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To cite this version:
Jacques Samarut, Michelina Plateroti, Elsa Kress, Jun Ichirou Mori. Thyroid Hormone Receptor alpha-1 Directly Controls Transcription of the beta-Catenin Gene in Intestinal Epithelial Cells. Molecular and Cellular Biology, American Society for Microbiology, 2006, 26 (8), pp.3204-3214.

HAL Id: ensl-00000005
https://hal-ens-lyon.archives-ouvertes.fr/ensl-00000005
Submitted on 7 Apr 2006

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Thyroid Hormone Receptor α1 Directly Controls Transcription of the β-Catenin Gene in Intestinal Epithelial Cells†

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Received 29 July 2005/Returned for modification 30 September 2005/Accepted 17 January 2006

The mammalian gut develops from the association of the endoderm and the splanchnic mesoderm. The adult features are acquired during well-defined fetal and postnatal developmental steps, which are completed at the weaning (16, 18). The pluristratified gut endoderm is remodeled to form a monolayer epithelium is its continuous cell renewal, which is fuelled by cell division, migration, and villus cell differentiation must be tightly controlled to ensure epithelial homeostasis (49).

Several pathways have been described to play an important role in the control of epithelial proliferation in the intestine, during early development as well as during the continuous renewal in adulthood (3, 49). Among these pathways, the Wnt/β-catenin pathway has received major attention, as it is involved in the normal and in the pathological proliferation of the gut (4, 50). The signal transducing components of the Wnt pathway are receptors belonging to the frizzled and LRP protein families (35, 42). One target of the Wnt pathway is the β-catenin protein. In the absence of Wnt signal, β-catenin is destabilized by phosphorylation from a cytoplasmic complex comprising axin, adenomatous polyposis coli, and glycogen synthase kinase 3β. The phosphorylated β-catenin is finally degraded by the proteasome. The action of this complex can be antagonized by disheveled, a cytoplasmic protein that is activated by the frizzled receptors. In addition to Wnt, some other factors have been shown to act in the stabilization of β-catenin, such as insulin-like growth factor, epidermal growth factor, and human growth factor, through their respective thyrosin kinase-associated receptors (35). The main characteristic of the stabilized β-catenin is its accumulation in the cytoplasm and migration into the nucleus, where it acts as a transcriptional coactivator by associating with transcription factors of the Tcf/Lef family (35). Intestinal tumors characterized by actively proliferating cells display high levels of nuclear β-catenin (42).

Recently, van de Wetering et al. (52) described the presence of β-catenin in the nuclei of crypt cells, strongly supporting its role in the control of cell proliferation. The complex β-catenin/Tcf has been shown to control the expression of several genes involved in cell cycle/proliferation control like cyclin D1, c-myc, and cdx1 (54) as well as, indirectly, cyclin D2 (20). It is worth noting that mutations of the different partners of this pathway are responsible for tumorigenesis in humans, as they constitutively activate uncontrolled cell proliferation (42, 50). In fact, activation of this pathway as well as that of the ras oncogene are sufficient to induce colon tumorigenesis (3). Surprisingly, to date, very little is known on the transcriptional regulation of the β-catenin gene.

Several observations suggested that thyroid hormones (TH), T3 and T4, are known regulators of intestine development. The best characterized example is the remodeling of the gastrointestinal tract during amphibian metamorphosis. Thyroid hormones act via nuclear receptors, the TRs, which are T3-dependent transcription factors. We previously showed that intestinal epithelial cell proliferation is controlled by thyroid hormones and the TRα gene. To analyze the mechanisms responsible, we studied the expression of genes belonging to and/or activated by the Wnt/β-catenin pathway, a major actor in the control of physiological and pathological epithelial proliferation in the intestine. We show that T3-TRα1 controls the transcription of the β-catenin gene in an epithelial cell-autonomous way.

This is parallel to positive regulation of proliferation-controlling genes such as type D cyclins and c-myc, known targets of the Wnt/β-catenin. In addition, we show that the regulation of the β-catenin gene is direct, as TR binds in vitro and in chromatin in vivo to a specific thyroid hormone-responsive element present in intron 1 of this gene. This is the first report concerning in vivo transcriptional control of the β-catenin gene. As Wnt/β-catenin plays a crucial role in intestinal tumorigenesis, our observations open a new perspective on the study of TRs as potential tumor inducers.

† Supplemental material for this article may be found at http://mcb.asm.org.

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cytes at weaning (15). The action of TH is mediated by T3 binding to thyroid hormone nuclear receptors, the TRs, which belong to the nuclear hormone receptor family of transcription factors (21). The TRs are encoded by two genes, TRα and TRβ (22), each of which produces several protein isoforms (10). More recently, using congenital hypothyroid and TR knockout mice, we pointed out that TH act on the intestinal postnatal development mainly through the control of the proliferation of the intestine epithelial progenitor cells. This function depends on the TRα receptor (9, 13, 41).

To analyze the molecular targets of T3-activated TRα in the intestine, we focused here on the regulation of the Wnt/β-catenin pathway. We showed that T3-TRα controls the expression of the murine β-catenin gene, catnb, by binding on the chromatin of epithelial cells to a functional TRE sequence in intron 1. This effect is parallel to the activation of the Wnt/β-catenin target genes, including type D cyclins and c-myc. This work then provides the first demonstration of the direct control of transcription of the β-catenin gene.

MATERIALS AND METHODS

Animal treatment and tissue preparation. TRα−/− (13), TRβ−/− (12), Pax6−/− (28), and the respective wild-type animals have been used in this study. Mice were housed and maintained with approval from the animal experimental committee of the Ecole Normale Supérieure de Lyon (Lyon, France). TH deficiency in pups was induced by feeding the mothers a low-iode diet supplemented with 0.15% propylthiouracil (PTU; Harlan/Teklad) for 2 weeks. To induce TH deficiency in adult animals, they were fed the low-iode diet supplemented with 0.15% PTU and 0.05% methimazole (Sigma) in the drinking water for 3 weeks. Hyperthyroidism was induced by intraperitoneal injections of a mixture of T4 and T3 (2.5 mg/kg T4 and 0.25 mg/kg T3 in 100 μl of phosphate-buffered saline) for the indicated length of time. Control animals were fed standard mouse chow. Animals were sacrificed at the indicated ages, and the intestine was quickly removed. The proximal and distal parts of the small intestine were fixed in 10% formaldehyde for immunohistochemistry or frozen in liquid nitrogen and used for RNA and/or protein extraction. Blood was also recovered for serum preparation, and the levels of free T3 and T4 were analyzed by a VIDAS enzyme-linked assay kit (Biemerieux). The data on T3 and T4 measurements are reported in Tables S1 and S2 in the supplemental material.

Primary cell culture of intestine epithelial cells. Primary intestinal cell cultures were derived from 10-day postnatal mice. After sacrifice, the whole small intestine was removed. The epithelium was isolated as intact organoids by enzymatic treatment to 1,000 bp, by using a Vibra Cell by Sonics and Materials, Inc.) were incubated, addition of glycine to a final concentration of 125 mM, followed by two washes of cold phosphate-buffered saline (Invitrogen) containing protease inhibitors (Roche). ChIP was conducted using the kit from Upstate according to the manufacturer’s instructions. The chromatin immunoprecipitation (ChIP) study was performed on collagenase-dispase-separated epithelial fragments from 3- to 5-day-old mouse intestines. The monolayer of cells was incubated with 1% formaldehyde in DMEM for 5 min at 37°C. The reaction was blocked by the addition of glycine to a final concentration of 125 mM, followed by two washes of cold phosphate-buffered saline (Invitrogen) containing protease inhibitors (Roche). ChIP was conducted using the kit from Upstate according to the manufacturer’s instructions. Briefly, 500 ng of sonicated DNA (fragments of 200 to 1,000 bp, by using a Vibra Cell by Sonics and Materials, Inc.) were incubated, respectively, with 10 μl of anti-TRα, anti-TRβ (Affinity Bioreagents), and anti-β-casein sera overnight at 4°C. At the end of the reaction and washing steps, DNA was extracted by phenol-chloroform and suspended in 30 μl of H2O. Four microliters of samples was used for conventional PCR (Taq from Eurogentec). For quantitative PCR we used the Lightcycler apparatus (Bio-Rad Laboratories) and the SYBR green PCR master mix from QIAGEN. The primers are listed in Table S3 in the supplemental material. The data from the PCR were normalized to that of 36B4 levels in each sample. Semiquantitative reverse transcription (RT)-PCR was used to analyze TRα expression; HPRT has been used as internal control. The primers were as follows: TRα1, 5′-GGAGAGATGGCTCAGTTGCA and 5′-CGACCT TCAGGGAGAAGAACAG (product size, 720 bp); HPRT, 5′-CCTGTTGAAGAG ACCTTC and 5′-CACGGGCTGAAACCTCGC (product size, 240 bp).

Cell line culture and transfection. Cos1 cells were cultured in DMEM supplemented with 5% heat-inactivated fetal calf serum. The different constructs were cloned in the pGl2 basic expression vector (Promega). The wild-type or mutated 36 bp of the catnb gene comprising TRE-int1 (from bp 2140 to 2175 of intron 1) were cloned upstream of a simian virus 40 minimal promoter (29). The cells were transfected using the Exgen transfection reagent (Euromedex). After transfection, cells were maintained in thyroid hormone-depleted serum (45), T3 (10−7 M) or vehicle alone was added to the culture medium 24 h before the end of the culture. Luciferase activity was measured 48 h after transfection using the luciferase dual system (Promega).

Electrophoretic mobility shift assay (EMSA). (i) In vitro protein synthesis. Full-length cDNA cloned in pSG5 (Stratagene) coding for mouse TRα1, rat TRβ1, and mouse RXRα were transcribed/translated in vitro using the TNT kit (Promega). Each cDNA was under the control of the T7 promoter. (ii) EMSA. The single-strand oligonucleotides were labeled with T4 polynucleotide kinase (Fermentas) in the presence of [γ-32P]ATP. After annealing with the complementary cold oligonucleotides, the probes were purified on a 10% acrylamide gel and the specific bands were eluted overnight at 4°C in Tris-EDTA. Binding reactions were performed for 20 min using the radiolabeled DNA probes (20,000 cpm) and the in vitro-transcribed TR and/or RXR in 10% glycerol/0.15 mM spermidine/4 mM spermine/5 mM dithiothreitol, 1 mM Na3PO4, and poly(dC-dC) (1.5 μg). Unspecific and nonspecific competitor oligonucleotides were included at the indicated molar excess in the binding reactions. Where indicated, anti-TRα antibodies (antibodies raised against a C-terminal peptide and affinity purified with the same peptide) have been included in the reaction mix for 30 min on ice. After binding, reaction samples were loaded on a 5% nondenaturing polyacrylamide gel and electrophoresed for 2 h at 180 V, followed by gel fixation, drying, and exposure to X-ray film.

Western blot and immunolabeling. Whole-protein extracts were obtained by homogenizing mouse intestine in 5 mM HEPES (pH 7.9), 26% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. NaCl was added to a final concentration of 300 mM before centrifugation. Supernatant proteins were separated on a 10% acrylamide–bis acrylamide (29:1) gel and transferred to a nitrocellulose membrane (Hybond ECL) before incubation with the first antibody. This was followed by an incubation with secondary anti-rabbit or anti-mouse immunoglobulin G–horseradish peroxidase (Promega). The signal was analyzed by using the enzymatic chemiluminescence detection kit (Amersham).

Antibodies. We used the following primary antibodies: mouse monoclonal antibody against β-catenin (Santa Cruz), rabbit anti-β-catenin (Santa Cruz), rabbit anti-ε-catenin (gift from Calame Kathryn, Columbia University College of Physicians and Surgeons, New York), and mouse anti-β-actin (Sigma). Immunohistochemical analysis of β-catenin (Santa Cruz), TRα1 (anti-bodies raised against a C-terminal peptide and affinity purified with the same peptide), and Ki67 (Novocastra) was performed on 10% buffered formalin-fixed, 5-μm sections. Secondary fluorescent antibodies were from Jackson Laboratories. Confocal analysis has been performed on a Zeiss Axiosvert microscope, with fluorescence microscopy on a Zeiss Axioplan microscope.

ChIP and DNA analysis. The chromatin immunoprecipitation (ChIP) study was performed on collagenase-dispase-separated epithelial fragments from 3- to 5-day-old mouse intestines. The monolayer of cells was incubated with 1% formaldehyde in DMEM for 5 min at 37°C. The reaction was blocked by the addition of glycine to a final concentration of 125 mM, followed by two washes of cold phosphate-buffered saline (Invitrogen) containing protease inhibitors (Roche). ChIP was conducted using the kit from Upstate according to the manufacturer’s instructions. Briefly, 500 ng of sonicated DNA (fragments of 200 to 1,000 bp, by using a Vibra Cell by Sonics and Materials, Inc.) were incubated, respectively, with 10 μl of anti-TRα, anti-TRβ (Affinity Bioreagents), and anti-β-casein sera overnight at 4°C. At the end of the reaction and washing steps, DNA was extracted by phenol-chloroform and suspended in 30 μl of H2O. Four microliters of samples was used for conventional PCR (Taq from Eurogentec). For quantitative PCR we used the Lightcycler apparatus (Bio-Rad Laboratories) and the SYBR green PCR master mix from QIAGEN. The primers are listed in Table S3 in the supplemental material.
RESULTS

TRα gene controls gut epithelial proliferation starting early after birth. Our previous study indicated the major involvement of the TRα gene in the control of epithelial proliferation in the intestine during weaning time (40, 41). We extended here this observation and analyzed the rate of cell proliferation in the TRα0/0 knockout mice (13) compared to the wild type (WT) throughout fetal and postnatal intestinal development until adulthood (Fig. 1A). In TRα0/0 mutants, we observed a 25% decrease in the number of proliferating cells at postnatal day 1 and in 8-week-old adult animals and a decrease of nearly 50% at 7, 14, and 21 days after birth compared with the respective WT animals. This was observed in both the proximal (Fig. 1A) and distal small intestine (not shown). Looking for the expression of the TRα1 receptor, we could observe that TRα1 mRNA was expressed during this same period (Fig. 1B). Moreover, TRα1 protein, analyzed by an immunohistochemical approach, was specifically present in the crypt cells, as shown for the 14-day-old intestine (Fig. 1C to H; see Fig. S1C to H in the supplemental material).

Thyroid hormone controls the expression of the Wnt/β-catenin pathway. Focusing on 2-week-old animals, we studied the expression of several components of the Wnt pathway, including β-catenin; β-catenin destabilizers adenomatous polyposis coli, glycogen synthase kinase 3β, and axin; β-catenin stabilizer disheveled; β-catenin transcription cofactor TCF4 and its repressors Groucho and Clybby (1, 51). Using RNAs extracted from euthyroid, hypothyroid, and hyperthyroid intestines, we observed that the expression of these genes was unaffected by the TH status (not shown), except for β-catenin. In fact, chemical-induced or congenital hypothyroidism resulted in a decrease of β-catenin mRNA and protein (Fig. 2A and B). TH injections for 2 days resulted in an increase of both mRNA and protein levels. We also analyzed the expression of the downstream targets of this pathway, cyclin D1 and c-myc (1), as well as the indirect target cyclin D2 (20), in animals of different TH status (Fig. 2A and B). All of these genes, expressed mainly in the epithelial organoids, this model could only be used for in vitro studies showed a hierarchy in the induction of gene expression by TH. If the induction of the Wnt/β-catenin target genes by T3-TRα1 depends on the previous induction of the β-catenin, the use of in vitro model could help to answer to this question. For this reason, we treated the cells with cycloheximide and/or T3 for 24 h and analyzed the expression of β-catenin and cyclin D1. In non-cycloheximide-treated cells, we observed an induction of both mRNAs compared to the control (Fig. 3C). The addition of cycloheximide specifically suppressed the induction by T3 of cyclin D1 expression. On the
FIG. 1. Analysis of crypt cell proliferation and TRα1 expression in the small intestine. (A) Study of cell proliferation during development in the proximal small intestine. Ki67 immunolabeling was performed as previously described (41). The number of Ki67-positive nuclei has been evaluated on well-oriented sections from 4 to 5 different animals per age. Histograms represent means ± standard deviations. Statistical analysis was conducted by using two-tailed Student t test. *, *P < 0.05; **, P < 0.001. P, postnatal day; W, weeks. (B) Analysis of TRα1 expression by semiquantitative RT-PCR during intestine development. HPRT was used as an internal control. E, embryo; PN, postnatal. (C to H) TRα1 protein immunolabeling (E and H) on a 2-week-old mouse intestine. In panel C, the phase-contrast picture is shown. All nuclei were stained with Hoechst (D). In panels F and G, the merging of each simple staining is shown. Controls for TRα1 antibody specificity are illustrated in Fig. S4 in the supplemental material. Bars, 15 μm (C to F); 7 μm (G and H). Abbreviations: ve, villus epithelium; ce, crypt epithelium. The white dotted bar defines the limit between the crypt and the villus compartments. Some villi (red dots) and crypts (yellow dots) are highlighted in panels C to E.
contrary, cycloheximide had no effect on β-catenin up regulation by T3 (Fig. 3C). The small decrease of gene expression in the cycloheximide-treated cells is probably due to a generalized action of the drug. These data demonstrate that the positive regulation of the β-catenin gene expression does not need protein synthesis, strongly suggesting a direct effect on β-catenin but not on the cyclin D1 gene.

Characterization of a functional TRE in intron 1 of the β-catenin gene: in vitro and in vivo studies. The data on the regulation of β-catenin gene by TH and TRα1, in vivo and in vitro, suggested that this gene may be a direct target of the TRα1 receptor. Looking in silico for prediction of TRE sequences in the mouse β-catenin gene (catnb), we found a sequence of 36 bp (referred as TRE-int1), spanning from

FIG. 2. Study of TH action in vivo. (A) Representative Northern blot analysis of the indicated mRNAs in 2-week-old intestine of euthyroid (Pax8+/+) (Ctr), congenital hypothyroid (Pax8-/-) (Hypo), and hyperthyroid (Pax8-/- TH-injected) (Hyper) animals. HPRT mRNA was used as an internal control. (B) Representative Western blot analysis of indicated proteins in animals as described for panel A. (C) Kinetic analysis of gene induction by TH evaluated by Northern blotting. PTU-treated animals were injected with TH for the indicated time. 18S RNA was the internal control. Histograms (means ± standard deviations) summarize densitometry analyses from three independent experiments. (D) Quantitative real-time RT-PCR analysis of β-catenin mRNA in intestines of TRα0/0, TRβ-/-, and WT animals displaying different TH status. The picture is representative of two independent experiments, with 3 animals/condition. Histograms represent means ± standard deviations. (E) Representative Western blot analysis of indicated proteins in animals as described for panel D. Statistical analysis was conducted by using two-tailed Student t test. **, \( P < 0.001 \). C, control; P, PTU treated; T, TH injected (inj).
nucleotide 2140 of the first intron and containing four putative TRE sites (Fig. 4A). While the TRE-A is a perfect DR4 (6), the sites TRE-B, TRE-C, and TRE-D show degenerate DR4 structures. To evaluate whether the TRα1 receptor binds in vitro to these TREs, we performed EMSAs. Compared to a synthetic positive control DR4 (29), the TRE-int1 has the same ability to bind to TRα1 as a monomer, homodimer, and TRα1/RXR heterodimer (Fig. 4C and D). These complexes were supershifted after incubation with an antibody against TRα1 (Fig. 4D). We also tested by EMSA different mutant TRE-int1 sequences (Fig. 4B). Mutant 2,5, mutant 6, and to a lesser extent, mutant 3 retained their ability to bind to TRα1 as a monomer, homodimer, and heterodimer (Fig. 4E). Mutant 1,4 had a lower ability, and mutant 2,3,4 was unable to bind to TRα1.

Next, we analyzed the enhancer properties of the β-catenin TREs in transient-transfection experiments. We cloned the 36-bp TRE-int1 sequence upstream of a minimal simian virus 40 promoter (29) in a luciferase reporter vector and then transfected it in Cos1 cells (Fig. 5). The expression of the luciferase was analyzed in the presence or absence of either TRα1 expression vector and/or T3. As Cos1 cells contain low levels of endogenous TRα1, induction of the DR4 positive control by T3 was observed only in cultures cotransfected with the TRα1 expression vector. A similar response was observed with the TRE-int1 reporter, showing that it is functionally responsive to T3. When we compared the different TRE-int1 mutant constructs, it was clear that only those preserving the TRE-A, alone or in combination with TRE-B and C, displayed a responsiveness to T3 similar to that of the TRE-int1. Altogether, these data strongly suggested that TRE-A is the functional binding site for TRα1.

To check whether the TRE-int1 is a binding site for TRα1 in vivo, we investigated the binding by in vivo ChIP assay. We performed these experiments on collagenase-dispase-isolated fragments of the intestinal epithelium, freshly prepared from newborn animals. Figure 6 summarizes the set up and the results of these experiments. The sonicated chromatin has been incubated with anti-TRα1, anti-TRβ1, or preimmune sera. The precipitated DNA from these different experimental conditions and in the starting inputs was then analyzed by conventional and real-time PCR to reveal the presence of specific DNA templates. All of the amplified products have been controlled by cloning and sequencing (not shown). Interestingly, using the Catnb1 and Catnb2 primers, which amplify DNA fragments of the catnb gene, including the TRE-int1 (Fig. 6A), we showed a specific band only in the samples incubated with the anti-TRα1 antibody (Fig. 6B, lane b). A band of the same size was obviously present in the starting input samples (Fig. 6B, lane d). To verify the specificity of this result, we performed a PCR analysis using the Catnb3 and Catnb4 primers, which amplify fragments of the intron 1 outside the TRE (Fig. 6A). Moreover, we used primers specific to promoters of the phosphoriboprotein gene 36B4 and of the villin gene, for which no regulation by TH has been described. Using these oligonucleotides, we only amplified DNA fragments in the starting inputs (Fig. 6B, lanes d), indicating that the anti-TRα1 antibody does not bind the chromatin aspecifically. To quantify the amount of the DNA precipitated by the

FIG. 3. In vitro study of TH action. (A) Rate of cell proliferation in epithelial primary cultures from WT and TR knockout intestines, after BrdU incorporation and immunolabeling. Total nuclei stained with propidium iodide and BrdU-positive nuclei were counted under the microscope. Histograms (means ± standard deviations) represent results from three independent experiments conducted (each) on 12 1-cm wells. (B) β-Catenin mRNA quantification by real-time RT-PCR, evaluated at different times of T3 treatment. The results (means ± standard deviations) are the summary of those for four independent experiments conducted in duplicate. (C) β-Catenin and cyclin D1 mRNA expression in primary cultures maintained during 24 h in the presence (+) or absence (−) of T3 and/or cycloheximide (Cyclohex). Data (means ± standard deviations) summarize the results of two independent experiments conducted in triplicate. Two-tailed Student t test was used. *, P < 0.05; **, P < 0.001; ***, P < 0.0001.
TRα1 antibodies, we also performed real-time PCR. Using the Catnb1 (Fig. 6C) and Catnb2 (not shown) primers, we amplified specific DNA fragments. The amounts are expressed as percentages of the starting inputs. No specific amplification could be observed in the samples incubated with the anti-TRα1 or preimmune sera or in the different samples when we used the Catnb3 (Fig. 6C), Catnb4, and p36B4 (not shown) primers. These data clearly demonstrated that, in intestinal epithelial cells in vivo, TRα1 binds to the β-catenin gene at the level of the TRE-int1.

FIG. 4. Molecular analysis of the thyroid hormone responsive element present in the catnb gene. (A) Structure of the four putative DR4 found in intron 1. Divergences from canonical DR4 are indicated in boldface type. (B) WT TREint-1, showing the GT sequences (1 to 6) substituted in the different TRE mutants. (C to E) EMSA analysis using the labeled probes indicated and in vitro-transcribed/translated proteins indicated. The specificity of the binding was assessed by adding specific or nonrelated cold sequences at the indicated molar excess (Comp. 10× or Comp. 100×). +, present; −, absent.

DISCUSSION

In this paper and in previous reports, we pointed out that T3-activated-TRα1 receptor controls the proliferation of the intestinal epithelial cells in vivo and in vitro. In an attempt to identify the molecular basis of this control, we focused here on the analysis of the Wnt/β-catenin pathway, since it plays a central role in the control of normal and pathological cell proliferation in the gut (42). Interestingly, we observed that the TRα1 receptor directly controls the transcription of the β-cate-
nin gene, the central actor of this pathway. In fact, in vivo and in vitro models treated with TH displayed an increase in $\beta$-catenin mRNA and protein expression. This was parallel to the up-regulation of cyclins D1 and D2 and c-myc, well-known proliferation controlling genes (5, 25, 52) and targets of the Wnt/$\beta$-catenin pathway (1, 20). Several findings support the assertion that the $\beta$-catenin gene, but not cyclin D or c-myc, is directly regulated by TH. First, induction of the $\beta$-catenin mRNA in vivo and in vitro is achieved after a short time of treatment by TH. Second, the cycloheximide addition to the culture medium does not prevent the up-regulation of the $\beta$-catenin mRNA in vitro by T3, as it is the case for cyclin D1 mRNA. Third, TH injections to TR$\alpha$0/0 animals, which are unable to stimulate $\beta$-catenin expression, also fail to increase the cyclin D1 mRNA compared to the WT animals (data not shown). Finally, we also provided the experimental proof that in epithelial cells of the intestine in vivo, TR$\alpha$ is fixed on the TRE-int1 present in the catnb gene. This is the first report of in vivo ChIP analysis which shows the occupancy of a TRE by a TR receptor in mammals. A binding of TRE by TRs has already been reported for *Xenopus laevis* target genes (44).

The regulation of the transcription of the $\beta$-catenin gene by T3 is likely to go through the TRE-int1. In fact, it is the unique responsive element found within the promoter environment. Moreover, its intronic location is not surprising, since several reports described TREs located downstream of the promoters (7, 38, 46). We also showed that the TRE-int1 is composed of several TREs organized in tandem, reminiscent of the TREs present in the rat growth hormone gene (37).

Despite the well-described posttranslational control of the $\beta$-catenin, very little is known at the gene transcription level. A recent paper described the cloning and analysis of the rat and human $\beta$-catenin regulatory regions of this gene (23). Interestingly, several AP1 binding sites and one TCF binding site have been described, suggesting that several putative factors may influence the transcription of this gene. However, no physiological functions for these regulatory regions have been reported until now. We showed here that the TRE in intron 1 of the catnb gene is functional in vivo and in vitro.

$\beta$-Catenin is a central actor in gut epithelial cell homeostasis, and its subcellular localization defines specific functions in cell adhesion (membrane bound) or in target gene activation (nuclear presence) (36, 52). We reported in our study that TH deprivation or supplementation in animals and in cells affects $\beta$-catenin gene and protein expression. More specifically, TH treatment allows an increase of its levels. The availability of free $\beta$-catenin, which migrates into the nuclei, is controlled by several mechanisms involving stabilizing and destabilizing phosphorylations as well as destabilizing dephosphorylations (36). In TH-stimulated models, we observed an increase of $\beta$-catenin-positive nuclei (not shown), strongly suggesting that this treatment positively affects its stabilization. However, the exact mechanisms responsible for this remain to be clarified and will be the subject of future investigations.

In the nucleus, $\beta$-catenin activates the transcription of target genes, as it binds to the Tcf/Lef family of transcription factors (35). Here we show that some of its targets, activators of cell proliferation, are positively regulated by TH in vivo and in vitro. This strongly suggests that the increase of cell proliferation induced by TH may be dependent on the activation of $\beta$-catenin.

![FIG. 5. Transient-transfection analysis in Cos1 cells. Two hundred nanograms of the luciferase expression vector was transfected alone or in combination with 200 ng of pSG5-hTR$\alpha$1 expression vector. Cells were treated or not with $10^{-7}$ M T3 for 16 h. Bars represent means ± standard deviations ($n = 6$).](image-url)
β-catenin expression. Is the increase of the β-catenin expression per se sufficient to induce cell proliferation? This is a crucial question. Several reports indicated that increasing the level of β-catenin leads to increased cell proliferation in various systems, such as the intestine (47, 55), liver (32, 34), and vestibular cells (19). This strongly supports our own data showing that the increase of β-catenin expression is one key molecular event related to the activation of cell proliferation. However, it is worth pointing out that a negative control by TH of the Wnt pathway was described in an in vitro model of pituitary cells (33). In this model, the positive action of the TH on cell proliferation was parallel to a decrease of β-catenin expres-
sion. However, there was no indication of a direct negative control of the β-catenin gene by TRs in these cells. The different model systems analyzed and differences in the cellular and molecular microenvironment may account for the discrepancy with our results. It is worth pointing out that our results are consistent with the well-established fact that the Wnt/β-catenin pathway is a positive regulator of the intestine cell proliferation.

Our observations suggested that TH signaling in the intestine is essential throughout intestine development and in adulthood. However, a major role is played during postnatal development, when an extensive growth takes place (39). Indeed, this period also corresponds in the mouse to a surge of TH, which declines starting from the third week to reach a steady-state adult level (14). According to this, TRα knockout mice show more intestinal impairment during the first 3 weeks after birth than in adulthood. Moreover, the absence of TH production during early postnatal development, as in the congenital hypothyroid Pax8−/− mice (28), has dramatic effects on intestine development, much more so than in TRα mutants (9). This can be explained by the aporeceptor function of TRα1 in absence of TH, acting as a transcription repressor of target genes (9, 26). In this light, the repression of β-catenin and of its downstream targets observed in hypothyroid animals (both congenital and chemical induced), may help explain some traits of the intestine phenotype. Indeed, removal of the TRα receptor in Pax8−/− animals was previously reported to induce a recovery of intestine development (9).

Together, these data indicated that T3-activated TRα1 plays an essential role in postnatal intestine development as well as in adult homeostasis. Our present data show that the action on the β-catenin gene control is one of the molecular processes involved.

Until now few studies pointed to a function of TH and TR in gastrointestinal pathophysiology (11, 15, 17, 27, 31). Some data showed a correlation between altered levels of TH and the incidence of breast and colon cancer in humans (43). On the other hand, it has been reported that hyperthyroxinemia may influence the rate of colon cancer in an experimental model of carcinogenesis in rats (24). Finally, in some cases, the absence (30) or the mutation (53) of the TRs have been associated with gastrointestinal tumors. In our study, we show that TH and TRα1 receptor control epithelial cell proliferation and the expression of components of the Wnt pathway. As β-catenin and some of its target genes are involved in intestinal tumorigenesis, our data open new perspectives in the function of the TRα1 receptor as a potential tumor inducer, as is the case for other nuclear receptors (2).

ACKNOWLEDGMENTS

We thank the animal (PBES) and microscopy (PLATIM) facilities of the IFR 128 Biosciences. We also thank the ANIGENE platform of Rhône-Alpes Génopole. We gratefully acknowledge Nadine Aguilera for animal handling. We thank N. Davidson, K. Gauthier, and B. Pain for critical reading of the manuscript and O. Bakker for help in immunolabeling for TRα1. We are grateful to A. Rezza for participation in experiments with adult animals.

This work was supported by a grant from the Ligue Nationale contre le Cancer to J.S. (Equipe Labellisée). E.K. was a fellow of the Ligue Nationale contre le Cancer.

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Together, these data indicated that T3-activated TRα1 plays an essential role in postnatal intestine development as well as in adult homeostasis. Our present data show that the action on the β-catenin gene control is one of the molecular processes involved.

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