasmid CMV-FmTR $\Delta\alpha$ 1, 0.3  $\mu$ g) as analyzed by Western blotting. HeLa cells were lysed after 48 h of transient transfection. The no. 21 antibody diluted 1:1,000 revealed both TR $\alpha$ 1 and FTR $\Delta\alpha$ 1 proteins as indicated by the arrows. (B) Effects of increasing amounts of the expression vector pSG5hTR $\alpha$ 2 (0, 1.5, 5, and 15  $\mu$ g) on the TR $\Delta\alpha$ 1 protein (plasmid CMV-FmTR $\Delta\alpha$ 1, 0.3  $\mu$ g) as analyzed by Western blotting. (C) Effect of cotransfection in HeLa cells of 15  $\mu$ g of pSG5hTR $\alpha$ 1 and 0.3  $\mu$ g of CMV-FmTR $\Delta\alpha$ 1 on FTR $\Delta\alpha$ 1 mRNA expression analyzed by RT-PCR. RNA was prepared from HeLa cells after 48 h of transient transfection. HPRT was used as internal control. (D) Effect of lactacystin addition ( $10^{-5}$  M) in culture medium of HeLa cells transiently transfected with 0.3  $\mu$ g of CMV-FmTR $\Delta\alpha$ 1 and/or 15  $\mu$ g of pSG5hTR $\alpha$ 1 expression vectors. The crude protein lysate was analyzed by Western blotting and the FTR $\Delta\alpha$ 1-specific band was revealed with an anti-Flag antibody diluted 1:1,000. The bands in the middle and upper parts of the picture represent nonspecific staining. In transient transfection experiments a total of 15  $\mu$ g of DNA/dish has been used; when necessary the pSG5 empty vector has been added.

minor or compensated for by TR $\alpha$ 1. Surprisingly, the comparison between body temperature deficits observed in TR $\alpha$ 1 $^{-/-}$  $\beta$  $^{-/-}$  ( $-0.5^{\circ}\text{C}$ ) and TR $\alpha^{0/0}\beta$  $^{-/-}$  ( $-4^{\circ}\text{C}$ ) mice reveals potentially important thermogenic functions for the TR $\alpha$ 2 isoform. Since no difference in body temperature is observed between TR $\alpha$ 1 $^{-/-}$  and TR $\alpha^{0/0}$  mice, this function is unraveled only in the absence of functional TR. The above data lead to the conclusion that the effects of TH on basal body temperature are mediated by the TR $\alpha$  and TR $\beta$  nuclear receptors, that TR $\alpha$ 1, TR $\beta$ s, and TR $\alpha$ 2 isoforms exercise redundant functions in the control of thermogenesis, and that the persistence of only one of these isoforms is sufficient to maintain body temperature within a physiologic range.

**TR $\alpha$ 2 is essential for normal body growth and bone maturation.** Previous publications and our data have shown that in TR $\alpha^{-/-}\beta$  $^{-/-}$ , TR $\alpha$ 1 $^{-/-}\beta$  $^{-/-}$ , and TR $\alpha^{0/0}\beta$  $^{-/-}$  mice, the total absence of functional TH receptors results in a reduced growth rate and delayed bone maturation, independently of the presence of non-TH-binding isoforms from the TR $\alpha$  locus. This consensus is in agreement with the bone maturation delay

observed in hypothyroid rats (34). This simple observation contrasts with the complexity resulting from the analysis of the functions of individual TR $\alpha$  and TR $\beta$  isoforms. Previous reports indicate that TR $\beta$  $^{-/-}$  and TR $\alpha$ 1 $^{-/-}$  mice have normal growth rates and bone maturation (10, 14, 16, 39). In contrast, combining some of the TR mutations results in a profound disruption of these processes, revealing functional redundancies between TR isoforms. One redundancy clearly occurs with TR $\alpha$ 1 and TR $\beta$ , which are both expressed in bone (2, 5, 31, 34) and which in combined but not individual knockouts result in bone maturation delay. The selective abolishing of TR $\alpha$ 1 in TR $\alpha$ 1 $^{-/-}$  mice does not result in obvious impairment of linear growth or bone maturation. However, in TR $\alpha^{-/-}$  (12) and TR $\alpha^{0/0}$  (this paper) mice, devoid of both TR $\alpha$ 1 and TR $\alpha$ 2 isoforms, growth retardation and delayed skeletal development are observed. The skeletal phenotype of TR $\alpha^{0/0}$  mice includes retarded ossification, failure of progression of hypertrophic chondrocyte differentiation, and disorganization of epiphyseal growth plate architecture, all features similar to those seen in hypothyroidism (34). Importantly, circulating

basal TH concentrations in TR $\alpha^{0/0}$  mice were only mildly impaired and GH concentrations were unchanged compared to those of wild-type mice, supporting the notion that the deletion of the TR $\alpha$  gene fully accounts for this phenotype. On consideration of the TR isoforms that are expressed in the different knockout strains (Fig. 2C), the skeletal phenotypes described can be proposed to result primarily from the abolishing of TR $\alpha$ 2, or the concomitant abolishing of both TR $\alpha$ 1 and TR $\alpha$ 2. Only when data concerning the TR $\alpha$ 2-specific mutation are available will it be possible to test this hypothesis further. Nevertheless, our data reveal the important role of TR $\alpha$ 2 in body growth and maturation of long bones. An interesting observation is that in bone, abolishing TR $\alpha$ 2, an isoform that is known to weakly inhibit T3 responses *in vitro*, paradoxically results in a phenotype which is similar to the skeletal consequences of profound hypothyroidism. This situation contrasts with the increased T3 response observed in the pituitary of TR $\alpha^{0/0}$  mice (25). The alteration of growth and bone maturation observed in TR $\alpha$ 1 $^{-/-}$  $\beta$  $^{-/-}$  and TR $\alpha^{0/0}$  $\beta$  $^{-/-}$  mice could be attributed to impaired GH synthesis since these animals exhibit decreased amounts of GH transcripts in pituitary (reference 16 and the present study). In TR $\alpha^{0/0}$  and TR $\alpha^{-/-}$  mice, however, the GH transcript is expressed at a normal level; therefore, the bone phenotype may be the direct consequence of the TR $\alpha$  mutation within bone cells.

**Deafness caused by loss of the TR $\beta$  gene is not rescued by deletion of the TR $\alpha$  gene.** In addition to confirming the importance of TR $\beta$  in achieving normal hearing function, the present studies demonstrate that TR $\alpha$  may also play a role in the development of normal hearing. In fact, a reduced hearing ability was observed in TR $\alpha^{0/0}$  mice compared to that of wild-type mice at higher frequencies. Although some elevation of ABR thresholds at 24 kHz is typical of C57BL/6J mice older than 2 to 3 months, the observed hearing loss in the TR $\alpha^{0/0}$  mice is highly significant. Moreover, the TR $\alpha^{0/0}$  $\beta$  $^{-/-}$  mouse was more severely deaf than the TR $\beta$  $^{-/-}$  mouse. While these observations confirm the important role of TR $\beta$  for the development of auditory function (12), they have also identified a contribution of TR $\alpha$  to the full integrity of hearing.

**Unbalanced expression of TR $\Delta\alpha$  isoforms results in improper intestinal development and precocious lethality.** We have previously described the lethal phenotype of TR $\alpha^{-/-}$  mice. The TR $\alpha^{-}$  mutation abolishes the TR $\alpha$ 1 and TR $\alpha$ 2 transcripts but retains TR $\Delta\alpha$  transcripts. We show in this study that TR $\Delta\alpha$  transcripts are expressed in the small intestines of wild-type and TR $\alpha^{-/-}$  but not of TR $\alpha^{0/0}$  mice. Accordingly, using anti- $\alpha$ 1 C-terminal antibody, we detect the TR $\Delta\alpha$  isoforms in the intestinal epithelial cells of wild-type and TR $\alpha^{-/-}$  but not TR $\alpha^{0/0}$  mice. This is the first time that the specificity of the staining observed in an immunocytochemical analysis of TRs is assessed by genetic arguments. Therefore, analysis of the products of the TR $\alpha$  locus reveals that the only molecular difference between the TR $\alpha^{-/-}$  and TR $\alpha^{0/0}$  mutations is the expression of the TR $\Delta\alpha$  isoforms. Immunocytochemical analysis further shows that the expression of the TR $\Delta\alpha$ 1 protein is expanded in the TR $\alpha^{-/-}$  mice, extending all along the crypt and the villus. *In vivo* observations show that abolishing TR $\alpha$ 1 and TR $\alpha$ 2 in TR $\alpha^{-/-}$  mice confers deleterious effects on the TR $\Delta\alpha$  isoforms, suggesting that, under normal conditions (wild-type mice), the TR $\alpha$  products down-modulate the activity of the

TR $\Delta\alpha$  isoforms. Transfection experiments suggest that one mechanism by which TRs could control the activity of TR $\Delta\alpha$  proteins is by triggering their rapid removal from the cells through the proteasome-mediated degradation. This mechanism would explain the increased intensity and extended expression domain of the TR $\Delta\alpha$ 1 protein in the absence of TR $\alpha$ 1 in the intestines of TR $\alpha^{-/-}$  mice. The mechanism by which TR $\Delta\alpha$  proteins exert their toxic effects in the developing intestine is unclear. The persistence of a severe intestinal phenotype upon inactivation of the TR $\beta$  gene in TR $\alpha^{-/-}$  $\beta$  $^{-/-}$  mice implies that the molecular targets of the TR $\Delta\alpha$  proteins are not only the TRs. We have previously suggested that other nuclear receptors, such as retinoic acid receptors, could be antagonized by TR $\Delta\alpha$  products.

At the level of the whole organism, the lethal phenotype observed in TR $\alpha^{-/-}$  and TR $\alpha^{-/-}$  $\beta$  $^{-/-}$  mice is indeed due to the persistence of TR $\Delta\alpha$  isoforms since it can be rescued by their abolition in TR $\alpha^{0/0}$  and TR $\alpha^{0/0}$  $\beta$  $^{-/-}$  mice. Thus, the severe intestinal abnormalities and the lethality observed in TR $\alpha^{-/-}$  and TR $\alpha^{-/-}$  $\beta$  $^{-/-}$  mice are consequences of the persistence of TR $\Delta\alpha$  isoforms, which are no longer under the control of the TR $\alpha$ 1 protein. These genetic and molecular analyses suggest that the TR $\Delta\alpha$  isoforms have powerful, dose-dependent toxic effects and that their level of expression must be tightly controlled. Altogether, these genetic analyses show that the non-T3-binding isoforms encoded from the TR $\alpha$  locus have important developmental and homeostatic functions.

In addition, our present and previously published data allow the prediction that a potential homozygous mutation in the TR $\alpha$  locus in humans, which would abolish all TR $\alpha$  isoforms, would be expected to result in a mild phenotype mainly characterized by (i) increased sensitivity to TH in the thyrotroph and liver and (ii) reduced sensitivity to TH in heart and bone, manifested as bradycardia, growth retardation, and bone maturation delay. Mutations restricted to the N-terminal moiety of the gene, sparing the TR $\Delta\alpha$  isoforms, are expected to have severe pathologic consequences.

#### ACKNOWLEDGMENTS

We thank Christelle Morin, Nadine Aguilera, and Djamel Belgarbi for animal handling, J. P. Magaud, J. Ghysdael, P. Brulet, and S. Omura for kindly providing genomic libraries, antibodies, plasmids, and lactacystin, respectively. We thank F. Flamant and J. M. Vanacker for helpful advice.

This work was supported in part by CNRS and grants from Fondation pour la Recherche Médicale and Human Frontier Scientific Program, and by grants from NIH (DK 15070 [S.R.], AG07554 [J.F.W.], MH61090 [J.F.W.]) and the Seymour J. Abrams Thyroid Research Center. G.R.W. was supported by an MRC Career Establishment grant (G9803002) and a Wellcome Trust Project grant (050570). M. Hara was supported by an FRM fellowship.

#### REFERENCES

- Abel, E. D., M.-E. Boers, C. Pazos-Moura, E. Moura, H. Kaulbach, M. Zakaria, B. Lowell, S. Radovick, M. C. Liberman, and F. Wondisford. 1999. Divergent roles for thyroid hormone receptor  $\beta$  isoforms in the endocrine axis and auditory system. *J. Clin. Investig.* **104**:291-300.
- Abu, E. O., S. Bord, A. Horner, V. K. K. Chatterjee, and J. E. Compston. 1997. The expression of thyroid hormone receptors in human bone. *Bone* **21**:137-142.
- Arpin, C., M. Pihlgren, A. Fraichard, D. Aubert, J. Samarut, O. Chassande, and J. Marvel. 2000. Effects of T3R $\alpha$ 1 and T3R $\alpha$ 2 gene deletion on T and B lymphocyte development. *J. Immunol.* **164**:152-160.
- Baas, D., C. Fressinaud, M. E. Ittel, A. Reeber, D. Dalençon, J. Puymirat, and I. L. Sarlièvre. 1994. Expression of thyroid hormone receptor isoforms

- in rat oligodendrocyte cultures. Effect of 3,5,3'-triiodo-L-thyronine. *Neurosci. Lett.* **176**:47–51.
5. **Ballock, R. T., B. C. Mita, X. Zhou, D. H.-C. Chen, and L. M. Mink.** 1999. Expression of thyroid hormone receptors isoforms in rat growth plate cartilage in vivo. *J. Bone Miner. Res.* **14**:1550–1556.
  6. **Chassande, O., A. Fraichard, K. Gauthier, F. Flamand, C. Legrand, P. Savatier, V. Laudet, and J. Samarut.** 1997. Identification of transcripts initiated from an internal promoter in the *c-erbA $\alpha$*  locus that encode inhibitors of retinoic acid receptor- $\alpha$  and triiodothyronine receptor activities. *Mol. Endocrinol.* **11**:1278–1290.
  7. **Desbois, C., D. Aubert, C. Legrand, B. Pain, and J. Samarut.** 1991. A novel mechanism of action for v-erbA: abrogation of the inactivation of transcription factor AP-1 by retinoic acid and thyroid hormone receptors. *Cell* **67**: 731–740.
  8. **Dussault, J. H., and J. Ruel.** 1987. Thyroid hormones and brain development. *Annu. Rev. Physiol.* **49**:321–334.
  9. **Fenteany, G., R. F. Standaert, W. S. Lane, S. Choi, E. J. Corey, and S. L. Schreiber.** 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**:726–731.
  10. **Forrest, D., E. Hanebuth, R. J. Smeyne, N. Everds, C. L. Stewart, J. M. Wehner, and T. Curran.** 1996. Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: evidence for tissue-specific modulation of receptor function. *EMBO J.* **15**:3006–3015.
  11. **Forrest, D., L. C. Erway, L. Ng, R. Altschuler, and T. Curran.** 1996. Thyroid hormone receptor beta is essential for development of auditory function. *Nat. Genet.* **13**:354–357.
  12. **Fraichard, A., O. Chassande, M. Plateroti, J. P. Roux, J. Trouillas, C. Dehay, C. Legrand, K. Gauthier, M. Kedinger, L. Malaval, B. Rousset, and J. Samarut.** 1997. The *TR $\alpha$*  gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production. *EMBO J.* **16**:4412–4420.
  13. **Franklyn, J. A.** 2000. Metabolic changes in hypothyroidism, p. 833–836. *In* L. E. Braverman and R. D. Utiger, (ed.), *The thyroid*. Lippincott, Williams and Wilkins, Philadelphia, Pa.
  14. **Gauthier, K., O. Chassande, M. Plateroti, J. P. Roux, C. Legrand, B. Pain, B. Rousset, R. Weiss, J. Trouillas, and J. Samarut.** 1999. Different functions for the thyroid hormone receptors *TR $\alpha$*  and *TR $\beta$*  in the control of thyroid hormone production and post-natal development. *EMBO J.* **18**:623–631.
  15. **Glass, C. K., and M. G. Rosenfeld.** 2000. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* **14**:121–141.
  16. **Göthe, S., Z. Wang, L. Ng, J. M. Kindblom, A. Campos Barros, C. Ohlsson, B. Vennström, and D. Forrest.** 1999. Mice devoid of all known thyroid hormone receptors are viable but exhibit disorders of pituitary-thyroid axis, growth, and bone maturation. *Genes Dev.* **13**:1329–1341.
  17. **Hashimoto, K., F. H. Curty, P. P. Borges, C. E. Lee, E. D. Abel, J. K. Elmquist, R. N. Cohen, and F. E. Wondisford.** 2001. An unliganded thyroid hormone receptor causes severe neurological dysfunction. *Proc. Natl. Acad. Sci. USA* **98**:3998–4003.
  18. **Johansson, C., S. Gothe, D. Forrest, B. Vennstrom, and P. Thoren.** 1999. Cardiovascular phenotype and temperature control in mice lacking thyroid hormone receptor-beta or both alpha1 and beta. *Am. J. Physiol.* **276**:H2006–H2012.
  19. **Kaneshige, M., K. Kaneshige, X.-G. Zhu, A. Dace, L. Garrett, T. A. Carter, R. Kazlauskaitė, D. G. Pankratz, A. Wynshaw-Boris, S. Refetoff, B. Weintraub, M. C. Willingham, C. Barlow, and S.-Y. Cheny.** 2000. Mice with a targeted mutation in the thyroid hormone  $\beta$  receptor gene exhibit impaired growth and resistance to thyroid hormone. *Proc. Natl. Acad. Sci. USA* **97**:13209–13214.
  20. **Langlois, M.-F., K. Zanger, T. Monden, J. D. Safer, A. N. Hollenberg, and F. E. Wondisford.** 1997. A unique role of the b2 thyroid hormone receptor isoform in negative regulation by thyroid hormone. *J. Biol. Chem.* **272**: 24927–24933.
  21. **Lazar, M. A., R. A. Hodin, and W. W. Chin.** 1989. Human carboxyl-terminal variant of  $\alpha$ -type *c-erbA* inhibit *trans*-activation by thyroid hormone receptors without binding thyroid hormone. *Proc. Natl. Acad. Sci. USA* **86**:7771–7774.
  22. **Lazar, M. A.** 1993. Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocr. Rev.* **14**:184–193.
  23. **Legrand, J.** 1986. Thyroid hormone effects on growth and development, p. 503–534. *In* G. Hennemann (ed.), *Thyroid hormone metabolism*. Dekker, Rotterdam, The Netherlands.
  24. **Lezoualc'h, F., A. H. Hassan, P. Giraud, J. P. Loeffler, S. L. Lee, and B. Demeneix.** 1992. Assignment of the  $\beta$ -thyroid hormone receptor to 3,5,3'-triiodothyronine-dependent inhibition of transcription from the thyrotropin-releasing hormone promoter in chick hypothalamic neurons. *Mol. Endocrinol.* **6**:1797–1804.
  25. **Macchia, P. E., Y. Takeuchi, T. Kawai, K. Cua, K. Gauthier, O. Chassande, H. Seo, Y. Hayashi, J. Samarut, Y. Murata, R. E. Weiss, and S. Refetoff.** 2001. Increased sensitivity to thyroid hormone in mice with complete deficiency of thyroid hormone receptor  $\alpha$ . *Proc. Natl. Acad. Sci. USA* **98**:349–354.
  26. **Meunier, P. J.** 1983. A yearly survey of developments in the field of bone and mineral metabolism. W. A. Peck, Amsterdam, The Netherlands.
  27. **Nuclear Receptors Committee.** 1999. A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97**:161–163.
  - 27a. **Plateroti, M., K. Gauthier, C. Domon-Dell, J.-N. Freund, J. Samarut, and O. Chassande.** 2001. Functional interference between thyroid hormone receptor  $\alpha$  (*TR $\alpha$* ) and natural truncated *TR $\Delta\alpha$*  isoforms in the control of intestine development. *Mol. Cell. Biol.* **21**:4761–4772.
  28. **Plateroti, M., O. Chassande, A. Fraichard, K. Gauthier, J.-N. Freund, J. Samarut, and M. Kedinger.** 1999. Involvement of *T3R $\alpha$* - and  $\beta$ -receptor subtypes in mediation of *T3* functions during post-natal murine intestinal development. *Gastroenterology* **116**:1367–1378.
  29. **Pohlenz, J., A. Maqueem, K. Cua, R. E. Weiss, J. Van Sande, and S. Refetoff.** 1999. Improved radioimmunoassay for measurement of mouse TSH in serum: strain differences in TSH concentration and thyrotroph sensitivity to thyroid hormone. *Thyroid* **9**:1265–1271.
  30. **Refetoff, S.** 2000. Resistance to thyroid hormone, p. 1028–1043. *In* L. E. Braverman and R. D. Utiger (ed.), *The thyroid*. Lippincott, Williams and Wilkins, Philadelphia, Pa.
  31. **Robson, H., T. Siebler, D. A. Stevens, S. M. Shalet, and G. R. Williams.** 2000. Thyroid hormone acts directly on growth plate chondrocytes to promote hypertrophic differentiation and inhibit clonal expansion and cell proliferation. *Endocrinology* **141**:3887–3897.
  32. **Savatier, P., H. Lapillonne, L. van Grunsven, B. B. Rudkin, and J. Samarut.** 1996. Withdrawal of differentiation of inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene* **12**:309–322.
  33. **Scanlon, M. F., and A. D. Toft.** 1996. Regulation of thyrotropin secretion, p. 220–240. *In* L. E. Braverman and R. D. Utiger (ed.), *Werner and Ingbar's the thyroid: a fundamental and clinical text*, 7th ed. Lippincott-Raven, New York, N.Y.
  34. **Stevens, D. A., R. P. Hasserjian, H. Robson, T. Siebler, S. M. Shalet, and G. R. Williams.** 2000. Thyroid hormones regulate hypertrophic chondrocyte differentiation and expression of parathyroid hormone-related protein and its receptor during endochondral bone formation. *J. Bone Miner. Res.* **15**: 2431–2442.
  35. **Turner, J. G., and J. F. Willott.** 1998. Exposure to an augmented acoustic environment alters progressive hearing loss in DBA/2J mice. *Hear. Res.* **118**:101–113.
  36. **Weiss, R. E., and S. Refetoff.** 1996. Effect of thyroid hormone on growth: lessons from the syndrome of resistance of thyroid hormone. *Endocrinol. Metab. Clin. N. Am.* **25**:719–730.
  37. **Weiss, R. E., D. Forrest, J. Pohlenz, K. Cua, T. Curran, and S. Refetoff.** 1997. Thyrotropin regulation by thyroid hormone in thyroid hormone receptor beta-deficient mice. *Endocrinology* **138**:3624–3629.
  38. **Weiss, R. E., Y. Murata, K. Cua, Y. Hayashi, H. Seo, and S. Refetoff.** 1998. Thyroid hormone action on liver, heart and energy expenditure in thyroid hormone receptor  $\beta$  deficient mice. *Endocrinology* **139**:4945–4952.
  39. **Wikström, L., C. Johansson, C. Salto, C. Barlow, A. Campos Barros, F. Baas, D. Forrest, P. Thoren, and B. Vennström.** 1998. Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor  $\alpha$ 1. *EMBO J.* **17**:455–461.
  40. **Williams, G. R., H. Robson, and S. M. Shalet.** 1998. Thyroid hormone actions on cartilage and bone: interactions with other hormones at the epiphyseal plate and effects on linear growth. *J. Endocrinol.* **157**:391–403.
  41. **Williams, G. R.** 2000. Cloning and characterization of two novel thyroid hormone receptor  $\beta$  isoforms. *Mol. Cell. Biol.* **20**:8329–8342.