

# Role of Liver-Enriched Transcription Factors in the Down-Regulation of Organic Anion Transporting Polypeptide 4 (Oatp4; Oatplb2; *Slc21a10*) by Lipopolysaccharide

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## ABSTRACT

Lipopolysaccharide (LPS) administration is a model of cholestasis. Organic anion transporting polypeptide 4 (Oatp4; *Slc21a10*) is almost exclusively expressed in liver. Therefore, it was hypothesized that LPS would down-regulate mouse Oatp4 and that this action is due to a decrease in nuclear binding activity of one or more liver-enriched transcription factors to mouse *Oatp4* promoter. The present study indicates a time-dependent decrease in mouse Oatp4 mRNA levels by LPS. Moreover, LPS produced a rapid and profound decrease in nuclear binding activity to the mouse *Oatp4* putative response elements for hepatocyte nuclear factor (HNF) 1, CAAT/enhancer binding protein (C/EBP), HNF3, and heterodimers of retinoid X receptor (RXR) and retinoic acid receptor (RAR). Maximal decrease in nuclear binding activity to these response elements preceded a significant reduction of Oatp4 mRNA

levels. HNF1 $\alpha$  bound to the *Oatp4* HNF1 response element as a homodimer. Multiple copies of the *Oatp4* HNF1 $\alpha$  response element, inserted upstream of a minimal promoter, were sufficient to mediate reporter activity and responded to the coexpression of HNF1 $\alpha$  in mouse hepatoma cells. Moreover, HNF1 $\alpha$  dose dependently activated the *Oatp4* promoter (–4.8 kilobases to +30 bp). Therefore, HNF1 $\alpha$  is a potent *trans*-activator of the mouse *Oatp4* promoter. In addition, Oatp4 mRNA levels were markedly decreased (95%) in HNF1 $\alpha$ -null mice as compared with wild-type mice, suggesting that HNF1 $\alpha$  levels are critical for the constitutive expression of the Oatp4 gene. Taken together, these findings suggest that the LPS-induced down-regulation of Oatp4 is likely due to reduction in the binding of HNF1 $\alpha$ , C/EBP, HNF3, and RXR:RAR to the *Oatp4* promoter.

Maintenance of bile flow and elimination of endo- and xenobiotics are facilitated by the coordinated regulation of hepatic transport proteins at the sinusoidal and canalicular membranes of hepatocytes. Some transport proteins are selectively or preferentially synthesized in liver. Expression of these transporters varies in response to a number of stimuli, such as the stage of liver development, or various pathophysiologic conditions, such as sepsis. Gene expression in liver is largely determined at the level of transcription initiation (Tavoloni and Berk, 1993).

Numerous genes are transcribed at much higher rates in hepatocytes than in other cell types, whereas “housekeeping” genes are transcribed at similar rates in many cell types (Powell et al., 1984). For example, the transcription rate of albumin is 1000-fold higher in liver than in other tissues (Powell et al., 1984; Liu et al., 1988). The high degree of liver-specific transcription is conferred by its regulatory se-

quence (Ott et al., 1984; Pinkert et al., 1987). The regulatory sequence of albumin contains response elements for several liver-enriched transcription factors involved in liver-specific expression, such as CAAT/enhancer binding protein (C/EBP), C/EBP-related protein, hepatocyte nuclear factor (HNF) 1, and HNF3 (Cereghini et al., 1987; Lichtsteiner et al., 1987; Herbst et al., 1989; Liu et al., 1991). Na<sup>+</sup>/taurocholate-cotransporting polypeptide (Ntcp; *Slc10a1*) is another specifically liver-expressed gene. Ntcp is regulated by HNF1 $\alpha$ , C/EBP, and heterodimers of retinoid X receptor (RXR)  $\alpha$  and retinoic acid receptor (RAR)  $\alpha$  (Trauner et al., 1998; Denson et al., 2000). It is widely accepted that activation domains of these liver-enriched transcription factors directly facilitate the assembly of ubiquitous transcription factors and RNA polymerase II onto the promoter (Ptashne and Gann, 1990). These transcription factors function in unique combinations to synergistically stimulate hepatocyte-specific transcription.

Organic anion-transporting polypeptide 4 (Oatp4; *Slc21a10*) is expressed almost exclusively in liver of rats

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**ABBREVIATIONS:** bDNA, branched DNA; C/EBP, CAAT/enhancer binding protein; HNF, hepatocyte nuclear factor; LPS, lipopolysaccharide; Oatp, organic anion transporting polypeptide; RAR, retinoic acid receptor; RXR, retinoid X receptor; Ntcp, Na<sup>+</sup>/taurocholate-cotransporting polypeptide; ATCC, American Type Culture Collection; nt, nucleotide; kb, kilobase(s); bp, base pair(s); RLU, relative light unit(s); EMSA, electrophoretic mobility shift assay.

The purpose of the present study was to determine whether LPS treatment decreases *Oatp4* mRNA levels and, if so, whether LPS decreases the binding activity of one or more of the liver-enriched transcription factors (HNF1 $\alpha$ , C/EBP, HNF3, and RXR:RAR) to the mouse *Oatp4* promoter. The present study further characterized the HNF1 isoform bound to the mouse *Oatp4* HNF1 response element. Moreover, the effect of HNF1 $\alpha$  on an intact or heterologous promoter, as well as constitutive expression of the mouse *Oatp4* gene, was investigated in the present studies.

**Materials.** DNA restriction enzymes, calf alkaline intestinal phosphatase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). PfuUltra HF DNA polymerase was obtained from Stratagene (La Jolla, CA). [ $\gamma$ - $^{32}\text{P}$ ]Adenosine 5'-triphosphate (6000 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). MiniQuick spin oligo columns were obtained from Roche Applied Science (Indianapolis, IN). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Cell culture reagents, medium, and fetal bovine serum were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). *N,N*'-Methylene-bis-acrylamide (2%), acrylamide (40%), lipopolysaccharide (serotype *Escherichia coli* 0111:B4), and protease inhibitors were purchased from Sigma-Aldrich, St. Louis, MO). Routine chemicals and reagents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

**Animals and Treatments.** Male C3H/OuJ mice (20–30 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed according to the American Animal Associations Laboratory Animal

**RNA Isolation.** Total RNA was isolated from mouse liver using RNazol B reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. The concentration of total RNA in each sample was determined spectrophotometrically at 260 nm. The integrity of each RNA sample was evaluated by formaldehyde-agarose gel electrophoresis before analysis.

**Preparation of Nuclear Extracts.** Mouse liver nuclear extracts were prepared by the method of Ganguly et al. (1997). Briefly, protease inhibitors antipain, pepstatin, and chymotrypsin inhibitor (2  $\mu\text{g/ml}$  each), leupeptin and aprotinin (5  $\mu\text{g/ml}$  each), trypsin inhibitor (10  $\mu\text{g/ml}$ ), and phenylmethylsulfonyl fluoride (0.1 mM), as well as phosphatase inhibitors NaF (10 mM) and  $\text{Na}_3\text{VO}_4$  (1 mM) were constituents of the homogenization buffer. After extraction in the buffer [10 mM Hepes (pH 7.6), 400 mM KCl, 1 mM EDTA, 1 mM  $\text{Na}_3\text{VO}_4$ , 10% glycerol, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride], the precipitate formed during dialysis was removed by centrifugation at 10,000g for 5 min, after which the extracts were aliquoted, snap frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Protein concentration was determined by the BCA protein assay obtained from Pierce Chemical (Rockford, IL).

**Electrophoretic Mobility Shift Assays.** The DNA sequence of the sense strand of each oligonucleotide is listed in Table 1. Oligonucleotides representing putative response elements of the mouse *Oatp4* promoter for several *trans*-activators, namely HNF1 (nt -71 to -49), C/EBP (nt -317 to -298), and RXR:RAR (nt -717 to -691), were designed based on computer analysis ([www.gene-regulation.com](http://www.gene-regulation.com)), and HNF3 (nt -146 to -126) based on a previous report (Ogura et al., 2000). Nuclear extracts (5–20  $\mu$ g) were incubated with 2  $\mu$ g of poly(dI-dC):poly(dI-dC) and 2 to 5  $\times 10^4$  cpm of  $^{32}$ P-end-labeled oligonucleotides in the binding reaction mixtures. The binding reaction was performed on ice for 30 min, and then the entire sample was electrophoresed through a nondenaturing 4% polyacrylamide gel in Tris-glycine buffer at 12 V/cm at 4°C. Competition for binding specificity was performed using an appropriate amount of unlabeled specific oligonucleotides in the binding mixtures, along with the labeled oligonucleotides. For supershift experiments, nuclear extracts were preincubated with antibodies specific to HNF1 $\alpha$  or HNF1 $\beta$  (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) on ice for 2 h, and the binding reaction was performed as described above.

**Plasmid Construct.** A 15-kb KpnI fragment (−4.8 kb to +10.2 kb) containing 4.8 kb of the 5′ flanking region and 10.2 kb of the downstream sequence of the mouse *Oatp4* gene was cloned by Dr. Kenichiro Ogura (Department of Drug Metabolism and Molecular Toxicology, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan). Preparation of the 1.4 kb of the 5′ flanking region (nt −1424 to +30) entailed amplification of the fragment by polymerase chain reaction, using the downstream primer 5′-CTGAACCCGGGCTTC-TACAACAAGTGTGGC-3′ engineered with an SmaI site, and the upstream primer 5′-GGATCACAGGGCCCCCAATTGAGG-3′ containing an ApaI site (nt −1423 to −1418). A reporter construct, p4.8kbOatp4-Luc (nt −4.8 to +30) was created by ligating the PCR-



Name	Sequence (5' → 3')
HNF3 (nt -146 to -126)	CTAGATCTTTGTTTGCTTCTT
RXR:RAR (nt -717 to -691)	ATCTCTGACCTCGAGCTGACTACAGA
C/EBP (nt -317 to -298)	ATACTAATGTGCAACCAATA
C/EBP (consensus)	TGCAGATTGCGCAATCTGCA
HNF1 (nt -71 to -49)	AGATAGTTAATCATCACTGAACT
HNF1 tandem	TCGAGAAGAGATAGTTAATCATCACTGAACTG

amplified fragment (ApaI-SmaI), the double digested fragment (KpnI-ApaI), and the luciferase vector pGL3-Basic (Promega, Madison, WI). Three or five copies of the HNF1 response element (32 bp) were inserted into a luciferase vector (pLuc-MCS) containing a minimal promoter (Stratagene) to create p3xHNF1-Luc and p5xHNF1-Luc, respectively. The mouse HNF1 $\alpha$  expression plasmid (pBJ5-HNF1 $\alpha$ ) was kindly provided by Dr. Gerald Crabtree (Stanford University School of Medicine). Host strain XL1-Blue competent bacterial cells (Stratagene) were used to propagate all plasmids. QIAGEN endotoxin-free Maxiprep kits (QIAGEN, Valencia, CA) were used to prepare plasmids for transient transfections. All plasmids were analyzed by restriction enzyme digestion, and DNA sequencing was performed at the Biotechnology Support Facility of the University of Kansas Medical Center. The 4.8-kb 5' flanking region was further confirmed by alignment with the mouse genomic database (www.ensembl.org).

**Cell Culture and Transient Transfections.** Mouse hepatoma HEPA1-6 cells were purchased from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% fetal bovine serum (ATCC). Cells were seeded at 85 to 90% density in 24-well plates. To normalize for transfection efficiency, pRL-TK plasmid (Promega) coding *Renilla* luciferase under the control of a thymidine kinase promoter was cotransfected. For transient transfections, a DNA/lipid mix containing 3  $\mu$ l of LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and 1  $\mu$ g of plasmid DNA per well was used. For cotransfection of various amounts of pBJ5-HNF1 $\alpha$ , the pBJ5 vector was used as carrier DNA. Cells were lysed with passive lysis buffer (Promega) 24 to 48 h after transfection. Luciferase activities were analyzed using a Dual Luciferase Reporter System (Promega) and were quantified in a Lumat LB 9507-2 luminometer (Berthold Technologies, Bad Wildbad, Germany).

**Branched DNA (bDNA) Assays.** Oatp4 mRNA levels in liver were determined by the quantitative branched DNA signal amplification assay (QuantiGene bDNA Signal Amplification Kit; Bayer Corp.-Diagnostics Div., Tarrytown, NY) (Hartley and Klaassen, 2000). Mouse Oatp4 gene sequence was accessed from GenBank (accession number AB031959). A multiple oligonucleotide probe set (capture, label, and blocker probes) specific to the mouse Oatp4 transcript (Table 2) was designed using ProbeDesigner software, version 1.0 (Bayer Corp.-Diagnostics Div.). Each probe developed in ProbeDesigner was submitted to the National Center for Biotechnology Information (Bethesda, MD) for nucleotide comparison by the basic local alignment search tool, to ensure minimal cross-reactivity with other known mouse sequences and expressed sequence tags. Any oligonucleotide with a high degree of similarity (>80%) to other mouse gene transcripts was eliminated from the design. Probes were designed with a melting temperature of approximately 63°C, enabling hybridization conditions to be held constant (i.e., 53°C) during each hybridization step. All probes were synthesized by QIAGEN Operon (Alameda, CA). Total RNA (1  $\mu$ g/ $\mu$ l) was added to each well (10  $\mu$ l/well) of a 96-well plate containing 50  $\mu$ l of capture hybridiza-

tion buffer and 50  $\mu$ l of each diluted probe set, and allowed to hybridize to the probe set overnight at 53°C. Subsequent hybridization steps were carried out according to the manufacturer's protocol. Luminescence from 96-well plates was analyzed with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex data management software, version 5.02 (Bayer Corp.-Diagnostics Div.). The luminescence for each well was reported as relative light units (RLU) per 10  $\mu$ g of total RNA.

**Statistical Analysis.** Differences between control and treatment groups were analyzed by analysis of variance, followed by Duncan's multiple range post hoc test. Reporter activities are presented as the mean  $\pm$  S.E.M. of a minimum of three independent transfections. Differences among experimental groups were analyzed by the Student's *t* test. Statistical significance was set at *p* < 0.05.

## Results

**Time-Response Relationship between LPS and Mouse Oatp4 mRNA Levels.** A single dose of LPS (5 mg/kg i.p.) was used to determine whether there is a time-related effect on mouse Oatp4 mRNA levels. As shown in Fig. 1, LPS produced a rapid and marked reduction of mouse Oatp4 mRNA levels in liver. Mouse Oatp4 mRNA levels were decreased 80% by 12 h after LPS administration and returned to control level by 48 h. This result indicates that LPS produced a time-dependent decrease in mouse Oatp4 mRNA levels, and the effect of a single LPS treatment on Oatp4 mRNA levels was reversible.

**Binding to Putative Response Elements of the Mouse Oatp4 Promoter in Untreated Liver Nuclear Extracts.** To examine binding activity to putative response elements of the mouse *Oatp4* promoter for *trans*-activators, namely HNF1, C/EBP, HNF3, and RXR:RAR in untreated mouse liver nuclei, electrophoretic mobility shift assays (EMSAs) were performed utilizing mouse liver nuclear extracts. As shown in Fig. 2, DNA-protein complexes were formed in the binding reaction containing mouse liver nuclear proteins and oligonucleotides representing putative response elements of the mouse *Oatp4* promoter for HNF1, C/EBP, HNF3, and RXR:RAR (Table 1). The specificity of the DNA and protein interactions was demonstrated by competition assays, using between a 20- and 200-fold molar excess of unlabeled specific competitors. The result was reduction of each DNA-protein complex. These data indicate that each complex was specific to the respective response elements of the mouse Oatp4 gene.

**Time Course of Nuclear Binding Activity to Putative Response Elements of the Oatp4 Promoter after LPS Administration.** To examine whether the LPS-induced decrease in mouse Oatp4 mRNA levels is associated with an

TABLE 2

List of oligonucleotide probes generated for analysis of mouse Oatp4 mRNA levels by QuantiGene signal amplification

Target region refers to the sequence of the mRNA transcript as enumerated in the GenBank file. Function relates to the function of the oligonucleotide probe in the QuantiGene assay (i.e., CE, capture probe; LE, label probe; BL, blocker probe).

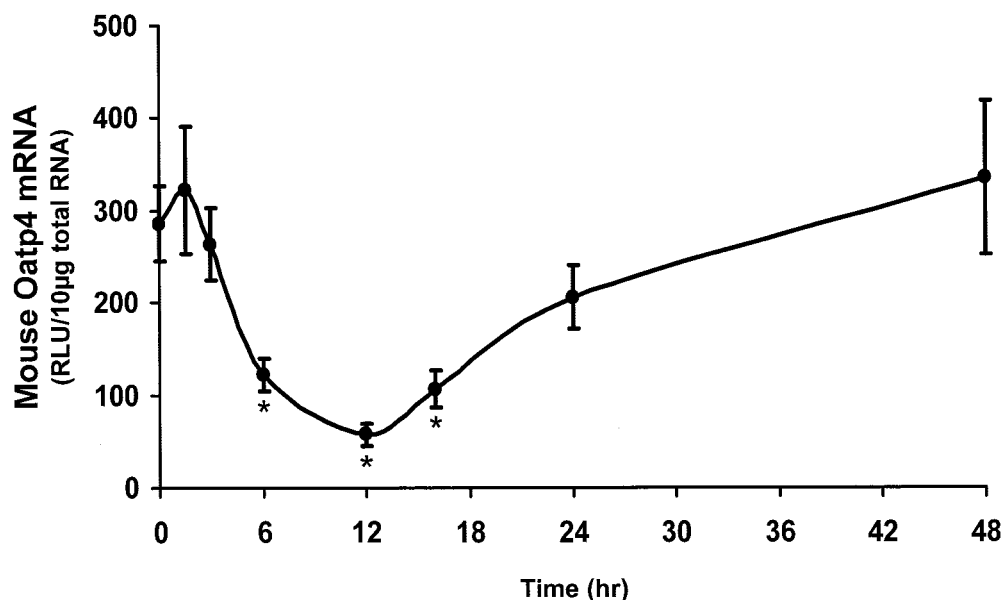
Oatp4 Probe	Target Region	Function	Sequence	Accession No.
M001	497–517	CE	atactcccaatgcccatgatgTTTTTctcttggaagaaagt	AB031959
M002	538–562	CE	gcataacctgtaatatcccatgaagaTTTTTctcttggaagaaagt	AB031959
M003	615–640	CE	gttcacagtgagtgatgtagtttgattTTTTTctcttggaagaaagt	AB031959
M004	476–496	LE	aagcaaccagttccaatcagcTTTTTaggcataggaccctgtct	AB031959
M005	518–537	LE	aatgtggcaacgcagtcagaTTTTTaggcataggaccctgtct	AB031959
M006	563–589	LE	tgtagagaactgatgtcattttctgttTTTTTaggcataggaccctgtct	AB031959
M007	590–614	LE	gactaaacaggtcaacgtggagttTTTTTaggcataggaccctgtct	AB031959
M008	641–664	LE	cctttctccattatctcaggtgagTTTTTaggcataggaccctgtct	AB031959
M009	686–711	LE	ccatcaagacataaatccaggtgatTTTTTaggcataggaccctgtct	AB031959
M010	712–732	LE	ctatcccacgaagcatgttccTTTTTaggcataggaccctgtct	AB031959
M011	665–685	BL	gagttggacccttttcacaa	AB031959



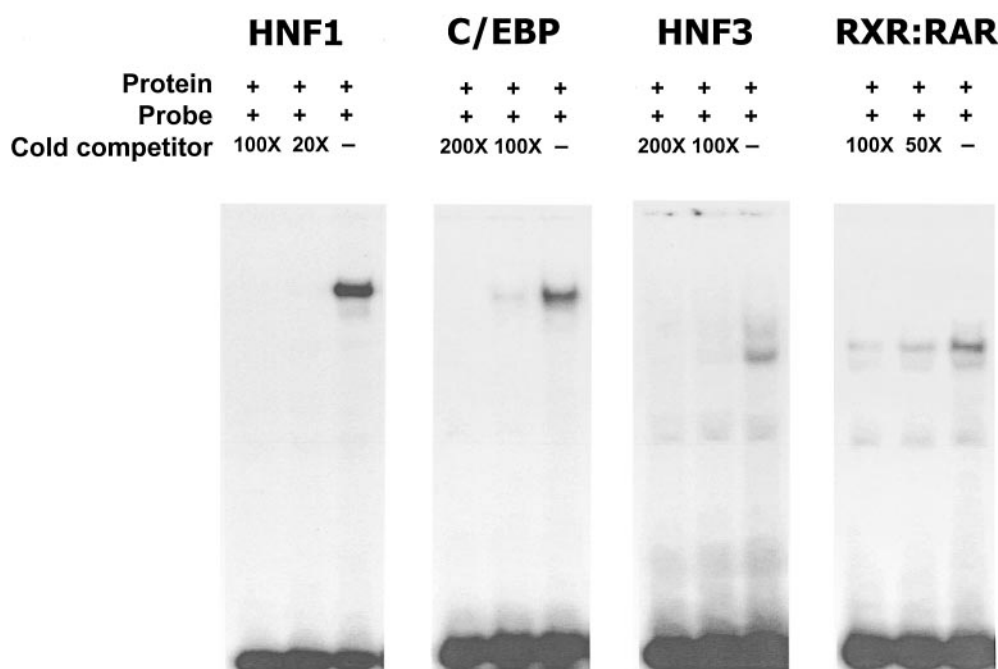
effect of LPS on nuclear binding activity, the binding activity to mouse *Oatp4* putative response elements for HNF1, C/EBP, HNF3, and RXR:RAR was performed using liver nuclear extracts prepared at various times after LPS administration. As shown in Fig. 3A, nuclear binding activity to these putative binding sites exhibited a time-dependent decrease and returned to control levels thereafter. The binding activity to putative response elements for HNF1 and C/EBP decreased maximally at 1.5 and 3 h after LPS administration, respectively, and returned to control levels by 12 h. Similarly, HNF3 and RXR:RAR response element exhibited a maximal decrease in nuclear binding activity to their putative response elements by 1.5 and 3 h after LPS treatment, respectively, and returned to control levels by 6 h after LPS administration. A consensus C/EBP oligonucleotide (Table 1)

exhibited the same binding pattern as the *Oatp4* C/EBP putative response element (Fig. 3A).

The time course of nuclear binding activities to the putative response elements of these transcription factors (Fig. 3) was compared with the time course of *Oatp4* mRNA levels after LPS administration (Fig. 1). The maximal decrease in nuclear binding activities preceded the maximal decrease in mouse *Oatp4* mRNA levels by about 9 to 10 h (Fig. 3). Moreover, the nuclear binding activities recovered before the mouse *Oatp4* mRNA levels returned to control levels after LPS treatment. For example, as shown in Fig. 3B, LPS produced a rapid and profound decrease in nuclear binding to the *Oatp4* HNF1 response element, which is prior to the LPS-induced decrease in *Oatp4* mRNA levels (Fig. 1). The HNF1 binding activity returned to control levels around 12 h



**Fig. 1.** Time course of mouse *Oatp4* mRNA levels after LPS administration. C3H/OuJ mice were treated with LPS (5 mg/kg i.p.). Livers were excised at 0, 1.5, 3, 6, 12, 16, 24, and 48 h after LPS administration ( $n = 5/\text{time}$ ). Total RNA from livers was analyzed by bDNA for *Oatp4* mRNA levels. Values are expressed as the mean  $\pm$  S.E.M. \*,  $p < 0.05$  versus control.



**Fig. 2.** Nuclear binding activity to putative response elements for the mouse *Oatp4* HNF1, C/EBP, HNF3, and RXR:RAR. Liver nuclear proteins (5–20  $\mu\text{g}$ ) were incubated with radiolabeled double-stranded oligonucleotides representing putative binding sites for HNF1, C/EBP, HNF3, and RXR:RAR. The entire binding reaction was electrophoresed through a 4% nondenaturing polyacrylamide gel and autoradiographed. Unlabeled specific oligonucleotides were included at 20- to 200-fold molar excess and added along with probes.

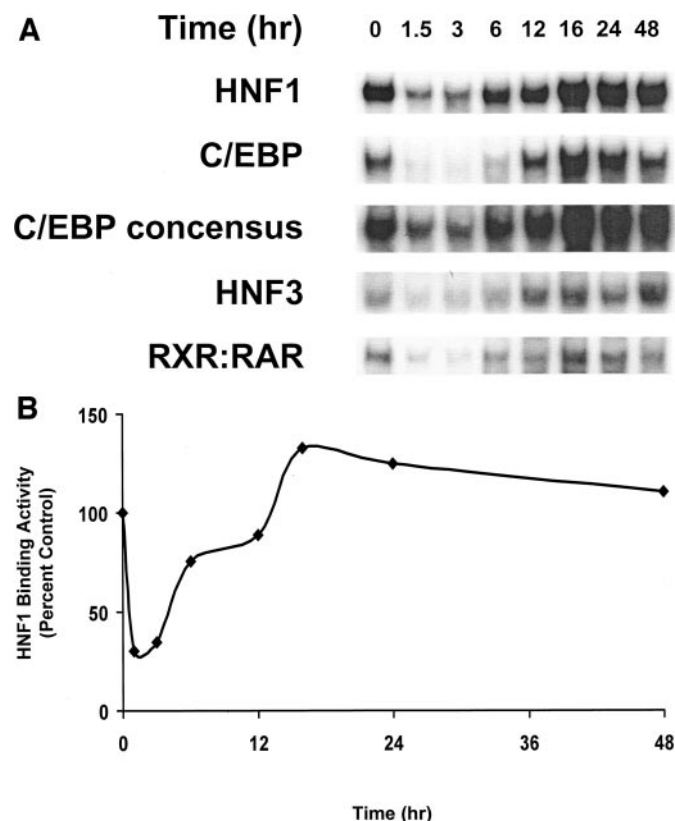
after LPS administration (Fig. 3B), and from that time the *Oatp4* mRNA levels started to recover (Fig. 1).

**The *Oatp4* HNF1 Response Element Is Bound by HNF1 $\alpha$  Rather than HNF1 $\beta$ .** HNF1 $\alpha$  and HNF1 $\beta$  are transcription factors of the variant homeodomain family (Baumhueter et al., 1990; Chouard et al., 1990). They have similar DNA binding specificity, although they are encoded by different genes. To determine the isoform of HNF1 in the DNA-protein complex with the *Oatp4* HNF1 binding sequence, supershift experiments were performed with antibodies specific to HNF1 $\alpha$  or HNF1 $\beta$ . As shown in Fig. 4, a 100-fold molar excess of unlabeled specific oligonucleotide completely abolished the DNA-protein complex, suggesting that the DNA-protein complex was specific for the *Oatp4* HNF1 response element. When antibodies specific to HNF1 $\alpha$  were preincubated with liver nuclear extracts, they caused a further shift of the DNA-protein complex, whereas HNF1 $\beta$  antibodies did not (Fig. 4). Moreover, the entire band was supershifted by HNF1 $\alpha$  antibodies.

**The *Oatp4* HNF1 $\alpha$  Response Element Confers HNF1 $\alpha$  Responsiveness.** To investigate whether the *Oatp4* HNF1 $\alpha$  response element was sufficient to mediate reporter activity, mouse hepatoma HEPA1-6 cells were transfected with a construct containing three or five copies of the *Oatp4*

HNF1 $\alpha$  response element (p3xHNF1-Luc or p5xHNF1-Luc) inserted upstream of the minimal promoter of the pLuc-MCS luciferase vector. The p3xHNF1-Luc and p5xHNF1-Luc constructs exhibited 11- and 14-fold higher reporter activities, respectively, compared with the vector pLuc-MCS containing the minimal promoter alone (Fig. 5). To further confirm that mouse HNF1 $\alpha$  is able to activate reporter activity of p3xHNF1-Luc or p5xHNF1-Luc, mouse hepatoma HEPA1-6 cells were cotransfected with an HNF1 $\alpha$  expression plasmid (pBJ5-HNF1 $\alpha$ ) and either p3xHNF1-Luc or p5xHNF1-Luc. Coexpression of mouse HNF1 $\alpha$  had no effect on reporter activity of the vector pLuc-MCS but up-regulated reporter activity of p3xHNF1-Luc or p5xHNF1-Luc by 30- and 40-fold, respectively. Moreover, five copies of the mouse *Oatp4* HNF1 $\alpha$  response element elicited a greater response to the coexpression of HNF1 $\alpha$  than did three copies.

**The Mouse *Oatp4* Promoter Is trans-Activated by HNF1 $\alpha$ .** Previous studies indicate that an *Oatp4* promoter (m4-137) was activated after cotransfection of HNF1 $\alpha$  (Jung et al., 2001). The effect of HNF1 $\alpha$  on a full-length *Oatp4* promoter (−4.8 kb to +30 bp), containing numerous additional *trans*-acting factors, should further elucidate the importance of HNF1 $\alpha$ . Mouse hepatoma HEPA1-6 cells were cotransfected with p4.8kb*Oatp4*-Luc (nt −4.8 kb to +30 bp) and pBJ5-HNF1 $\alpha$ . As depicted in Fig. 6, cotransfected pBJ5-HNF1 $\alpha$  (150 and 350 ng) increased the reporter activity (−4.8 kb to +30 bp) by 5- and 6-fold, respectively. These data indicate that HNF1 $\alpha$  activated *Oatp4* promoter in a dose-dependent manner in mouse hepatoma cells through the 5'



**Fig. 3.** A, time course of nuclear binding activity to the putative binding sites for the mouse *Oatp4* HNF1, C/EBP, HNF3, and RXR:RAR after LPS administration. Liver nuclear proteins were prepared from mouse livers excised at various times after a single injection of LPS (5 mg/kg i.p.). Nuclear proteins (5–20  $\mu$ g) were incubated with radiolabeled double-stranded oligonucleotides representing putative binding sites for HNF1, C/EBP, HNF3, and RXR:RAR, respectively. The entire binding reaction was electrophoresed through a 4% nondenaturing polyacrylamide gel and autoradiographed. B, densitometric analysis of nuclear binding activity to *Oatp4* HNF1 response element. Gel was quantified by PhosphorImager analysis and expressed as percentage of control.

Nuclear Protein	+	+	+	+	–
HNF1 Probe	+	+	+	+	+
Antibodies	–	–	$\alpha$	$\beta$	–
Cold competitor (100X)	+	–	–	–	–



**Fig. 4.** Characterization of HNF1 isoform bound to the mouse *Oatp4* HNF1 response element. Unlabeled specific oligonucleotides were included at 100-fold molar excess and added along with the probe. Nuclear proteins (5  $\mu$ g) were preincubated with 2  $\mu$ g of polyclonal antibodies specific to HNF1 $\alpha$  or HNF1 $\beta$  for 2 h before addition of the probe. The entire reaction was electrophoresed through a 4% nondenaturing polyacrylamide gel and autoradiographed.

flanking region (−4.8 kb to +30 bp), which contains numerous *cis*-acting sequences.

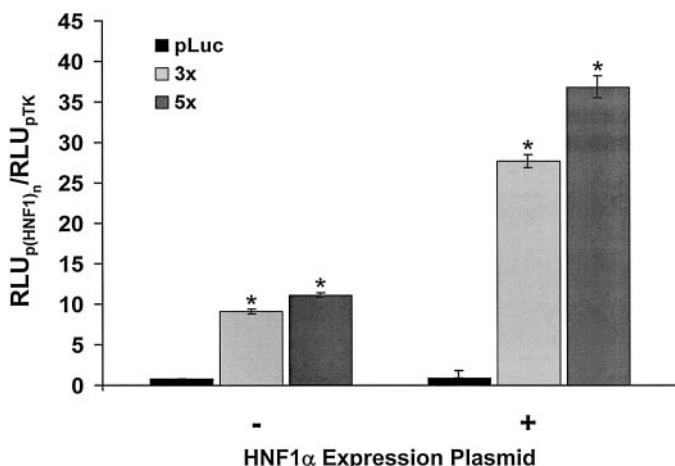
**Mice Lacking HNF1 $\alpha$  Exhibit a Marked Decrease in Oatp4 mRNA Levels.** HNF1 $\alpha$ -null mice were engineered by removing the first exon and the 5' sequence of the first intron of the HNF1 $\alpha$  gene (Lee et al., 1998). The dimerization domain of HNF1 $\alpha$  is encoded by the first exon, which is indispensable for DNA binding of HNF1 $\alpha$ . HNF1 $\alpha$ -null mice exhibit impaired bile acid homeostasis, including bile acid

synthesis and hepatic uptake of bile acids, as well as ileal and renal absorption of bile acids (Shih et al., 2001). As shown in Fig. 7, HNF1 $\alpha$ -null mice exhibited a marked decrease (95%) in Oatp4 mRNA levels, compared with wild-type mice.

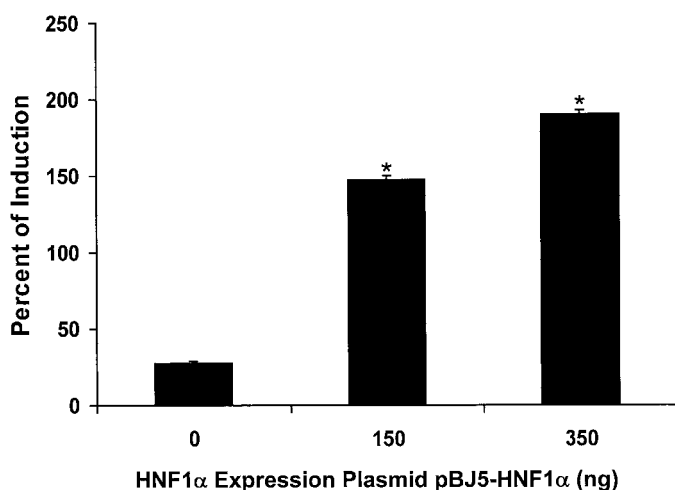
## Discussion

Previous studies suggest that LPS-induced cholestasis is mediated by impairment of the hepatobiliary transporting systems involved in the formation of bile. To date, several genes encoding hepatobiliary transporters localized to sinusoidal or canalicular membranes of hepatocytes have been cloned. It has been shown that LPS-mediated repression of hepatobiliary transporters is principally the result of down-regulation of gene expression (Green et al., 1996; Moseley et al., 1996; Trauner et al., 1997, 1998; Vos et al., 1998). Consistent with these published studies on other transporters, the present data demonstrate that LPS treatment also produces a time-dependent decrease in mouse Oatp4 mRNA levels (Fig. 1). Oatp4 mRNA levels return to control values after a single injection of LPS, indicating that the effects of LPS on Oatp4 mRNA are reversible in this model. Among the rat Oatps, Oatp4 mRNA levels have been shown to be relatively high in liver (Li et al., 2002). Oatp4 mediates Na<sup>+</sup>-independent transport of bile acids and other organic anions across the sinusoidal membrane of hepatocytes (Kakyo et al., 1999; Cattori et al., 2000). Finally, LPS reduces Na<sup>+</sup>-independent transport of organic anions in rodents (Bolder et al., 1997). Taken together, these studies suggest that Oatp4 might be a key player in the LPS-mediated reduction of Na<sup>+</sup>-independent hepatic uptake of bile acids and other organic anions.

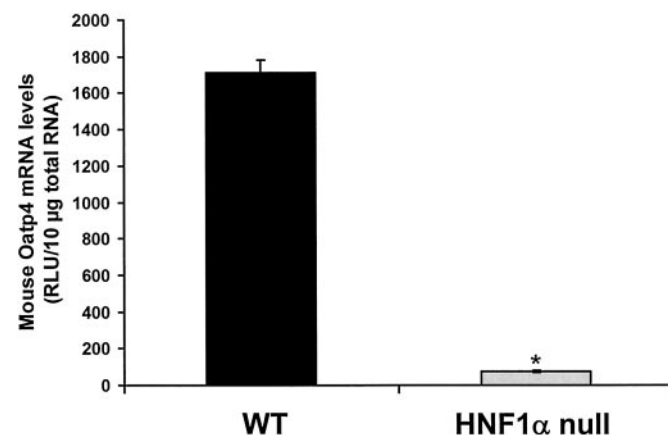
Previous studies have shown that Oatp4 mRNA is expressed almost exclusively in liver of mice (Ogura et al., 2000) and rats (Li et al., 2002). However, numerous putative transcription factor binding sites are predicted by the analysis of the 5' flanking region of mouse Oatp4 gene (Ogura et al., 2000). In the present study, mouse liver nuclear extracts exhibit binding activities to the putative response elements of four positive *trans*-acting factors (HNF1, C/EBP, HNF3, and RXR:RAR) (Fig. 2). Moreover, LPS decreases the nuclear binding activities to the HNF1 $\alpha$  response element, and pu-



**Fig. 5.** The mouse Oatp4 HNF1 $\alpha$  response element is sufficient to mediate promoter activation by mouse HNF1 $\alpha$ . Three or five copies of HNF1 $\alpha$  response element were inserted upstream of the minimal promoter in the pLuc-MCS luciferase vector to create p3xHNF1 $\alpha$ -Luc and p5xHNF1 $\alpha$ -Luc, respectively. The construct (p3xHNF1 $\alpha$ -Luc or p5xHNF1 $\alpha$ -Luc) was transfected with or without pBJ5-HNF1 $\alpha$  (150 ng) into mouse hepatoma HEP1-6 cells. Lysates were analyzed as described under *Materials and Methods*. Black solid bars represent reporter activities produced by pLuc-MCS containing the minimal promoter. Less filled bars represent reporter activities produced by p3xHNF1 $\alpha$ -Luc containing three copies of mouse Oatp4 HNF1 $\alpha$  response element in tandem. More filled bars represent reporter activities produced by p5xHNF1 $\alpha$ -Luc containing five copies of mouse Oatp4 HNF1 $\alpha$  response element. +, with coexpression of mouse HNF1 $\alpha$  with reporter constructs; −, without coexpression of mouse HNF1 $\alpha$  with reporter constructs. Values are expressed as the mean (RLU<sub>p(HNF1 $\alpha$ )</sub>/RLU<sub>pTK</sub>)  $\pm$  S.E.M. of a minimum of three independent transfections. \*,  $p < 0.05$  versus control.



**Fig. 6.** The mouse Oatp4 promoter is activated by coexpression of mouse HNF1 $\alpha$ . The reporter construct p4.8kbOatp4-Luc (50 ng) was cotransfected with 0, 150, and 350 ng of pBJ5-HNF1 $\alpha$ . Lysates were analyzed as described under *Materials and Methods*. Values are expressed as the mean (percentage of induction)  $\pm$  S.E.M. of a minimum of three independent transfections. \*,  $p < 0.05$  versus control.



**Fig. 7.** HNF1 $\alpha$ -null mice exhibit a marked decrease in Oatp4 mRNA levels compared with wild-type mice. Total RNA from livers of wild-type and HNF1 $\alpha$ -null mice ( $n = 5$ ) was analyzed by bDNA for Oatp4 mRNA levels. Values are expressed as the mean  $\pm$  S.E.M. \*,  $p < 0.05$  versus wild-type mice.



tative C/EBP, HNF3, and RXR:RAR response elements. The maximal decreases in nuclear binding activities to these response elements occur prior to the maximal reduction of *Oatp4* mRNA levels and return to control levels before *Oatp4* mRNA levels start to recover (Fig. 3). These data suggest that the LPS-induced down-regulation of mouse *Oatp4* mRNA might result from the reduction of *trans*-activation of *Oatp4* promoter by HNF1 $\alpha$ , C/EBP, HNF3, and RXR:RAR. It is widely accepted that liver-specific expression of genes is mediated by the concerted action of several liver-enriched transcription factors in a unique combination (Tronche et al., 1989). The present data suggest that HNF1 $\alpha$ , C/EBP, HNF3, and RXR:RAR might be involved in the constitutive expression of the mouse *Oatp4* gene, as well as the LPS-induced decrease in *Oatp4* mRNA levels.

Liver-enriched transcription factors regulate liver-specific expression of numerous genes (e.g., albumin, transthyretin, Ntcp) (Maire et al., 1989; Costa and Grayson, 1991; Tronche et al., 1994; Trauner et al., 1998; Denson et al., 2000). Recent studies indicate that HNF1 $\alpha$  is a key regulator of the hepatic uptake transporters (Jung et al., 2001; Shih et al., 2001; Jung and Kullak-Ublick, 2003). HNF1 $\alpha$  and HNF1 $\beta$  exhibit equal binding specificity with HNF1 response elements. Moreover, the HNF1 response element can be bound by HNF1 $\alpha$  homodimers, HNF1 $\alpha$ -HNF1 $\beta$  heterodimers, and HNF1 $\beta$  homodimers (De Simone et al., 1991; Rey-Campos et al., 1991). Therefore, it was of interest to determine whether HNF1 $\alpha$  and/or HNF1 $\beta$  bind to the HNF1 response element of the mouse *Oatp4* promoter. The complex of DNA with HNF1 $\beta$  homodimers migrates faster than HNF1 $\alpha$  homodimers, whereas the mobility of heterodimers is intermediate. The EMSA described in the present study indicates that there was predominantly a single band, and antibodies specific to HNF1 $\alpha$  caused a supershift of the entire band, whereas antibodies specific to HNF1 $\beta$  did not. This suggests that the protein component of the DNA-protein complex is exclusively HNF1 $\alpha$  (Fig. 4). Therefore, the present studies demonstrate that HNF1 $\alpha$  binds to the *Oatp4* HNF1 $\alpha$  response element as a homodimer, and LPS decreases HNF1 $\alpha$  binding to the mouse *Oatp4* promoter.

The present study indicates that three or five copies of the *Oatp4* HNF1 $\alpha$  response element upstream of a minimal promoter are sufficient to elevate basal reporter activity over that of the minimal promoter alone, probably due to the basal levels of HNF1 $\alpha$  in mouse hepatoma cells (Fig. 5). Coexpression of mouse HNF1 $\alpha$  increased the reporter activity of p3xHNF1-Luc from 11- to 30-fold and p5xHNF1-Luc from 14- to 40-fold. Moreover, five copies of the *Oatp4* HNF1 $\alpha$  response element (p5xHNF1-Luc) conferred more responsiveness to mouse HNF1 $\alpha$  than did three copies (p3xHNF1-Luc). Therefore, reporter activity linked to the multiple copies of *Oatp4* HNF1 $\alpha$  response element was dependent on the amount of mouse HNF1 $\alpha$ , as well as the copy number of *Oatp4* HNF1 $\alpha$  response elements. In addition to the effect of HNF1 $\alpha$  on the heterologous promoters containing the proximal HNF1 $\alpha$  binding site and a TATA box, HNF1 $\alpha$  activated the mouse *Oatp4* promoter (−4.8 kb to + 30 bp) in a dose-dependent manner (Fig. 6). Taken together, these data clearly demonstrate that HNF1 $\alpha$  is a potent *trans*-acting factor of the mouse *Oatp4* promoter.

The importance of HNF1 $\alpha$  in the expression of the mouse *Oatp4* gene was further confirmed in HNF1 $\alpha$ -null mice. The

present studies indicate that *Oatp4* mRNA levels were decreased by approximately 95% in HNF1 $\alpha$ -null mice, as compared with wild-type mice (Fig. 7). This finding clearly demonstrates the importance of HNF1 $\alpha$  for expression of mouse *Oatp4* gene in vivo.

HNF1 $\alpha$  mRNA is present in liver, kidney, intestine, pancreas, and stomach of adult mice (Baumhueter et al., 1990; Blumenfeld et al., 1991; Pontoglio et al., 1996). Therefore, the restricted expression of the *Oatp4* gene to liver is probably not a function solely of HNF1 $\alpha$ . In fact, it is accepted that liver-specific expression requires the concerted action of a unique combination of liver-enriched transcription factors, together with ubiquitous transcription factors (Tronche and Yaniv, 1992). The present studies suggest that C/EBP, HNF3, and RXR:RAR might be required for the constitutive expression of the mouse *Oatp4* gene in addition to HNF1 $\alpha$ . Further investigation would be needed to determine the role of these additional transcription factors in constitutive *Oatp4* expression in liver.

In summary, the present data indicate that LPS decreases mouse *Oatp4* mRNA levels in a time-dependent manner. The LPS-induced decrease in *Oatp4* mRNA levels in liver occurs subsequent to the reduction of nuclear binding activity to the response elements of HNF1 $\alpha$ , C/EBP, HNF3, and RXR:RAR, suggesting that these transcription factors might be involved in the LPS-induced down-regulation of *Oatp4* mRNA. This observation supports the concept that liver-specific gene expression requires the concerted action of a unique combination of liver-enriched and ubiquitous transcription factors. Additionally, the present studies clearly demonstrate that HNF1 $\alpha$  is a potent *trans*-activator of the mouse *Oatp4* promoter and activates the *Oatp4* promoter as a homodimer. HNF1 $\alpha$  levels are critical for the constitutive expression of the mouse *Oatp4* gene. Together with a rapid and profound decrease in HNF1 $\alpha$  binding to the mouse *Oatp4* HNF1 $\alpha$  response element after LPS treatment, these data suggest that HNF1 $\alpha$  plays an important role in the LPS-induced down-regulation of mouse *Oatp4*.

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