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Comparative Quantification of Two Hepatic UDP-Glucuronosyltransferase Bilirubin Isoforms mRNAs in Various Thyroid States in Rat

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ABSTRACT. The study was designed to compare the effects of 3,5,3′ triiodo-L-thyronine (L-T3) on the levels of hepatic mRNAs encoding two UDP-glucuronosyltransferase bilirubin isoforms (UGT1*1 and UGT1*0) in rats, by reverse transcription and quantitative polymerase chain reaction (RT-PCR). The administration of L-T3 decreased the UGT1*0 mRNA by 2.2-fold and that of UGT1*1 by only 1.4-fold. In contrast, thyroidectomy increased the UGT1*0 mRNA level by twofold but did not change that of the UGT1*1 isoform significantly. Interestingly, treatment with a known inducer of UGT bilirubin, ciprofibrate, induced the hepatic mRNA levels encoding for the UGT1*0 isoform by 3.5-fold and for the UGT1*1 isoform by only twofold. The results indicate for the first time that, although UGT1*1 mRNA is indeed a major transcript, its level is weakly affected by these compounds. In contrast, the minor UGT1*0 form is much more sensitive both to the action of this drug and to changes in thyroid status. The data support the notion that the various members of exon1 of the UGT1 locus have their own individual regulatory region. BIOCHEM PHARMACOL 53;7:1013–1017, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. UDP-glucuronosyltransferase; gene expression; 3,5,3′-triiodo-L-thyronine; bilirubin; glucuronidation; rat liver

Bilirubin IXα, the product of haemoprotein catabolism, is produced in large amounts in human and induces toxicity when it accumulates within the body. Glucuronidation is the only pathway to clear bilirubin from the body. The reaction is catalysed by UDP-glucuronosyltransferases (UGTs,§ EC 2.4.1.17), which transfer glucuronic acid from the high-energy donor UDP-glucuronic acid to one or two propionic acid side chains of bilirubin, leading to the formation of water-soluble mono- and diglucuronides excreted in bile [1]. In humans and rats, UGT bilirubins are encoded by the bilirubin/phenol UGT1 gene complex, which contains a different exon 1 unique to each enzyme and four common exons (exons 2 to 5) [2]. A recent report on the rat UGT1 gene showed that the variable region consisted of 4 UGT phenol exons (A1–A4) and 5 UGT bilirubin-like exons (B1–B5) [3]. Although encoded by a single gene, the expression of the individual UGT1 isoenzymes in rats and possibly in humans is subject to different control mechanisms. In previous work, we and others demonstrated that the expression of UGT bilirubin in rat liver microsomes was regulated in an opposite way when animals were treated either with 3,5,3′ triiodo-L-thyronine (L-T3) or fibrate derivative such as ciprofibrate [4–7]. Bilirubin glucuronidation was selectively increased by these hypolipidaemic drugs, whereas it was decreased by the thyroid hormone, while the reverse was true in hypothyroidism. Using quantitative reverse transcription-polymerase chain reaction (RT-PCR) and specific probes of the exon B2 of UGT bilirubin, we showed that these variations were related to concomitant changes in mRNA levels [8]. Because of the pivotal role played by the UGT bilirubins, it is necessary to better understand how these enzymes are regulated, especially by drugs and hormones, and to elucidate the molecular organization of the bilirubin/phenol UGT1 gene. The purpose of this work was to evaluate in more detail the expression of two forms of UGT bilirubin (UGT1*1 and UGT1*0) after modification of the thyroid status or administration of ciprofibrate in rat liver [9].

MATERIALS AND METHODS

Chemicals

Ciprofibrate (2-[4-(2-2 dichlorocyclopropyl) phenoxyl]-2 methyl propionic acid) was a gift from Sterling-Winthrop

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

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(Dijon, France). 3.5.3′ triiodo-L-thyronine and the silver stain kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MMLV-reverse transcriptase, ribonuclease inhibitor, random primers, DNA size standards (*phi* × 174 digested with *Hinf* 1) were purchased from Promega Corporation (Lyon, France). Taq polymerase and dNTP were from Bioprobe Systems (Montreuil-sous-Bois, France). All other chemicals were of reagent grade and purchased from standard suppliers.

Treatment of Rats and RNA Isolation

Male Wistar rats from an SPF husbandry (Iffa Credo, St. Germain l'Arbresle, France) and weighing 80–100 g were used. They were kept at constant temperature (26°) and were provided food and drinking water *ad libitum*. The rats were divided into five groups of eight animals. They received the following treatments: the hypothyroid group (THX) was surgically thyroidectomized upon weighing 80 g. The hypothyroid state was controlled by measurement of free L-thyroxine (F-T4) by radioimmunoassay (Amersham, France). The hyperthyroid (L-T3) and normothyroid groups (N) were sham-operated. The hyperthyroid group was obtained by intraperitoneal administration, 3 weeks after surgery, of L-T3 dissolved in 0.9% NaCl at a dose of 50 μg/kg/day for 8 days. The same volume of 0.9% NaCl was delivered to the normothyroid and thyroidectomized rats. One group received ciprofibrate (2 mg/kg/day) in corn oil by gastric intubation for 5 days. A control group received the vehicle only. Rats were sacrificed by decapitation 16 hr after the last injection of L-T3, ciprofibrate, or vehicles. The livers were removed and immediately frozen in liquid nitrogen and subsequently stored at −70°C. Total RNA from rat liver was purified by the RNA QUICK II kit (Bioprobe Systems) according to the protocol suggested by the supplier.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Complementary DNA (cDNA) was made by RT from 1 μg of total RNA for 60 min at 37°C using random primers, and a small fraction (1/10th) of the resulting cDNA was subjected to PCR using designated specific primers, as described [8]. The following primers from rat liver were used. For the UGT1*1 isoform, the sense primer B5′ (GTGATCCCCATAGATGGCAG, 102–122 bp) and the antisense primer B3′ (GTATGTTTTAACCACACGCAGCA, 348–324 bp) were employed, according to the sequence of Coffman *et al.* [10]. For the UGT1*0 isoform and the internal standard β-actin, the sequences of primers were as reported previously [8]. Quantitative PCR analysis was performed as described by Ozawa *et al.* [11]. The PCR reaction (10 μL) was sampled after 19 to 32 amplification cycles. The amount of material amplified after each cycle was quantified by an Ultroscan XL enhanced laser densitometer (LKB, Bromma, Sweden). The proportion of UGT bilirubin mRNA to β-actin mRNA was calculated using the formula described by Chelly *et al.* [12]. The annealing temperatures were 60 and 52°C for UGT1*1 and UGT1*0, respectively.

RESULTS AND DISCUSSION

PCR coamplification of reverse-transcribed hepatic UGT bilirubin mRNA and β-actin mRNA produced PCR products of expected size: 249 bp for exon 1 (UGT1*1), 303 bp for exon 2 (UGT1*0), and 222 bp for the internal standard (β-actin). These products were easily separated from each other by polyacrylamide gel electrophoresis (Fig. 1A). The logarithms of the densities of the bands were plotted against the number of cycles (Fig. 1B). We found that the rate of amplification was exponential for 19 to 29 cycles for UGT1*1 and to 32 cycles for UGT1*0, after which it decreased drastically and reached a plateau. The slope of the linear regression line reflects the efficiency of amplification. The same kinetic pattern was found after coamplification of UGT bilirubin and β-actin transcripts in rat liver. Thus, parallel slopes indicate equal efficiencies of amplification of the two targets, thereby allowing mRNA quantitation. The reproducibility of the quantitative RT-PCR procedure determined by three independent analyses of the same sample gave a standard deviation of the mean of 5 to 10%.

Figure 2 shows the levels of mRNAs encoding each UGT bilirubin isoform in rat liver. The proportion of UGT mRNA was estimated by comparing it to that of β-actin mRNA known to be unmodified by thyroid [13] and diet conditions [14]. The mRNA encoding the UGT1*0 isoform was fourfold more abundant than the mRNA in sham-operated animals. A similar situation was also observed after thyroidectomy or treatment with L-T3. The two populations of mRNA were present, but at different levels. Interestingly, the thyroid state modulated the mRNA concentration in opposite ways (Fig. 2A). The absence of thyroid hormone in thyroidectomized animals was associated with a significant twofold increase in UGT1*0 mRNA levels in rat liver (0.42 ± 0.075), whereas this state induced no significant increase in UGT1*1 mRNA levels (1.1 ± 0.09) in comparison with the normothyroid state (0.21 ± 0.057; 0.93 ± 0.13, for UGT1*0 and UGT1*1, respectively). Conversely, administration of L-T3 led to a significant 2.2-fold decrease in UGT1*0 mRNA levels (0.1 ± 0.017), and a mere 1.4-fold decrease (0.65 ± 0.05) for the other isoform. Thus, the expression of UGT1*0 was more affected than that of UGT1*1 in rat liver, because the amount of UGT1*0 mRNA varied by 4.2-fold, depending on the presence or absence of thyroid hormone, while that of UGT1*1 was modified only 1.7-fold. To illustrate clearly the magnitude of this differential modulation, we calculated the UGT1*1 mRNA/UGT1*0 mRNA ratios (exon 1/exon 2 mRNA) in various thyroid states. An unequivocal dose–response relationship between thyroid hormones and the exon 1/exon 2 mRNA ratio was observed, because this ratio

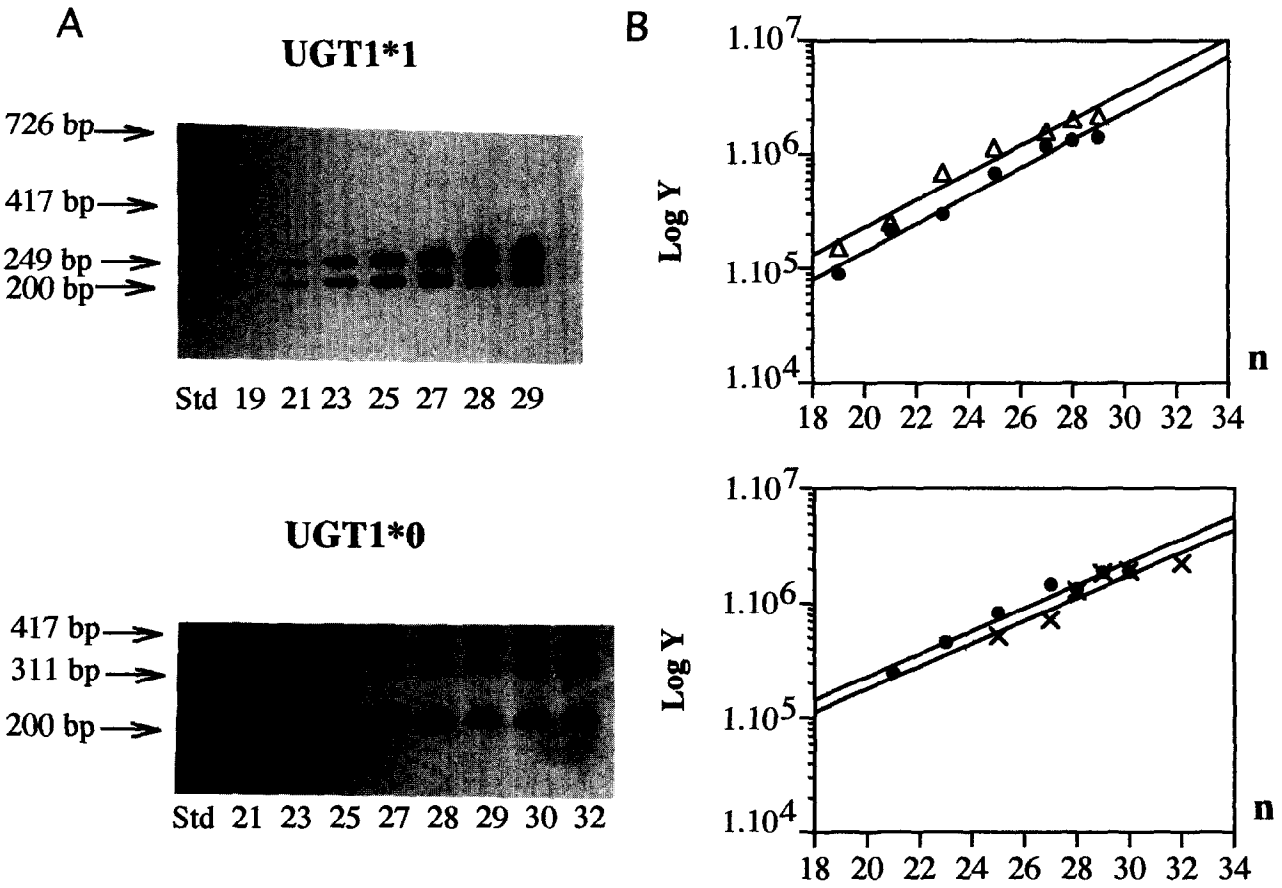

FIG. 1. Kinetics analyses of PCR products from UGT1B1, UGT1B2, and β actin mRNAs. Acrylamide gels of PCR coamplified products of rats treated with ciprofibrate of UGT1*1 (249 bp), UGT1*0 (303 bp), and β actin (220 bp) gene (A). Quantified PCR amplification curves as determined by counting the intensity of the fragments visualized in (A). Semi-logarithmic representations of the relative extent of amplification were plotted against the number of PCR amplification cycles (B). Straight lines with different slopes thus obtained were used for determination of the proportion of UGT bilirubin mRNA to β actin mRNA (for other details, see Materials and Methods). (△) UGT1*1; (x) UGT1*0: (●) β actin. *n* is the number of PCR cycles. Y is the intensity of staining. Std lanes refer to DNA molecular weight markers (from 24 to 726 bp).

was 2.6, 4.4, and 6.2 in hypothyroid, normothyroid, and hyperthyroid animals, respectively. Thus, the circulating thyroid hormones modulate the expression of the UGT1*0 isoform much more intensively than that of the UGT1*1 isoform, although the expression of the latter appears greater under basal conditions and represents the constitutive isoform expressed in rat liver [3, 15]. On the other hand, treatment with the specific inducer, ciprofibrate, significantly increased the levels of the two UGT bilirubin mRNAs when compared with those measured in the control group. Interestingly, the mRNA encoding the UGT1*0 isoform was enhanced more than threefold by the drug (0.82 ± 0.1), whereas the UGT1*1 isoform mRNA was increased by only 1.8-fold (1.93 ± 0.36) in comparison with control animals (0.24 ± 0.06; 1.06 ± 0.15, for UGT1*0 and UGT1*1, respectively) (Fig. 2). Thus, this hypolipidaemic agent appears to be a more potent inducer of the UGT1*0 isoform than of the UGT1*1 isoform. Interpretation of this differential sensitivity of the multiple first exons in the UGT1 gene complex toward thyroid hormones as well as ciprofibrate, in terms of physiological significance, requires further investigation. Nevertheless, two hypotheses can be proposed. One is that the UGT1*0 isoform could play a supporting role when the UGT1*1 isoform is not functional. The UGT1*1 mRNA encodes a major functional protein in rat liver and in man, and a mutation in exon 1 may cause unconjugated hyperbilirubinemia. In humans, the Crigler-Najjar type II syndrome appears to be caused by mutations in exon 1 (HUG-Br1) that result in partial inactivation of bilirubin glucuronidation [16, 17]. A second hypothesis is that UGT bilirubins are involved in glucuronidation of thyroid hormones [18]. Thus, we suggest that the UGT1*1 isoform is an important form for the metabolic clearance of thyroid hormones and that it is expressed temporarily in response to all metabolic changes in thyroid status. Therefore, the thyroid hormones may take part in their own metabolism.

In conclusion, the most important finding of this present report is that the mRNA levels of UGT bilirubin isoforms are differently affected by L-T3 and ciprofibrate. This em-

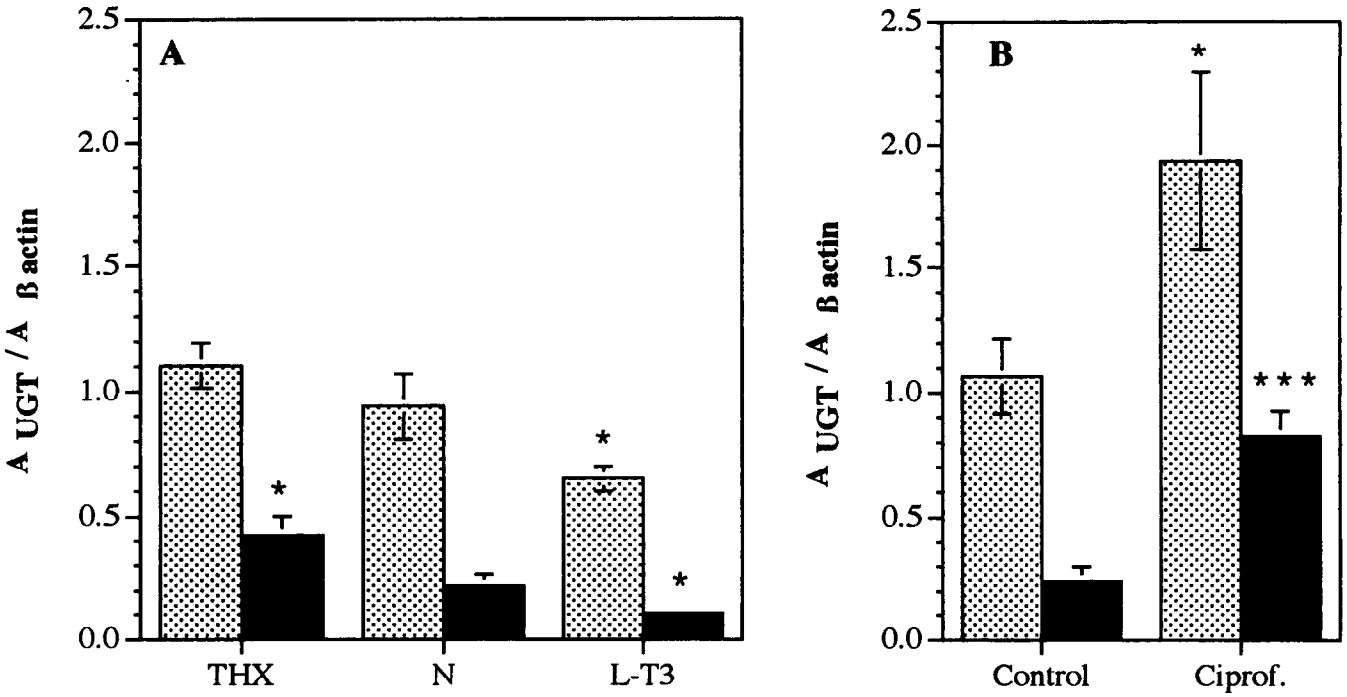

FIG. 2. Effects of the thyroid status (A) and of ciprofibrate (B) on hepatic levels of UGT1*1 (light bar) and UGT1*0 (dark bar) mRNA in rats. The proportion of UGT mRNA to β actin mRNA was calculated in thyroidectomized rats (THX), sham-operated rats (N), in animals treated with L-T3 (L-T3) or ciprofibrate (Ciprof.), and in control rats (Control), as described previously in Materials and Methods. Each value represents the mean ± SD of three separate measurements of eight rats; A_{UGT} and $A_{\beta\ actin}$ are absolute values of UGT mRNA and β actin mRNA. *Significantly different ($P < 0.05$, *t*-test Student) from N (A) or control (B) rats.

phasizes the complexity of the regulation of this gene and supports the concept that the various members of exon 1 in the UGT1 locus have their own regulatory region. However, the real physiological role of UGT1*0 remains to be elucidated, and further experiments will be necessary.

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