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Differential Effect of Hypophysectomy and Growth Hormone Treatment on Hepatic Glucuronosyltransferases in Male Rats: Evidence for an Action at a Pretranslational Level for Isoforms Glucuronidating Bilirubin

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ABSTRACT. The influence of growth hormone (GH) on 4-nitrophenol, bilirubin, testosterone, androsterone and estrone glucuronidation activities was studied in fully activated male rat hepatic microsomes. Sham-operated and hypophysectomized animals were injected with two different dosages of GH, mimicking either the male or female GH secretion pattern. Half the animals received thyroxine and cortisol in concentrations chosen to compensate for the lack of thyroid hormones and glucocorticoids in hypophysectomized rats. GH induced a decrease in several glucuronidation activities: bilirubin glucuronidation in both sham-operated and cortisol/thyroxine-treated hypophysectomized rats in a dose-dependent manner, testosterone glucuronidation in hypophysectomized animals, and androsterone and estrone glucuronidation in cortisol/thyroxin-treated hypophysectomized rats. 4-nitrophenol glucuronidation was not affected by GH treatment. A hypothetical "feminizing" effect of GH (due to an almost continuous secretion) could not be invoked to explain these results, contrary to what has been observed elsewhere for other hepatic enzyme activities. Hypophysectomy altered all the activities tested, with bilirubin the most modified (a 200% enhancement). Restoration of control values was achieved in hypophysectomized animals with cortisol/thyroxine replacement together with a low dosage of GH (mimicking a male GH secretion pattern), except for androsterone glucuronidation activity where both GH and cortisol/thyroxine treatments reinforced the decreasing effect of hypophysectomy. Variations in protein amounts were correlated to variations in bilirubin, testosterone and androsterone conjugation activities induced by hypophysectomy and GH treatment. Reverse transcription-polymerase chain reaction (RT-PCR) mRNA analysis of bilirubin cluster isoforms or uridine diphosphate glucuronosyltransferase 1B1 (UGT1B1), UGT1B2 and UGT1B5 showed that GH controlled the different isoforms involved in bilirubin glucuronidation differentially at a pretranslational level. BIOCHEM PHARMACOL 53;11:1637–1647, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. liver glucuronidation; growth hormone; hypophysectomy; pulsatility; bilirubin; haem metabolism

Glucuronidation is a major metabolic pathway by which a vast array of lipophilic endogenous (such as bilirubin and steroid hormones) and xenobiotic compounds are converted into more water-soluble derivatives, which are more readily excreted than the parent substrates [1]. This reaction is catalysed by uridine diphosphate glucuronosyltransferases [EC 2.4.1.17] (UGTs), which constitute a superfamily of closely related isozymes that require uridine diphosphate glucuronic acid (UDPGA) as co-substrate. These enzymes exist mainly in the liver, where they are deeply embedded in the endoplasmic reticulum membrane [1]. The different isoforms show overlapping substrate specificity for xenobiotics but rather distinct specificities for endogenous substrates [2]. UGTs are divided into two families and a total of three subfamilies [3]. Isoforms belonging to the first family (UGT1) are responsible for the glucuronidation of bilirubin and small planar phenolic compounds such as 4-nitrophenol. These isoforms derive from the *UGT1* gene complex by the splicing of a unique first exon to the commonly used exons. In fact, the *UGT1* locus consists of nine first exons (five for the bilirubin cluster B and four for the phenol cluster A) encoding an isoform-specific sequence and a single set of four exons encoding the common sequence of all UGT1 isoforms

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Abbreviations: GH, growth hormone; UGT, uridine diphosphate glucuronosyltransferase; UDPGA, uridine diphosphate glucuronic acid; 4-NP, 4-nitrophenol; BIL, bilirubin; TESTO, testosterone; ANDRO, androsterone; E_1, estrone; CT, cortisol and thyroxine; RT-PCR, reverse transcription-polymerase chain reaction; dNTP, dinucleotidetriphosphate; DTT, dithiothreitol.

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[4–6]. Nevertheless, 4-nitrophenol was shown to be glucuronidated by several isoforms belonging to both the UGT1 and UGT2 families [2, 7, 8], while bilirubin was glucuronidated by a major isoform (UGT1 isoform B1*) in the liver of untreated rats [6]. The UGT2 family is responsible for the glucuronidation of some xenobiotics and of endogenous compounds such as steroid hormones. Androsterone is conjugated by one isoform (UGT2B2) [9, 10] while testosterone is glucuronidated by three isoforms (primarily UGT2B3, UGT2B1, which is inducible by phenobarbital, and UGT2B6 [11]). Estrone is conjugated by a separate isoform [12]. These different isoforms differ by their developmental expression [13, 14] and their susceptibility to different drug and xenobiotic stimulations [15, 16]: 3-methylcholanthrene induces 4-nitrophenol but not bilirubin glucuronidation, while clofibric acid derivatives induce bilirubin but not 4-nitrophenol glucuronidation [17]. Although the regulation of UGTs by hormones has been less extensively studied than the inductive effect of xenobiotic compounds, some authors have shown that thyroid hormones regulate the different isoforms differentially [18, 19, 20], in particular enhancing 4-nitrophenol and decreasing bilirubin and testosterone glucuronidation in a dose-dependent manner. The effects of other pleiotropic hormones, such as growth hormone (GH), on the different glucuronidation activities has not been investigated in rats. However, this hormone was shown to control other hepatic metabolisation activities such as sex-dependent steroid hydroxylations *via* several cytochromes P-450 [21, 22] or the expression of different glutathione-S transferase isoforms [23]. GH exerts its effects on these enzymes *via* its secretion pattern or pulsatility, which differs in the male and the female rat.

It would be of interest to determine whether GH could also modify UGT activities and thus be responsible for some sex differences already observed in some of these activities, namely a higher rate of glucuronidation of 4-nitrophenol in the male than in the female [24] and the opposite observation for estrone glucuronidation [25]. In order to further examine the dependence of UGT activities on GH status, sham-operated and hypophysectomized male rats were treated with two different dosages of GH, a low dosage mimicking the male secretion pattern in hypophysectomized animals, and a high dosage mimicking the female one. Their glucuronidation capabilities towards several substrates were measured in liver microsomes. The conditions of restitution of control values for these glucuronidation activities following hypophysectomy were also studied, using the same concentrations of GH with or without coadministration of physiological doses of thyroxine and glucocorticoids. Protein quantification was achieved by immunoblotting. As bilirubin glucuronidation appeared to be the most dependent on GH status, analysis of the mRNA expression of the isoforms that conjugate this substrate (mainly UGT1B1 but also UGT1B2) or that are closely related (UGT1B5) [6] was realized using reverse transcriptase-polymerase chain reaction (RT-PCR) in order to further investigate the molecular mechanism by which GH regulates bilirubin UGT expression.

* The nomenclature used in this work refers to Ikushiro *et al.* [16]

MATERIALS AND METHODS

Chemicals

Unlabelled 4-nitrophenol (4-NP), testosterone (TESTO), estrone (E_1), androsterone (ANDRO), bilirubin (BIL), androsterone-3α-glucuronide, 4-nitro[U-^{14}C]phenol (437 MBq/mmol), coenzymes and biochemicals were purchased from Sigma (St-Quentin Fallavier, France). [α-^{32}P]dCTP (<110 TBq/mmol) was purchased from Amersham (Les Ulis, France). [4-^{14}C]testosterone (1.67–2.22 GBq/mmol) and [4-^{14}C]estrone (1.67–2.22 GBq/mmol) were provided by DuPont NEN (Les Ulis, France). [4-^{14}C]androsterone was synthesized enzymatically in the laboratory from [4-^{14}C]testosterone. Briefly, [4-^{14}C]testosterone (1.67–2.22 GBq/mmol) was incubated for 30 min at 37° with 2 mg of female rat hepatic microsomal protein in presence of 1 mM NADH, in order to obtain 5-α reduced metabolites. After being extracted with CH_2Cl_2, the steroids were subjected to a 10-min oxidation using CrO_3 (7% in water w/v, 800 μL) and H_2SO_4 (65% v/v, 200 μL). NaOH (5N, 1 mL) was added and the steroids were extracted with CH_2Cl_2 and then purified on a C18 cartridge (Supelclean LC-18 SPE, Supelco, Bellefonte, PA, USA). Purified steroids were subsequently incubated with 2 units of 3α-hydroxysteroid-dehydrogenase in presence of 3 mM NADH for 60 min at 37°. The radiolabelled androsterone was purified by HPLC on an ODS2 column (Interchim, Montluçon, France) with a Philips model PU4100 apparatus (Argenteuil, France). Unlabelled androsterone was obtained using the same methodology from unlabelled testosterone. Labelled and unlabelled androsterone have the same migration on silica gel TLC plates as the reference steroid (R_f = 0.5 with two repeated migrations on the same dimension with the following system: $CHCl_3/CH_3CN$: 9/1, v/v). The structure of unlabelled androsterone obtained in this way was confirmed by GC-MS on a Nermag apparatus (model R10-10T, Argenteuil, France) by comparison with the reference compound mass spectrum. The structure of [4-^{14}C] androsterone-3α-glucuronide, obtained from microsomal incubations of radioactive androsterone (see below) and purified by HPLC on an ODS2 column, was confirmed by FAB-MS by comparison with the reference compound mass spectrum. Moloney murine leukemia virus (MMLV)-reverse transcriptase was from Boehringer Mannheim (Meylan, France). Ribonuclease inhibitor, random primers, and DNA size standards (ΦX 174 digested with Hinf 1) were purchased from Promega Corporation (Charbonnières-les-Bains, France). Taq polymerase (Bio Taq), and deoxynucleotidetriphosphate (dNTP) were from Bioprobe Systems (Montreuil-sous-Bois, France). The oligonucleotide primers were provided by Bioprobe Systems.

Animals

Male Sprague–Dawley rats, hypophysectomized or sham-operated at 6 weeks of age, and female rats were purchased from Iffa-Credo (L'Arbresle, France). Animals were individually housed under standard conditions and were maintained *ad lib.* on a standard diet. After allowing them to recover for two weeks, hypophysectomized and sham-operated animals were randomly divided into 12 groups of 8 according to a factorial arrangement. The effectiveness of hypophysectomy was verified by the absence of weight gain over this period. The animals were given an s.c. injection of recombinant human growth hormone (GH1: 0.25 IU/kg of body weight; GH2: 2 IU/kg of body weight) twice daily while the controls (GH0) received only vehicle (25 mM sodium carbonate buffer, pH 9.5, 0.9% NaCl). Recombinant human growth hormone was a generous gift from Sanofi Recherches (Labège, France). Half the animals were daily s.c. injected cortisol (400 μg/kg of body weight) [22] and L-thyroxine (T_4) (50 μg/kg of body weight) (Sigma) as described elsewhere [26]. This T_4 concentration was found to render hypophysectomized rats euthyroid [26]. The remaining animals received only vehicle (0.9% NaCl). This cortisol/thyroxine treatment (CT) started 2 days prior to GH treatment and lasted throughout the experimental period. Weight gain during the treatment period showed that hormone replacement was effective. Control females received only GH0 treatment. After 16 days of treatment, the animals were slaughtered. Blood was collected and the liver was immediately perfused with Ringer solution *via* the portal vein, excised, weighed and placed in liquid nitrogen before storage at −80°. For microsomal preparations, portions of livers were minced and homogenized at 4° in 0.1 M sodium phosphate pH 7.4. Homogenates were centrifuged sequentially at 11000 *g* (15 min) and 105000 *g* (60 min) at 4° on a Centrikon T-1045 ultracentrifuge (Kontron Instruments, St-Quentin-en-Yvelines, France) in order to prepare the microsomal pellets. Microsomal membranes were resuspended in the homogenization buffer containing 20% glycerol and were stored at −80° until use. The amount of microsomal protein was determined by the method of Lowry [27] using BSA as a standard. By using an immunoradiometric assay (monoclonal antibodies) (hGH "coatria", bioMérieux, Marcy-l'Etoile, France), plasmas of GH2-treated animals were checked for the presence of a significant amount of recombinant human growth hormone at least 12 hr after the last injection, which determined a continuous exposure of GH in these animals.

Enzyme Assays

UGT activities towards 4-NP, TESTO, E_1, ANDRO and BIL were measured in fully detergent-activated microsomes. For each substrate, the optimal concentration of detergent (Triton X-100 in all cases except for BIL glucuronidation activity, for which digitonin was used) was determined after testing different concentrations of detergent on microsomes of rats that had received different treatments (hypophysectomy or GH2 treatment) in the event of a possible effect of the hormonal status on the activation characteristics of UGTs. UGT activity towards 4-NP was assayed radiochemically by the procedure of Tukey *et al.* [28] with 250 μM of substrate, 2.5 mg of microsomal protein and Triton X-100 (0.15 mg/mg microsomal protein) at 37° for 30 min. UGT activities towards TESTO, E_1 and ANDRO were assayed radiochemically in silanized glass tubes. Reactions were carried out in 0.05 M Tris/HCl buffer, pH 8, containing 5 mM $MgCl_2$, 100 μM of substrate, Triton X-100 (0.4, 0.2 and 0.5 mg/mg of microsomal protein for TESTO, E_1 and ANDRO glucuronidation, respectively), 0.5 mg of microsomal protein for TESTO and ANDRO assays and 1 mg for E_1 assay. Final incubation volume was 1 mL. After 5 min of preincubation, incubations were started with 1 mM UDPGA and lasted 30 min for TESTO and ANDRO assays and 60 min for E_1 assay at 37°. Glucuronidation rates were found to be linear with respect to incubation time and microsomal protein amount. The reactions were stopped with 1 mL ice-cold CH_2Cl_2 and unconjugated substrates were extracted twice by 4 mL CH_2Cl_2. Radioactivity in the aqueous phase was estimated on a Packard scintillation analyzer (model Tricarb 2200CA, Meriden, CT, USA). UGT activity towards BIL was assayed as described by Van Roy and Heirwegh [29]. Incubations were carried out with 3.75 mM UDPGA, 1 mg of microsomal protein and 2% digitonin for 30 min at 37°. Final incubation volume was 400 μL. Determination of BIL glucuronide in incubation mixtures was done spectrophotometrically at 530 nm using the diazonium salt of ethyl anthranilate [29].

Immunoblot Analysis

Microsomal preparations were subjected to SDS-PAGE according to Laemmli [30] in 7.5% polyacrylamide gels. Immunoblotting was conducted according to the method of Towbin *et al.* [31] using nitrocellulose membranes. Immunodetection was performed using an ECL Western blotting detection system (Amersham) with a polyclonal antibody raised in rabbits against hepatic UGTs of rats treated with 3-methylcholanthrene, kindly provided by Dr. J. Magdalou, and a horseradish peroxidase-linked goat anti-(rabbit IgG) IgG. The UGT isoforms recognized by the antibody used in this work were previously determined from their molecular weight and the specific increase in their expression by inducers: (kDa ± 1) bilirubin UGT (55), androsterone (53) and testosterone (51) [20, 32]. Protein weight markers were purchased from Sigma (SigmaMarkers; ovalbumin (45 kDa), glutamic dehydrogenase (55 kDa), BSA (66 kDa)).

Analysis of mRNA Expression

Total RNA was prepared from 200 mg of liver of hypophysectomized or not and GH0- or GH2-treated rats using an extraction kit (RNA Quick II, Bioprobe Systems). Expression of mRNA species was analysed by RT-PCR. Comple-

mentary DNA was synthesized from total RNA samples by mixing 1 μg RNA, 100 pmol of random hexamers, in presence of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 200 units of MMLV reverse transcriptase, 40 units of ribonuclease inhibitor and 1 mM of each dNTP in a final volume of 20 μL. The mixture was incubated for 1 hr at 37°. Reverse transcriptase was inactivated by heating at 95° for 5 min. Volume was completed to 100 μL with water and a 10 μL aliquot was used for subsequent PCRs. PCRs were carried out in a final volume of 50 μL containing 2 units BioTaq DNA polymerase in presence of 20 mM Tris-HCl pH 8.55, 16 mM $(NH_4)_2SO_4$, 150 μg/mL BSA, 0.2 mM of each dNTP, 50 pmol of each bilirubin UGT specific primer (UGT1B1, UGT1B2 or UGT1B5) and 25 pmol of each internal standard primer (β-actin). $MgCl_2$ concentration was 0.5, 1.5 and 2.5 mM for UGT1B1, UGT1B2 and UGT1B5 specific set of primers, respectively. Each mixture was overlayed with one drop of mineral oil and incubated in a programmable thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer Cetus, Norwalk, CT, USA) at 94° for 5 min and immediately cycled 30 times (or 29 cycles for UGT1B2 specific set of primers). Cycles consisted in denaturation at 94° for 45 sec; annealing at 60°, 52° and 55° for UGT1B1, UGT1B2 and UGT1B5, respectively, for 60 sec; and elongation at 72° for 60 sec. Following the final cycle, a 5-min elongation step at 72° was included. A 10 μL aliquot was resolved through an agarose gel (0.8% regular; 1.75% low melting) and the PCR products were then transferred onto nylon membranes (Genescreen Plus, NEN Research Products, Boston, MA, USA) by capillary action. Membranes were baked for 2 hr at 65°. The different membranes obtained, one for each UGT isoform mRNA expression analysis, were incubated for 20 min at 65° in hybridization buffer containing Denhardt's solution, 0.5% SDS and salmon sperm DNA, followed by hybridization for 2 hr at 65° with the corresponding UGT1B1, UGT1B2, UGT1B5 or β-actin specific probe. These probes were obtained by RT-PCR as described above and labelled using a kit (Ready-To-Go DNA Labelling Beads (−dCTP), Pharmacia Biotech, Orsay, France) and [α-^{32}P]dCTP. These probes were then 100% homologous to the amplification products. Membranes were then washed and radioactivity quantitated using storage phosphor imaging technology of radioactive samples (PhosphorImager, model 445SI, Molecular Dynamics, Sunnyvale, CA, USA). The sequences of forward (F) and reverse (R) primers were designed by using "Oligo 4, primer analysis software", published by the National Bioscience Inc., Plymouth, MA, USA, from the sequences published by Coffman *et al.* [33], Sato *et al.* [4], Emi *et al.* [6] and Nudel *et al.* [34] for those corresponding to UGT1B1, UGT1B2, UGT1B5 and β-actin, respectively. **B1**: (F) 5′-GTGATCCCCATAGATGGCAG; (R) 5′-GTATGTTTTAACCACACGCAGCAG; **B2**: (F) 5′-GAAGAATATCAGCGGAAATA; (R) 5′-CGGACA TTGTGTAGCCTCA; **B5**: (F) 5′-ATGTGACCCTGCAA GGATTAGCTG; (R) 5′-TGTCTTCACATAGTTGCCT GTTTCA and **β-actin**, (F) 5′-TGCAGAAGGAGATTAC TGCC; (R) 5′-CGCAGCTCAGTAACAGTCC. Their specificity was checked by using the "Blast Search Program" from the GCG package, version 8, Madison, WI, USA. Controls with no retrotranscription were made in order to check for the absence of any crossed reaction with exonic genomic DNA. The UGT mRNA levels were compared after normalization against the expression of β-actin [35].

Statistical Analysis

The effects of hormonal treatments on glucuronidation activities were analyzed using the GLM procedure of SAS [36] on the completely randomized design with a 2 × 3 × 2 factorial arrangement of treatments (hypophysectomy, GH and CT, respectively). Data were transformed into square root to correct for unequal variances. When a three-factor ANOVA was used (complete additive model: hypophysectomy*GH*CT), some first order and second order interactions between treatments most often proved to be highly significant. Thus, the effect of each treatment was also tested in the following reduced groups of animals: GH treatment in sham-operated CT untreated, sham-operated CT-treated, hypophysectomized CT untreated and hypophysectomized CT-treated rats, separately; hypophysectomy in GH untreated animals; and CT treatment in sham-operated GH untreated rats. Inside these different groups, the statistical analysis of differences between the means was performed using the Student–Newman–Keuls test. In order to further examine the conditions of hormonal restoration of control values after hypophysectomy, a one-factor ANOVA was done followed by mean comparisons between the 12 groups of animals using the Student–Newman–Keuls test. The analysis of the effects of GH2 treatment and hypophysectomy on UGT mRNA expression was performed by a two-factor ANOVA. In order to simultaneously assess the relative effects of the different physiological factors on the five glucuronidation activities, a multivariate variance analysis (MANOVA) was done, completed by a discriminant analysis of the different groups of animals. This latter descriptive statistical methodology was first intended to give a hierarchy between the effect of hypophysectomy and the cortisol/thyroxine restitution treatment on the one hand and the GH treatment on the other, and second, to test *a posteriori*, simultaneously for the five glucuronidation activities, the hormonal conditions chosen to restitute control hormonal situation from hypophysectomized animals. The discriminant analysis is equivalent to a principal component analysis done on the barycenters of the different groups of animals, one which maximizes the distances between these groups and minimizes the distances between individuals inside these groups [37–39]. This analysis can reduce the total variance (or information) given by initial variables to some principal components (or discriminant axes) which summarize this information and gives an explanation of these linear discriminants by the correlation they have with the initial

variables. The factorial analysis was performed by using the DISCRIM procedure of SAS [36].

RESULTS

Effects of GH and CT Treatments, Hypophysectomy and Hormone Replacement and Sex on Different UGT Activities

In sham-operated animals, GH treatment significantly decreased ($P = 0.02$) BIL glucuronidation activity (by 30% with GH2 treatment) but only tended to decrease TESTO glucuronidation activity (by 14 and 20% with GH1 and GH2 treatments, respectively, $P = 0.09$) (Fig. 1). In these animals, GH did not significantly decrease 4-NP nor ANDRO glucuronidation. By contrast, GH1 (but not GH2) treatment significantly enhanced E_1 UGT activity by 33%. Hypophysectomy significantly altered all the glucuronidation activities tested but in a contrasting manner depending on the substrate: E_1 and BIL UGT activities were increased by 77 and 200%, respectively, while 4-NP, TESTO and ANDRO UGT activities were decreased by 43, 23 and 67%, respectively (Fig. 1). GH2 treatment tended to counterbalance the effect of hypophysectomy on ANDRO and BIL conjugation activities (+47% and −42% for ANDRO and BIL UGT activities, respectively). By contrast, this treatment intensified the depressing effect of hypophysectomy on TESTO glucuronidation activity by 23%. Restoration of control values after hypophysectomy was achieved with concomitant treatment of the animals with CT and GH1 for all the activities tested, except for ANDRO UGT activity for which the control values could not be restituted. Restitution was also achieved with concomitant treatment with GH2 and CT for TESTO and E_1 UGT activities, with GH1 treatment administered alone for TESTO UGT activity and CT treatment administered alone for 4-NP and TESTO UGT activities. In hypophysectomized CT-treated rats, GH1 and GH2 treatments significantly decreased BIL (by 25 and 52%, respectively), ANDRO (by 60 and 57%, respectively) and E_1 (by 43 and 45%, respectively) UGT activities (Fig. 1). In these animals, 4-NP and TESTO UGT activities were not affected by GH treatments.

CT treatment in sham-operated rats decreased all the activities tested (by 18, 56, 51 and 16% for TESTO, BIL, ANDRO and E_1 glucuronidation, respectively) except 4-NP glucuronidation, which was enhanced by 18% by this treatment (Table 1). Concomitant treatment with GH did not modify the CT effect on the activities tested, except for ANDRO UGT activity in which GH tended to abolish the depressing effect of CT. 4-NP and TESTO UGT activities were significantly lower in females as compared to male rats (Fig. 1). The opposite situation was found for E_1 glucuronidation. BIL and ANDRO UGT activities presented no sex difference.

Immunoblot Analysis

Immunoblots of UGTs were realized with polyclonal anti-rat liver UGT antibody, which recognizes at least three bands (Fig. 2). The first band (55 kDa), whose intensity was increased in response to clofibrate treatment on a control animal, was strongly related to bilirubin glucuronidation activity. Compared to controls, this band intensity was greatly increased with hypophysectomy and decreased with GH2 treatment in both sham-operated and hypophysectomized animals. This decrease was even more striking in hypophysectomized rats treated with GH2 and CT. The second band (53 kDa), whose intensity was related to ANDRO glucuronidation activity, was diminished in hypophysectomized animals, particularly those that had been treated with CT. The third band (51 kDa) seemed to be related to TESTO glucuronidation activity, with a slight decrease in intensity with GH2. Another band (57 kDa) could be visualized only in hypophysectomized untreated rats or in a clofibrate-treated control animal.

Effect of GH2 Treatment and Hypophysectomy on UGT1B1, UGT1B2 and UGT1B5 mRNA Expression

mRNA expression of three different isoforms belonging to the bilirubin cluster was analyzed using RT-PCR with a set of primers specific for each UGT1 first exon, the other exons being the same for all UGT1 isoforms. The relative semi-quantitation of UGT mRNA expression was estimated after normalization against β-actin mRNA expression, the two cDNA being co-amplified in the same assay. β-actin mRNA expression is known to be unmodified by GH or thyroid status [35, 40]. The proportion of UGT1B1 mRNA, which represents the main constitutive isoform that glucuronidates bilirubin, was increased by 63% by hypophysectomy (Fig. 3). GH2 treatment decreased the expression of this mRNA in hypophysectomized rats (by 45%) and tended to decrease it in sham-operated ones (by 12%). Expression of UGT1B2 mRNA was greatly influenced by hypophysectomy and GH treatment. Hypophysectomy dramatically increased this mRNA expression by 445%. GH2 treatment had opposite effects in hypophysectomized animals, where it counterbalanced the increase due to hypophysectomy by 43%, and in sham-operated animals where it increased this mRNA expression by 180%. Expression of UGT1B5 mRNA was not affected by hypophysectomy nor by GH treatment.

Statistical Multivariate Analysis of the Hormonal Restitution Conditions of Glucuronidation Activities

Manova done on these five UGT activities is highly significant (Wilks lamda = 19.95, 55 ddl, $P < 0.0001$). The pseudo-F value associated with this multivariate analysis is larger than any F value associated with the univariate analysis of UGT activities, thus enabling us to pursue a discriminant analysis on this set of variables. The first

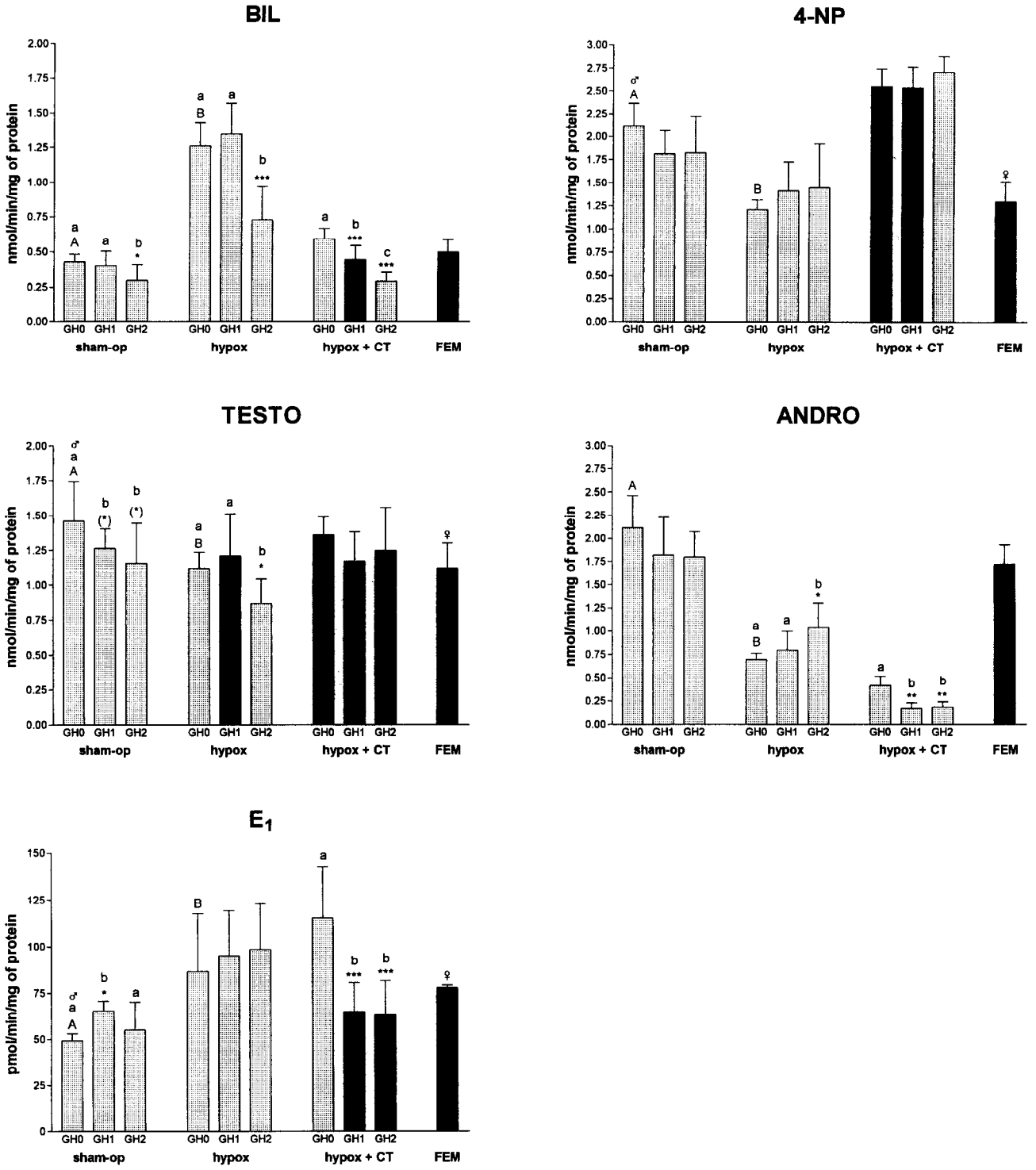

FIG. 1. Effect of GH treatment, hypophysectomy and cortisol/thyroxine treatment on UGT activities towards bilirubin (BIL), 4-nitrophenol (4-NP), testosterone (TESTO), androsterone (ANDRO) and oestrone (E_1) in male rats. Activities were measured in fully activated microsomes as described in the Materials and Methods section. Values are the mean of eight individual determinations ± SD. Abbreviations: CT, cortisol and thyroxine treatment; GH, growth hormone treatment; GH0, no GH treatment; GH1, low dosage treatment; GH2, high dosage treatment. Hypox, hypophysectomized rats; sham-op, sham-operated rats; FEM, females. *, ** and *** show a significant effect of GH treatment in the following groups of animals: sham-operated rats, hypophysectomized CT untreated rats and hypophysectomized CT-treated rats with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. (*) shows statistical differences at $P < 0.1$. a, b and c show significant differences between GH treatments within each group at $P < 0.05$ (Student–Newman–Keuls test). A and B show a significant effect of hypophysectomy at $P < 0.05$. Black bars represent the necessary combination of hormonal treatments of hypophysectomized animals for a restitution of the control values (statistically not different from sham-operated untreated animals) with Student–Newman–Keuls tests. ♂ and ♀ show a significant difference between male and female rats at $P < 0.05$.

TABLE 1. Effect of CT treatment and combined CT/GH treatment on different glucuronidation activities

UGT activity (nmol/min/mg prot)	Sham-operated	Sham-operated + CT		
	GH0	GH0	GH1	GH2
PNP-UGT	2.12 ± 0.25	2.49 ± 0.37†	2.49 ± 0.23	2.39 ± 0.28
BIL-UGT	0.43 ± 0.05	0.19 ± 0.03†	0.21 ± 0.07	0.20 ± 0.05
TESTO-UGT	1.46 ± 0.28	1.19 ± 0.12†	1.23 ± 0.08	1.25 ± 0.23
ANDRO-UGT	2.11 ± 0.35	1.04 ± 0.31†	1.41 ± 0.19‡	1.76 ± 0.52‡
E_1-UGT	0.049 ± 0.004	0.041 ± 0.007†	0.048 ± 0.010	0.051 ± 0.016

Glucuronidation activities were measured in sham-operated rats as described in the Materials and Methods section. CT, cortisol and thyroxine treatment; GH, growth hormone treatment; GH0, controls for GH treatment; GH1, low dosage treatment; GH2, high dosage treatment.

† Represents a significant effect of CT treatment at $P < 0.05$. ‡ Represents a significant effect of GH treatment in CT-treated rats at $P < 0.01$.

factorial map (Fig. 4) constructed from the first and second linear discriminants accounts for 97.7% of the total variance (or information) (first component: 66.5% and second component: 31.2%). The third and fourth linear discriminants were significant but were excluded from the interpretation because of the insufficient amount of information they summarize. The first principal component is well explained by BIL UGT and 4-NP UGT activities, the correlations being measured to −95.1% and +80.3% respectively (these two activities are anticorrelated), and to a lesser extent by E_1 UGT activity (correlation: −47.5%). The second linear component is only explained by ANDRO UGT activity (correlation: −96.5%). The third and fourth axes are mainly correlated to E_1 and TESTO UGT activities respectively (corresponding correlations: 31.9% and 68.0%). Analysis of the projection of individuals on the first linear component gives a clear opposition between the group of hypophysectomized animals on the one hand and the groups of sham-operated CT-treated or untreated animals and hypophysectomized CT-treated animals on the other (Fig. 4). Yet, there is no confusion between the group of hypophysectomized CT-treated animals and the groups of sham-operated animals (CT-treated or not) when they are projected on the second factorial axis. Mahalanobis distances (Euclidean distances corrected for correlations between initial variables) between the means of the different subgroups of animals show that hypophysectomy is the major element in the perturbation of UGT activities. Although highly significant, the overall effect of CT on UGT activities of sham-operated animals is lower than hypophysectomy, as shown by the distances between CT-treated and untreated animals. Inside each subgroup of rats, the overall effect of GH on UGT activities appears to be significant only for GH2 treatment in sham-operated animals as compared to GH0 or GH1 animals, but the effects of GH1 or GH2 as compared to GH0 animals are well evidenced in hypophysectomized rats. Finally, from the projection of individuals on the factorial map, we can observe that if CT treatment seems to be sufficient in getting hypophysectomized animals superimposed to sham-operated control animals when they are projected on the first linear discriminant, this treatment is insufficient to recover ANDRO UGT control activities because of a very significant discrimination on the second linear discriminant. Moreover, we can notice that the female group (projected as supplementary individuals) is not superimposed to the group of sham-operated GH2-treated males. This confirms the default of a "feminization" process of the studied glucuronidation activities, related to continuous GH exposure.

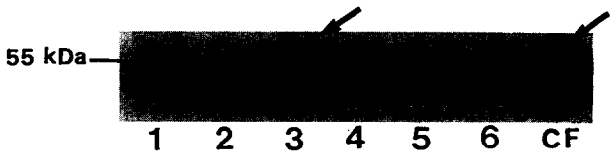

FIG. 2. Expression of bilirubin UGT, androsterone UGT and testosterone UGT in sham-operated untreated (track 1), sham-operated GH2-treated (track 2), hypophysectomized untreated (track 3), hypophysectomized GH2-treated (track 4), hypophysectomized CT-treated (track 5), hypophysectomized GH2 and CT-treated (track 6) and clofibrate-treated (track CF) male rats. Hepatic microsomes (20 μg of protein) were subjected to SDS-PAGE and the assignation of bands to the different UGT isoforms was achieved as described in the Materials and Methods section. Arrows show a supplementary band (57 kDa) on track 3 and CF. Glutamic deshydrogenase from bovine liver is used as molecular weight marker protein (55 kDa).

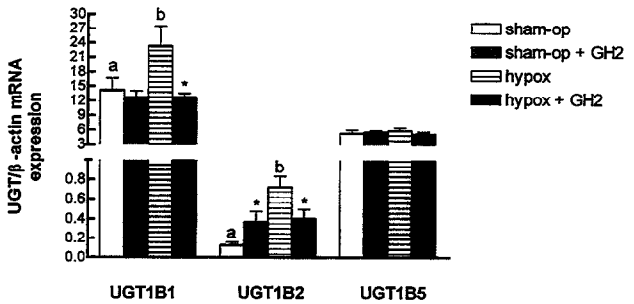

FIG. 3. Effect of GH treatment on UGT1B1, UGT1B2 and UGT1B5 mRNA expression in sham-operated and hypophysectomized male rats. Values are the mean of three individual determinations ± SD and are expressed as the ratio UGT/β-actin mRNA expression analysed by RT-PCR. *Shows a significant effect of GH in sham-operated or in hypophysectomized animals at $P < 0.05$; a and b show a significant effect of hypophysectomy at $P < 0.01$. Hypox, hypophysectomized rats; sham-op, sham-operated rats; GH2, growth hormone high dosage treatment.

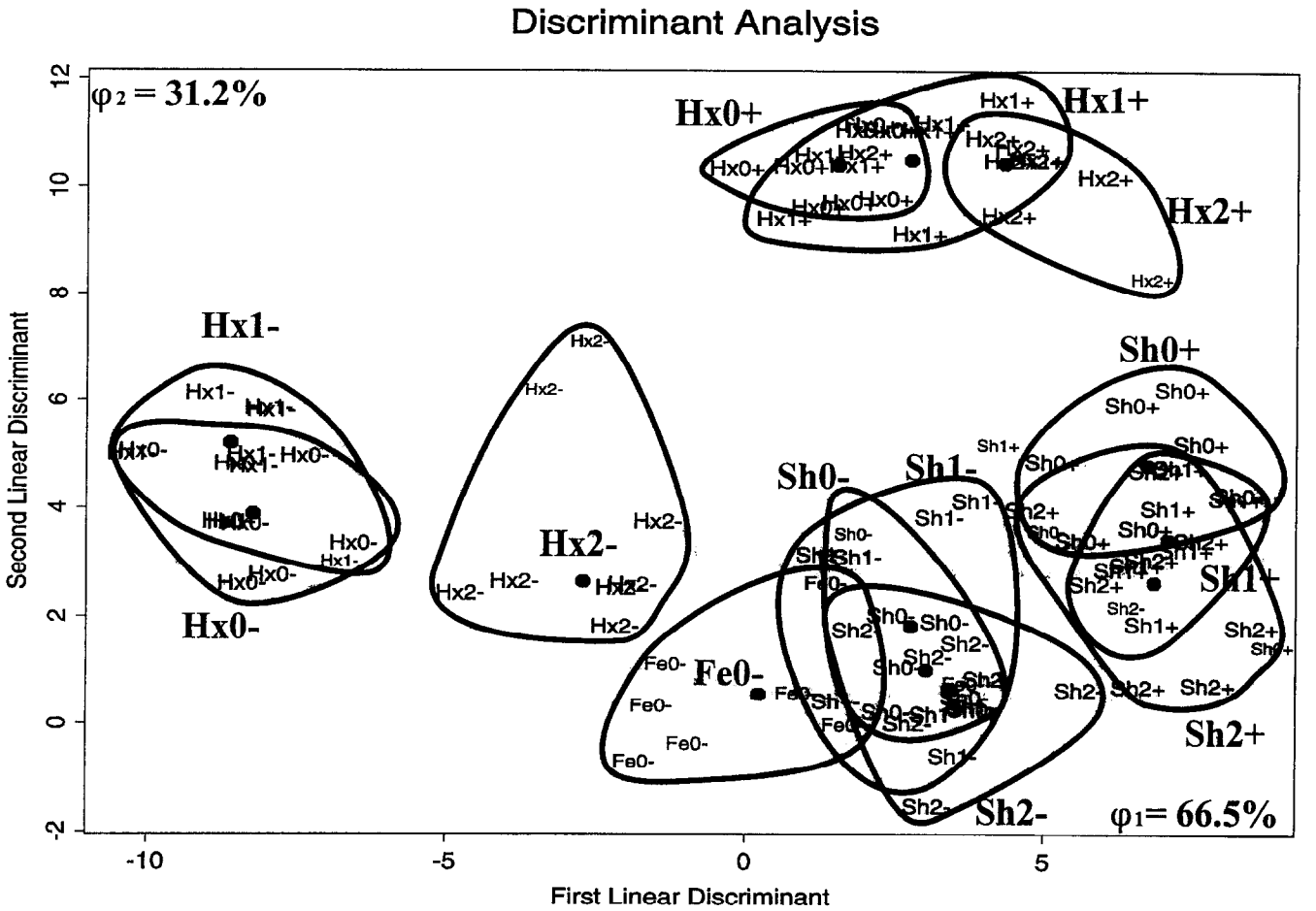

FIG. 4. Plot on the first factorial map (first and second linear discriminants) of the projection of individuals characterized by their belonging to the different treatment groups: The first and the second components explain 66.5 and 31.2% of the total variance, respectively. Black squares represent the location of group means. The female rat group is projected on the factorial map as a supplementary rat group. Sh represents sham-operated rats; Hx, hypophysectomized rats; Fe, female rats. 0 represents control rats for GH treatment (GH0); 1, rats treated with the low GH dosage treatment (GH1); 2, rats treated with the high GH dosage treatment (GH2). + Represents rats treated with cortisol/thyroxine (CT); − represents control rats for CT treatment.

DISCUSSION

GH is a Moderate but Necessary Hormonal Regulator of BIL and E_1 UGT Activities

In the present study, fully detergent-activated microsomes were used in order to estimate effects of hormonal treatments and hypophysectomy on UGT activities that are representative of enzyme concentrations. Consequently, this methodology enabled us to discard any eventual interference due to an effect of the hormonal status on the membrane environment of these enzymes (i.e. latency), since it was observed at the same time that GH treatment induced modifications in the fatty acid composition of the microsomal membrane†. The discriminant analysis procedure applied to the set of UGT activity data was performed to synthesize the most important criteria (UGT activities and physiological treatments) that explain the major part of information (variance) provided by the factorial experimental design. We observed that a substantial reduction in the initial representation space of individuals (a 5-dimension space corresponding to the five UGT activities) to a 2-dimensional space did not significantly alter the total amount of information. Thus, the first linear discriminant is explained by BIL, 4-NP and E_1 UGT activities. The main constitutive isozymes involved in BIL and 4-NP UGT activities belong to the UGT1 family (UGT1B1 and UGT1A1 respectively [6]). Yet, the strong positive correlation of E_1 UGT activity with BIL UGT activity and the negative correlation with 4-NP UGT activity could be related to an almost common mechanism of regulation of expression of these isozymes. Even though BIL and E_1 UGT activities are significantly anticorrelated to the 4-NP UGT activity, all these activities are highly sensitive to hypophysectomy, the control values being conditionally recovered in these animals by an almost physiological CT treatment supplemented with a male GH dosage (Figs. 1 and 4). For any of these three activities, control values cannot be restored in the absence of CT administration in GH-treated hypophysectomized animals. However, for BIL and E_1 UGT activities, adjonction of GH to CT treatment of hypophysectomized animals is a necessary condition of recovering

† F. Guéraud and A. Paris, unpublished results.

values of sham-operated untreated animals. By contrast, ANDRO UGT activity, which explained the second linear discriminant, is also highly sensitive to hypophysectomy, but the CT restitution treatment of these animals greatly reinforces the decreasing effect of hypophysectomy. Moreover, GH treatment of CT treated hypophysectomized animals also amplifies this decreasing effect (Fig. 1). That none of the hormone replacement tested could achieve a restoration of control values after hypophysectomy for ANDRO UGT activity could be explained by the fact that hypophysectomy alters also gonadal steroid secretion due to pituitary gonadotropin suppression. As sex hormones are known to regulate also their own glucuronidation [1], the secretion of these gonadal steroids could be of importance for the regulation of this activity. TESTO and ANDRO UGT activities, which result from the activity of isoforms that belong to the UGT2 family [3] are not correlated. The effects of hypophysectomy are similar to those observed on BIL and 4-NP UGT activities following thyroidectomy [19, 20]. However, it is important to bear in mind that thyroidectomy dramatically reduces GH secretion [41] and consequently that thyroid hormone regulation may be both direct and indirect. In sham-operated rats, cortisol and thyroxine treatments have their own effect on all UGT activities; these effects were similar to those of hyperthyroidism on 4-NP and BIL UGT activities [19, 20], underlying the importance of thyroid hormone in this cortisol/thyroxine effect.

Our results show that GH effects, although less important than those of hypophysectomy or even those of CT treatment, are still significant within each every subgroup of animals. In fact, the GH1 dosage, in conjonction with CT treatment of hypophysectomized animals, is necessary to give a complete restoration of control values of BIL and E_1 UGT activities. Moreover, when we evaluate the specific GH treatment effect, we can observe a significant effect of GH in hypophysectomized CT-treated rats for BIL, E_1 and ANDRO UGT activities. Nevertheless, our results show for the first time that GH decreased BIL UGT activity in male rats by 30% in fully activated microsomes. GH is known to be responsible for many of the sex differences observed in the hepatic metabolism of many drugs and steroids. In fact, some steroid hydroxylase and steroid 5α A-ring reductase activities [42], some glutathione-S transferase isoforms [23] and the cortisol sulfotransferase activity [43] were reported to be under the influence of GH. This hormone exerts its effect on these enzymes *via* its secretion pattern, which shows long periods of trough after peak surge in male rats, but an almost continuous secretion pattern in females [44]. In the present study, physiological dosages of GH were chosen in order to restitute a male-like or female-like secretion pattern of GH in hypophysectomized rats [45]. As a GH1 dosage administered subcutaneously is commonly used to mimic the pulsatile GH pattern of male rat [44], a GH2 dosage, which is 8 times higher than GH1, was chosen in order to determine a continuous GH exposure as is the case in females. This GH2 dosage was sufficient to mimic a female secretion profile in males, as shown by a female-predominant steroid metabolisation activity (Δ_4-3-oxosteroid 5α-reductase) obtained in GH2-treated animals‡. Surprisingly, GH2 treatment reduced BIL glucuronidation activity that presented no sex differences, but did not affect 4-NP UGT activity that is lower in female than in male rats. Only E_1 UGT activity, which was slightly enhanced by GH1 in sham-operated animals, was shown to be higher in females than in males. Our results concerning sex differences in UGT activities are in accordance with those previously reported [24, 25]. However, sex differences of glucuronidation activities cannot be attributed to gender differences of GH secretion.

GH Differentially Controls the Different Isoforms that Belong to the Bilirubin Cluster at a Pretranslational Level

BIL glucuronidation activity appears to be under the control of GH regulation to a greater extent than the other activities tested in sham-operated as well as in hypophysectomized CT-treated or untreated animals (Fig. 1). Due to the low abundance of UGT mRNAs, which represent less than 0.1% of rat liver mRNAs [46], the RT-PCR technique was chosen in order to determine the mRNA expression of some of the different UGT1 isoforms that derive from the bilirubin cluster [6]. All these isoforms, as well as those that are derived from the phenol cluster, result from an alternate splicing of specific and common exons localised on the same *UGT1* locus [6]. The effect of hormonal status on the mRNA expression of different UGT1B was studied in order to determine a possible influence of hormones on the alternate splicing of *UGT1* transcripts. UGT1B1, which corresponds to the main isoform conjugating BIL, is constitutively expressed in rat liver [6, 16]. Its mRNA expression (Fig. 3) is strongly related to enzyme concentration (Fig. 2) and activity (Fig. 1), indicating that GH exerts its effect on this isoform at a pretranslational level. UGT1B2, which corresponds to the isoform previously named bilirubin-UGT (UGT1*0) [3], was in fact previously shown to be poorly expressed in hepatic microsomes [6, 16]. Our results show that this expression is less important than that of UGT1B1 in the liver but is greatly influenced by GH status, particularly by the absence of pituitary hormones, since hypophysectomy induced a 5-fold increase in UGT1B2 mRNA. This increase was partly reversed by GH treatment to a level similar to that obtained after GH treatment of sham-operated animals. Surprisingly, GH treatment had an opposite effect on sham-operated rats, inducing an almost 3-fold increase. This increase in UGT1B2 mRNA expression with hypophysectomy could account for the band being detected only in hypophysectomized rats (57 kDa) after immunoblotting of the UGTs (Fig. 2). As reported elsewhere, the UGT1B2 isoform is known to migrate more slowly than UGT1B1 on an SDS-PAGE gel [16]. However,

‡ F. Guéraud and A. Paris, unpublished results.

as UGT1B2 is not supposed to be influenced by clofibrate treatment [6], the band observed at 57 kDa in a parallel immunoblot from a clofibrate-treated animal could correspond to UGT1B5, which is also known to migrate more slowly than UGT1B1 on an SDS-PAGE gel and which was reported to be induced by clofibrate [6]. Consequently, the band observed at 57 kDa could correspond to two different UGT isoforms: UGT1B2 in hypophysectomized rats and UGT1B5 in the clofibrate-treated rat. Our data show that UGT1B5 is not dependent on GH status. These results indicate that the expression of some different UGT1 isozymes that are encoded by a single *UGT1* gene locus can be differentially regulated by GH and consequently, that alternate splicing of transcripts deriving from this unique *UGT1* gene locus is probably under hormonal control. The mechanism by which GH exerts its effect on UGT1 mRNA expression is not known and could be different from the one already proposed by Waxman *et al.* [47], in which intermittent GH pulsation (male GH secretion pattern) could trigger tyrosine phosphorylation and the nuclear translocation of a DNA binding protein, since GH effects on UGTs do not seem to be related to GH pulsatility. The effects of GH treatment and hypophysectomy on bilirubin glucuronidation activity could be of importance for the regulation of hemoprotein catabolism, since bilirubin glucuronide is the terminal product of this catabolism. The increased BIL UGT activity following hypophysectomy may be correlated to the decreased hematocrit and hemoglobin rate observed by others in this condition [48]. Yet, the relationship between GH treatment, hypophysectomy and modifications of haem catabolism remains to be established.

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