

Hepatic Expression of the *UGT1A9* Gene Is Governed by Hepatocyte Nuclear Factor 4 α

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ABSTRACT

UDP-glucuronosyltransferase (UGT) enzymes catalyze the glucuronidation reaction, which is a major pathway in the catabolism and elimination of numerous endo- and xenobiotics. Among the UGT enzyme family members, the UGT1A7, UGT1A8, UGT1A9, and UGT1A10 isoforms are issued from a single gene through differential splicing. However, these enzymes display distinct tissue-specific expression patterns. Indeed, UGT1A7, UGT1A8, and UGT1A10 are exclusively expressed in extrahepatic tissues, whereas UGT1A9 transcripts are found at high concentrations in liver. In the present study, we report that the liver-enriched hepatocyte nuclear factor 4 (HNF4)- α controls the hepatic expression of the UGT1A9 enzyme. Liver-specific disruption of the *HNF4 α* gene in mice

drastically decreases liver UGT1A9 mRNA levels. Furthermore, an HNF4 α response element (HNF4 α RE) was identified in the promoter of human UGT1A9 at position -372 to -360 base pairs by transient transfection, electrophoretic mobility shift assays, and chromatin immunoprecipitation experiments. It is interesting that this response element is absent in the proximal UGT1A7, UGT1A8, and UGT1A10 gene promoters. In conclusion, the present study identifies HNF4 α as a major factor for the control of UGT1A9 hepatic expression and suggests that the absence of UGT1A7, UGT1A8, and UGT1A10 expression in the liver is caused by, at least in part, a few base pair changes in their promoter sequences in the region corresponding to the HNF4 α RE of the *UGT1A9* gene.

Conjugation with glucuronic acid represents one of the major detoxification pathways for numerous endo- and xenobiotics in mammals, and liver is one of the primary sites of this reaction (Dutton, 1980). Glucuronidation is catalyzed by members of the UDP-glucuronosyltransferase (UGT) enzyme family (Dutton, 1980). On the basis of the homology of primary structures, the UGT proteins have been categorized into two major families, UGT1 and UGT2, with the UGT2 family further divided into two subfamilies, UGT2A and UGT2B (Mackenzie et al., 1997). In humans, members of the UGT1A subfamily are encoded by a complex gene that contains at least 17 exons spanning over

200 kb (Gong et al., 2001). Located on chromosome 2q37, the UGT1A gene complex leads to the production of nine functional proteins and encodes four pseudogenes by differential splicing; whereas exons 14 to 17 are shared between all UGT1A isoforms, the 13 first exons encode the amino terminal part of each protein and share between 37 and 90% amino acid sequence identity (King et al., 2000). Furthermore, the 5' flanking region of each first-exon cassette in this locus contains appropriate promoter elements that would attract PolII polymerase and the transcriptional initiation factors, and every UGT1A isoform is initiated at the isoform-specific promoter (Tukey and Strassburg, 2000). Belonging to the UGT1A subfamily, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 form a cluster of genes encoding highly homologous enzymes in both humans and rodents, because the first exon nucleotide sequences of these enzymes are more than 70% homologous (Gong et al., 2001). Moreover, the recent cloning of the UGT1A8, UGT1A9, and UGT1A10 promoters also revealed a high degree of sequence

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HNF, hepatocyte nuclear factor, ChIP, chromatin immunoprecipitation; RE, response elements; wt, wild type; mt, mutant; bp, base pair(s); kb, kilobase(s); Cdx2, caudal-related homeodomain protein 2; apo CIII, apolipoprotein CIII gene promoter; HA, hemagglutinin; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction.

similarity (>75%) within their proximal 1-kb sequence (Gong et al., 2001; Gregory et al., 2003). It is interesting that these enzymes possess different tissue distribution expression patterns. UGT1A8 and UGT1A10 enzymes are found exclusively in extrahepatic tissues and particularly in the gastrointestinal tract (small intestine and colon) (Strassburg et al., 1997, 1998; Cheng et al., 1998; Mojarrabi and Mackenzie, 1998). In contrast, human UGT1A9 is highly expressed in the liver, kidney, and intestine, whereas the UGT1A7 transcript is detected principally in the human esophagus and stomach (Strassburg et al., 1997, 1998, 1999; Albert et al., 1999). This isoform-specific expression suggests that genes of the UGT1A7 to UGT1A10 cluster may be regulated by tissue-specific transcription factors. Indeed, Gregory et al. (2003, 2004) recently demonstrated that the UGT1A8, UGT1A9, and UGT1A10 genes are differentially regulated by the hepatocyte nuclear factor (HNF)-1 α and the intestine-specific transcription factor caudal-related homeodomain protein 2 (Cdx2) in colon carcinoma Caco2 cells through an initiator element in their promoter regions. In contrast, the specific factor(s) that allows the expression of UGT1A9 in liver, the main site for glucuronidation, has not been identified to date.

Liver-specific gene expression in adult hepatocytes relies on four families of transcription factors that are liver-enriched: the CCAAT enhancer-binding proteins and HNF1, HNF3, and HNF4 (Cereghini, 1996). HNF4 α (NR2A1) is a highly conserved member of the steroid/thyroid superfamily of transcription factors, which is involved in the control of lipid and glucose homeostasis (Hayhurst et al., 2001). It is expressed at the highest levels in the liver, kidney, pancreas, and intestine (Xanthopoulos et al., 1991; Drewes et al., 1996). Generation of mice lacking hepatic HNF4 α expression demonstrated that this receptor is central in the maintenance of hepatocyte differentiation and is a major regulator of genes involved in the control of lipid homeostasis (Hayhurst et al., 2001). In addition, HNF4 α controls the expression in human hepatocytes of numerous metabolizing enzymes, including cytochrome P450 enzymes such as CYP3A1, 3A4, 3A5, 2A6, 2B6, 2C9, and 2D6 (Jover et al., 2001; Kamiya et al., 2003). In the present study, we hypothesized that HNF4 α may also control the hepatic UGT1A9 expression. To verify whether HNF4 α controls the expression of UGT1A9 in liver, UGT1A9 mRNA levels were analyzed in tissues from wild-type or hepatic HNF4 α -null mice. Our results indicate that HNF4 α gene disruption provokes a drastic decrease in hepatic UGT1A9 expression. Furthermore, a functional HNF4 α response element (HNF4 α RE) located at position -372 to -360 base pairs (bp) in the promoter region of the *UGT1A9* gene was identified. The observation that this element is not conserved in the UGT1A7, UGT1A8, and UGT1A10 genes may explain, at least in part, the lack of hepatic expression of these UGTs.

Materials and Methods

Animal Studies. Liver-specific HNF4 α -deficient mice were obtained as described previously (Hayhurst et al., 2001). Livers were collected and frozen until RNA analyses.

Materials. Restriction enzymes and other molecular biology reagents were from New England Biolabs (distributed by Ozyme, Saint-Quentin, France), Stratagene (La Jolla, CA), Promega (Charbonnières, France), and Roche Diagnostics (Mannheim, Germany). [γ -³²P]ATP was purchased from PerkinElmer Life and Analytical

Sciences (Boston, MA). Cell-culture reagents were from Invitrogen (Carlsbad, CA). ExGen 500 was from Euromedex (Souffelwey-sheim, France).

RNA Purification and Reverse Transcription. Total RNA was isolated from 100 mg of wet mouse livers using TRIzol as specified by the supplier (Invitrogen). Total RNA (2 μ g) from mouse livers was reverse-transcribed in a final volume of 30 μ l of first-strand buffer (250 mM Tris-HCl, pH 8.0, 375 mM KCl, and 15 mM MgCl₂) using random hexamer primers, 40 units of RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), and 200 units of the Moloney murine leukemia virus reverse transcriptase, as recommended by the supplier (Invitrogen).

Real-Time PCR. For real-time PCR analyses of reverse-transcribed murine UGT1A9 and 28S cDNAs, reverse transcriptase products were diluted 1/10 and 1/200, respectively, and 2 μ l of dilution was used as amplification template using the previously described specific primers for mUGT1A9 and 28S (Clausel et al., 2002; Barbier et al., 2003). PCR amplifications were performed on an MX 4000 apparatus (Stratagene) in a volume of 25 μ l containing a 100 nM concentration of each primer, 4 mM MgCl₂, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene), and SYBR Green 0.33X (Sigma-Aldrich). The conditions were 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, as reported previously (Barbier et al., 2003). mUGT1A9 mRNA levels were subsequently normalized to 28S mRNA. A nonparametric Mann-Whitney test was used to analyze for statistical differences between the different experimental groups.

Plasmid Cloning and Site-Directed Mutagenesis. The 1A9p1wt- and 1A9pDR1mt-pGL3 (in which the direct repeat 1 sequence was mutated) constructs were obtained as described previously (Barbier et al., 2003). The 1A9p0.5wt-pGL3 reporter construct was generated by digestion of 1A9p1wt-pGL3 with NcoI and subsequent ligation into an NcoI-digested pGL3 plasmid. The proximal 616-, 610-, and 601-bp genomic fragment of the UGT1A7, UGT1A8, and UGT1A10 promoters, respectively, were amplified by PCR, using primers listed in Table 1. The PCR amplifications were performed in a final reaction volume of 50 μ l under the following conditions: denaturation at 94°C for 3 min, 35 cycles of 15 s at 94°C, 40 s at 60°C for the UGT1A7 promoter; and at 61°C for UGT1A8 and UGT1A10, and extension for 1 min at 72°C, followed by a final extension at 72°C for 7 min. The PCR products were cloned into the XhoI and HindIII restriction sites of the pGL3-basic vector to generate the 1A7p0.6wt, 1A8p0.6wt, and 1A10p0.6wt reporter constructs. Mutations [respective change of alanine to glycine and threonine to cysteine as reported previously (Fraser et al., 1998)] were introduced in the HNF4 α response element to generate the 1A9p1mt and 1A9p0.5mt constructs by using the QuikChange site-directed mutagenesis kit (Stratagene) and primers as described in Table 1.

Cell Culture and Transient Transfection Assays. Human hepatoblastoma HepG2 cells were from the American Type Culture Collection (Rockville, MD) and were grown as described previously (Clausel et al., 2002; De Tomassi et al., 2002). HepG2 cells (60 \times 10³ cells/well of 24-well plates) were transfected with 100 ng of the indicated luciferase reporter plasmids, 50 ng of the pCMV- β -galactosidase expression vector, and the indicated concentrations of pSG5-HNF4 α or empty pSG5. All samples were complemented with pBS-SK+ plasmid (Stratagene) to an identical amount of 500 ng/well. Cells were transfected with ExGen 500 reagent for 6 h at 37°C and subsequently cultured for 24 h in 10% fetal bovine serum. At the end of the experiment, the cells were washed twice with ice-cold phosphate-buffered saline, and lysed in 100 μ l of lysis buffer (1% Triton X-100, 25 mM glycyl-glycine, 15 mM MgSO₄, and 4 mM EGTA) for 30 min at room temperature. Cell lysates (10 μ l) were assayed for luciferase activity by using an LB9507 LUMAT luminometer (Berthold, France) in the presence of 100 μ l of luciferase assay buffer (25 mM glycyl-glycine, 15 mM MgSO₄, 5 mM ATP, and 6.25 μ M D-luciferin). Transfection efficiency was monitored by measuring the β -galactosidase activity (optical density at 405 nm) of cell

lysates (20 μ l) after 2-h incubation at 37°C in the presence of 200 μ l of assay buffer (80 mM Na₂HPO₄, 8 mM KCl, 800 μ M MgSO₄, and 1 mg/ml *O*-nitrophenyl β -D-galactopyranoside).

Electrophoretic Mobility Shift Assays. The HNF4 α protein was synthesized *in vitro* using the TNT Quick-Coupled Transcription/Translation System (Promega, Madison, WI). Sense and antisense oligonucleotides (2.5 μ g each) encompassing the different HNF4 α response elements were annealed at a final concentration of 100 ng/ μ l in a final volume of 100 μ l. One microliter of double-stranded oligonucleotides were end-labeled with [γ -³²P]ATP using T4-polynucleotide kinase to produce radiolabeled probes. The HNF4 α protein was incubated for 15 min at room temperature in a total volume of 20 μ l containing 2.5 μ g of poly(dI-dC) and 1 μ g of herring sperm DNA in binding buffer as described previously (Pineda Torra et al., 2002). The radiolabeled probes 1A9HNF4 α REwt, 1A9HNF4 α REmt (which contains the same mutation as introduced in the 1A9p1mt and 1A9p0.5mt promoter constructs), apolipoprotein CIII gene promoter (apo CIII) (Mietus-Snyder et al., 1992), 1A7 -368/-355, 1A8 -384/-371, or 1A10 -369/-356 (Table 1) were added, and the binding reaction was incubated for a further 15 min at room temperature. The protein complexes were resolved by 4% nondenaturing polyacrylamide gel electrophoresis in 0.25 \times Tris-Borate-EDTA at room temperature. For supershift experiments, the HNF4 α protein was incubated for 15 min at room temperature in the same buffer as above, and the anti-HNF4 α antibody (0.2 μ g) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added and incubated at room temperature for 20 min before the addition of the radiolabeled probe and a final 15-min incubation at room temperature. For competition experiments, the indicated excess quantities of unlabeled oligonucleotides were added to the binding reaction just before the labeled probes.

Chromatin Immunoprecipitation Assays. Chromatin Immunoprecipitation Assay (ChIP) experiments were performed as described previously (Caudel et al., 2003). In brief, DNA was sonicated 15 times for 15 s with 45-s intervals on ice. A volume of sonicated DNA corresponding to 20 \times 10⁶ cells was then immunoprecipitated using 4 μ g of an anti-HNF-4 α (Santa Cruz Biotechnology) or an anti-HA antibody as negative control. All immunoprecipitations were subjected to a round of preclearing with an excess of protein A-Sepharose to ensure the specificity of the reaction. Precipitated and nonprecipitated (input) genomic DNA was then purified and

resuspended into 100 μ l of H₂O. One tenth of the DNA preparations was PCR-amplified for 35 cycles (30 s at 95°C, 30 s at 58°C, and 30 s at 72°C), using the primers listed in Table 1. One fifteenth of the input and one fifth of the amplified PCR products were separated on an ethidium bromide-stained 2% agarose gel.

Results

HNF4 α Gene Disruption Decreases Hepatic Expression of Murine UGT1A9. A recent study identified HNF4 α as an important regulator of hepatic metabolism (Kamiya et al., 2003). To investigate whether HNF4 α also controls the hepatic expression of UGT1A9, liver mRNA levels were determined in floxed wild-type and hepatic HNF4 α -deficient (HNF4 α -/-) mice. It is interesting that a 73% reduction in UGT1A9 transcripts was observed in the liver of HNF4 α -deficient mice compared with the controls (Fig. 1), indicating that HNF4 α is a major determinant for the expression of UGT1A9 in mouse liver.

HNF4 α Activates the UGT1A9 Gene Promoter. To determine whether the activity of the human UGT1A9 gene promoter can be modulated by HNF4 α , a pGL3-luciferase reporter construct containing a 1.1-kb fragment of the human UGT1A9 gene promoter (1A9p1wt) (Barbier et al., 2003) was transfected into HepG2 cells in the presence or absence of a pSG5-HNF4 α expression vector. A dose-dependent induction of luciferase activity was observed in the presence of increasing concentrations of this nuclear receptor, whereas the empty pSG5 plasmid failed to induce promoter activity (Fig. 2). This indicates that HNF4 α activates the proximal UGT1A9 promoter.

The UGT1A9 promoter contains a DR1 sequence at position -719 to -706 (Barbier et al., 2003). Because HNF4 α is able to bind such a response element (Fraser et al., 1998) and to test whether this site could mediate the induction by HNF4 α , mutations were introduced in the context of the 1.1-kb UGT1A9 promoter construct (1A9p1DR1mt) (Barbier et al., 2003). Mutation of this site did not affect the induction

TABLE 1

Oligonucleotides used in this study

These oligonucleotides were used to amplify UGT1A7, UGT1A8, or UGT1A10 promoters, to mutate the HNF4 α response element in the UGT1A9 promoter, and for EMSA or ChIP analyses. Underlined nucleotides represent restriction sites used to clone the genomic PCR fragments, whereas bases in boldface type correspond to the HNF4 α response element, and nucleotides in italic correspond to mutated bases. The apo CIII probe derives from Mietus-Snyder et al. (1992).

Promoter cloning	
UGT1A7p (Xho I) sense	5'-CTAGCACTCGAGCGAGACCAGCCTGG
UGT1A7p (HindIII) antisense	5'-CTAGCTGAAGCTTATCAGAGAAGCTTCAGCCC
UGT1A8/10p (XhoI) sense	5'-CTAGCACTCGAGCAGGGTTGTCAATGTCATTTC
UGT1A8p (HindIII) antisense	5'-CTAGCTGAAGCTTATGAGAGAAGCTGCAGCCC
UGT1A10p (HindIII) antisense	5'-CTAGCTGAAGCTTATGAGAGAAGCTGCAGCCC
Site-directed mutagenesis	
1A9p1mt and 1A9p0.5mt	5'-TTTGCTCTGGGACGGGCTTGA AAAAA ATTAG
1A9pmt to 1A8	5'-TTGCTCTGGGA TGAATTCC AAAAA
1A8pmt to 1A9	5'-TTGCTTTGGGACAAATTCCAAAAA
EMSA	
1A9HNF4 α REwt	5'-TTTGCTCTGGGACAAATTCCAAAAAATTAG
1A9HNF4 α REmt	5'-TTTGCTCTGGGACGGGCTTGA AAAAA ATTAG
1A7 -368/-355	5'-TTTCTTTGTGACAAATTC CAAAAT TATTAGG
1A8 -364/-371	5'-TTTGCTTTGGGATGAATTCCAAAAATATTAGC
1A10 -369/-356	5'-TTTGCTTTGGGATAAATTCCAAAAATATTAGC
Apo CIII	5'-CAGCAGGTGACCTTTGCCAGCAGCCC
ChIP	
UGT1A9 -386 (sense)	5'-TGAGTTGCCATCTTCTCTGG
UGT1A9 -241 (antisense)	5'-ATGCTTTTGGACCTTGAAGGT
UGT1A9 -2050 (sense)	5'-GATTACAGGCATGCACCACACCT
UGT1A9 -1890 (antisense)	5'-CTCACACCTGTAGTCCCAGCAC
β -actin forward	5'-CGAGCCATAAAGGCAACTTTCG
β -actin reverse	5'-AGGAAGAGGAGGAGGGAGATT

of UGT1A9 promoter activity by HNF4 α (Fig. 3), suggesting that HNF4 α -dependent activation of the promoter occurs independently of the DR1. To confirm this observation, a 0.5-kb reporter construct of the human UGT1A9 promoter, which does not contain the DR1 sequence, was generated (1A9p0.5wt). As for the longer construct, HNF4 α dose-dependently induced the activity of the 0.5-kb promoter fragment (Fig. 3), thus demonstrating that HNF4 α stimulates

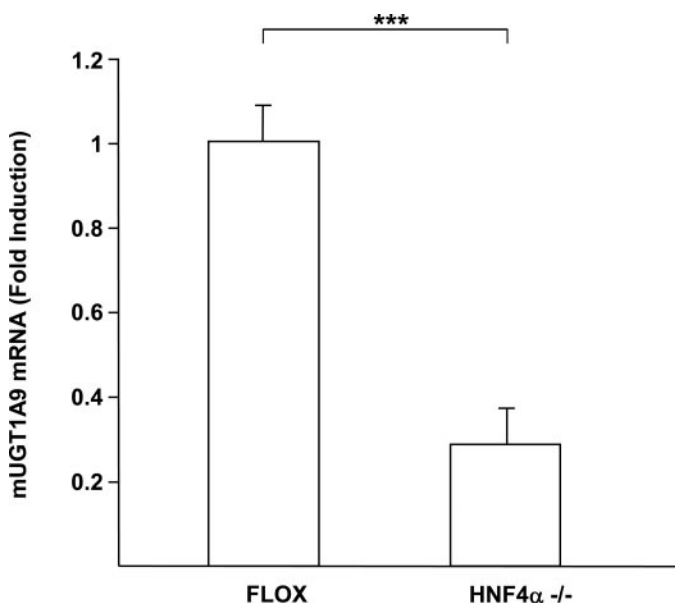


Fig. 1. HNF4 α gene disruption decreases basal UGT1A9 expression in mouse liver. UGT1A9 mRNA levels in livers from hepatic HNF4 α -null (HNF4 α -/-) mice were analyzed by real-time reverse-transcriptase PCR and compared with those from wild-type (FLOX) mice ($n = 3$ per group). Values are expressed as means \pm S.D. relative to the control group set as 1. Statistically significant differences between the groups are indicated by asterisks (Mann-Whitney test: ***, $p < 0.001$).

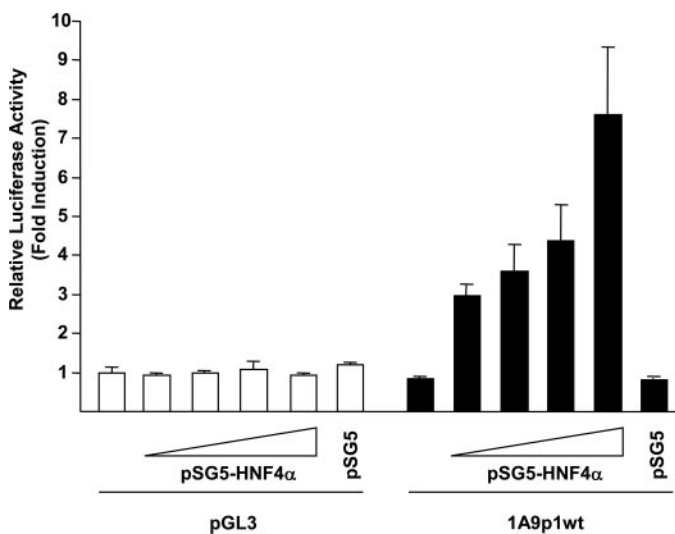


Fig. 2. HNF4 α activates the UGT1A9 promoter. HepG2 cells were transfected with the control pGL3 or the human UGT1A9 promoter-driven luciferase reporter plasmid (100 ng) in the absence or presence of increasing concentrations (5, 10, 15, and 30 ng) of pSG5-HNF4 α or empty pSG5 (30 ng). Cells were subsequently cultured for 24 h, and luciferase and β -galactosidase activities were measured. Values are expressed as fold induction of the control (pGL3) set at 1 and normalized to internal β -galactosidase activity as described under *Materials and Methods*. Values represent the means \pm S.D.

UGT1A9 promoter activity through a response element located in its proximal 500-bp sequence.

Computer-assisted analysis of the UGT1A9 gene promoter region revealed the presence of a 5'-GGGACAAATTCCAA sequence located at position -372 to -360, which resembles closely the HNF4 α RE consensus sequence (Fraser et al., 1998; Odom et al., 2004). To test whether this site mediates the induction by HNF4 α , mutations were introduced in the context of the 1.1- and 0.5-kb UGT1A9 promoter constructs (1A9p1mt and 1A9p0.5mt, respectively). Mutation of this potential HNF4 α RE abolished the HNF4 α -dependent induction of luciferase activity of both reporters (Fig. 3). Taken together, these data indicate that the UGT1A9 promoter contains a functional HNF4 α RE, which controls the induction of the human UGT1A9 promoter activity in the presence of this nuclear receptor.

HNF4 α Binds the cis-Acting Element at Position -372 to -360 in the UGT1A9 Gene Promoter. To determine whether human HNF4 α binds to this response element in the UGT1A9 promoter, electrophoretic mobility shift assays (EMSAs) were performed using the wild-type or mutated HNF4 α RE of the UGT1A9 promoter (1A9HNF4 α REwt and 1A9HNF4 α REmt, respectively) or the HNF4 α RE of apo CIII (positive control) as radiolabeled probes (Fig. 4). Although no binding to any probes was observed in the absence of HNF4 α (Fig. 4a, lanes 1 and 4), the receptor bound both 1A9HNF4 α REwt and apo CIII probes (lanes 2 and 5). Furthermore, these complexes were supershifted by the anti-HNF4 α antibody (lanes 3 and 6). The presence of the anti-HNF4 α antibody resulted in a much lower binding of the receptor to the 1A9HNF4 α REwt probe (lane 3) compared with the apo CIII probe (lane 6). However, lower exposure of the shift revealed a similar decrease of the binding onto the apo CIII probe in the presence of the antibody (data not shown), and a similar reduction of the binding of HNF4 α to DNA probes in the presence of the antibody was reported in various studies (Stauffer et al., 1998; Stroup and Chiang, 2000; Wang et al., 2000; Bartoov-Shifman et al., 2002). However, no protein-DNA complex was observed when using the mutated HNF4 α RE probe (Fig. 4b, lane 10). For competition experiments, increasing amounts (1-, 10-, 50-, and 100-fold excesses) of unlabeled oligonucleotides encompassing either the apo CIII, 1A9HNF4 α REwt, or 1A9HNF4 α REmt sites were added to the binding reactions (Fig. 4c). HNF4 α binding to the 1A9HNF4 α REwt was strongly competed by the apo CIII site and to a lower extent by the 1A9HNF4 α REwt itself (Fig. 4c, lanes 13 to 20), which indicate that HNF4 α binds to the apo CIII probe with a higher affinity compared with the 1A9HNF4 α REwt. In contrast, the mutated RE did not efficiently compete for HNF4 α binding to the probe encompassing the wild-type sequence (lanes 21 to 24). Taken together, these data demonstrate that HNF4 α binds to the UGT1A9 HNF4 α RE site at position -372 to -360 in vitro.

Occupancy of the UGT1A9 promoter by HNF4 α in living cells was analyzed by using ChIP assays performed on DNA from HepG2 cells (Fig. 5). Chromatin DNA was precipitated using either the anti-HNF4 α or anti-HA antibodies or was incubated for the same period in the presence of protein A Sepharose alone, and the sequence encompassing the HNF4 α RE was amplified using specific primers (Fig. 5A, lane 4). The results clearly indicate that HNF4 α occupies this region of the UGT1A9 promoter. As a negative control, amplification of

an equivalent amount of genomic DNA precipitated with a nonrelevant anti-HA antibody or incubated with protein A Sepharose alone only slightly amplified the 145-bp fragment containing the response element (Fig. 5A, lanes 2 and 3). Moreover, when the same DNA samples were PCR-amplified with primers covering a region 1600-bp upstream of the HNF4 α RE, no signal was observed (Fig. 5B). Finally, PCR amplification with oligonucleotides for β -actin, as a negative control for the immunoprecipitation, did not result in any

signal (Fig. 5C). Taken together, these results demonstrate that the HNF4 α RE is immunoprecipitated by the anti-HNF4 α antibody, thus indicating the binding of the nuclear receptor to this DNA region in living cells.

HNF4 α Does Not Bind to and Fails to Activate the Human UGT1A7, UGT1A8, and UGT1A10 Gene Promoters. To investigate whether HNF4 α also affects human UGT1A7, UGT1A8, and UGT1A10 promoter activities, 0.6-kb fragments of these promoters were cloned into the

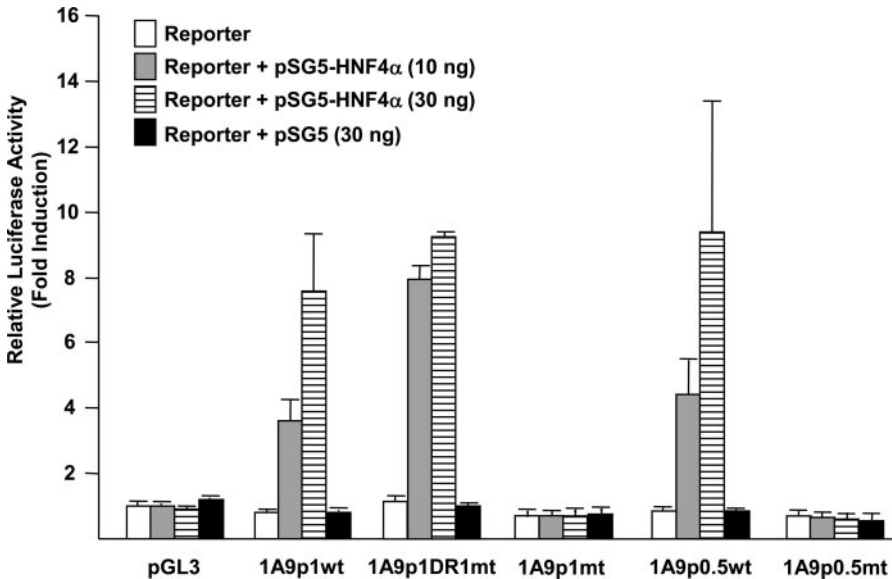


Fig. 3. HNF4 α activates UGT1A9 promoter activity through a response element located at position -372 to -360. HepG2 cells were transfected with the indicated human UGT1A9 promoter-driven luciferase reporter plasmids (100 ng) in the absence or presence of pSG5-HNF4 α (10 and 30 ng) or pSG5 (30 ng). Cells were subsequently cultured for 24 h, and luciferase and β -galactosidase activities were measured. Values are expressed as fold induction of the control (pGL3) set at 1 and normalized to internal β -galactosidase activity as described under *Materials and Methods*. Values represent the means \pm S.D.

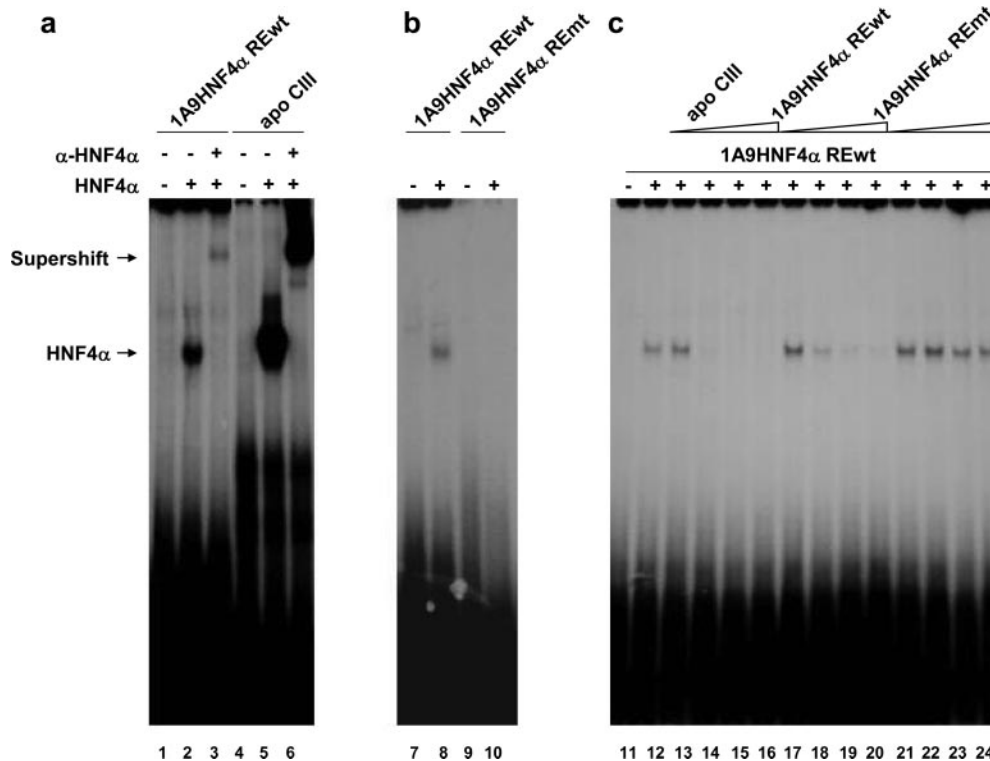


Fig. 4. HNF4 α binds to the HNF4 α RE in the UGT1A9 promoter. EMSAs were performed with end-labeled wild-type or mutated 1A9HNF4 α RE or apo CIII probes in the presence of unprogrammed reticulocyte lysate or HNF4 α proteins as indicated. a, supershift experiments with 1A9HNF4 α RE or apo CIII probes were carried out using an anti-HNF4 α antibody (0.2 μ g). b, an equal amount of wild-type or mutated 1A9HNF4 α RE probes were assayed for binding with unprogrammed reticulocyte lysate or HNF4 α as described under *Materials and Methods*. c, competition EMSAs on radiolabeled 1A9HNF4 α RE probe were performed by adding 1-, 10-, 50-, or 100-fold molar excess of the indicated unlabeled apo CIII, 1A9HNF4 α REwt, or 1A9HNF4 α REmt oligonucleotides in EMSA with unprogrammed reticulocyte lysate or HNF4 α .

luciferase reporter plasmid pGL3. These constructs and the 1A9p0.5wt and 1A9p0.5mt plasmids (as positive and negative controls, respectively) were subsequently transfected into HepG2 cells in the presence of two concentrations of the pSG5-HNF4 α plasmid (10 or 30 ng). As described above, a dose-dependent induction of the 1A9p0.5wt-driven reporter by HNF4 α was observed, whereas the 1A9p0.5mt was unresponsive to HNF4 α (Fig. 6a). It is interesting to note that HNF4 α failed to modulate the activity of the UGT1A7 promoter construct, whereas 50 and 65% reductions in UGT1A8 and UGT1A10 promoter activities, respectively, were obtained when the corresponding constructs were cotransfected with the pSG5-HNF4 α plasmid (Fig. 6a). Overall, these results demonstrate that HNF4 α specifically activates the UGT1A9 promoter, whereas those of the UGT1A7, UGT1A8, and UGT1A10 genes are not affected or down-regulated by the receptor.

Alignment of the UGT1A7, UGT1A8, UGT1A9, and UGT1A10 promoter nucleotide sequences indicated that the HNF4 α RE found in the *UGT1A9* gene resembles more closely the consensus HNF4 α response element sequence than the corresponding site in the other genes, which present at least one additional nucleotide change (Fig. 6b). This observation suggests that the absence of UGT1A7, UGT1A8, and UGT1A10 promoter activation by HNF4 α , as observed in transient transfection experiments, is caused by an inability of the receptor to bind these promoters. To verify this hypothesis, EMSAs were performed using the wild-type or mutated HNF4 α RE of the UGT1A9 promoter (as positive and negative controls, respectively) or the corresponding sequences (Table 1) in the UGT1A7, UGT1A8, and UGT1A10 promoters as radiolabeled probes (Fig. 6c). No binding to any of the probes was observed in the absence of HNF4 α , although, as expected, the receptor bound the 1A9HNF4 α REwt probes (lanes 2). In contrast, no protein-DNA complex formation was observed when using the 1A9 HNF4 α REmt, 1A7 -368/-355, 1A8 -384/-371, or 1A10 -369/-356 probes (Fig. 6c).

To further ascertain that few changes in the HNF4 α RE sequence are able to knock down the HNF4 α -dependent ac-

tivation of UGT1A7, UGT1A8, and UGT1A10 promoters, the 1A9p0.5mt to 1A8 and 1A8p0.6mt to 1A9 reporter constructs were generated by the respective replacement of the -2 and -1 nucleotides in 1A9p0.5wt (CA to TG) and 1A8p0.6wt (TG to CA) (Fig. 6b). These constructs were subsequently transfected into HepG2 cells in the same conditions as described above (Fig. 6d). It is interesting that the luciferase activity of the mutated 1A9p0.5mt to 1A8 plasmid was drastically reduced, with only a 1.4-fold activation when cotransfected with HNF4 α . In contrast, the 1A8p0.6mt to 1A9 plasmid displayed a 2.5-fold activation in the presence of HNF4 α (30 ng) instead of a down-regulation, as observed with the wild-type promoter. The drastic reduction of HNF4 α -dependent activation of the mutated UGT1A9 promoter demonstrates the importance of the HNF4 α RE located at position -372 to -360. The 2.5-fold activation of the mutated UGT1A8 promoter construct observed in the presence of HNF4 α indicates that introducing the HNF4 α RE from the UGT1A9 allows an activation of the promoter. However, the lower HNF4 α -dependent induction of the mutant UGT1A8 promoter compared with the wild-type UGT1A9 (2.5- and 7.8-fold, respectively) indicates that the HNF4 α RE is insufficient to drive hepatic expression of UGT enzymes under the control of HNF4 α .

Overall, these data demonstrate that HNF4 α binds and activates specifically the UGT1A9 promoter, whereas the UGT1A7, UGT1A8, and UGT1A10 promoters are either unaffected or inhibited by this transcription factor.

Discussion

In the present study, we identify UGT1A9 as a hepatic target gene of HNF4 α . Although Odom et al. (2004) recently reported the presence of HNF4 α RE in promoter regions of UGT2B11 and UGT2B15 genes, the present study is the first demonstration that HNF4 α regulates the expression of a UGT enzyme in the liver. However, the similar patterns of tissue-specific expression shared by UGT1A9 and HNF4 α (Xanthopoulos et al., 1991; Albert et al., 1999) suggested that UGT1A9 could be an HNF4 α target gene. On the other hand, Metz et al. (2000) previously reported that HNF4 α does not regulate UGT1A7 expression in rat hepatocytes. Consistent with this result, we observed that HNF4 α activates the UGT1A9 promoter without affecting the UGT1A7 promoter, thus suggesting that the human and rat UGT1A7 enzymes are regulated similarly.

We previously identified a functional peroxisome proliferator-activated receptor response element, which corresponds to a direct repeat of the hexamer AGGTCA sequence separated by one nucleotide (DR1) at position -719 to -706 bp in the UGT1A9 promoter (Barbier et al., 2003). Although it was previously shown that HNF4 α binds to DR1 sequences (Fraser et al., 1998; Odom et al., 2004), we found that the peroxisome proliferator-activated receptor response element is not involved in the HNF4 α -dependent activation of the UGT1A9 promoter. However, various studies demonstrated that an adenosine nucleotide as spacer in the direct repeat sequence creates a CAAAG core motif to which HNF4 α binds with high affinity (Fraser et al., 1998; Odom et al., 2004). Thus, the presence of a deoxyguanosine as the spacing base in the DR1 of the UGT1A9 promoter may explain why this response element is not involved in the regulation of

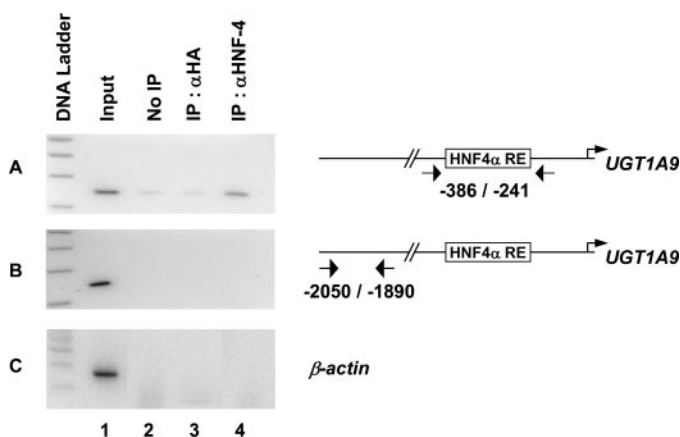


Fig. 5. HNF4 α binds to the HNF4 α RE in the UGT1A9 promoter in cells. Soluble chromatin was prepared from HepG2 cells as described under *Materials and Methods* and immunoprecipitated with anti-HNF4 α (lane 4) or anti-HA (lane 3, negative control) antibodies. Immunoprecipitated, incubated with protein A-Sepharose (lane 2, negative control), or extracted DNA (lane 1, input) was amplified using pairs of primers covering the HNF4 α RE (A), a distal region of the UGT1A9 promoter (B), or the β -actin gene (C) as negative control.

UGT1A9 by HNF4 α (Barbier et al., 2003). This observation demonstrates that few base-pair changes in the HNF4 α response element reduce in a drastic manner the ability of the receptor to bind DNA. In line with this is the observation that minimal nucleotide changes between the HNF4 α RE of the UGT1A9 promoter and the corresponding regions in the UGT1A7, UGT1A8, and UGT1A10 promoters abolish the binding of HNF4 α .

The human UGT1A7, UGT1A8, UGT1A9, and UGT1A10 genes possess highly conserved sequences in both coding and promoter regions (Cheng et al., 1998; King et al., 2000; Gregory et al., 2003). However, among this cluster, only the *UGT1A9* gene is expressed in the liver, whereas UGT1A7, UGT1A8, and UGT1A10 isoforms are expressed exclusively in extrahepatic tissues, particularly in the tissues of the gastrointestinal tract (Strassburg et al., 1998, 1999; Albert et al., 1999; King et al., 2000). Results presented here suggest that HNF4 α plays a major role for the hepatic expression of UGT enzymes, and it is tempting to speculate that the absence of UGT1A7, UGT1A8, and UGT1A10 expression in human liver is caused by the absence of HNF4 α -dependent activation of these genes. However, the residual expression of

UGT1A9 in livers from hepatic HNF4 α -deficient mice and the low HNF4 α -dependent activation of the mutated UGT1A8 promoter construct suggest that other transcription factors may participate, with HNF4 α , in the differential control of UGT1A9 expression in this tissue. Therefore, it could not be excluded that UGT1A7, UGT1A8, and UGT1A10 promoters also lack response elements for such transcription factors. Such a hypothesis is supported by the observation that introducing the HNF4 α -responding region of the *UGT1A9* gene in the UGT1A8 promoter context only allows a slight induction of its activity in the presence of HNF4 α (Fig. 6d). Furthermore, despite that HNF4 α does not bind or activate the examined UGT1A7, UGT1A8, and UGT1A10 promoters, we cannot exclude that response elements for HNF4 α are present in other more distal regions of these promoters.

On the other hand, we observed that UGT1A8 and UGT1A10 promoter activities are reduced in the presence of HNF4 α , suggesting that the receptor may negatively regulate the expression of these genes. To the best of our knowledge, this is the first observation to suggest that HNF4 α can negatively regulate gene promoter activity. However, we can-

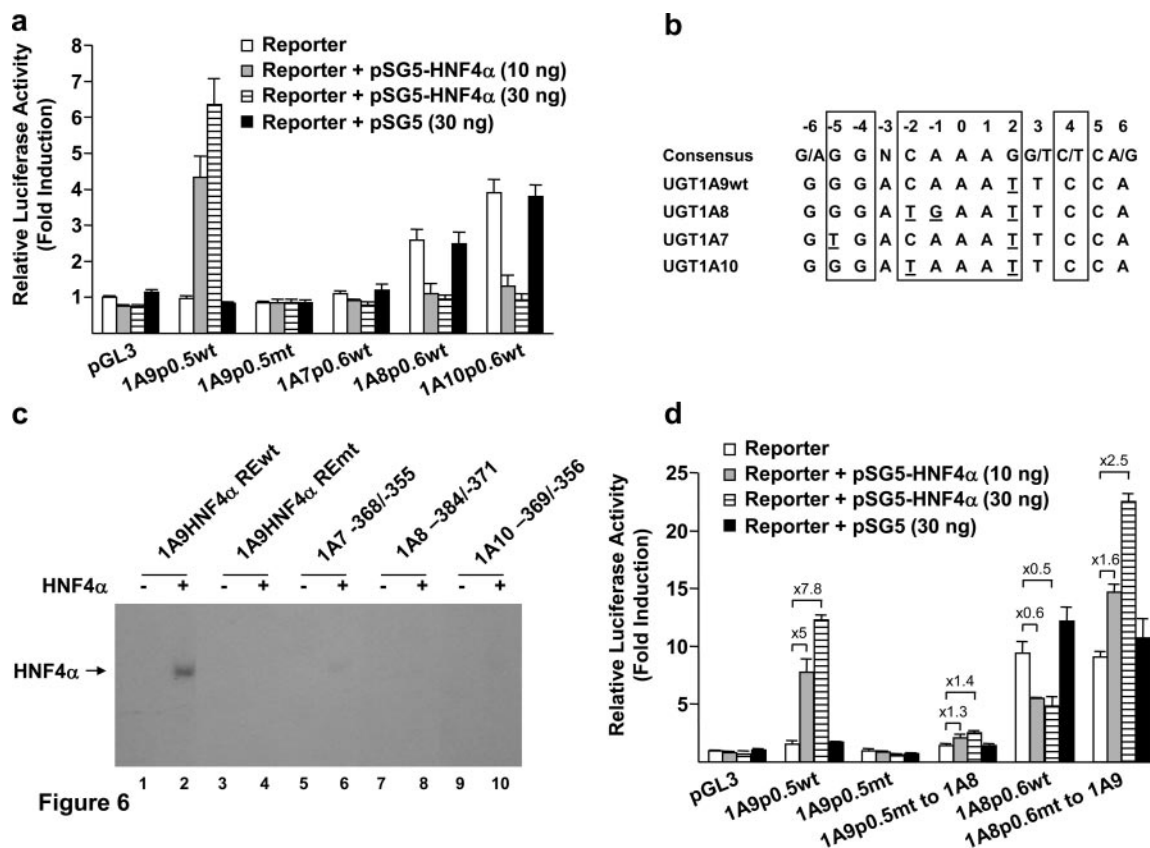


Fig. 6. HNF4 α activates and binds to the UGT1A9, but not UGT1A7, UGT1A8, and UGT1A10 promoters. **a**, HepG2 cells were transfected with the indicated luciferase reporter plasmids (100 ng) in the absence or presence of pSG5-HNF4 α (10 and 30 ng) or pSG5 (30 ng). Cells were subsequently cultured for 24 h, and luciferase and β -galactosidase activities were measured. Values are expressed as fold induction of the controls (pGL3) set at 1 and normalized to internal β -galactosidase activity as described under *Materials and Methods*. Values represent the means \pm S.D. **b**, sequence alignment of the UGT1A7, UGT1A8, UGT1A9, and UGT1A10 gene promoters revealed that the -372 to -360 site is closer to the consensus sequence than the corresponding sequence in UGT1A7, UGT1A8, and UGT1A10 promoters. Underlined nucleotides represent mismatches between the UGT sequences and the consensus HNF4 α RE, whereas boxes correspond to bases governing the binding by HNF4 α . **c**, EMSAs were performed with end-labeled wild-type or mutated 1A9HNF4 α RE, the corresponding 1A7 -368/-355, 1A8 -384/-371, and 1A10 -369/-356 probes in the presence of unprogrammed reticulocyte lysate or HNF4 α proteins as indicated. **d**, HepG2 cells were transfected with the indicated luciferase reporter plasmids (100 ng) in the absence or presence of pSG5-HNF4 α (10 and 30 ng) or pSG5 (30 ng). Cells were subsequently cultured for 24 h, and luciferase and β -galactosidase activities were measured. Values are expressed as fold induction of the controls (pGL3) set at 1 and normalized to internal β -galactosidase activity as described under *Materials and Methods*. Values represent the means \pm S.D. The values indicated represent the HNF4 α -dependent activation of each reporter construct.

not exclude that this observation is caused by an artifact of the transient transfection assay, such as the squelching of transcription machinery proteins by overexpressed HNF4 α protein. The expression of the medium chain acyl-CoA dehydrogenase is increased in livers from HNF4 α -null mice compared with wild-type animals, suggesting that HNF4 α may negatively regulate this gene also (Hayhurst et al., 2001; Jung and Kullak-Ublick, 2003). However, the molecular mechanism(s) of such negative regulation has not yet been studied.

It is interesting that Gregory et al. (2003) recently reported that the UGT1A8 and UGT1A10 promoters possess an 8-fold higher basal activity compared with the UGT1A9 promoter when transfected into intestinal Caco2 cells and that this difference is caused by two base-pair differences in an Sp1 binding site in the UGT1A9 promoter sequence. The same authors also demonstrated that the intestine-specific transcription factor Cdx2 bound to and activated the UGT1A8 and UGT1A10 promoters but could not activate the UGT1A9 promoter (Gregory et al., 2004). In addition, Cdx2 was shown to cooperate with HNF1 α to synergistically activate the UGT1A8, -1A9, and -1A10 promoters (Gregory et al., 2004). Taken together, these data demonstrate that only small nucleotide divergences between the UGT promoter sequences may drastically affect their promoter activity, and consequently the level of UGT protein expression in a given tissue. Furthermore, an increasing number of single nucleotide polymorphisms (SNPs) have been reported in human UGT genes and that some of these mutations are found in the promoter regions of these genes (Tukey and Strassburg, 2000; Gagné et al., 2002; Guillemette, 2003; Yamanaka et al., 2004); thus, it is tempting to speculate that SNPs located in the HNF4 α response element of the *UGT1A9* gene could partially explain the great variability of UGT1A9 expression and activity as observed in the human liver (Congiu et al., 2002; Gagné et al., 2002; Nakajima et al., 2002; Ramirez et al., 2002). Although this hypothesis remains to be clearly established, it is supported by the recent identification of an SNP in the AT-rich region of the UGT1A9 promoter, which provokes a 2.6-fold higher luciferase activity of the promoter in HepG2 cells (Yamanaka et al., 2004). On the other hand, it is reasonable to speculate that minor base-pair changes in the UGT1A7, UGT1A8, and UGT1A10 promoters may create functional binding sites for hepatic transcription factors, thus resulting in a polymorphic expression of these UGT enzymes in the liver.

In addition to UGT1A9, which participates in the metabolic pathways of a huge variety of endo- and xenobiotics, HNF4 α regulates the expression of numerous other xenobiotic- and drug-metabolizing enzymes and transporters, including CYP3A1, 3A4, 3A5, 2A6, 2B6, 2C9, and 2D6 (Jover et al., 2001; Kamiya et al., 2003). Among these target genes, the CYP3A enzymes share similar regulatory pathways with UGT1A9 (Tirona et al., 2003). Indeed, as for the UGT enzyme, *HNF4 α* gene disruption results in a drastic reduction of CYP3A11/13/16 expression in adult mouse liver, whereas an induction is found in both wild-type and HNF4 α -null livers after treatment with pregnenolone-16 α -carbonitrile (Tirona et al., 2003). It is interesting that the CYP3A4/5/7 and UGT enzyme families catalyze, respectively, the metabolic phase I and II reactions of approximately 35% of all clinically used molecules (Evans and Relling, 1999). These

observations indicate that the expression of major cytochrome P450- and drug-metabolizing enzymes is regulated in a coordinated fashion to reach an optimized response against the introduction of potentially toxic compounds in the organism.

In conclusion, this study identifies UGT1A9 as a novel HNF4 α target gene and demonstrates a role for this transcription factor in the hepatic expression of UGT1A9. Because the identified HNF4 site is not conserved in the UGT1A7, UGT1A8, and UGT1A10 genes, these data also provide a likely explanation for the absence of expression of these genes in liver.

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