Opposite regulation of bilirubin and 4-nitrophenol UDP-glucuronosyltransferase mRNA levels by 3,3′,5 triiodo-L-thyronine in rat liver

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Abstract The effects of 3,3′,5 triiodo-L-thyronine (L-T3) on the constitutive levels of hepatic mRNA encoding two UDP-glucuronosyltransferase (UGT) isoforms implicated in the glucuronidation of planar phenolic substrates (UGT1*06) and bilirubin (UGT1*0) were investigated in rat liver. The amount of UGT mRNA was quantitated by reverse transcription and amplification methods (RT-PCR). Treatment with L-T3 significantly increased UGT1*06 and decreased UGT1*0 mRNA levels by 41% and 54%, respectively. The opposite situation was observed in thyroidectomised animals. A good relationship observed between UGT activity toward 4-nitrophenol and bilirubin and mRNA levels emphasizes the key role played by the thyroid hormone L-T3 on UGT expression.

Key words: UDP-glucuronosyltransferase; Liver (rat); Gene expression; 3,3′,5 Triiodo-L-thyronine; Bilirubin and 4-nitrophenol glucuronidation

1. Introduction

UDP-glucuronosyltransferase (UGTs, EC 2.4.1.17), which are encoded by a superfamily of genes, catalyze the conjugation of glucuronic acid to a large variety of structurally unrelated compounds with hydroxyl-, carboxyl-, amine or sulfhydryl groups [1]. Drugs or endogenous compounds that are important for cell differentiation and growth (e.g. steroid and thyroid hormones, fatty acids, retinoic acid) are substrates of these enzymes [2,3]. Thyroid hormones are both substrates and inducers of UGTs. They are glucuronidated on the phenolic or carboxyl group and excreted as inactive metabolites into bile [4]. On the other hand, we and others demonstrated that thyroid hormones differentially affected the glucuronidation by rat liver microsomes of various substrates supported by separate UGTs [5,6]. Interestingly, we previously reported, using thyroidectomised animals, that treatment with L-T3 and analog compounds increased, in a dose-dependent fashion, the glucuronidation of 4-nitrophenol, whereas that of bilirubin and the monoterpenoid nopol were decreased and unchanged, respectively. These opposite changes in enzyme activities were observed in detergent-activated and in native microsomal preparations, without any variation of the lipid fluidity of the microsomal membranes [7]. Therefore a modification of the expression of the individual UGT isoforms upon thyroid hormone treatment, rather than variations of enzyme latency, was suspected. The aim of the present study was to explain the modulation, in opposite ways, of the activity of two UGT isoforms implicated in the glucuronidation of 4-nitrophenol (UGT1*06) and bilirubin (UGT1*0) by thyroid hormones. These enzymes are encoded by two members of family 1 of the UGT gene superfamily. The asterisk indicates that these isoforms derive by alternative splicing from a primary transcript common to several isoforms encoded by the *UGT1* locus. The constitutive levels of hepatic mRNA encoding the two enzymes were measured by RT-PCR method using highly specific primers which hybridize two corresponding cDNAs. Their expression variations were followed in hypo-, normo- and hyperthyroid animals. The inducing effect of 3-methylcholanthrene and that of the peroxisome proliferator, ciprofibrate, that are known to stimulate the glucuronidation of 4-nitrophenol and bilirubin, respectively [8,9], were also monitored as positive controls. Our results clearly show that the opposite changes observed in glucuronidation of 4-nitrophenol and bilirubin could be explained by variations of the levels of the corresponding mRNAs.

2. Materials and methods

4-Nitrophenol, bilirubin and detergents for enzyme activation, Triton X-100 and digitonin were purchased from Merck (Darmstadt, Germany). Ciprofibrate [2-(4-(2-2dichlorocyclopropyl)phenoxyl-2methylpropionic acid)] was a gift from Sterling-Winthrop (Dijon, France). 3,3′,5 Triiodo-L-thyronine (L-T3), goat anti-rabbit IgG, silver stain kit and 3-methylcholanthrene (3-MC) were purchased from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) were obtained from GIBCO BRL (Eragny, France). Moloney murine leukemia virus (MMLV) reverse transcriptase, ribonuclease inhibitor, random primers, DNA size standards (phi X 174 digested with *Hin*fI) were purchased from Promega Corporation (Lyon, France). Taq polymerase, dNTP were from Bioprobe Systems (Montreuil-sous-Bois, France). All other chemicals were reagent grade. The oligonucleotide primers were synthesized using an Applied Biosystem Model 394-08 DNA synthesizer (Bioprobe Systems), and PCR reactions were performed in a thermal controller incubator (Models PTC-150-16 & -25 DNA). The gels were scanned with an Ultroscan XL enhanced laser densitometer from LKB (Bromma, Sweden).

2.1. Treatment of rats and UGT assays

Male Wistar rats from a SPF husbandry (Iffa Credo, St. Germain l'Arbresle, France), were used. Except for a control group, the thyroid

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Abbreviations: UGT, UDP-glucuronosyltransferase; L-T3, 3,3′,5-triiodo-L-thyronine; RT-PCR, reverse transcription-polymerase chain reaction; 3-MC, 3-methylcholanthrene.

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gland was surgically removed when the animals weighed 80 g. Each group of rats was composed of eight animals. A control and a group of thyroidectomised rats did not receive any compound. One group of rats was treated intraperitoneally with L-T3 dissolved in 0.9% NaCl at a dose of 50 μg/kg/day for eight days. The other groups were treated with ciprofibrate (2 mg/kg/day) by gastric intubation for five days or with 3-MC by one single intraperitoneal injection of the compound dissolved in corn oil and administered 48 h before killing at a dose of 80 mg/kg body weight. Rats were killed by decapitation 16 h after the last injection of L-T3 or ciprofibrate. The liver of each rat was removed, weighed and perfused with an ice cold 0.154 M KCl solution. A part of the liver (200 mg) was collected from each rat, immediately frozen in liquid nitrogen and stored at −70°C for RNA extraction. Liver microsomes preparation, protein concentration and UGT activity towards bilirubin and 4-nitrophenol were performed as previously reported [7].

2.2. Western blotting analysis

The microsomal proteins were subjected to SDS-PAGE according to Laemmli [10]. After transfer of proteins onto a Hybond C membrane, Western blot was performed by the method of Towbin et al. [11]. UGT proteins were detected using two antibodies: a polyclonal antibody raised in rabbits against hepatic UGTs of rats treated by 3-MC, which recognizes, at least, four different UGT proteins, including 4-nitrophenol and bilirubin UGTs [12]; a monospecific antibody raised in rabbits against the N-terminal end of the human liver UGT1*6, which is specific for one UGT protein active toward planar phenols, only [13]. This antibody cross-reacts with the rat liver 4-nitrophenol UGT. Precipitated bands were detected by addition of a second antibody conjugated with rabbit alkaline phosphatase in the presence of BCIP and NBT.

2.3. RNA analysis

Total RNA was extracted from hepatic tissues by the RNA QUICK II kit (Bioprobe Systems) as described by the supplier. Complementary DNA was synthesized from RNA samples by mixing 1 μg total RNA, 100 pmol of random hexamer in the presence of 50 mM Tris-HCl buffer, (pH 8.3); 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiothreitol; 200 U of MMLV reverse transcriptase; 40 U of RNase inhibitor and 1 mM of each dNTP in a total volume of 20 μl. Samples were incubated at 37°C for 60 min and then diluted to 100 μl with sterile diethylpyrocarbonate-treated H_2O. The enzyme was inactivated by heating at 95°C for 5 min. A 10 μl aliquot was then used for subsequent PCR and added to a reaction mixture containing 20 mM Tris-HCl buffer (pH 8.5), 16 mM $(NH_4)_2SO_4$, 150 μg/ml bovine serum albumin, 0.2 mM of each dNTP, 50 pmol of each primer and 25 pmol of each internal standard primer in the same conditions. 2 U of Taq polymerase [14] and $MgCl_2$ at optimal concentration determined for each set of primers (1.5 mM and 0.5 mM for bilirubin UGT and 4-nitrophenol UGT, respectively), in a total volume of 50 μl were also added. Each mixture (50 μl) was overlayed with 50 μl of mineral oil and incubated in a thermal cycler at 94°C for 5 min and immediately cycled 28 to 30 times through a 30-s denaturing step at 94°C, a 60-s annealing step at 52°C for bilirubin UGT and 55°C for 4-nitrophenol UGT and a 90-s elongation step at 72°C. Following the final cycle, a 5-min elongation step at 72°C was included.

Two sets of primers were designed to specifically amplify bilirubin and 4-nitrophenol UGTs (Table 1). They were 100% homologous with the sequences described by Sato et al. [15] and Iyanagi et al. [16], and were chosen in order to avoid any crosslinking with other known sequences recorded in data banks. Absence of genomic DNA contamination was verified by using a control tube supplemented with 10 μg of RNase. Measurement of mRNA encoding β-actin was simultaneously performed using two specific primers, according to Nudel et al. [17], as internal standard (Table 1). A highly specific PCR would generate only one product of the correct size that is the intended target sequence. Utilizing sequence information from data bank, the computer program Gene Joker was used to predict the restriction enzyme map for each expected PCR fragment. Specific PCR fragments were digested by various restriction enzymes for 1 h at 37°C to confirm the PCR products.

For quantitation purpose, 10 μl of the PCR reaction were sampled after 22 to 30 amplification cycles [18], and the co-amplified fragments were separated by electrophoresis on 5% polyacrylamide gel. The bands were visualized by silver staining and quantified by densitometry. To minimize errors, the scanning procedure was repeated three times, and the mean value for each area intensity was determined. The intra- and interassay coefficients of variation were 5% and 7.5%, respectively.

3. Results

Fig. 1 shows the glucuronidation activity measured with 4-nitrophenol (Fig. 1A) and bilirubin (Fig. 1B) as substrates by liver microsomes of rats that had been thyroidectomised or treated with L-T3, 3-MC or ciprofibrate. Compared to controls, thyroidectomy significantly reduced by 2-fold the glucuronidation of 4-nitrophenol, whereas L-T3 or 3-MC administration increased the activity by 1.5- or 3-fold, respectively. The opposite situation was found for bilirubin glucuronidation, as far as the effects of L-T3 are considered; thyroidectomy significantly increased the activity; by contrast, administration of L-T3 markedly decreased this parameter. On the other hand, as expected, treatment with ciprofibrate enhanced the glucuronidation rate of bilirubin 3-fold.

The variations in the glucuronidation of 4-nitrophenol and bilirubin upon treatment with L-T3 and other inducers were strongly related to the expression of the corresponding UGT proteins, as revealed by Western blot, using two different antibodies (Fig. 1C,D). The intensity of the 54 kDa band corresponding to the isoform which glucuronidates bilirubin was increased in response to ciprofibrate treatment and thyroid ablation (Fig. 1C). On the other hand, an increase in the staining of the 4-nitrophenol UGT (56 kDa) was also found in rats administered with L-T3 or 3-MC, when compared to control or thyroidectomised animals (Fig. 1D).

The proportion of UGT mRNA was estimated from comparison to that of β-actin mRNA known to be unmodified by thyroid [19] and diet conditions [20]. In Fig. 2 is reported the analysis of RT-PCR products on polyacrylamide gels: 507 bp for the 4-nitrophenol UGT fragment (UGT1*06) and 303 bp for the bilirubin UGT fragment (UGT1*0). The specificity of the amplification obtained was checked by restriction site anal-

Table 1
Synthesized primers used for PCR

Isoforms	Primers	5′3′ sequence	Complementary site
Bilirubin UGT*	A	GAAGAATATCAGCGGGAAATA	250 to 270
(UGT1*0)	B	CGGACATTGTGTAGCCTCA	555 to 537
4-Nitrophenol UGT**	C	TTGCCTTCTTCCTGCTGC	6 to 23
(UGT1*06)	D	TCTGAAGAGGTAGATGGAAGGC	513 to 492
β-Actin***	β1	TGCAGAAGGAGATTACTGCC	2818 to 2837
	β2	CGCAGCTCAGTAACAGTCC	3153 to 3135

*From rat liver according to the sequence of Sato et al. [15].
**From rat liver according to the sequence of Iyanagi et al. [16].
***From rat cytoplasmic β-actin gene according to the sequence of Nudel et al. [17].

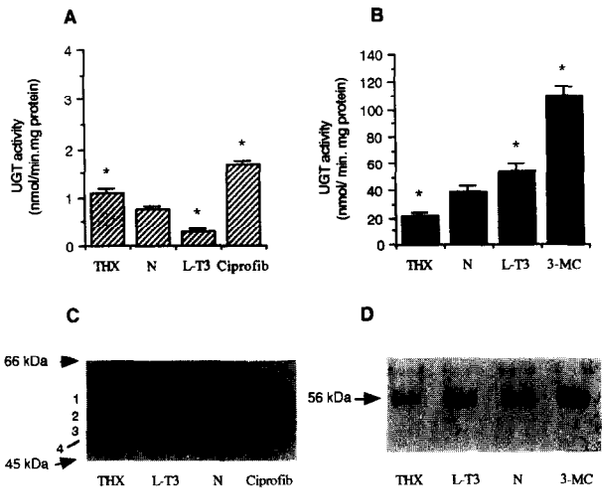

Fig. 1. Effect of the thyroid status on the expression of bilirubin UGT and 4-nitrophenol UGT in rat liver. The glucuronidation of bilirubin (A) and 4-nitrophenol (B) was measured in liver microsomes of thyroidectomised (THX), normal (N), treated with 3,3',5 triiodo-L-thyronine (L-T3) or with ciprofibrate (Ciprofib) and 3-methylcholanthrene (3-MC). Values are the mean ± S.D. of eight animals. *Significantly different ($P < 0.01$) from normal rats. Immunoblot of UGT with (C) polyclonal anti-rat liver UGT antibodies which recognize 4-nitrophenol UGT (1, 56 kDa), bilirubin UGT (2, 54 kDa), androsterone UGT (3, 52 kDa) and testosterone UGT (4, 50 kDa); with (D) monospecific antibodies raised against the human liver UGT1*6, which recognize only one UGT which glucuronidates planar phenols including 4-nitrophenol (56 kDa).

ysis: the 303 bp bilirubin UGT and the 507 bp 4-nitrophenol UGT sequences detected in rat liver, once digested with *Alu*I–*Eco*RI and *Hin*dIII–*Pvu*II, respectively, (lanes 2, 3 and 5, 6) gave fragments of the expected size.

After a first step of reverse transcription allowing the production of cDNA from the different groups of rats, co-amplification of β-actin cDNA and UGT cDNA was performed using specific primers (see Table 1). PCR reactions were stopped after different amplification cycles and the reaction products were separated by electrophoresis on acrylamide gels (Fig. 3A). Logarithms of area intensity were plotted against the number of PCR cycles. Straight lines with different slopes were obtained as shown in Fig. 3B. The proportion of UGT mRNA to β-actin mRNA was calculated using the formula: $A_{UGT}/A_{act} = Y_{UGT}(1 + R_{act})^n / Y_{act}(1 + R_{UGT})^n$ according to Chelly et al. [21], where A is the initial amount of material, R the efficiency, n the number of cycles and Y the area intensity; R can be deduced from the slope of the semi-log plot: $\log(1 + R)$. The proportion of bilirubin UGT mRNA to β-actin mRNA was decreased by 54% in rats treated with L-T3, but increased about 1-fold in thyroidectomised animals (Fig. 4). Treatment with the specific inducer ciprofibrate enhanced this content about 2- to 3-fold. The opposite situation was observed for 4-nitrophenol UGT mRNA. Compared to control rats, the levels were increased 41% in L-T3-treated rats and decreased by 70% in thyroidectomised animals. The specific inducer 3-MC increased the 4-nitrophenol UGT mRNA more than 3-times (Fig. 4).

4. Discussion

The modulation of UGT expression by thyroid hormones is a new fascinating area of investigation, which is still a pioneering work due to lack of specific probes necessary to follow the formation of these proteins. This study reports, for the first time, the effect of the thyroid hormone L-T3 on the mRNA levels of two rat liver UGTs (UGT1*06, UGT1*0). Interestingly, L-T3 differentially affected the expression of both these proteins, by decreasing the glucuronidation of bilirubin, and conversely by increasing that of 4-nitrophenol. The absence of L-T3 in thyroidectomised animals led to the opposite situation. Such results support the hypothesis that L-T3 plays a regulatory role on this enzyme system. The relationship observed between enzyme activities and expression of the two UGT isoforms as a function of the presence of L-T3 further emphasized the key role played by this hormone. Indeed, in this work, thyroidectomy increased bilirubin UGT and decreased 4-nitrophenol UGT mRNA levels concomitantly. The reverse was true in hyperthyroid animals.

The mechanism by which L-T3 modifies the mRNA levels encoding UGT1*06 and UGT1*0 in opposite way is not known, but it is clear that it may depend on a transcriptional or post-transcriptional process. Subsequent studies of other genes known to be positively regulated by thyroid hormone have also demonstrated a significant degree of control at the transcriptional level. These genes include the hepatic spot 14, the malic enzyme and the rat α-myosin heavy chain gene. Transcriptional inhibition by thyroid hormone has also been documented for the α- and β-subunits of thyroid stimulating hormone genes [22 and ref. herein]. Preliminary results by run-on analysis obtained in our laboratory suggested a transcriptional effect of L-T3 toward UGT1*06, eventhough a post transcriptional effect could not be ruled out. On the other hand, no data are available concerning the regulation of UGT1*0. It has been clearly established that UGT1*06 and UGT1*0 are the result of alternate splicing of specific and common exons localised on the same locus [23]. Although encoded by a single UGT1 gene locus, the expression of the individual UGT1 isoenzymes in rats and also possibly in man could be under different control mechanisms involving specific nuclear receptors of thyroid hormones (TRs), which are considered as transcriptional factors. Moreover it has been found that TRs bind to L-T3 responsive elements (TREs) as monomers, homodimers or heterodimers with other nuclear receptors of the same superfamily, especially with the retinoid X receptors (RXRs) and the

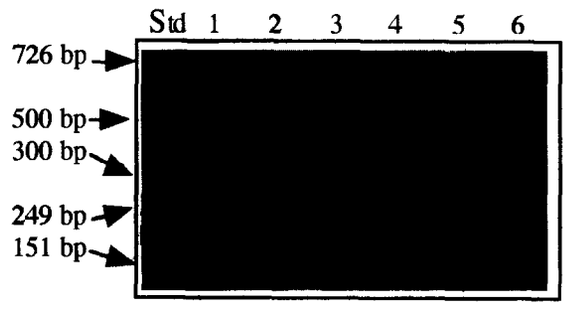

Fig. 2. The specificity of the amplification products obtained was monitored by restriction site analysis. Lanes 1 and 4 show the bilirubin UGT (303 bp) and 4-nitrophenol UGT (507 bp) isoforms, respectively; lanes 2, 3 and lanes 5, 6 show the fragments obtained upon digestion of the 303 bp product with *Alu*I, *Eco*RI, and of the 507 bp product with *Hin*dIII, *Pvu*II, respectively. Std lanes refer to DNA molecular weight markers (from 24 to 726 bp).

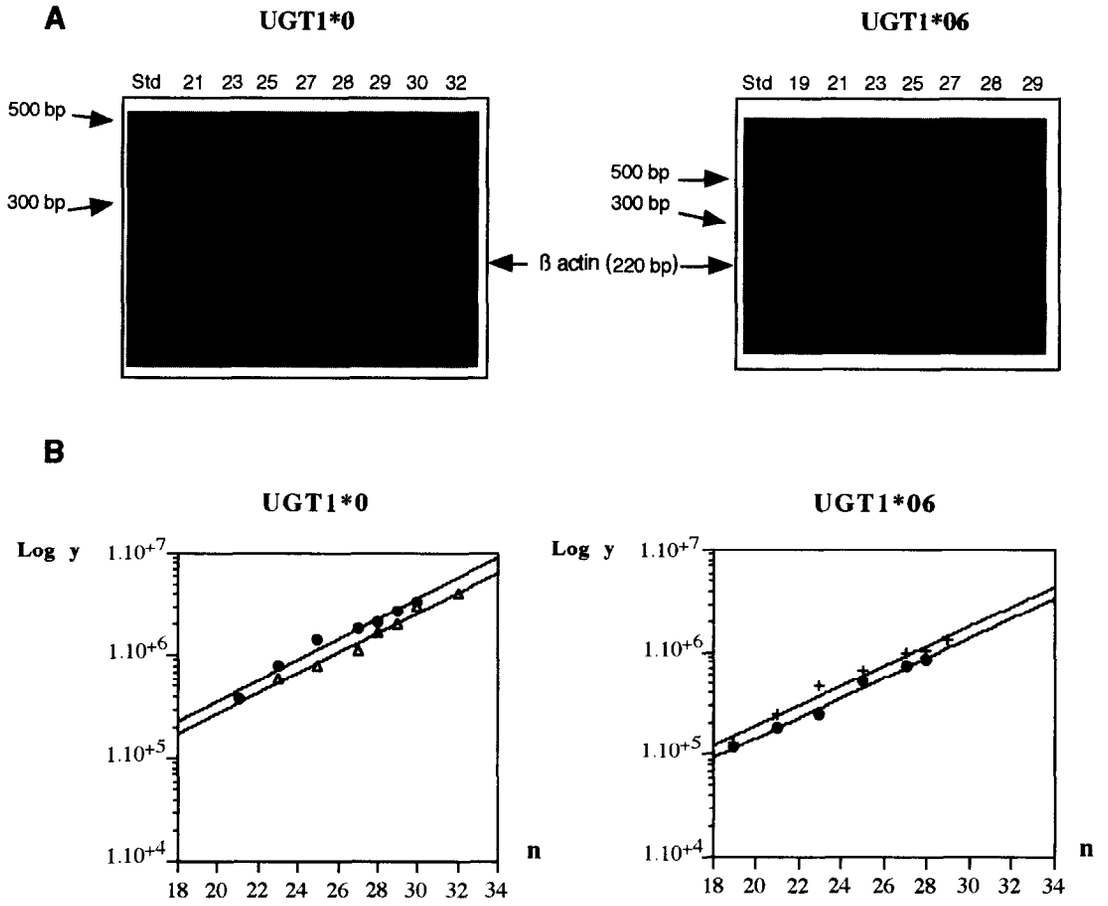

Fig. 3. Kinetic analyses of PCR products from bilirubin UGT (UGT1*0), 4-nitrophenol UGT (UGT1*06) and β-actin mRNAs. Photography of acrylamide gel performed on PCR co-amplified products of rats of bilirubin UGT (303 bp), 4-nitrophenol UGT (507 bp) and β-actin (220 bp) gene (A). Semi-logarithmic representation of the relative extent of amplification in (B) measured by counting the intensity of the fragments visualized in (A): (△) bilirubin UGT; (+) 4-nitrophenol UGT; (●) β-actin. *n* is the number of PCR cycles; *Y* is the intensity of staining.

peroxisome proliferator-activated receptors (PPARs), and a large number of structurally related proteins, termed orphan receptors, for which no ligand has yet been identified [24–26].

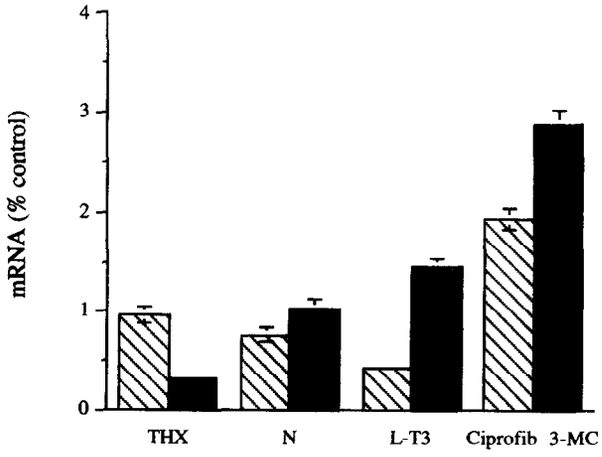

Fig. 4. Effects of treatment by L-T3 and specific inducers on hepatic bilirubin UGT (light columns) and 4-nitrophenol UGT (dark columns) mRNA levels. Each point represents the mean ± S.D. of three animals. For other details, see legend of Fig. 1.

On binding their respective ligands, they can activate transcription, but only at certain binding sites. Elsewhere or without a ligand, they act as transcriptional repressors, but the mechanism responsible is unknown. The diversity provided by heterodimerization within the subfamily of nuclear hormone receptors offers additional levels of regulation. For example, PPAR has been reported to be able to positively or negatively modulate TR activity depending on the type of TRE [27]. In addition, Thomas Perlmann et al. [28] recently described a new transcriptional co-repressor which binds to the receptor for thyroid hormone. On binding ligand, however, the DNA-bound form undergoes a conformational change which displaces the co-repressor, explaining the effect of the ligand on transcription. We think that such interactions could potentially have important consequences on the alternate modulation of the UGT gene. This hypothesis, which is being investigated in our laboratory, could bring further insight in the knowledge of the molecular mechanisms of gene regulation of the UGT1 family by thyroid hormones, retinoic acids and peroxisome proliferators.

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