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Functional Interference between Thyroid Hormone Receptor α (TRα) and Natural Truncated TRΔα Isoforms in the Control of Intestine Development

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Thyroid hormone is known to participate in the control of intestine maturation at weaning. Its action is mediated by the thyroid hormone nuclear receptors, encoded by the *TR*α and *TR*β genes. Since previous studies have shown that *TR*β plays a minor role in the gut, we focused here our analysis on the *TR*α gene. The *TR*α locus generates the TRα1 receptor together with the splicing variant TRα2 and the truncated products TRΔα1 and TRΔα2, which all lack an intact ligand binding domain. The TRΔα isoforms are transcribed from an internal promoter located in intron 7, and their distribution is restricted to a few tissues including those of the intestine. In order to define the functions of the different isoforms encoded by the *TR*α locus in the intestinal mucosa, we produced mice either lacking all known TRα products or harboring a mutation which inactivates the intronic promoter. We performed a detailed analysis of the intestinal phenotypes in these mice and compared it to that of the previously described TRα$^{-/-}$ mice, in which TRα isoforms are abolished but the TRΔα isoforms remain. This comparative analysis leads us to the following conclusions: (i) the TRα1 receptor mediates the T3-dependent functions in the intestine at weaning time and (ii) the TRΔα products negatively control the responsiveness of the epithelial cells to T3. Moreover, we show that TRΔα proteins can interfere with the transcription of the intestine-specific homeobox genes *cdx1* and *cdx2* and that their activity is regulated by TRα1. Altogether these data demonstrate that cooperation of TRα and TRΔα products is essential to ensure the normal postnatal development of the intestine and that mutations in the *TR*α locus can generate different phenotypes caused by the disruption of the equilibrium between these products.

The intestinal mucosa is composed of smooth muscle and connective tissues, two mesodermal derivatives, and of epithelial tissue derived from the endoderm (18). The epithelium is characterized by continuous cell renewal from multipotent stem cells within the crypts. The epithelial cells acquire differentiated phenotypes (enterocyte, goblet, or enteroendocrine) during a vertical migration toward the villus tip, and finally they die and are exfoliated into the lumen. The Paneth cells, the other cytotype composing the intestinal epithelium, migrate to the crypt base and represent the only resident differentiated cell type of this compartment (10). Small intestine mucosal thickness depends on the rate of crypt cell division and migration as well as the lifespan of the villus cells. All these parameters change markedly during normal development and in response to various environmental, dietary, and hormonal factors (19). The enterocytes represent the most abundant cell type composing the small intestine epithelium (95% [10]), and their function in nutrient uptake and metabolism takes part in ensuring the organism's homeostasis. Their differentiation is characterized by functional polarization, i.e., the expression of digestive enzymes integrated in the apical brush border membranes (18). At weaning, the passage from milk to solid diet is characterized in rodents by a switch in the expression of lactase to various α-glucosidases such as sucrase (13) and increased expression of other brush border enzymes such as alkaline phosphatase (14). Thyroid hormones have been shown to take part in the developmental processes responsible for intestinal postnatal maturation, such as the increase in the number of crypts and proliferating cells as well as the onset of the adult-type digestive enzyme expression (3, 15; reviewed in references 12 and 13). The molecular mechanisms as well as the direct regulation of these processes by the thyroid hormone remain under discussion. It has been shown that *cdx1* and *cdx2* homeobox genes control intestinal epithelial cell proliferation as well as the expression of enterocyte differentiated markers (reviewed in reference 8). This and a previous work (25) suggest that the thyroid hormone signaling pathway could involve these major regulators of intestinal homeostasis.

The action of T3, the active form of thyroid hormones, is mediated by its binding to thyroid hormone receptors (TR), which belong to the nuclear hormone receptor family of transcription factors (22). The activity of TRs is modulated by the T3 binding that leads to the activation or the repression of target genes (22). The TRs are encoded by two genes, *TR*α and *TR*β (N1RA1 and N1RA2 according to the Nuclear Receptors Nomenclature Committee [1999]) (26, 31). The *TR*β locus encodes the TRβ1, TRβ2, and TRβ3 receptors and the TRΔβ3 truncated isoform, generated by different promoter usage and alternative splicing (31, 32). The *TR*α gene has been shown to encode the T3 receptor TRα1 and several other isoforms unable to bind T3 (5, 20, 21). The TRα2 isoform, produced by

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alternative splicing of the primary transcript, retains DNA recognition capacity and can act as an antagonist of the T3 receptors, TRα1, TRβ1, and TRβ2 in vitro (20). Recently, we have demonstrated that the *TR*α gene also produces transcripts from an internal promoter located in intron 7, TRΔα1 and TRΔα2 (5), whose expression is restricted to a few organs including the lung and the small intestine (7). The sequences of these isoforms are identical to the C-terminal sequences of TRα1 and TRα2, respectively. They lack the DNA binding domain and part of the ligand binding domain, leading to their inability to bind DNA and T3. In vitro experiments showed that they can repress the transactivation activity of TR and retinoic acid receptors (5), suggesting that these truncated isoforms could act as modulators of nuclear hormone receptor activities in the tissues in which the different proteins are coexpressed. However, the molecular basis of their action as well as their physiological role is still unknown. Several in vivo studies aimed at clarifying the functions of the different isoforms encoded by the *TR*α gene have been performed in different laboratories. By using the homologous recombination technology, we and others have generated strains of mice lacking the expression of either TRα1 or both TRα1 and TRα2, as well as the respective double mutants combining the deletion in *TR*α and *TR*β genes (reviewed in reference 6). The TRα$^{-/-}$ animals, which lack both the TRα1 and TRα2 isoforms but still express the TRΔα isoforms, display growth retardation, become progressively hypothyroid by weaning time, and die thereafter. In addition, they show bone and small intestine developmental alterations (7). In contrast, TRβ$^{-/-}$ mice do not display intestinal development retardation, suggesting that *TR*α is the only locus involved in postnatal small intestine development (25). However, as the TRα$^{-/-}$ mice retain the expression of the TRΔα isoforms we cannot exclude that in these animals the unbalanced expression of the TRα/TRΔα products is one of the causes of the strong phenotype observed in these animals. To investigate this possibility we have generated new *TR*α mutant mice. The *TR*α^{0} mutation was designed to abolish the expression of all transcripts from the *TR*α locus (9a.). The *TR*α^{7} mutation described here was aimed at selectively suppressing the TRΔα transcripts while retaining the normal expression of the TRα transcripts. As all TRα products are expressed in the small intestine, our study focused on the morphofunctional comparative analysis between mice harboring different mutations in the *TR*α locus. We demonstrate that (i) removing only the TRα1 and TRα2 products in TRα$^{-/-}$ mice results in a severe alteration of intestinal development and function, (ii) further abolishing of the expression of the TRΔα isoforms in TRα$^{0/0}$ mice generates a milder phenotype, and (iii) specifically deleting the intronic promoter to prevent the expression of the TRΔα isoforms in TRα$^{7/7}$ mice results in an enhanced response of the epithelial cells to T3. In conclusion, these data demonstrate that the balance between the TRα and TRΔα isoforms is critical to ensure normal postnatal intestinal development.

MATERIALS AND METHODS

Construction of *TR*α-targeting vector. To construct the *TR*α^{7} targeting vector, the 5′ and 3′ homology fragments were generated by PCR, using VIS/PIAS and Pr3S/RevS as primer pairs, respectively, and Expand High Fi reagent, and were successively inserted into pBSKII (Stratagene). The *loxP* PGKNeo^{r} PGKTK *loxP* cassette was inserted between the 5′ and 3′ arms in sense orientation.

Targeted disruption of *TR*α gene. ENS embryonic stem (ES) cells were electroporated with 40 μg of linearized targeting vector and then selected with 250 μg of G418 (Gibco-BRL) per ml and 0.2 μM ganciclovir. The *TR*α^{7} allele was obtained in two steps. Upon electroporation with the recombination vector and selection with G418, the cells containing the targeted [*loxP* NeoTK *loxP*] cassette were identified by PCR-based screening using 6S1 and pT102AS as oligonucleotide primers. Positive clones were subsequently transfected with a Cre-expressing vector. To identify clones having excised the *loxP* flanked selection cassette among the ganciclovir-resistant colonies, DNA was cut by *Pst*I and submitted to Southern blot analysis, using a probe hybridizing to the 3′ end of intron 7 and to exon 8. To determine the promoter activities of the −270 to +237, −114 to +237, and −51 to +237 portions of intron 7, the corresponding fragments were inserted upstream of a chloramphenicol acetyltransferase (CAT) reporter gene into a BAS-CAT vector (Promega). Cells were cotransfected with the CAT-expressing vector and a plasmid containing the beta-galactosidase gene under the control of the PGK promoter.

Animals and tissues. The small intestine was dissected from the ligament of Treitz to the rectum. We collected the first (proximal jejunum) and the last (distal ileum) fourths of the small intestine as well as the proximal part of the colon. The intestinal segments were immediately fixed for morphological analysis or frozen in liquid nitrogen and stored at −80°C until they were used for enzymatic analysis or RNA extraction.

The epithelium and connective and smooth muscle tissues were isolated from proximal jejunums, distal ilea, and proximal colons of three 12-day-old wild-type mice, as described in reference 25.

Experimental hypothyroidism and hyperthyroidism. TH deficiency was induced in wild-type, TRα$^{0/0}$, and TRα$^{7/7}$ mice by a low-iodine diet supplemented with 0.15% propylthiouracil (PTU) purchased from Harlan/Teklad. The animals were treated from birth until they were sacrificed. Hyperthyroidism was induced in one-half of PTU-treated animals by daily injection of 0.25 μg of L-T3 per mouse for 2 days. For each experimental condition at least three animals per genotype were used. The levels of free T4 and T3 in serum were measured using a VIDAS enzyme-linked immunosorbent assay kit (Biomérieux).

Purification of RNAs, RNase protection assay, and RT-PCR analysis. Total RNA from intestine was isolated by the improved acid-guanidine-phenol-chloroform method. The RNase protection assay was performed as described in reference 9a.

Reverse transcription (RT) was performed as described in reference 7. cDNA (0.05 μg) was used for each PCR with Eurobio *Taq*. To perform the detection of TRΔα1 and TRΔα2 we used oligonucleotide pairs α7S/α1A and α7S/α2A, respectively. The primers used for Cdx1 and Cdx2 mRNA detection and semiquantitative RT-PCR conditions are described in reference 25.

Sequences of oligonucleotides used. All the primers are from MWG (Ebersberg, Germany) and have the following sequences: VIS, GGAGATGATTCGC TCACTGCAG; PIAS, GTCATGCTGCCTGCAGATAG; Pr3S, GGTGTG GAGAGGATGCACTGAAG; RevS, GGAGGTGGTAGAGTTTGCCAAAC; α7S, GTGTGGAGAGGATGCACTGAAGT; 6S1, GGTTCTAGATGATTCG AAGCGG; α1A, CGACTTTCATGTGGAGGAAG; α2A, CCTGAACAACAT GCATTCCGA; α15A, CAGCCTGCAGCAGAGCCACTTCCGT; pT102AS, CCTCGAGCGGCCATAACTTCG.

Morphological staining and immunohistochemistry. Three-week-old mice were killed by cervical dislocation, and their small intestines were immediately removed. Proximal jejunums and distal ilea were collected separately and fixed in 4% paraformaldehyde overnight at 4°C. They were then embedded in paraffin, and 5-μm sections were applied to polylysine-coated slides. For morphological observations, after dewaxing and rehydration, the slides were stained with Schiff's reagent or hematoxylin and eosin. Immunohistochemistry experiments were performed with a monoclonal antibody (Novocastra Laboratories) for Ki67 detection in proliferating cells (27), a polyclonal PA1-211 (Affinity Bioreagents, Inc.) for TRα1 and TRΔα1 protein localization, and a polyclonal antibody recognizing the N-terminal part of both TRα1 and TRα2 proteins (generous gift of D. Baas [1]). These antibodies were used in combination with secondary biotinylated antibody and a 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.

Cells, plasmids, and transfections. The Caco2-TC7 cell line (kindly gift of A. Zweibaum, Paris, France [4]) corresponds to a highly differentiated subclone of the human colonic adenocarcinoma Caco2 cell line that exhibits spontaneous enterocyte-like differentiation in culture (11). The cells were maintained in Dulbecco's modified Eagle medium (Biomedia) supplemented with 10% of heat

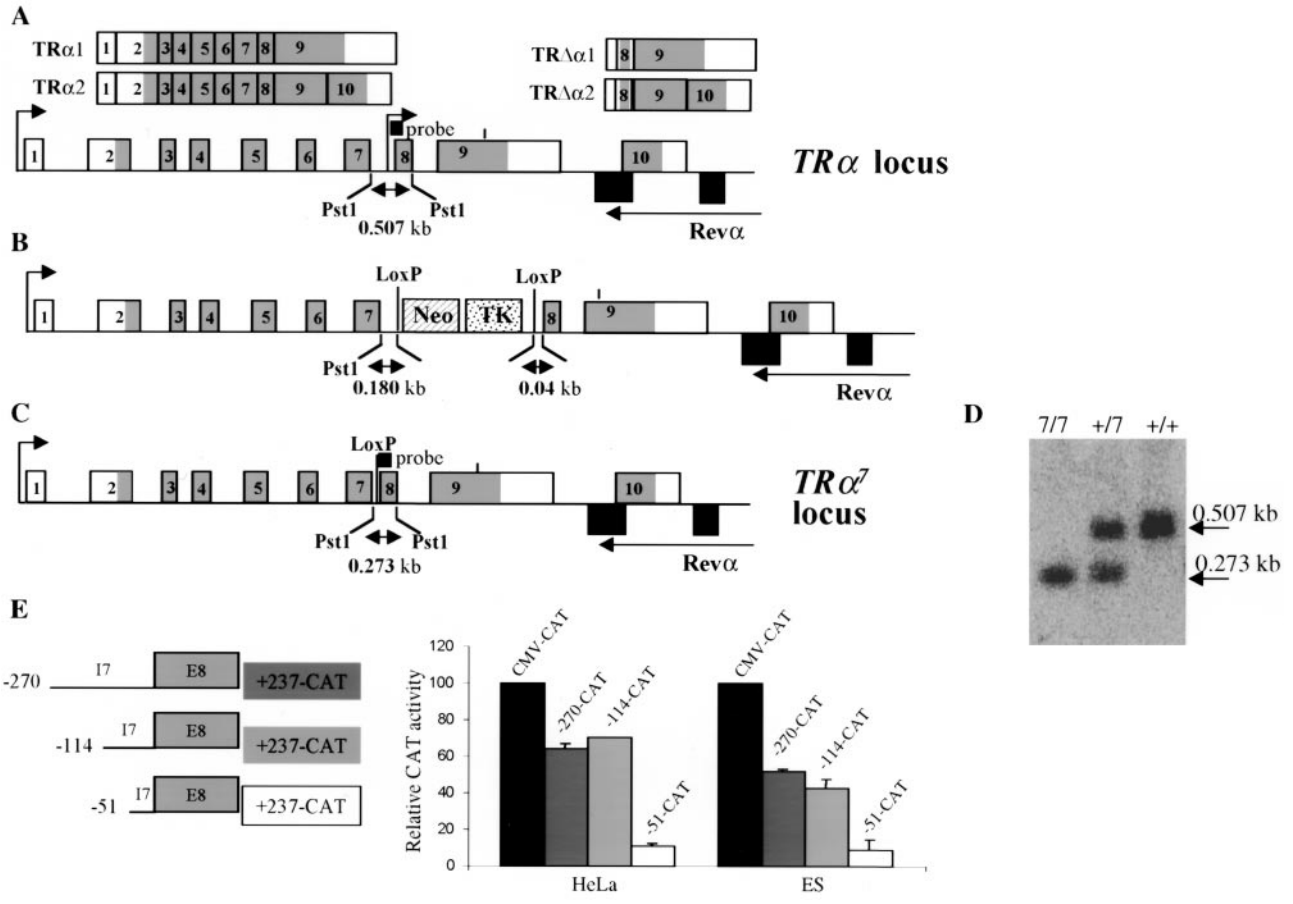


FIG. 1. Targeted mutagenesis of the *TR*α gene by homologous recombination. (A) Structure of the *TR*α gene. The upper arrows indicate the two transcription start sites. The differential splice site in exon 9 is indicated by a vertical bar. Exons are numbered starting downstream to the distal promoter. Grey-shaded areas represent the coding regions. Structures of the transcripts are shown at the top. (B) *TR*α mutated locus in which the *loxP* Neo^r TK *loxP* cassette replaces a 257-bp fragment of intron 7. Cells expressing this construct have then been transfected with a plasmid encoding the Cre recombinase to obtain the *TR*α^7 locus. (C) Structure of the targeted allele *TR*α^7 containing a 257-bp deletion of intron 7, corresponding to the active portion of the internal promoter. The probe used for Southern blot analysis and the size of the fragment detected after digestion with *Pst*I are indicated. (D) Southern blot analysis showing wild-type (+/+), heterozygous (+/7), and homozygous (7/7) littermates. (E) Promoter activities of the −270 to +237, −114 to +237 and −51 to +237 fragments of intron 7 have been tested in HeLa and ES cells. Results are mean values ± SD of two independent experiments conducted in duplicate.

inactivated fetal calf serum (Biomedia). The reporter pCdx1-4Luc and pCdx2-1Luc plasmids containing promoter fragments of the murine *Cdx1* and *Cdx2* genes have already been described (23). The plasmids containing the cDNA coding for human TRα1 (pSG5hTRα1), for mouse TRΔα1 (pSG5mTRΔα1), and for mouse TRΔα2 (pSG5mTRΔα2) have already been reported (5). Caco2TC7 cells have been transfected as described (23) using the Exgen transfection reagent (Euromedex). Luciferase activity was measured 48 h after transfection using the luciferase assay system (Promega).

Enzymatic activities. Sucrase and lactase enzymatic activities were measured in the purified brush border membrane as previously described (28). The samples were incubated with the appropriate substrate in 0.1-mol/liter maleate buffer (0.056 mol of sucrose per liter, pH 6.25; 0.056 mol of lactose per liter, pH 5.8), and liberated glucose was measured. Enzyme activities were expressed as milliunits per milligram of brush border protein. One unit hydrolyzes 1 mmol of substrate per min at 37°C.

Statistics. Numerical results are presented as means ± standard deviations (SD). Groups were compared using the Student *t* test, with *P* values of <0.05 considered significant.

RESULTS

Generation of the TRα$^{7/7}$ mutant mice. To introduce the *TR*α^7 mutation in the *TR*α locus (Fig. 1A), we deleted a limited portion of intron 7 in order to abolish the activity of the internal promoter. The extent of this deletion was determined after assaying the transcriptional activities of different truncated fragments of intron 7 following transfection in HeLa and ES cells (Fig. 1E). We thereby identified a 234-bp fragment whose deletion abolished 80% of the transcriptional activity in this assay and designed a vector in which this fragment was replaced by a Neo^r-TK cassette flanked by *loxP* sites. ES cell clones screened for homologous recombination of this construct (Fig. 1B) were transfected with a vector expressing the Cre recombinase fused to a nuclear localization signal under the control of a PGK promoter. ES cells which had excised the *loxP*-Neo^R-TK-*loxP* fragment (Fig. 1C) were selected by ganciclovir resistance and identified by Southern blot screening (Fig. 1D). Mice homozygous for the *TR*α^7 mutation were named TRα$^{7/7}$.

To further characterize the new mutant mice, we compared the expressions of the different RNA products of the *TR*α locus in the distal ilea of TRα$^{7/7}$ mice to those of wild-type mice by

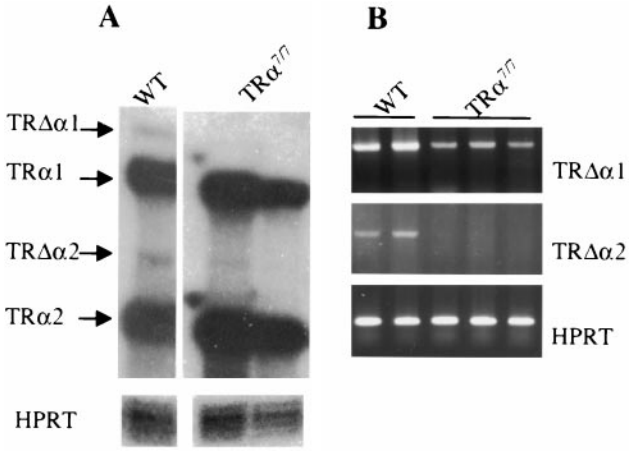
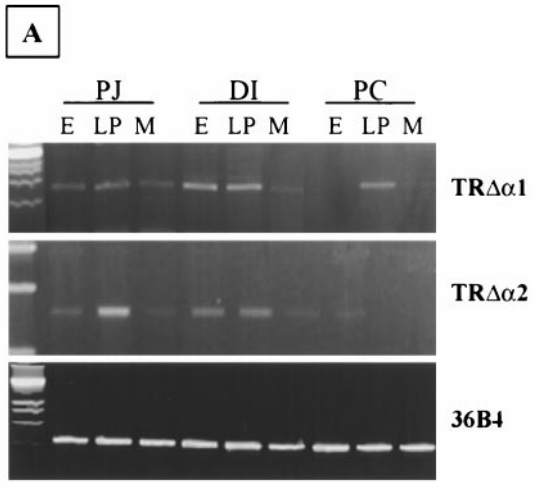


FIG. 2. Expression of the different transcripts encoded by the *TR*α locus in the distal ilea of wild-type and TRα$^{7/7}$ mutant mice. (A) An RNase protection assay was performed using RNA isolated from the distal ilea of animals carrying the indicated genotypes. The protected fragments are indicated on the left. (B) Semiquantitative RT-PCR analysis to selectively detect TRΔα1 and TRΔα2 mRNAs in the distal ilea of wild-type (WT) and TRα$^{7/7}$ mice. For the semiquantitative RT-PCR, a preliminary assay was conducted to define the appropriate range of cycles consistent with an exponential increase of the amount of the PCR products in each experimental condition. For both panels, HPRT was used as internal control.



B

mRNA	Tissues		
	E	LP	M
TRα1*	+	-/+	++
TRα2*	+	-	-
TRΔα1	+	+	-/+
TRΔα2	+	+	-/+

FIG. 3. Expression of TRΔα1 and TRΔα2 in the gut. (A) Semiquantitative RT-PCR analysis was performed to study the longitudinal expression of the two transcripts in the different tissue compartments of 12-day-old wild-type mice. 36-B4 was used as internal control. Standard molecular masses for DNA size are in the first lane. (B) Summary of the expression pattern of the four mRNAs encoded by the *TR*α gene in the different tissues composing the distal ileum mucosa. E, epithelium; LP, lamina propria; M, muscle; PJ, proximal jejunum; DI, distal ileum; PC, proximal colon: *, described in reference 25.

RNase protection and RT-PCR analysis. Using specific probes in an RNase protection assay, we detected similar amounts of TRα1 and TRα2 mRNAs in wild-type and TRα$^{7/7}$ mice (Fig. 2A), showing that the mutation introduced in intron 7 did not affect the production of these transcripts. The same experiment also showed that the mutation reduced the mRNA expression of both TRΔα1 and TRΔα2. The reduced expression of the TRΔα isoforms was also confirmed by semiquantitative RT-PCR (Fig. 2B). Using a sense primer within intron 7, which has been shown to contain the transcription initiation site of TRΔα mRNAs (5), and either an α1-specific or an α2-specific oligonucleotide as antisense primer, we detected the TRΔα1 and TRΔα2 transcripts in wild-type mice. Under the PCR conditions used, we were unable to detect a TRΔα2 transcript in TRα$^{7/7}$ animals, while the TRΔα1 mRNA was strongly reduced compared to that of wild-type mice. These analyses indicate that TRα$^{7/7}$ animals display normal levels of TRα1 and TRα2 transcripts and decreased amounts of TRΔα transcripts. The decreased level of expression of the TRΔα isoforms in TRα$^{7/7}$ animals is consistent with the reduced promoter activity of the deleted intron 7.

Analysis of the TRΔα1 and TRΔα2 expression patterns in the intestinal mucosa. (i) mRNA expression. We performed RT-PCR analysis to evaluate the longitudinal expression of the TRΔα truncated isoforms on separated epithelia, laminae propriae, and muscle layers of 12-day-old wild-type mice (Fig. 3A and B). TRΔα1 mRNA is expressed in almost all tissues in each region analyzed, with a higher expression in the epithelium and lamina propria of the distal ileum and a very faint expression in the muscular layers of the distal ileum and proximal colon. These data are in agreement with the data concerning the protein immunostaining using specific antibody (see below). TRΔα2 mRNA shows a distribution similar to that of TRΔα1 except that the maximal expression is in the lamina propria of the proximal jejunum.

(ii) Protein expression. We used specific antisera to analyze the expression pattern of the *TR*α locus products in mice carrying different *TR*α mutations. Using the anti-Nα antiserum, which recognizes the N-terminal region common to TRα1 and TRα2 proteins, we observed a similar nuclear labeling in muscle layers of TRα$^{7/7}$ and wild-type distal ileum (Fig. 4D and H), consistent with the unmodified mRNA expression of these two isoforms in TRα$^{7/7}$ animals. In contrast, when using the anti-Cα1 antibody, which recognizes the C-terminal part common to TRα1 and TRΔα1 proteins, the signals in these two mouse strains were very different. While a strong nuclear signal in the differentiated epithelial cells of the villi and a fainter signal in the laminae propriae and muscle layers were detected in wild-type mice (Fig. 4J to L), no labeling was observed in TRα$^{7/7}$ mice (Fig. 4N to P). The loss of staining in TRα$^{7/7}$ mice definitely shows that the protein labeled by the anti-Cα1 reagent in wild-type mice is TRΔα1. These data also demonstrate that

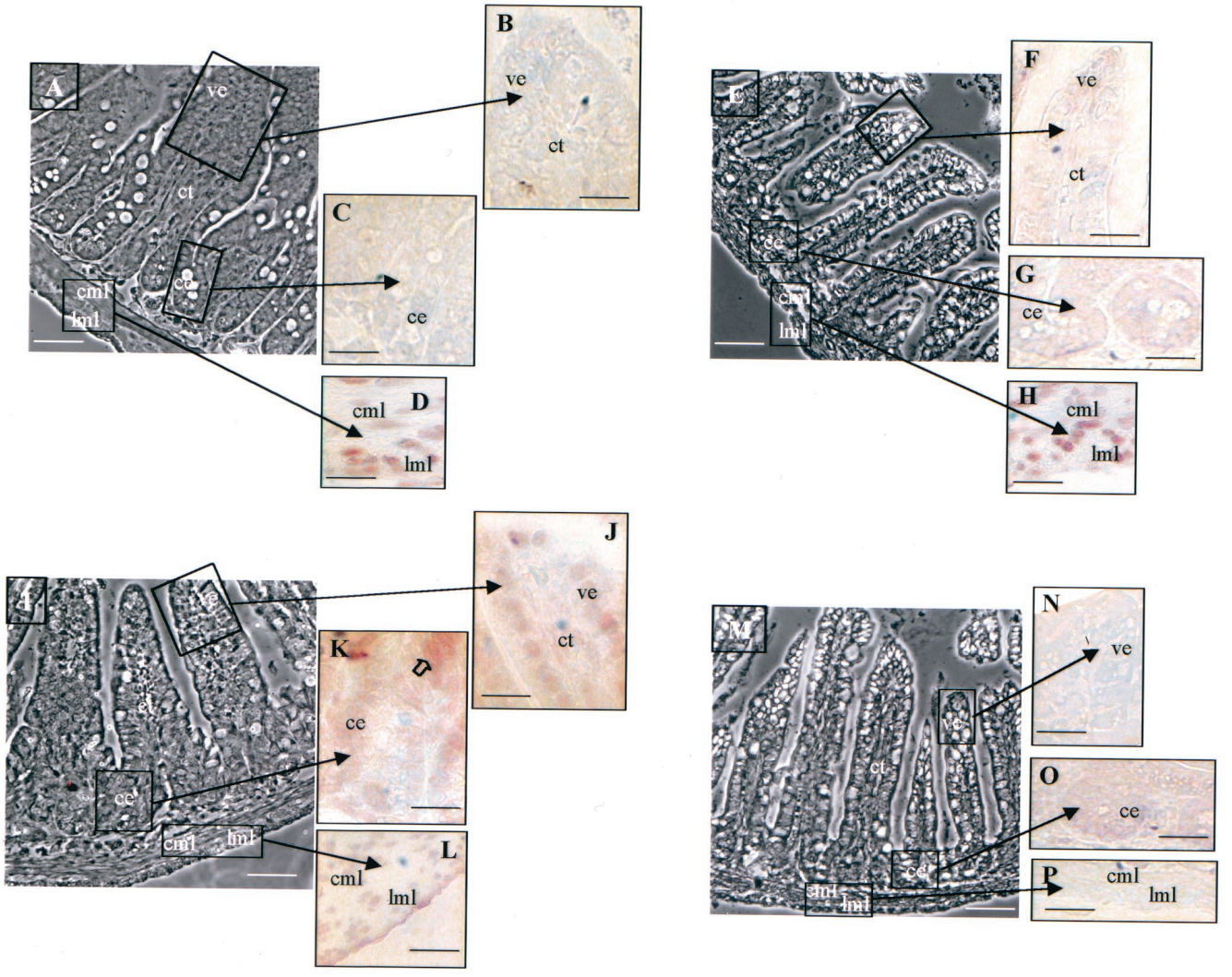


FIG. 4. Immunolocalization of TRα1/TRα2 and TRΔα1 proteins. We analyzed the expression patterns of TRα and TRΔα1 proteins on distal ileum paraffin-embedded sections (5 μm) of wild-type (A through D and I through L) and TRα$^{7/7}$ (E through H and M through P) intestines from 3-week-old mice. Two different antibodies were used, one (anti-Nα) recognizing the N-terminal part common to TRα1 and TRα2 on wild-type (A through D) and TRα$^{7/7}$ (E through H) mouse tissues and the other (anti-Cα1) recognizing the C-terminal part common to TRα1 and TRΔα1 on wild-type (I through L) and TRα$^{7/7}$ (M through P) mouse tissues. Technical controls included the use of phosphate-buffered saline or the antibody preincubated with an excess of the recognized peptide. Phase contrast low magnification (A, E, I, M) bar, 30 μm; bright field high magnification (B to D, F to H, J to L, N to P) bar, 70 μm; ce, crypt epithelium; ve, villus epithelium; ct, connective tissue; cml, circular muscle layer; 1ml, longitudinal muscle layer. The open arrow in K indicates the stronger nuclear staining of epithelial cells above the crypt-villus junction.

even though a small amount of TRΔα1 mRNA can be revealed by RNase protection assay in the distal ilea of TRα$^{7/7}$ mice, the amount of TRΔα1 protein is strongly reduced to below the detection limit. Using specific antibodies against TRα2 and TRΔα2 proteins, we could not reveal any staining (not shown). Table 1 summarizes the data concerning the expression of the proteins produced by the *TR*α locus in the intestinal mucosae of mice with different mutations in the TRα gene.

Morphological and functional parameters of the small intestine are more affected in TRα$^{-/-}$ mice than in TRα$^{0/0}$ and TRα$^{7/7}$ mice. It has previously been shown that the morphology of the small intestine, the proliferation of crypt epithelial cells, and their differentiation were altered in mice lacking TRα1 and TRα2 expression (TRα$^{-/-}$) and that this impairment was more severe in the distal part of the small intestine (25). Interestingly, mice lacking the expression of all the products of the *TR*α locus (TRα$^{0/0}$) display a milder alteration of the intestinal mucosa than the TRα$^{-/-}$ mice (9a). In order to determine the molecular basis of these differences, we examined morphofunctional parameters in the distal small intestines of TRα$^{0/0}$ and TRα$^{7/7}$ mice and compared them to those described for wild-type and TRα$^{-/-}$ mice.

(i) Morphology. As shown in Fig. 5, the thickness of the mucosa in TRα$^{0/0}$ mice was decreased compared to that of wild-type mice (Fig. 5B versus A), with a reduction of the villus height, resulting from a reduction of the number of epithelial cells per crypt-villus axis. This phenotype was milder than but comparable to the one observed in the TRα$^{-/-}$ ileum (Fig.

TABLE 1. Expression of the TRα isoforms in the different tissues composing the distal small intestine mucosa

Immunolabeling (isoform)	Expression[a] in indicated tissues[b] of TRα isoforms for mice with genotype:															
	Wild type				$TR\alpha^{-/-}$				$TR\alpha^{0/0}$				$TR\alpha^{7/7}$			
	Ce	Ve	LP	M	Ce	Ve	LP	M	Ce	Ve	LP	M	Ce	Ve	LP	M
N-TRα[c] (TRα1/α2)	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+
C-TRα[c] (TRα1/Δα1)	−/+	++	−/+	−/+	++	++	−/+	−/+	−	−	−	−	−	−	−	−

[a] The symbols − to ++ correspond to the relative intensities of the signals.
[b] Ce, crypt epithelium; Ve, villus epithelium; LP, lamina propria or connective tissue; M, muscle layers.
[c] See also reference 9a.

5C). No obvious morphological alteration was observed in the $TR\alpha^{7/7}$ distal ileum (Fig. 5D) compared to that of the wild type. A previous study described a decreased number of goblet cells in the distal ileum of 3-week-old $TR\alpha^{-/-}$ mice compared to the wild type (25). In order to investigate whether the other TRα mutants also show this alteration, we determined the number of mucus-producing goblet cells stained by Schiff's reagent as previously described (25). The $TR\alpha^{-/-}$ and $TR\alpha^{0/0}$ mice show similar significant decreases in the number of goblet cells compared to that of the wild type ($TR\alpha^{-/-}$, 4 ± 1 [mean ± SD]; $TR\alpha^{0/0}$, 6 ± 1; wild type, 14 ± 2); in contrast, the $TR\alpha^{7/7}$ mice displayed an increased amount (20 ± 3) statistically significantly different from that of the wild type ($P < 0.05$). The Paneth cells, a differentiated epithelial cytotype located in the intestinal crypts, displayed no altered proportions as demonstrated by specific staining or morphological criteria (data not shown).

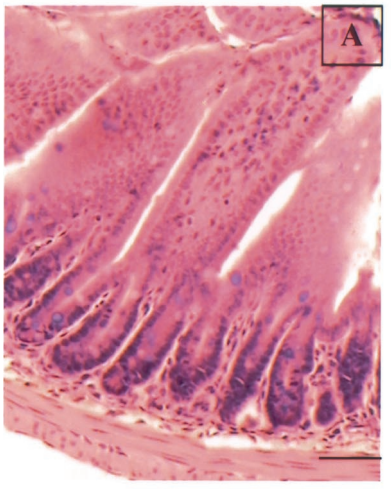
(ii) Epithelial proliferation. We then checked the effects of the *TR*α mutations on cell proliferation by determining the number of Ki67-positive cells per crypt (Fig. 5E). In $TR\alpha^{0/0}$ mice the number of Ki67-positive cells was significantly reduced, consistent with the reduction in the number of cells per axis described above. This decrease was more important in $TR\alpha^{-/-}$ mice. The proliferation in $TR\alpha^{7/7}$ mice was also slightly decreased, although this difference did not reach statistical significance compared to wild-type mice. This is consistent with the absence of obvious intestinal morphological alteration in $TR\alpha^{7/7}$ mice.

(iii) Epithelial differentiation. In order to analyze whether the functional polarization typical of differentiated enterocytes and the ontogenetic rise in sucrase expression occurring in mature enterocytes at weaning were affected by the different mutations of the *TR*α gene, we measured the enzymatic activities of lactase and sucrase in 3-week-old mutant mice (Fig. 5F). This study was conducted on brush border preparations from the proximal jejunal epithelium, as these enzymes are mainly expressed in this region of the small intestine (13). In $TR\alpha^{0/0}$ mice, both enzymatic activities were moderately decreased. In $TR\alpha^{-/-}$ mice, the reduction of both activities was more dramatic. Interestingly, in $TR\alpha^{7/7}$ mice the lactase activity was not affected but the sucrase activity was considerably decreased, to less than 35% of that of the wild type.

In summary, in $TR\alpha^{-/-}$ mice the epithelial proliferation was decreased and morphological (25) and biochemical parameters characteristic of enterocyte differentiation were impaired; in $TR\alpha^{0/0}$ mice, the proliferation was decreased but the differentiation was almost normal; in $TR\alpha^{7/7}$ mice, the functional polarization was normal but the expression of some markers specific for the mature differentiated epithelial cells was impaired. In addition, these data show that the *TR*α locus controls the fate of both enterocytes and goblet cells.

***cdx1* and *cdx2* gene expression is controlled by the TRΔα isoforms.** In an attempt to identify the molecular mechanisms responsible for the impaired proliferation and differentiation of the epithelial cells in *TR*α mutant mice, we examined the levels of expression of the Cdx1 and Cdx2 transcripts, which encode key homeoproteins involved in the control of intestinal epithelium homeostasis (reviewed in reference 8). The amounts of Cdx1 and Cdx2 mRNAs were estimated by semiquantitative RT-PCR analysis along the proximodistal intestinal axis. Figure 6A and a previous report (25) show that the increasing gradient of expression of these genes along the proximodistal axis is maintained in mice of each genotype. Moreover, the amounts of both transcripts were specifically decreased in each region of $TR\alpha^{-/-}$ mice but unchanged in $TR\alpha^{0/0}$ and $TR\alpha^{7/7}$ mice compared to the amounts in wild-type mice. These data suggest that the TRΔα products, which are still expressed in $TR\alpha^{-/-}$ mice, could have a negative regulatory role on the transcription of the *cdx* genes in the absence of TRα1. To challenge this assumption, we investigated the effects of TRΔα1 and TRΔα2 products on the activities of the *cdx1* and *cdx2* promoters by transient transfection assays in the human colonic cell line Caco2-TC7, using 5′ regulatory regions previously described (23). Figure 6 shows that both TRΔα1 and TRΔα2 repressed the activities of the *cdx1* (Fig. 6B) and *cdx2* (Fig. 6D) promoters in a dose-dependent manner.

FIG. 5. (A through D) Morphological appearance of distal small intestine in 3-week-old animals. Five-micrometer histological sections of wild-type (A), $TR\alpha^{0/0}$ (B), $TR\alpha^{-/-}$ (C), and $TR\alpha^{7/7}$ (D) mice were stained with hematoxylin and eosin. Bar, 30 μm. The numbers at the right of each picture indicate the number of epithelial cells per crypt-villus axis. (E) Analysis of proliferation of epithelial cells. The number of proliferating cells per crypt was evaluated after immunolabeling of cells expressing Ki67 antigen on distal ileum paraffin-embedded sections (5 μm). The Student *t* test indicated that in $TR\alpha^{0/0}$ and $TR\alpha^{-/-}$ mice the number of Ki67-positive cells is significantly decreased compared to the number in wild-type animals. For each pair a *P* value of <0.0001 (***) was obtained ($n = 15$). (F) Functional analysis of the differentiation markers of small intestine epithelial cells. Lactase and sucrase brush border activities were analyzed at the proximal jejunum level in 3-week-old wild-type and mutant animals. Enzymatic activities are represented as percentages of the respective wild-type levels. Statistical analysis was performed using the Student *t* test by comparing each group of mutant animals and the control. *, $P < 0.05$; **, $P < 0.001$ ($n = 4$).



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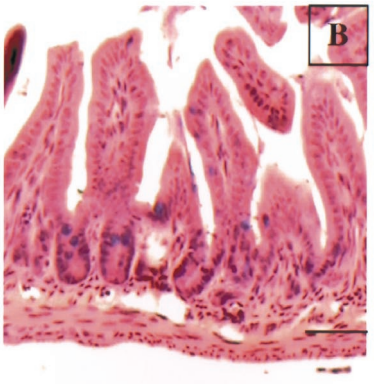

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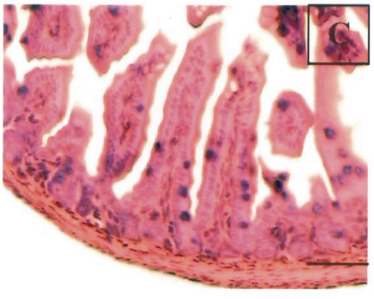

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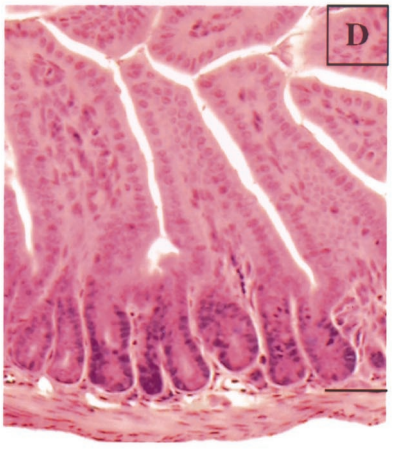

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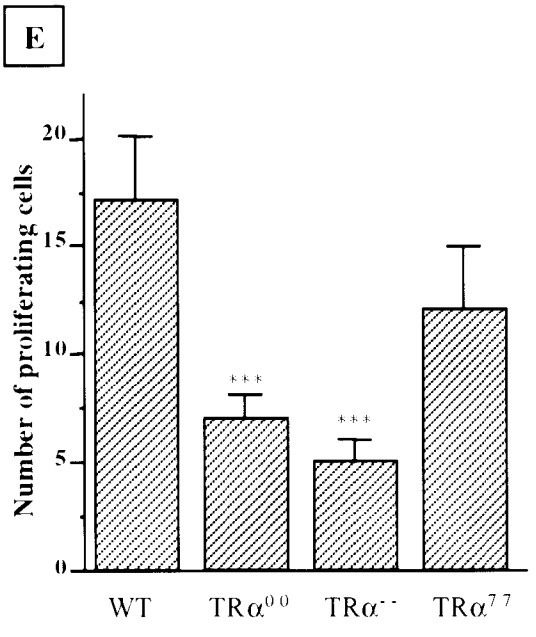
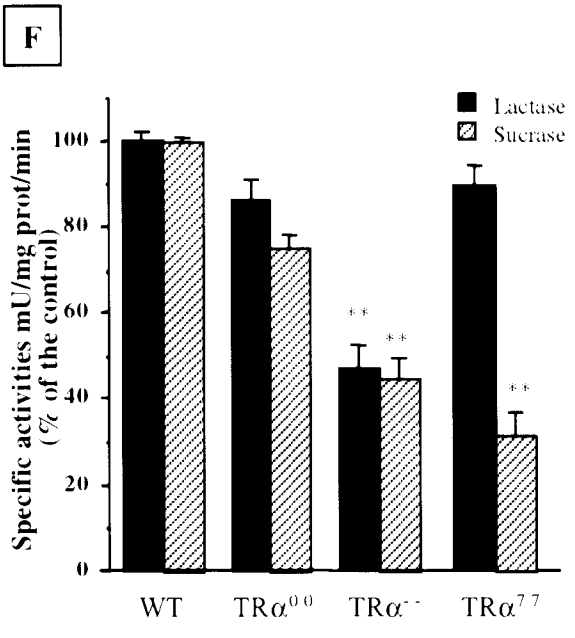




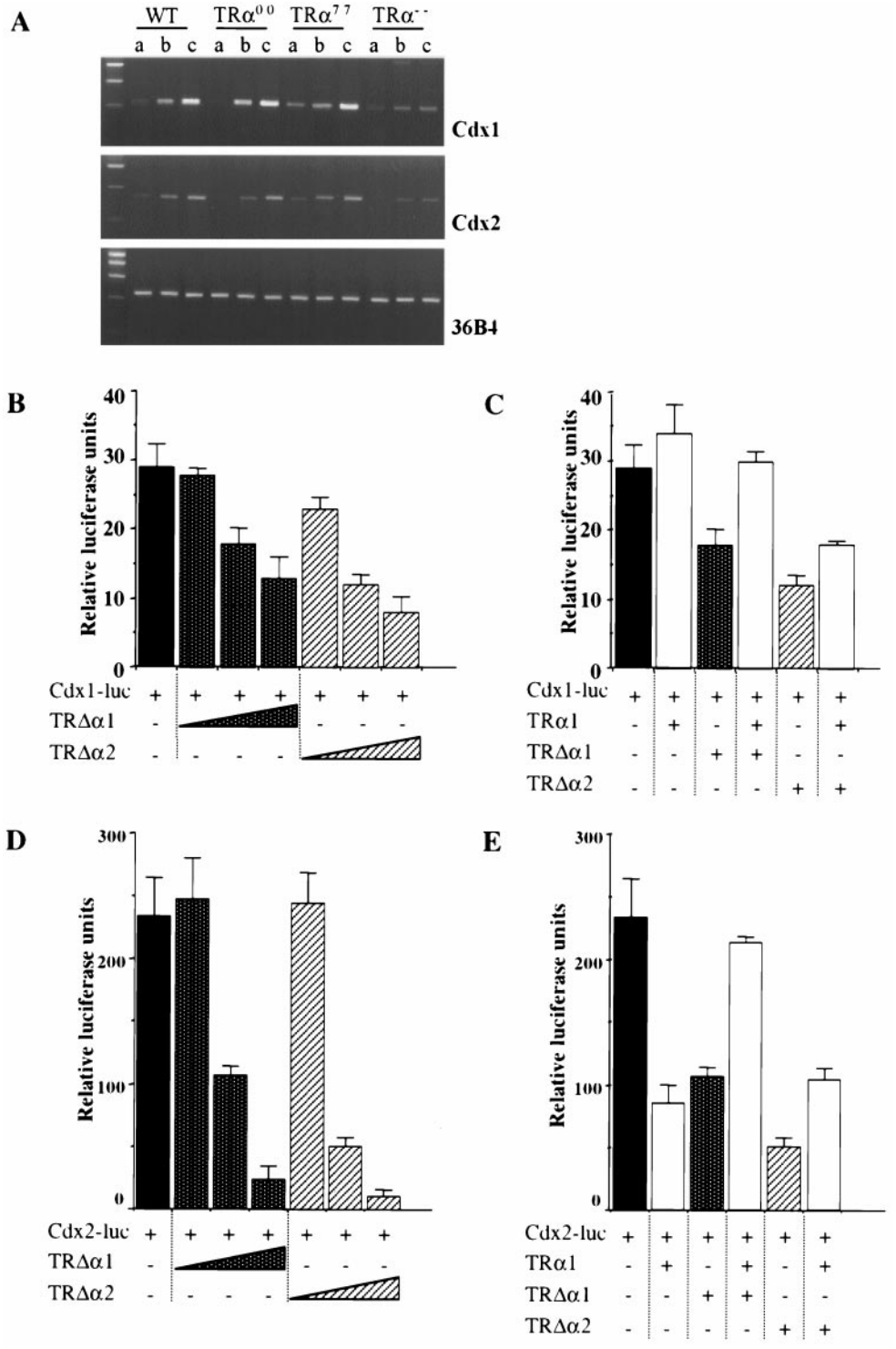


FIG. 6. Expression and transcriptional regulation of *cdx1* and *cdx2* homeobox genes. (A) Representative results of RT-PCR analysis of *cdx1* and *cdx2* gene expression along the intestinal proximo-distal axis of knockout and wild-type (WT) animals. 36-B4 was used as an internal control. (B) Relative levels of luciferase activity triggered by the *cdx1* promoter (1 μg of the pCdx1-4luc plasmid) in Caco2-TC7 cells cotransfected with increasing amounts of the expression vector pSG5mTRΔα1 or pSG5mTRΔα2 (10 ng, 100 ng, and 1 μg). (C) Relative levels of luciferase activity triggered by the *cdx1* promoter as in panel B in Caco2-TC7 cells cotransfected or not with 900 ng of the expression vector pSG5hTRα1 and 100 ng of either the pSG5mTRΔα1 or the pSG5mTRΔα2 plasmid. (D) Relative levels of luciferase activity triggered by the *cdx2* promoter (1 μg of the pCdx2-1luc plasmid) in Caco2-TC7 cells cotransfected with increasing amounts of the expression vector pSG5mTRΔα1 or pSG5mTRΔα2 (10 ng, 100 ng, and 1 μg). (E) Relative levels of luciferase activity triggered by the *cdx2* promoter as in panel D in Caco2-TC7 cells cotransfected or not with 900 ng of the expression vector pSG5hTRα1 and 100 ng of either the pSG5mTRΔα1 or the pSG5mTRΔα2 plasmid. The cells were transiently transfected with a total of 2 μg of DNA/well; when necessary, the empty vector pSG5 was added. Cells were lysed 48 h after transfection. Results are mean values ± SD of three independent experiments conducted in triplicate.

TRΔα1 did not affect the activity of a mouse β-actin promoter in Caco2-TC7 cells (data not shown) and has been previously shown to have no effect on various promoters in HeLa cells (5). TRΔα2 repressed the β-actin promoter activity by 50% when 1 μg of vector was transfected. TRΔα2 has previously been shown to exert transcriptional inhibition towards several promoters (5). Therefore, we assume that while the specificity of the TRΔα1 isoform appears tight, TRΔα2 can inhibit a wider spectrum of promoters. However, since TRΔα1 but not TRΔα2 was detected in the epithelium of distal ilea of wild-type and TRα$^{-/-}$ mice, this protein is likely to be responsible for the decreased levels of *cdx* transcripts observed in mutant mice. In vivo, the amount of *cdx* transcripts is reduced in TRα$^{-/-}$ mice, which lack the TRα1 and TRα2 isoforms. We have shown that TRα1 accelerated the degradation of the TRΔα1 protein in transfected HeLa cells (9a). We examined whether the expression of TRα1 in Caco2-TC7 cells was able to counteract the activity of the TRΔα isoforms. In the absence of T3, TRα1 did not affect the activity of the *cdx1* promoter but inhibited the *cdx2* promoter by 60% (Fig. 6C and E). The addition of T3 resulted in a slightly increased activity of the *cdx1* promoter and did not affect the *cdx2* promoter activity (data not shown). Remarkably, TRα1 in the absence (Fig. 6C) or in the presence (data not shown) of T3 partly alleviated the repression exerted by TRΔα1 and TRΔα2 on the activity of the *cdx1* promoter. The coexpression of TRα1 and TRΔα1 or TRΔα2 resulted in the alleviation of the inhibitory activity of each product taken separately and led to at least partial restoration of full transcriptional activity of the *cdx2* promoter (Fig. 6E). The TRα2 cotransfection did not modify the inhibitory activity of the TRΔα isoforms on the *cdx* promoters (data not shown). The modulation of the activity of the TRΔα products by TRα1 in transient transfection assays is consistent with the decreased levels of Cdx transcripts observed in vivo in the absence of TRs in TRα$^{-/-}$ mice (Fig. 6A). We conclude that transfection experiments and in vivo observations support the existence of reciprocal interactions between TRΔα products and the TRα1 protein. We propose that TRΔα products can alter the transcription of specific genes when they are not "buffered" by TRα1.

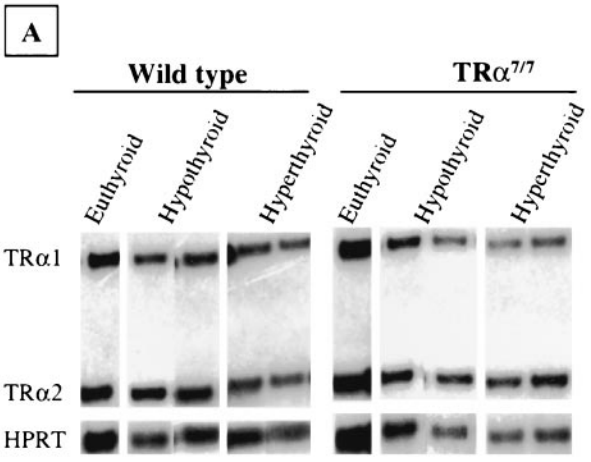
TRΔα products control responsiveness to T3. We have shown that the TRα$^{0/0}$ animals displayed a decreased number of proliferating crypt cells and that TRα$^{7/7}$ mice had a lower sucrase activity than did the wild type. To check whether altered T3 responsiveness could account for these phenotypes, we analyzed several morphological and functional parameters in wild-type, TRα$^{0/0}$, and TRα$^{7/7}$ mice in which experimental hypothyroidism or hyperthyroidism had been induced. Wild-type and TRα$^{7/7}$ animals following a low-iodine diet supplemented with PTU (hypothyroid) displayed a reduction of the mucosa thickness related to a reduction of the crypt-villus height. This correlates with a decrease in the number of the epithelial cells per crypt-villus axis compared to the respective euthyroid 3-week-old mice (data not shown). It is noteworthy that hypothyroid TRα$^{0/0}$ animals displayed the same morphological appearance as euthyroid animals of the same age. Indeed, the number of epithelial cells was not significantly decreased in TRα$^{0/0}$ hypothyroid animals compared to that in euthyroid animals (data not shown). Upon administration of T3, which induced a hyperthyroid status, the number of epithelial cells did not change significantly in any of the strains analyzed. This is not surprising as the complete renewal of the epithelium is accomplished in 3 to 4 days (10), whereas T3 was administered only 48 h before the animals were sacrificed. Instead, we measured the number of proliferating crypt cells in the same experimental conditions (Fig. 7C). It is worth noting that hypothyroidism strongly reduced the numbers of proliferating cells in wild-type and TRα$^{7/7}$ mice but was ineffective in TRα$^{0/0}$ mice compared to euthyroid animals of the corresponding genotypes (Fig. 7C). Administration of T3 stimulated the proliferation in wild-type but not in TRα$^{0/0}$ mice despite higher levels of serum T3 (Fig. 7B), demonstrating that the proliferative response to T3 is mediated by the TRα1 receptor. Surprisingly, T3-induced stimulation of proliferation was much larger in TRα$^{7/7}$ mice (threefold increase) than in wild-type animals (twofold increase), although similar blood concentrations of T3 were measured in both sets of mice (Fig. 7B). In order to check whether the hypothyroid or hyperthyroid status modified TRα1 and TRα2 mRNA expression in wild-type and TRα$^{7/7}$ animals, we quantified the relative abundance of the two mRNAs by RNase protection assay and densitometric analysis in both strains (Fig. 7A and B). The results clearly indicate that the relative amounts of TRα1 and TRα2 were not dependent on the thyroid hormone status.

This study indicates that in vivo, as in vitro (5), the TRΔα isoforms repress the responsiveness to T3 mediated by TRα1. In addition it enables us to definitively conclude that the TRα1 receptor is the only mediator of the T3-stimulated proliferation in the intestinal epithelium.

DISCUSSION

Thyroid hormone has long been suggested to be a regulator of the intestine developmental changes occurring at weaning (reviewed in reference 13). Indeed, the thyroid hormone-dependent intestinal remodeling during amphibian metamorphosis is well characterized (17, 29). The actions of the thyroid hormones are mediated by the TRs acting as transcription factors. Previous work and our more recent findings have led to the identification of four transcripts generated by the *TR*α locus. As all the isoforms produced by the *TR*α locus are expressed in the intestine, we analyzed morphofunctional parameters of this organ in mice with different TRα gene mutations. This enabled us to assign specific functions to the different TRα products. We demonstrated that the TRα1 receptor mediates the T3-dependent functions in the small intestine. We provide genetic evidence for the functions exercised by the TRΔα products in the postnatal intestinal development as well as in the control of T3 responsiveness.

The unbalanced expression of TRα and TRΔα products accounts for the strong intestinal phenotype of *TR*α$^{-/-}$ mutants. It has been shown that in TRα$^{-/-}$ mice the expression of the TRΔα transcripts was maintained in the absence of the TRα transcripts and that severe alterations occurred, particularly in the distal small intestine (25). The intestinal phenotype is characterized by impairment of cell proliferation and differentiation, associated with the downregulation of *cdx1* and *cdx2* homeobox gene expression. In contrast, in the TRα$^{0/0}$ mice, which lack all TRα and TRΔα isoforms, only the proliferation



B

Genotype	TH status	FT3 (pmole/l)	TRα1/TRα2 mRNA ratio
Wild type	Euthyroid	5.8±1.1	1±0.02
TRα0/0	Euthyroid	ND*	
TRα7/7	Euthyroid	4.98±0.6	0.9±0.1
Wild type	Hypothyroid	2.6±0.6	0.9±0.001
TRα0/0	Hypothyroid	0.9±0.06	
TRα7/7	Hypothyroid	2.3±0.4	0.9±0.02

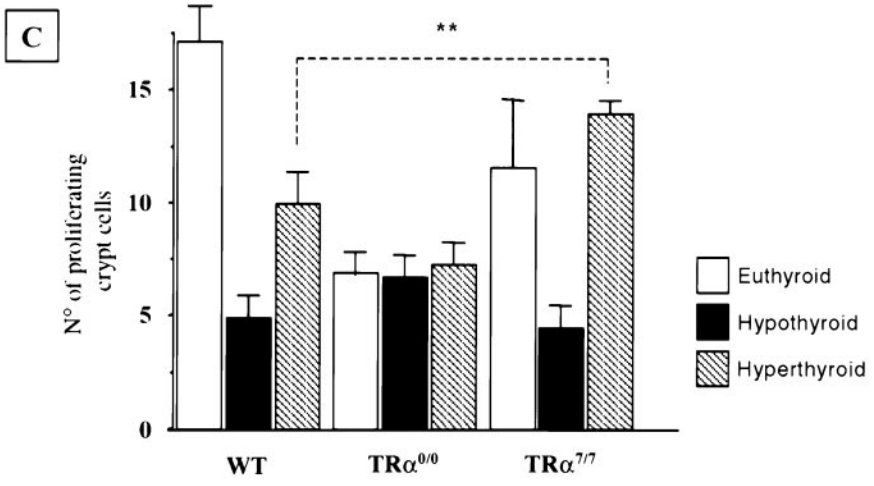


FIG. 7. Effects of hypothyroidism and hyperthyroidism induction on the small intestine. (A) Analysis of TRα1 and TRα2 expression by RNase protection assay on wild-type and TRα$^{7/7}$ hypothyroid and hyperthyroid animals. The protected fragments are indicated on the left; HPRT was used as internal control. (B) Free T3 (FT3) blood concentrations (in picomoles per liter) in hypothyroid, euthyroid, and hyperthyroid animals of the indicated genotypes and densitometric analysis of the protected bands in the RNase protection assay. The study was performed to quantify the relative amounts of TRα1 and TRα2 in animals of different thyroid hormone statuses. Results are mean values ± SD; $n = 3$ or 4. ND*, not determined. The total T3 concentration has been shown not to be different in TRα$^{0/0}$ and wild-type mice (24). (C) Analysis of epithelial proliferation. The numbers of proliferating cells per crypt in hypothyroid and hyperthyroid animals were evaluated after immunolabeling of cells expressing Ki67 antigen on distal ileum paraffin-embedded sections (5 μm). The Student t test indicated that in TRα$^{7/7}$ T3-treated animals the number of proliferating cells was significantly increased compared to that in wild-type T3-treated animals. **, $P < 0.001$.

of the epithelial cells is affected. These data establish that the *TR*α^{-} and the *TR*α^{0} mutations generate very different phenotypes. Theoretically, the severity of the phenotype in TRα$^{-/-}$ mice could be attributed either to the low concentration of thyroid hormones associated with this mutation (7) or to the unbalanced expression of the TRΔα and TRα products. The former hypothesis can be definitely ruled out since intestinal impairment is already observed in 2-week-old TRα$^{-/-}$ mice which retain normal TH concentrations at this preweaning age (25). Ruling out the difference in the thyroid hormone status as the factor accounting for the discrepancies between the phenotypes observed in TRα$^{-/-}$ and TRα$^{0/0}$ mice implies that the cause must be the altered balance between the TRα and TRΔα products of the *TR*α locus.

TRα1 mediates T3-dependent functions in intestinal epithelial cells. The data reported in the present study clearly show that the proliferation of epithelial cells in the crypts of the small intestine, which was shown to be enhanced at weaning (30, 33), is modulated by T3 through a TRα1-dependent pathway. The further deletion of the *TR*β genes does not worsen this phenotype (9a), demonstrating that it is exclusively mediated by the TRα1 receptor. Moreover, the experiment of induced hypothyroidism and hyperthyroidism in TRα$^{0/0}$ mice confirms that TRα1 is the only receptor which mediates the T3-dependent control of crypt cell proliferation, at least during the weaning period. We also showed that the numbers of mucus-producing goblet cells are decreased in similar proportions in TRα$^{-/-}$ and TRα$^{0/0}$ animals, indicating that the TRα1 receptor is indeed involved in the previously described role of T3 on goblet cell maturation (2). Finally, we show that in TRα$^{0/0}$ mice the epithelial differentiation is slightly but not significantly impaired, suggesting that the T3-TRα1 pathway is not a major regulator of intestinal epithelial differentiation.

Functional interference between TRα1 and TRΔα isoforms. In TRα$^{7/7}$ mice, the levels of expression of the TRα1 and TRα2 transcripts are unchanged, but the production levels of TRΔα1 and TRΔα2 transcripts and of the TRΔα1 protein are reduced. Despite the persistence of small amounts of TRΔα transcripts, a clear alteration in the intestinal epithelium is observed in these mice. This emphasizes the physiological importance of these proteins in wild-type animals. The enhancement of T3-stimulated proliferation as well as the increase in the number of goblet cells in untreated TRα$^{7/7}$ mice compared to wild-type mice suggests that TRΔα products down modulate the activity of T3. Since we have shown that the proliferation of crypt epithelial cells and the goblet cell maturation depend on T3-TRα1, this implies that TRΔα products regulate the activity of TRα1, in agreement with our previous observations in transient transfection experiments (5). Another striking feature of TRα$^{7/7}$ mice is the low specific activity of sucrase. In contrast to what is observed with TRα$^{-/-}$ mice, this is not the consequence of an impaired differentiation since lactase activity (this paper) and the number of sucrase-expressing cells, as assessed by immunohistochemical staining using antisucrase antisera (our unpublished data), are not affected in TRα$^{7/7}$ mice. Instead, it may reflect a delay in the maturation process at weaning. The colocalization of the TRΔα1 and sucrase proteins in the epithelial cells (13) of the villi in wild-type mice is consistent with a potential regulatory effect of TRΔα isoforms on sucrase gene expression. Since no significant reduction of sucrase activity is observed in the small intestines of TRα$^{0/0}$ mice, lacking not only TRΔα but also TRα products, the down-regulation of sucrase expression observed in TRα$^{7/7}$ mice is not due to an autonomous activity of TRΔα isoforms but might be the result of interference with the other *TR*α gene products, although the mechanisms involved are unknown. Altogether, our data support the assumption that TRΔα products modulate the activity of TRα proteins in vivo and that this modulation is essential to ensure the normal maturation of the small intestine.

Genetic and biochemical evidence for TRΔα functions. TRα$^{0/0}$ mice, which have lost both the *TR*α and *TR*Δα genes, do not exhibit the very severe phenotype shown by TRα$^{-/-}$ mice. We conclude that this phenotype is the consequence of the unbalanced expression of the *TR*Δα versus the *TR*α gene in TRα$^{-/-}$ mutants. We have demonstrated that the stability of the TRΔα1 protein is negatively controlled by the α1 receptor (9a), and that the inhibitory activity of TRΔα1 over the transcription of the *cdx1* and *cdx2* promoters was alleviated by the expression of TRα1. This suggests that, when TRα1 is present, the amount and hence the activity of TRΔα1 are blunted. In contrast, in the absence of the α1 receptor (i.e., in TRα$^{-/-}$ mice), the stability and thus the amount of TRΔα1 protein are increased, leading to deleterious effects. Careful examination of the expression pattern of the TRΔα1 protein in the intestinal epithelium supports this assumption. In wild-type mice, this protein is detected as a faint signal in the crypts compared to the stronger staining in the villi. In TRα$^{-/-}$ mice, the staining reaches the same intensity in both compartments, revealing increased amounts of the TRΔα1 protein in the crypts of these animals compared to amounts in the wild type. In striking correlation with this altered expression pattern, the cell proliferation in crypts and their further differentiation are obviated in TRα$^{-/-}$ mice. Interestingly, in vitro and in vivo in the absence of TRα1, the products of the *TR*Δα genes can repress the transcription of *cdx1* and *cdx2*, two genes which control intestinal cell proliferation and differentiation (8). The molecular mechanisms of such repression are not yet clear. The TRΔα products may act on the *cdx* promoters or may interfere in vivo with other nuclear receptors such as retinoic acid receptors, as suggested by transfection experiments (5; our unpublished observations) and by the direct activation of the *cdx1* promoter by retinoic acid (16). They may, however, also affect other signaling pathways.

In conclusion, we demonstrate here the physiological importance of TRΔα products. We show that these isoforms, in the absence of the TRα proteins, alter the transcription of specific genes that regulate intestinal homeostasis, impair cell proliferation and differentiation in the small intestine epithelial cells, and generate a severe, lethal phenotype. We show that a reduced expression of the *TR*Δα gene increases T3-mediated functions and leads to a decreased sucrase activity in enterocytes. Therefore, the TRΔα isoforms should interfere with T3-dependent and T3-independent pathways. We conclude that the *TR*α locus generates in the intestine TRΔα and TRα isoforms, which negatively control each other and whose balanced expression is critical for the correct development of the small intestine at weaning.

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