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Hormonal Regulation of Hepatic Organic Anion Transporting Polypeptides

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ABSTRACT

Organic anion transporting polypeptides (Oatp) mediate the transport of a wide variety of amphipathic organic substrates. Rat Oatp1b2 and human OATP1B3 are members of a liver-specific subfamily of Oatps/OATPs. We investigated whether prolactin (PRL) and growth hormone (GH) regulated Oatp1b2 and OATP1B3 gene expression via signal transducers and activators of transcription 5 (Stat5). Binding sites for Stat5 transcription factors were located in the promoters of *Oatp1b2* and *OATP1B3* at −209 to −201 (5′-TTCTGGGAA-3′) and −170 to −162 (5′-TTCTGAGAA-3′), respectively. In primary hepatocytes from female and male rats treated with PRL or GH, Oatp1b2 mRNA measured by real-time polymerase chain reaction was significantly induced 2-fold. HepG2 cells were transiently transfected with expression vectors containing Oatp1b2 or OATP1B3 promoter fragments, cDNAs for Stat5a, and the receptors for PRL ($PRLR_L$) or GH (GHR), and treated with PRL or GH. PRL and GH induction of Oatp1b2 and OATP1B3 promoter activity required cotransfection of Stat5a and $PRLR_L$ or GHR. Mutation of the Stat5 binding site in both promoters eliminated hormonal induction. In DNA binding assays, HepG2 cells transfected with cDNAs for Stat5a and $PRLR_L$ were treated with PRL, and nuclear extracts were probed with a ^{32}P-labeled oligomer corresponding to −177 to −157 of the OATP1B3 promoter. PRL enhanced the binding of Stat5a to the OATP1B3 promoter and DNA-protein binding was inhibited in competition assays by excess OATP1B3 and Stat5 consensus oligomers but not by mutant Stat5 oligomers. These findings indicate that PRL and GH can regulate Oatp1b2 and OATP1B3 gene expression via the Stat5 signal-transduction pathway.

The organic anion transporting polypeptides (rodents, Oatps; human, OATPs) make up an important superfamily of proteins that are involved in the membrane transport of endogenous and exogenous (xenobiotic) compounds. Although most Oatps/OATPs are expressed in a wide variety of tissues, including the brain, heart, intestine, kidney, lung, placenta, and testis, some Oatps/OATPs are selectively expressed in rodent and human livers (Tamai et al., 2000). These liver-specific transporters, localized to the basolateral (sinusoidal) plasma membrane in hepatocytes, are involved in the Na^+-independent hepatic transport of bile salts and nonbile salt organic anions from blood to bile. Included in the liver-specific subfamily OATP1B of Oatps/OATPs are human OATP1B3 [previously called OATP-8 (*SLC21A8*)], and its rat and mouse ortholog Oatp1b2 [previously called Oatp-4 (Slc21a10)] (Hagenbuch and Meier, 2004). Oatp1b2 seems to be the most abundantly expressed organic anion transporting polypeptide in rat liver (Li et al., 2002). In addition to bile salts, the preferred common substrates of OATP1B3 and Oatp1b2 include conjugated steroid hormones such as dehydryepiandrosterone-3-sulfate and estradiol-17β-D-glucuronide, bromosulfophthalein, and the toxins microcystin and phalloidin. Additional substrates specific to OATP1B3 include the gastrointestinal peptide hormone cholecystokinin, the cardiac glycosides digoxin and ouabain, the antineoplastic methotrexate, and the antibiotic rifampicin. Complete lists of Oatp/OATP substrates are noted in Hagenbuch and Meier (2003 and 2004) and Tirona and Kim (2002).

We have shown previously that prolactin (PRL) and growth hormone (GH), hormones secreted by the anterior pituitary, play an important role in regulating the expression

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ABBREVIATIONS: Oatp/OATP, organic anion transporting polypeptide; Ntcp, Na^+-taurocholate cotransporting polypeptide; PRL, prolactin; GH, growth hormone; $PRLR_L$, long form of the prolactin receptor; GHR, growth hormone receptor; Stat5, signal transducer and activator of transcription; FXR/BAR, bile acid receptor; PCR, polymerase chain reaction; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; o, ovine; r, rat.

of rat Na^+-taurocholate cotransporting polypeptide (Ntcp), the major basolateral, Na^+-dependent transporter of bile salts. Two signal transducers and activators of transcription (Stat5) binding sites are located in the promoter (−936 to −928 and −912 to −904) of the *Ntcp* gene (Ganguly et al., 1997). Cotransfection studies in HepG2 cells showed that PRL acts via the long form of the PRL receptor ($PRLR_L$) and Stat5 to induce *Ntcp* promoter activity (Ganguly et al., 1997). Studies in cultures of primary rat hepatocytes showed that PRL also activates Stat5 and induces Ntcp mRNA expression in this more physiological system (Cao et al., 2001a). These studies established a molecular basis by which PRL is able to influence transcriptional regulation of the *Ntcp* gene. Cotransfection studies in HepG2 cells and in primary rat hepatocyte cultures have also shown that GH acts via the GH receptor (GHR) and Stat5 to induce Ntcp expression (Cao et al., 2001), thus showing that GH and PRL are able to transcriptionally regulate Ntcp.

The transcriptional regulation of *Oatp/OATP* gene expression has not been examined thoroughly. Recent studies have shown that hepatic expression of OATP1B3, OATP1B1 (also a member of subfamily OATP1B), and mouse Oatp1b2 are dependent on the liver-enriched transcription factor hepatic nuclear factor-1α (Jung et al., 2001). Rat Oatp1a4 (formerly Oatp-2) gene expression is induced by pregnenolone-16α-carbonitrile and by lithocholic acid, a cholestatic bile acid, through several pregnane X receptor response elements located in the rat Oatp1a4 promoter (Staudinger et al., 2001; Xie et al., 2001; Guo et al., 2002). Rat Oatp1b2 gene expression is also induced by pregnenolone-16α-carbonitrile, although the results were not statistically significant (Li et al., 2002). Finally, OATP1B3 expression can be influenced by bile acids and the bile-acid nuclear receptor FXR/BAR (Jung et al., 2002). The induction of hepatic Oatps/OATPs via the pathways described above may function as an important adaptive mechanism to maintain hepatic transport and to detoxify endogenous and exogenous substrates.

We have identified Stat5 binding sequences (TTC/A PyNPu G/TAA) in the Oatp1b2 and OATP1B3 gene promoters at −209 to −201 (5′-TTCTGGGAA-3′) and −170 to −162 (5′-TTCTGAGAA-3′) relative to the transcription start site (+1), respectively, by sequence homology searches. In this study, we investigated whether Oatp1b2 and OATP1B3 expression is regulated by PRL and GH through the Stat5 signal-transduction pathway similar to *Ntcp,* as shown in our earlier studies. In our work, we used freshly isolated rat hepatocytes to demonstrate the induction of *Oatp1b2* mRNA by PRL and GH. We also examined the ability of PRL and GH to activate the rat *Oatp1b2* and human *OATP1B3* gene promoters via the Stat5 signal-transduction pathway using HepG2 cell transient cotransfection assays.

Materials and Methods

Materials. Ovine PRL (NIDDK-oPRL-21; AFP10692C) and rat GH (rGH) were provided by Dr. A. F. Parlow (National Hormone and Peptide Program, Harbor-University of California Los Angeles Medical Center, Torrance, CA). The expression vector containing the rat GHR (rGHR) cDNA, pcDNA-rGHR, and ovine Stat5a cDNA, pXM-Stat5a, were provided by Dr. B. Groner (Institute for Experimental Cancer Research, Freiburg, Germany). The cDNA for the $rPRLR_L$ was a gift from Dr. Paul Kelly (Institut National de la Santé et de la Recherche Médicale, Paris, France), and the pCMX-rFXR plasmid for FXR/BAR was a gift from Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). Plasmid pRSV-β-galactosidase was provided by Dr. Daniel Noonan (University of Kentucky, Lexington, KY). The OATP1B3 luciferase reporter construct, LUC-438, containing the OATP1B3 promoter (−438 to +38) was constructed as described previously (Jung et al., 2002). Plasmid DNA was extracted using QIAGEN maxicolumns (Valencia, CA). Unless otherwise noted, all other chemicals were of analytical grade or cell-culture grade and were from Fisher Scientific Co. (Pittsburgh, PA), Sigma-Aldrich (St. Louis, MO), or Invitrogen (Carlsbad, CA).

Plasmid Construction. A fragment of the 5′-region (−303 to +38) of the rat Oatp1b2 gene containing the Stat5 binding site (−209 to −201) was PCR-amplified by using genomic DNA obtained from rat liver as a template, with primers p-303 and p+38 (Table 1), and Platinum *Pfx* DNA polymerase. The p-303 primer contained an internal SacI restriction site and the p+38 primer contained an internal BglII site. The resulting PCR product was digested with SacI and BglII and ligated into the luciferase reporter vector pGL3-Basic that had been predigested with SacI and BglII, yielding the Oatp1b2 promoter construct LUC-303. A LUC-303–derived construct with a mutation in the Stat5 binding site was generated by a two-step PCR method as outlined previously (Barik, 1993), using primers p-303, p-190, and p+38 (Table 1), and Platinum *Pfx* DNA polymerase, yielding the promoter construct LUC-303mut. The OATP1B3 construct LUC-438 was prepared according to the procedure described by Jung et al. (2002). An analogous OATP1B3 LUC-438-derived construct with a mutation in the Stat5 binding site was generated with two complementary oligonucleotides, p-193 and p-128 (Table 1), and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions, yielding the promoter construct LUC-438mut. Sequence identity of all Oatp1b2 and OATP1B3 constructs was verified by sequence analysis (Elim Biopharmaceuticals, Hayward, CA). The nucleotide sequence of the 5′-flanking region of the rat Oatp1b2 gene from nucleotide −2299 to −1 has been published by Choudhuri et al. (2002). The nucleotide sequence of the 5′-flanking region of the human OATP1B3 from nucleotide −900 to the translation start site has been published by Jung et al. (2002).

Animals. Pregnant female Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). The rats had free access to food and

TABLE 1
Oligonucleotides used for plasmid construction and real-time PCR

Oligonucleotide	Sequence (5′ to 3′)
p-303	gtggagctccacatttggatgaacgct
p+38	gagagatctcttctagagcaagcgt
p-190	gtatttaaaacttaaccaaaagta
p-193	ctaatgttaacataatgtgtacatttggttaaattattaataaaatgtttaaaagataggcttctg
p-128	cagaagcctatcttttaaacattttattaataatttaaccaaatgtacacattatgttaacattag
Oatp1b2for	cctgttcaagttcatagagcagca
Oatp1b2rev	tgccatagtaggtatggttataattcctaa
18Sfor	gtaacccgttgaaccccatt
18Srev	ccatccaatcggtagtagcg

water and were maintained on an automatically timed 12-h light/dark cycle. The first 24 h after birth were designated as day 0. Pups were kept with their mother until weaning at day 21, after which they were separated into female and male groups and were housed separately. All protocols dealing with animals were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and followed the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Animals.

Isolation and Culture of Rat Hepatocytes. Hepatocytes were isolated from female and male rats at 4 weeks (~day 28) of age by a two-step perfusion method as described previously (LeCluyse et al., 1996). Dishes were precoated with rat-tail collagen I diluted in Hanks' buffered saline solution (125 μl/ml collagen I; 3 ml/60-mm dish), incubated overnight at 37°C, and washed with Williams' E medium before use. Viability of hepatocyte preparations was assessed by Trypan blue exclusion. Hepatocyte suspension was added to precoated dishes at a density of 2×10^6 cells/dish and was incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. After 2 to 3 h of incubation, the unattached cells were decanted, and the attached cells were exposed to various concentrations of hormones in Williams' E medium containing 5% fetal bovine serum (FBS) or blank medium for 2 or 3 h. At the end of the incubation period, hepatocytes were washed twice with phosphate-buffered saline (PBS), scraped into 1.5 ml of PBS, and centrifuged at 2000*g* for 30 s. Total RNA was isolated from the resulting cell pellet using TRIzol (Invitrogen). Otherwise, the cell pellet was resuspended in gel loading buffer for subsequent immunoblot analysis.

Immunoblot Analysis. For the determination of expression of Oatp1b2 protein in cultured primary hepatocytes, hepatocytes were resuspended in gel loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.1% bromphenol blue). Samples (20 μg, without boiling) were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with Tris-buffered 5% nonfat milk overnight at 4°C and then incubated with the anti-oatp4 antibody (1:2000 dilution) (Cattori et al., 2001) overnight at room temperature. The blots were washed three times (5 min each) in Tris-buffered saline/Tween 20 solution (25 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and incubated with a peroxidase-conjugated anti-rabbit secondary antibody (1:5000 dilution) for 1 h at room temperature. The blots were further washed three times (5 min each) in Tris-buffered saline/Tween 20 solution and were visualized using the enhanced chemiluminescence detection system (ECL+Plus; Amersham Biosciences Inc., Piscataway, NJ). The membrane was exposed to Storm 860 PhosphorImager and was quantified by measurement of blue fluorescence, according to the manufacturer's instructions.

Real-Time PCR. The expression of Oatp1b2 mRNA in primary hepatocytes exposed to various concentrations of hormones versus control was determined by real-time PCR analysis. cDNA was produced from 2 μg of total RNA by using the SuperScript preamplification system for first-strand cDNA synthesis according to the manufacturer's instructions (Invitrogen). Real-time quantitative PCR was performed on cDNA samples using the Roche LightCycler system (Roche Diagnostics, Indianapolis, IN). Primer pairs for Oatp1b2, Oatp1b2for, and Oatp1b2rev (Table 1) generated an amplified product of 150 base pairs. Primer pairs for 18S, 18Sfor, and 18Srev (Table 1) generated an amplified product of 150 base pairs. Individual glass capillaries were filled with a reaction mixture containing 2 μl of cDNA template, 1.6 μl of $MgCl_2$ (25 μM), 1.2 μl of specific primer pairs (10 μM each), 2 μl of SYBR Green Master solution, and 13.2 μl of distilled water. After a 5-min denaturation at 95°C, the amplification of Oatp1b2 cDNA was performed for 40 to 50 cycles according to the following steps: denaturation at 95°C (0 s), annealing at 54°C (15 s), and elongation at 72°C (15 s). The amplification of 18S cDNA was performed according to the following steps: denaturation at 95°C (0 s), annealing at 55°C (30 s), and elongation at 72°C (30 s). After each elongation step, the SYBR Green fluorescence was measured at 72°C. A no-template control was included in each real-time PCR and showed no amplification. At the end of the PCR, a melting-curve analysis was performed by gradually increasing the temperature from 72 to 95°C (0.1°C/s). At the end of some experiments, PCR products were removed from capillaries and analyzed by gel electrophoresis to confirm the generation of the product of interest. After completion of PCR, the SYBR green fluorescent signal was analyzed and converted into a relative number of copies of target molecules. The relative amounts of Oatp1b2 or 18S mRNA in each sample were calculated by using standard curves established from a series of successive dilutions of cDNA from normal rat liver. Oatp1b2 quantitative results were normalized to the amount of 18S RNA in each sample to minimize sample-to-sample variations that may have occurred during RNA extraction and quantification.

Cell Culture and Transient Transfections. HepG2 cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) medium, supplemented with 10% charcoal-stripped FBS (Hyclone Laboratories, Logan, UT), 3.58 mM glutamine, 55 μg/ml gentamicin, and 1 μg/ml insulin (Invitrogen). A day before transfection, cells were subcultured into 100-mm plates in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped FBS, glutamine, and gentamicin. Transfections were performed using Lipofectin reagent (Invitrogen) following the manufacturer's instructions. For luciferase assays, cells were transfected with 5 μg of luciferase constructs, LUC-303, LUC-303mut, LUC-438, or LUC-438mut, 5 μg of pXM-Stat5a, and 1 μg of the indicated receptors cDNA (pL3-$PRLR_L$ or pcDNA-rGHR). pRSV-βgal was included as a control to monitor transfection efficiency, and pUC19 was added as a carrier, to a total of 20 μg of DNA/100-mm plate. Five to six hours after transfection, the medium was removed and the cells were washed twice with PBS and replated in a 96-well plate. The cells were treated with medium alone or medium with the indicated concentrations of ligands (oPRL or rGH). In some experiments examining LUC-438 responsiveness, rat FXR/BAR (4 μg) was also contransfected, and chenodeoxycholic acid (100 μM) and 9-*cis*-retinoic acid (1 μM) were added along with oPRL to the medium.

For DNA binding assays, cells were transfected with 5 μg of pXM-Stat5a and 1 μg of pL3-$PRLR_L$. pUC19 was added as a carrier to a total of 20 μg of DNA/100-mm plate. Five to six hours after transfection, the medium was removed and the cells were washed twice with PBS. Fresh medium was added to the cells, and the cells were incubated for an additional 36 to 48 h. The cells were then treated with medium alone or medium with oPRL at the indicated concentrations for 1 h. After a single PBS wash, nuclear extracts were isolated using the NucBuster protein extraction kit (Novagen, Darmstadt, Germany), according to the manufacturer's instructions.

Luciferase and β-Galactosidase Reporter Assays. After incubation with the indicated hormones for 36 to 48 h, cells were washed with PBS and lysed with 50 μl of lysis buffer for 20 min at room temperature. The cell extract (20 μl) was combined with 100 μl of K-ATP/$MgCl_2$ buffer and assayed for luciferase activity (Microlumat LB 96P; PerkinElmer Life and Analytical Sciences, Boston, MA). The remaining cell extract (30 μl) was mixed with 200 μl of *o*-nitrophenyl β-galactopyranoside substrate solution and analyzed for β-galactosidase activity at 415 nm on an enzyme-linked immunosorbent assay plate reader (Bio-Rad Laboratories, Hercules, CA). The composition of the solutions and buffers used were as reported previously (Ganguly et al., 1997). The normalized luciferase activity was calculated as relative light units/β-galactosidase activity (absorbance at 415 nm)/min. Each ligand treatment was conducted in duplicate at minimum, and the mean was calculated for each data point. The hormone-dependent fold induction (relative to the no-hormone exposure as a control) is represented as mean ± S.E.M. from three to five independent transfections.

DNA Binding Assays. A double-stranded oligonucleotide probe containing the Stat5 sequence corresponding to the sequence found in the OATP1B3 gene promoter −177 to −157 (5′-gtgtacattct-

gagaaaattat-3′) was obtained by hybridizing single-stranded oligonucleotides (IDT, Coralville, IA). The probe was labeled with [^{32}P]ATP by using T4 polynucleotide kinase (Promega, Madison, WI) and purified using the Nucleotide Extraction Kit (QIAGEN, Valencia, CA). For DNA binding assays, 15 μg of nuclear extracts was incubated at room temperature for 30 min with ~100,000 cpm of radiolabeled probe in a 25-μl reaction containing 25 mM MOPS, 1 mM EDTA, 0.02% NaN_3, 10% glycerol, 0.8 M KCl, and 100 ng/μl salmon sperm DNA. In competition studies, 20- and 100-fold molar excess of unlabeled probe, oligomer containing the bovine β-casein Stat5 consensus binding motif (5′-agatttctaggaattcaatcc-3′), or mutant Stat5 oligomer (5′-agattt**agttt**aattcaatcc-3′) was added to the binding reaction. Free and protein-bound probes were separated on a 4% polyacrylamide gel in 0.5× Tris borate-EDTA buffer at 140 V for 2.5 h. The gel was dried and exposed to X-ray film at −80°C for ~3 days.

Statistical Analysis. Data are expressed as mean ± S.E.M. Statistical analysis was performed using one-way analysis of variance followed by the Bonferroni test. Values of $P < 0.05$ were considered statistically significant.

Results

PRL and GH Increase mRNA Expression of Oatp1b2 in Primary Rat Hepatocytes. We examined whether PRL or GH was able to induce Oatp1b2 gene expression in primary hepatocyte cultures from female and male rats. There was no difference in basal (untreated) expression levels of Oatp1b2 in female and male rats (data not shown). Oatp1b2 mRNA expression in female and male rats was increased after treatment with PRL and GH relative to nontreated controls (Fig. 1, A and B). In particular, in primary hepatocytes from female rats, 1.0 μg of oPRL and 0.1 μg/ml rGH maximally induced Oatp1b2 mRNA expression by 1.9- and 2.2-fold, respectively ($P < 0.05$). In primary hepatocytes from male rats, 1.0 μg of oPRL and 10 μg/ml rGH maximally induced Oatp1b2 mRNA expression by 2.1- and 2.3-fold, respectively ($P < 0.05$). To determine whether treatment with PRL increased the expression of Oatp1b2 protein, cells cul-

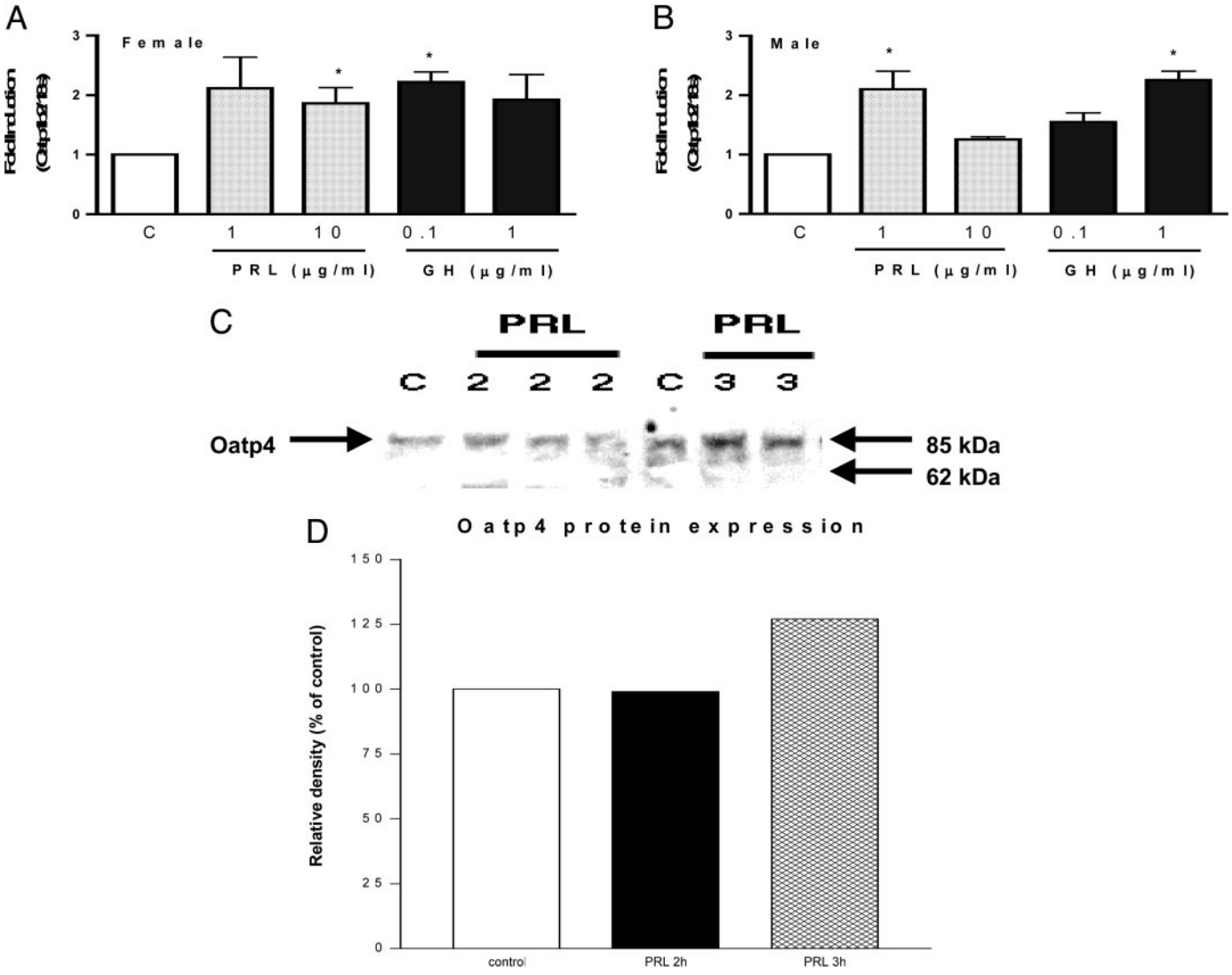

Fig. 1. Oatp1b2 expression is increased in rat primary hepatocyte cultures after treatment with PRL or GH. Total liver RNA isolated from control or hormone-treated hepatocyte cultures was reverse-transcribed to cDNA, and the relative amounts of mRNA coding for Oatp1b2 were determined by real-time PCR, as described under *Materials and Methods*. Oatp1b2 mRNA in hepatocyte cultures isolated from female (A) and male (B) rats was quantified by analyzing real-time PCR data using a simultaneously produced standard curve. Data were normalized to the amount of 18S present in the same sample. Data are expressed as mean ± S.E. of three to five preparations each in female and male rat. *, $P < 0.05$ versus control. C, immunoblot analysis of Oatp1b2 protein expression in female rat hepatocytes treated with PRL (1 μg/ml) for 2 and 3 h in culture. D, densitometric analysis of data shown in C.

tured for 2, 3, 4, or 6 h were examined using a specific Oatp1b2 antibody. A small (~25%) but consistent increase in Oatp1b2 protein expression was seen 3 h after treatment with PRL (Fig. 1, C and D), indicative of PRL-mediated increased expression. The protein was rapidly degraded in both control and PRL-treated hepatocytes, however, and had begun to degrade by 4 h; no Oatp1b2 protein was detected when cells were incubated for 6 h (data not shown).

PRL- and GH-Dependent Transcriptional Activation of the Oatp1b2 Promoter in HepG2 Cells. To determine whether the Oatp1b2 promoter is PRL- or GH-responsive, HepG2 cells were transiently transfected with the Oatp1b2 plasmid LUC-303 and with expression vectors containing cDNAs for the $PRLR_L$ or GHR and Stat5. The addition of oPRL or rGH increased luciferase activity in a dose-dependent manner when LUC-303 was cotransfected with both the $PRLR_L$ or rGHR and Stat5 expression vectors (Fig. 2, A and B). LUC-303 promoter activity was maximally induced by 5 μg/ml oPRL (1.7-fold) and 0.5 μg/ml rGH (1.9-fold). Exclusion of either $PRLR_L$, GHR, or Stat5 expression vectors abrogated PRL and GH induction (Fig. 2, A and B).

To further determine the importance of the Stat5 binding site for the PRL- and GH-dependent activation of the rat Oatp1b2 gene, cotransfection experiments were carried out using the Stat5 mutant construct LUC-303mut. The construct LUC-303mut failed to respond to oPRL and rGH treatment compared with the plasmid LUC-303 (Fig. 2, C and D).

PRL- and GH-Dependent Transcriptional Activation of the Human OATP1B3 Promoter in HepG2 Cells. To determine whether the human OATP1B3 promoter is PRL- or GH-responsive, HepG2 cells were transiently transfected with the plasmid LUC-438 and with expression vectors containing cDNAs for the $PRLR_L$ or GHR and Stat5. The addition of oPRL or rGH increased luciferase activity in a dose-dependent manner when LUC-438 was cotransfected with both the $PRLR_L$ or rGHR and Stat5 expression vectors (Fig. 3, A and B). LUC-438 promoter activity was maximally induced by 0.5 μg/ml oPRL (3.5-fold) and 1.0 μg/ml rGH (2.1-fold). Exclusion of either $PRLR_L$, GHR, or Stat5 expression vectors abrogated PRL and GH induction (Fig. 3, A and B). When cells were also cotransfected with FXR/BAR, and when chenodeoxycholic acid and 9-*cis*-retinoic acid were added to the media, no further increase in promoter activity was detected (data not shown).

To further determine the importance of the Stat5 binding site for the PRL- and GH-dependent activation of the human

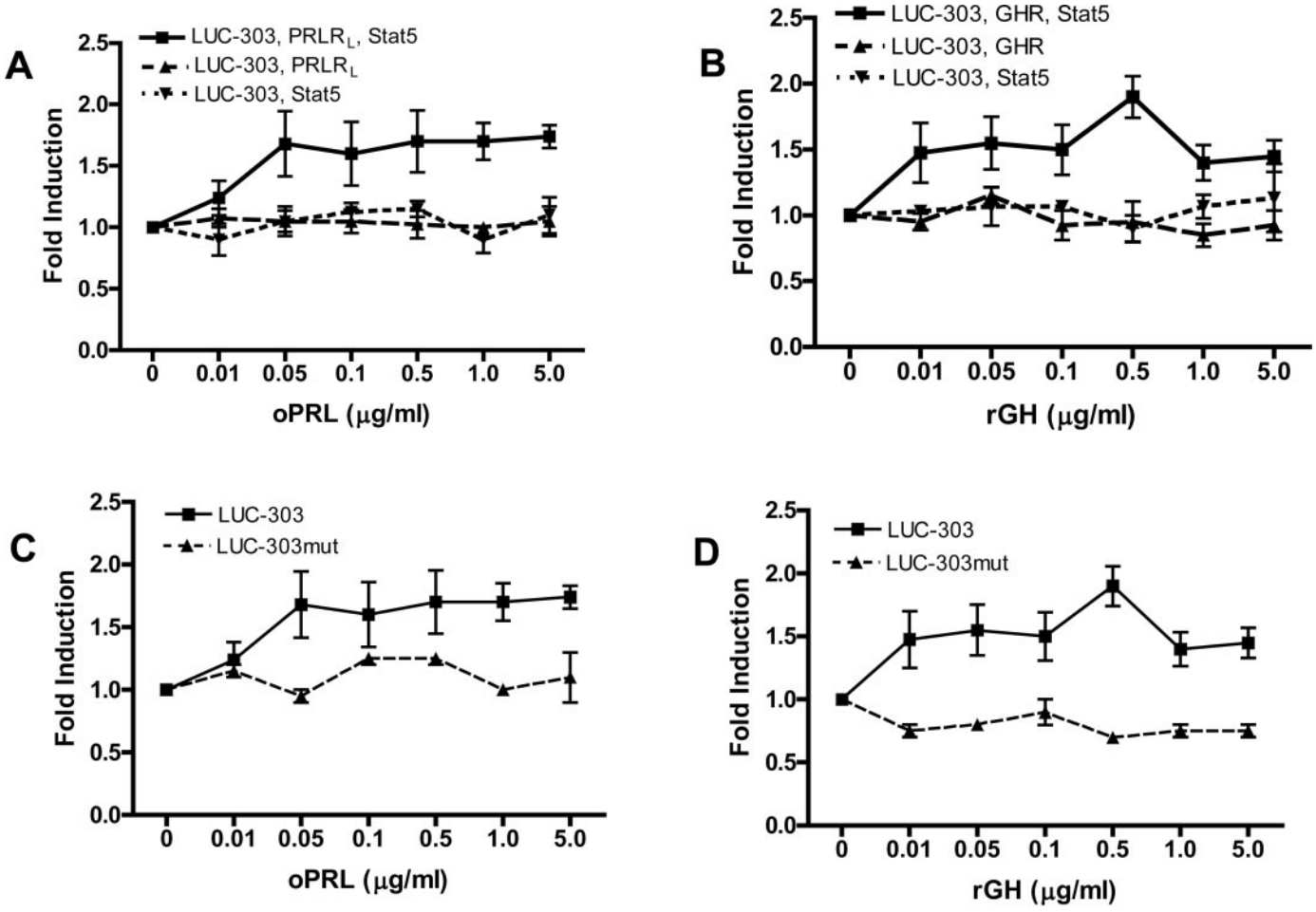

Fig. 2. A and B, hormone receptors ($PRLR_L$ or GHR) and Stat5 mediate PRL and GH inducibility of the Oatp1b2 promoter. HepG2 cells were cotransfected with a luciferase reporter for the Oatp1b2 promoter (LUC-303), pRSV-β-galactosidase, and expression vectors for $PRLR_L$, GHR, and Stat5 as indicated. C and D, the Stat5 binding site confers PRL and GH responsiveness to the Oatp1b2 promoter. HepG2 cells were cotransfected with LUC-303 or LUC-303mut, which contains a mutated Stat5 binding site, pRSV-β-galactosidase, and expression vectors for $PRLR_L$ or GHR and Stat5. oPRL and rGH dose-response curves were generated as described under *Materials and Methods* for all experiments. All data represent mean ± S.E. of at least three independent experiments.

OATP1B3 gene, cotransfection experiments were carried out using the Stat5 mutant construct LUC-438mut. The construct LUC-438mut failed to respond to oPRL and rGH treatment compared with the plasmid LUC-438 (Fig. 3, C and D).

PRL Treatment Leads to the Binding of Stat5 to the OATP1B3 Promoter in Transfected HepG2 Cells. A Stat5 binding site located at −170 to −162 (5′-ttctgagaa-3′) was identified in the human OATP1B3 promoter by a sequence homology search. DNA binding assays were performed to determine whether PRL was able to enhance DNA binding activity of nuclear proteins to a 21-oligomer containing the OATP1B3 Stat5 binding sequence. HepG2 cells were transfected with expression vectors for Stat5 and $PRLR_L$ and treated with oPRL (0.1 or 1.0 μg/ml) or blank medium. As shown in Fig. 4, a weak DNA-protein complex was detected in untreated HepG2 cell nuclear extract (lane 1). Stimulation of HepG2 cells with oPRL increased the DNA-protein complex in a dose-dependent manner (lanes 2 and 3). The formation of this complex was abolished by a 20- and 100-fold molar excess of unlabeled OATP1B3 oligomers (lanes 4 and 5), and consensus Stat5 oligomers (lanes 6 and 7), but not by mutant Stat5 oligomers (lanes 8 and 9), thus verifying the specificity of the binding.

Discussion

The present study provides clear evidence that PRL and GH can activate transcription of the Oatp1b2 and OATP1B3 genes. We have shown previously that PRL and GH increase the expression of *Ntcp* mRNA in freshly isolated rat hepatocyte cultures (Cao et al., 2001a). Therefore, we examined whether PRL or GH would increase the expression of *Oatp1b2* mRNA in rat hepatocyte cultures. The present studies show the increased expression of the organic anion transporting polypeptide Oatp1b2 in cultures of primary rat hepatocytes from both female and male rats, aged 4 weeks, within 2 h after treatment with oPRL and rGH (Fig. 1, B and C). There were no differences in basal (untreated) expression levels of Oatp1b2 in 4-week-old female and male rats (data not shown). These data are in agreement with Li et al. (2002) who also report no gender differences in Oatp1b2 expression during postnatal development. Within 1 h after treatment, oPRL and rGH also activated Stat5 protein expression in a dose-dependent manner in both female and male hepatocyte preparations (data not shown).

The binding of PRL and GH to their respective receptors has been shown to activate Stat proteins, including Stat1, -3,

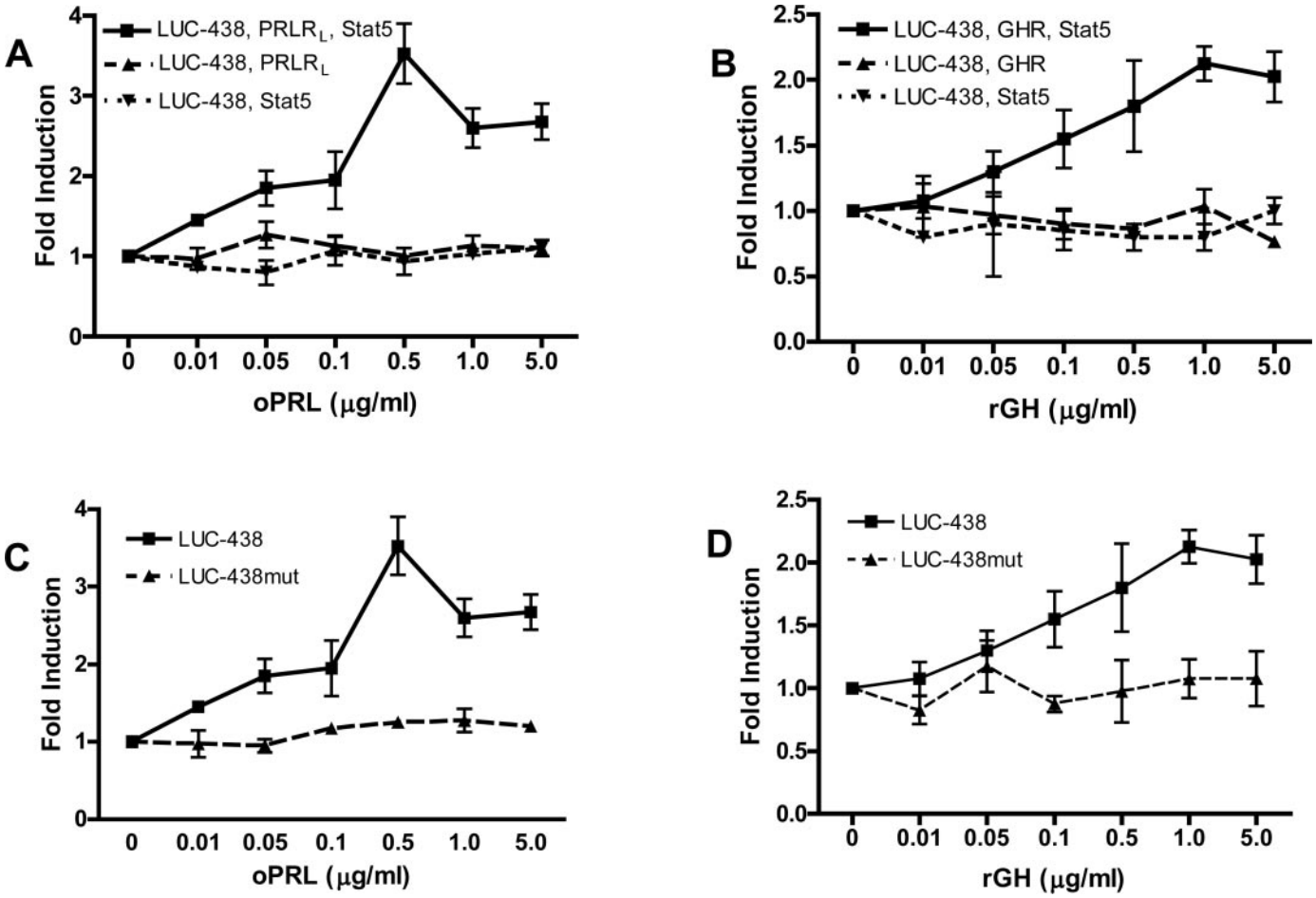

Fig. 3. A and B, Hormone receptors ($PRLR_L$ or GHR) and Stat5 mediate PRL and GH inducibility of the OATP1B3 promoter. HepG2 cells were cotransfected with a luciferase reporter for the OATP1B3 promoter (LUC-438), pRSV-β-galactosidase, and expression vectors for $PRLR_L$, GHR, and Stat5 as indicated. C and D, the Stat5 binding site confers PRL and GH responsiveness to the OATP1B3 promoter. HepG2 cells were cotransfected with LUC-438 or LUC-438mut, which contains a mutated Stat5 binding site, pRSV-β-galactosidase, and expression vectors for $PRLR_L$ or GHR and Stat5. oPRL and rGH dose-response curves were generated as described under *Materials and Methods* for all experiments. All data represent mean ± S.E. of at least three independent experiments.

and -5 (DaSilva et al., 1996; Ram et al., 1996). Transcriptional regulation by these activated Stat proteins is a key event in PRL and GH hormonal signaling. We used transiently transfected HepG2 cells to identify the mechanism by which *Ntcp* expression is induced by PRL in vivo and in cultures of primary hepatocytes, namely, the activation of Stat5 via the $PRLR_L$ (Ganguly et al., 1997; Cao et al., 2001a). We used this same approach to demonstrate that GH increases *Ntcp* expression by the activation of Stat5 via the GHR (Cao et al., 2001a). Having identified a Stat5 binding site in the *Oatp1b2* promoter located at −209 to −201 and a similar conserved Stat5 binding site in the *OATP1B3* promoter at −170 to −162, we used transiently transfected HepG2 cells to further examine whether PRL and GH upregulate the expression of *Oatp1b2*/*OATP1B3* via the Stat5 signal-transduction pathway. Dose-dependent PRL- or GH-induced increases in *Oatp1b2* and *OATP1B3* promoter activity were observed when expression vectors for Stat5 and $PRLR_L$ or GHR cDNA were cotransfected with *Oatp1b2* and *OATP1B3* promoter-luciferase reporter constructs (Fig. 2, A and B; Fig. 3, A and B). When either Stat5 or $PRLR_L$ cDNA was omitted, PRL- and GH-induced *Oatp1b2* and *OATP1B3* promoter activation was abrogated (Fig. 2, A and B; Fig. 3, A and B). This reinforces earlier observations from this laboratory (Ganguly et al., 1997; Cao et al., 2001a) that PRL- and GH-dependent promoter activation is a receptor-mediated event that requires the Stat5 protein. We also examined the possibility that the addition of chenodeoxycholic acid, a ligand for FXR/BAR, in the presence of cotransfected FXR/BAR would synergistically increase promoter activity. However, we were unable to detect an increased response under conditions optimized for responsiveness to PRL and GHR. Further studies are needed to determine whether these ligands might have important interactions under physiological conditions.

DNA binding assays showed that Stat5 in nuclear extracts from HepG2 cells that had been transfected with cDNA for Stat5 and $PRLR_L$ demonstrated increased binding to an oligomer containing the Stat5 binding site (−209 to −201) of the OATP1B3 promoter after treatment with increasing concentrations of oPRL (Fig. 4, lanes 1–3). Competition assays further confirmed the specificity of the Stat5 protein/Stat5 binding site interaction in that excess unlabeled *OATP1B3* (Fig. 4, lanes 4 and 5) and consensus Stat5 oligomers (Fig. 4, lanes 6 and 7), but not mutant Stat5 oligomer (Fig. 4, lanes 8 and 9), significantly inhibited binding. In further studies, however, incubation with specific anti-Stat5 antibody was unable to supershift the Stat5 protein/OATP1B3 complex under a variety of conditions. Instead, the DNA/protein complex formed a band at the same position that was significantly darker (data not shown) than the band formed by treatment with 1.0 μg/ml oPRL (Fig. 4, lane 3). It is unknown why the anti-Stat5 antibody was unable to supershift the DNA/protein complex.

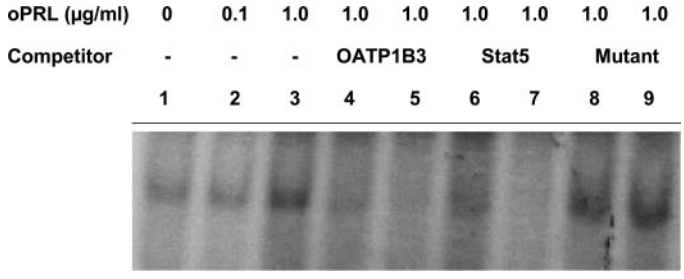

Fig. 4. PRL increases the DNA binding activity of Stat5a to the OATP1B3 promoter. HepG2 cells were transfected with expression vectors for $PRLR_L$ (1 μg) and Stat5a (5 μg) and were treated with the indicated concentrations of oPRL for 1 h. ^{32}P-labeled OATP1B3 promoter oligomer containing the Stat5 binding site was used as a probe. Labeled oligomer was incubated with nuclear extracts (15 μg) from untreated (lane 1) or treated (lanes 2 and 3) samples. A 20- and 100-fold molar excess of OATP1B3 (lanes 4 and 5), consensus Stat5 (lanes 6 and 7), and mutant Stat5 (lanes 8 and 9) were used in competition assays.

To provide further evidence that Stat5 is necessary for PRL- and GH-mediated activation of the *OATP1B3* promoter, we created a luciferase reporter construct, LUC-438mut, identical with the OATP1B3 LUC-438 plasmid but containing a mutated Stat5 binding site. We also generated a construct identical with Oatp1b2 LUC-303 but containing a mutated Stat5 binding site, LUC-303mut. When HepG2 cells were cotransfected with LUC-438mut or LUC-303mut and expression vectors for Stat5 and $PRLR_L$ or GHR cDNA, PRL and GH were unable to induce promoter activity (Fig. 2, C and D; Fig. 3, C and D). Taken together, these data provide strong evidence that the Stat5 binding sites in the *OATP1B3* and *Oatp1b2* promoters function in PRL- and GH-induced transcriptional regulation of these genes.

This study is the first to report the hormonal regulation of organic anion transporting polypeptides through the Stat5 signal-transduction pathway. Mesotten et al. (2003) infused a high dose of GH in male Sprague-Dawley rats over 4 days (250 μg/24 h), followed by administration of a sublethal dose of lipopolysacharide or saline on the fourth day. *Oatp1a4* mRNA levels were significantly decreased by GH alone at 6 and 12 h after saline administration. In contrast, *Oatp1b2* mRNA levels were significantly increased by GH alone to 160% of that in saline control animals 12 h after saline administration on the fourth day of GH administration. Although these studies did not examine Stat5 expression status after GH administration, they do provide evidence supporting the differential regulation of organic anion transporting polypeptides by GH. Previous studies from our laboratory showed that rat *Oatp1a4* mRNA was increased postpartum, although changes in protein were not significant. Oatp1a1 (formerly Oatp1) exhibited no change at the mRNA and protein levels postpartum (Cao et al., 2001b). These data again provide evidence of differential regulation of Oatps during the postpartum period, a time of marked increases in plasma PRL levels. The physiological significance of *Oatp1b2* and *OATP1B3* gene induction by Stat5 and PRL/GH is currently speculative. We have provided strong evidence that PRL and GH induce *Oatp1b2* and *OATP1B3* promoter activity via the activation of Stat5 in transient cotransfection studies. However, mechanisms obtained from cotransfection studies, in which receptors and their transducer proteins are overexpressed, cannot alone ensure the occurrence of similar mechanisms in vivo or under normal physiological conditions. Studies in freshly isolated hepatocytes provided the opportunity to study PRL and GH induction of *Oatp1b2* mRNA under near-normal physiological conditions. Modest increases in Oatp1b2 protein were detected after treatment with PRL, but these were not sustained, most likely because of the rapid degradation of the protein in culture. It remains to be seen whether protein levels of Oatp1b2 and transport of Oatp1b2 substrates are increased in vivo after PRL or GH treatment.

Studies are underway in our laboratory to characterize the expression of Oatp1b2 in postpartum lactating rats.

There are important physiological and pharmacological implications of the regulation of human OATP expression. A number of genetic polymorphisms in human liver *OATP*s have been identified in certain populations (Tirona and Kim, 2002). Although the in vivo significance of these polymorphisms is not known, functional polymorphisms in *OATP1B3*, for example, would be expected to alter the drug disposition of digoxin, because OATP1B3 is the only human OATP known to mediate its transport (Kullak-Ublick et al., 2001). In addition, rifamycin is an inhibitor of transport by all human liver OATPs, and rifampicin is an inhibitor of OATP1B1 and OATP1B3-mediated transport. Hagenbuch and Meier (2003) have suggested that coadministration of OATP inhibitors could increase bioavailability of other drugs (OATP substrates) with high first-pass clearance; conversely, the induction of OATP by rifampicin via the pregnane X receptor or by the present hormones could decrease bioavailability by increasing first-pass clearance. Examination of OATP1B3 expression, combined with examination of the transport of OATP1B3-specific substrates in patients with hypo- or hyperpituitarism or in patients treated with GH, could provide important human data on the effects of hormones on transporter expression and function. Finally, although OATP1B3 is predominately liver-specific under normal physiological conditions, OATP1B3 is also expressed in various human cancer tissues at levels significantly higher than those in liver (Abe et al., 2001). The pathophysiological significance of OATP1B3 expression in human cancer tissues is unknown, but introduction of the *OATP1B3* gene into mammalian cells potentiates sensitivity to methotrexate (Abe et al., 2001). The possibility that OATP1B3 may be induced by hormone treatment could be exploited to further increase the expression of OATP1B3 in cancer tissues and increase the delivery of chemotherapeutic methotrexate as a result.

In summary, the present data provide evidence for the transcriptional regulation of the rat *Oatp1b2* and human *OATP1B3* genes by the Stat5 signal-transduction pathway and for their activation by PRL and GH. Oatp1b2 and OATP1B3 are the first known organic anion transporting polypeptides identified as targets of Stat5-mediated induction. Hormonal induction of Oatp/OATP expression may provide an important mechanism for the regulation of transport and metabolism of organic anion substrates.

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