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Title Page

Hepatic expression of the UGT1A9 gene is governed by HNF4α.

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Running Title Page

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Abbreviations:

UGT: UDP-glucuronosyltransferase,

HNF: Hepatocyte Nuclear Factor,

ChIP: Chromatine Immunoprecipitation

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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 HNF4α gene in mice drastically decreases liver UGT1A9 mRNA levels. Furthermore, a HNF4α response element (HNF4α RE) was identified in the promoter of human UGT1A9 at position -372 to -360 bp by transient transfection, electrophoretic mobility shift assays and chromatin immunoprecipitation experiments. Interestingly, this response element is absent in the proximal UGT1A7, UGT1A8 and UGT1A10 gene promoters. In conclusion, the present study identifies HNF4α 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 due, at least in part, to few base pair changes in their promoter sequences in the region corresponding to the HNF4α RE of the UGT1A9 gene.

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Introduction

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) enzymes family (Dutton, 1980). Based on 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 sub-families, UGT2A and UGT2B (Mackenzie et al., 1997). In humans, members of the UGT1A sub-family are encoded by a complex gene, which 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 9 functional proteins and encodes 4 pseudogenes by differential splicing: while 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 sub-family, UGT1A7, UGT1A8, UGT1A9 and UGT1A10 form a cluster of genes encoding highly homologous enzymes in both humans and rodents, since 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 similarity (>75%) within their proximal 1kb sequence (Gong et al., 2001; Gregory et al., 2003). Interestingly, 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) (Cheng et al., 1998; Mojarrabi and Mackenzie, 1998; Strassburg et al., 1998; Strassburg et al., 1997). By contrast, human UGT1A9 is highly expressed in the liver, kidney and intestine, whereas the UGT1A7 transcript is detected principally in the human oesophagus and stomach (Albert et al., 1999; Strassburg et al., 1998; Strassburg et al., 1999;

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Strassburg et al., 1997). 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*. recently demonstrated that the UGT1A8, UGT1A9 and UGT1A10 genes are differentially regulated by the hepatocyte nuclear factor (HNF)1α 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 (Gregory et al., 2003; Gregory et al., 2004). By contrast, the specific factor(s) which allow(s) the expression of UGT1A9 in liver, the main site for glucuronidation, has (have) 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 (C/EBP) and the hepatocyte nuclear factors (HNF)1, HNF3 and HNF4 (Cereghini, 1996). HNF4α (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 (Drewes et al., 1996; Xanthopoulos et al., 1991). Generation of mice lacking hepatic HNF4α 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, HNF4α controls the expression in human hepatocytes of numerous metabolizing enzymes, including cytochrome P450 (CYP) 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 HNF4α may also control the hepatic UGT1A9 expression. To verify whether HNF4α controls the expression of UGT1A9 in liver, UGT1A9 mRNA levels were analyzed in tissues from wild type or hepatic HNF4α-null mice. Our results indicate that HNF4α gene disruption provokes a drastic decrease in hepatic UGT1A9 expression. Furthermore, a functional HNF4α response element (HNF4α RE) located at position -372 to -360 base

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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.

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Materials and methods

Animal studies. Liver specific HNF4α-deficient mice were obtained as previously described (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 Corp. (Charbonnieres, France) and Roche (Mannheim, Germany). [γ-^{32}P]ATP was purchased from NEN-Life Sciences (Paris, France). Pregnenolone-16α-carbonitrile was from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were from Invitrogen (Cergy-Pontoise, France). ExGen 500 was from Euromedex (Souffelweyersheim, France).

RNA purification and Reverse-Transcription (RT). Total RNA was isolated from 100 mg of wet mouse livers using Trizol as specified by the supplier (Invitrogen). Two micrograms of total RNA from mouse livers were reverse transcribed in a final volume of 30 μl of first strand buffer (250 mM Tris-HCl, pH8.0, 375 mM KCl, 15 mM $MgCl_2$) using random hexamer primers, 40 units of RNaseOUT Recombinant ribonuclease inhibitor (Invitrogen) and 200 units of the Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase as recommended by the supplier (Invitrogen).

Real-time PCR. For real-time PCR analyses of reverse transcribed murine UGT1A9 and 28S cDNAs, RT products were diluted 1/10 and 1/200, respectively and 2 μl of dilution were used as amplification template using the previously described specific primers for mUGT1A9 and 28S (Barbier et al., 2003; Claudel et al., 2002). PCR amplifications were performed on a MX 4000 apparatus (Stratagene) in a volume of 25 μl containing a 100 nM concentration of each primer, 4 mM $MgCl_2$, the Brilliant

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Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene), and SYBR Green 0.33X (Sigma). 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 previously reported (Barbier et al., 2003). mUGT1A9 mRNA levels were subsequently normalized to 28S mRNA. A non-parametric 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 previously described (Barbier et al., 2003). The 1A9p0.5wt-pGL3 reporter construct was generated by digestion of 1A9p1wt-pGL3 with Nco I, and subsequent ligation into a Nco I-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 µl under the following conditions: denaturation at 94 °C for 3 min, 35 cycles of 15 sec at 94 °C, 40 sec 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 Xho I and Hind III restriction sites of the pGL3-basic vector to generate the 1A7p0.6wt, 1A8p0.6wt and 1A10p0.6wt reporter constructs. Mutations (respective change of A to G and T to C as previously reported (Fraser et al., 1998)) were introduced in the HNF4α response element to generate the 1A9p1mt and 1A9p0.5mt constructs, by using the Quick Change 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 grown as described (Claudel et al., 2002; De Tomassi et al., 2002). HepG2 cells ($60x10^3$ cells per well of 24-well plates) were transfected with 100 ng of the indicated luciferase reporter plasmids, 50 ng of the pCMV-βgalactosidase expression vector and

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with the indicated concentrations of pSG5-HNF4α 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 (FBS). At the end of the experiment, the cells were washed twice with ice-cold PBS, and lysed in 100 µl of lysis buffer (Triton X100 1%, Glycyl-glycine 25 mM, $MgSO_4$ 15 mM, EGTA 4 mM) for 30 min at room temperature. Ten µl of cell lysates were assayed for luciferase activity by using an LB9507 LUMAT luminometer (Berthold, France) in presence of 100 µl of luciferase assay buffer (Glycyl-glycine 25 mM, $MgSO_4$ 15 mM, ATP 5 mM, D-Luciferin 6.25 µM). Transfection efficiency was monitored by measuring the β-galactosidase activity (OD at 405 nm) of cell lysates (20 µl) after 2h incubation at 37°C in the presence of 200 µl of assay buffer (Na_2HPO_4 80 mM, KCl 8 mM, $MgSO_4$ 800µM, ONPG 1mg/ml).

Electrophoretic Mobility Shift Assays (EMSA). The HNF4α protein was synthesized *in vitro* using the TNT Quick Coupled Transcription/Translation System (Promega, Madisson, WN). Sense and antisense oligonucleotides (2.5 µg each) encompassing the different HNF4α response elements were annealed at a final concentration of 100 ng/µl in a final volume of 100 µl. One microlitre of double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP using T4-polynucleotide kinase to produce radiolabeled probes. The HNF4α protein was incubated for 15 min at room temperature in a total volume of 20 µl containing 2.5 µg poly(dI-dC) and 1 µg herring sperm DNA in binding buffer as previously described (Pineda Torra et al., 2002). The radiolabeled probes 1A9HNF4α REwt, 1A9HNF4α REmt (which contains the same mutation as introduced in the 1A9p1mt and 1A9p0.5mt promoter constructs), 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% non-denaturing polyacrylamide gel electrophoresis in 0.25X Tris-Borate-EDTA (TBE) at room temperature. For supershift experiments, the HNF4α protein was incubated for 15

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min at room temperature in the same buffer as above and the anti-HNF4α antibody (0.2 µg) (Santa-Cruz Biotechnology, 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 (ChIP). ChIP experiments were performed as previously described (Claudel et al., 2003). Briefly, DNA was sonicated 15 times for 15 sec with 45 sec intervals, on ice. A volume of sonicated DNA corresponding to 20×10^6 cells was then immunoprecipitated using 4 µg of an anti-HNF-4α (Santa Cruz Biotech) 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 non-precipitated (input) genomic DNA was then purified and resuspended into 100 µl H_2O. One tenth of the DNA preparations were PCR-amplified for 35 cycles (30 sec at 95°C, 30 sec at 58°C and, 30 sec at 72°C), using 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

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Results

HNF4α gene disruption decreases hepatic expression of murine UGT1A9.

A recent study identified HNF4α as an important regulator of hepatic metabolism (Kamiya et al., 2003). To investigate whether HNF4α also controls the hepatic expression of UGT1A9, liver mRNA levels were determined in floxed wild type (FLOX) and hepatic HNF4α-deficient (HNF4α -/-) mice. Interestingly, a 73% reduction in UGT1A9 transcripts was observed in the liver of HNF4α-deficient mice compared to the controls (Figure 1), indicating that HNF4α is a major determinant for the expression of UGT1A9 in mouse liver.

HNF4α activates the UGT1A9 gene promoter.

To determine whether the activity of the human UGT1A9 gene promoter can be modulated by HNF4α, 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-HNF4α expression vector. A dose-dependent induction of luciferase activity was observed in presence of increasing concentrations of this nuclear receptor, whereas the empty pSG5 plasmid failed to induce promoter activity (Figure 2). This indicates that HNF4α activates the proximal UGT1A9 promoter.

The UGT1A9 promoter contains a DR1 sequence at position –719 to –706 (Barbier et al., 2003). Since HNF4α 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 of UGT1A9 promoter activity by HNF4α (Figure 3), suggesting that HNF4α-dependent activation of the promoter occurs independently of the DR1. To confirm this observation, a 0.5 kb reporter construct of the

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human UGT1A9 promoter which does not contain the DR1 sequence was generated (1A9p0.5wt). As for the longer construct, HNF4α dose-dependently induced the activity of the 0.5 kb promoter fragment (Figure 3), thus demonstrating that HNF4α stimulates 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 HNF4αRE consensus sequence (Fraser et al., 1998; Odom et al., 2004). To test whether this site mediates the induction by HNF4α, mutations were introduced in the context of the 1.1 kb and 0.5 kb UGT1A9 promoter constructs (1A9p1mt and 1A9p0.5mt, respectively). Mutation of this potential HNF4αRE abolished the HNF4α-dependent induction of luciferase activity of both reporters (Figure 3). Taken together, these data indicate that the UGT1A9 promoter contains a functional HNF4αRE**,** which controls the induction of the human UGT1A9 promoter activity in the presence of this nuclear receptor.

HNF4α binds the cis-acting element at position -372 to -360 in the UGT1A9 gene promoter.

To determine whether human HNF4α binds to this response element in the UGT1A9 promoter, EMSA were performed using the wild-type or mutated HNF4α RE of the UGT1A9 promoter (1A9HNF4α REwt and 1A9HNF4α REmt, respectively), or the HNF4α RE of the apolipoprotein CIII gene promoter (apo CIII, positive control) as radiolabeled probes (Figure 4). Whereas no binding to any probes was observed in the absence of HNF4α (Figure 4a lanes 1 & 4**)**, the receptor bound both 1A9HNF4α REwt and apo CIII probes (lanes 2 & 5). Furthermore, these complexes were supershifted by the anti-HNF4α antibody (lanes 3 & 6). The presence of the anti-HNF4α antibody resulted in a much lower binding of the receptor to the 1A9HNF4α REwt probe (lane 3) compared to the Apo CIII probe (lane 6). However, lower exposure of the shift revealed a similar decrease of the binding onto the Apo

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CIII probe in the presence of the antibody (data not shown), and a similar reduction of the binding of HNF4α to DNA probes in the presence of the antibody was reported in various studies (Bartoov-Shifman et al., 2002; Stauffer et al., 1998; Stroup and Chiang, 2000; Wang et al., 2000). However, no protein-DNA complex was observed when using the mutated HNF4α RE probe (Figure 4b, lane 10). For competition experiments, increasing amounts (1, 10, 50 and 100-fold excess) of unlabeled oligonucleotides encompassing either the Apo CIII, 1A9HNF4α REwt and 1A9HNF4α REmt sites were added to the binding reactions (Figure 4c). HNF4α binding to the 1A9HNF4α REwt was strongly competed by the apo CIII site, and to a lower extent by the 1A9HNF4α REwt itself (Figure 4c, lanes 13 to 20), which indicate that HNF4α binds to the Apo CIII probe with a higher affinity compared to the 1A9HNF4α REwt. By contrast, the mutated RE did not efficiently compete for HNF4α binding to the probe encompassing the wild-type sequence (lanes 21 to 24). Taken together, these data demonstrate that HNF4α binds to the UGT1A9 HNF4α RE site at position -372 to -360 *in vitro*.

Occupancy of the UGT1A9 promoter by HNF4α in living cells was analyzed by using ChIP assays performed on DNA from HepG2 cells (Figure 5). Chromatin DNA was precipitated using either the anti-HNF4α or anti-HA antibodies or was incubated for the same period in the presence of protein A sepharose alone, and the sequence encompassing the HNF4α RE was amplified using specific primers (Figure 5, panel A, lane 4). The results clearly indicate that HNF4α occupies this region of the UGT1A9 promoter. As a negative control, amplification of an equivalent amount of genomic DNA precipitated with a non-relevant anti-HA antibody or incubated with protein A sepharose alone only slightly amplified the 145 bp fragment containing the response element (panel A, lanes 2&3). Moreover, when the same DNA samples were PCR-amplified with primers covering a region 1600 bp upstream of the HNF4α RE, no signal was observed (panel B). Finally, PCR-amplification with oligonucleotides for β-actin, as negative control for the immunoprecipitation, did not result in any signal (panel C). Taken together these results

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demonstrate that the HNF4α RE is immunoprecipitated by the anti-HNF4α antibody, thus indicating the binding of the nuclear receptor to this DNA region in living cells.

HNF4α does not bind to and fails to activate the human UGT1A7, UGT1A8 and UGT1A10 gene promoters.

To investigate whether HNF4α also affects human UGT1A7, UGT1A8 and UGT1A10 promoter activities, 0.6 kb fragments of these promoters were cloned into the 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-HNF4α plasmid (10 or 30 ng). As above, a dose-dependent induction of the 1A9p0.5wt driven reporter by HNF4α was observed, whereas the 1A9p0.5mt was unresponsive to HNF4α (Figure 6a). Interestingly, HNF4α failed to modulate the activity of the UGT1A7 promoter construct, while 50 and 65% reductions in UGT1A8 and UGT1A10 promoter activities, respectively, were obtained when the corresponding constructs were co-transfected with the pSG5-HNF4α plasmid (Figure 6a). Overall, these results demonstrate that HNF4α 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 HNF4α RE found in the UGT1A9 gene resembles more closely the consensus HNF4α response element sequence, than the corresponding site in the other genes, which present at least one additional nucleotide change (Figure 6b). This observation suggests that the absence of UGT1A7, UGT1A8 and UGT1A10 promoter activation by HNF4α, as observed in transient transfection experiments, is due to an inability of the receptor to bind these promoters. To verify this hypothesis, EMSA were performed using the wild-type or mutated HNF4α RE of the UGT1A9 promoter (as positive

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and negative control, respectively), or the corresponding sequences (table 1) in the UGT1A7, UGT1A8 and UGT1A10 promoters as radiolabeled probes (Figure 6c). No binding to any of the probes was observed in the absence of HNF4α, whereas, as expected, the receptor bound the 1A9HNF4α REwt probes (lanes 2). By contrast, no protein-DNA complex formation was observed when using the 1A9 HNF4α REmt, 1A7 –368/-355, 1A8 –384/-371 or 1A10 –369/-356 probes (Figure 6c).

To further ascertain that few changes in the HNF4α RE sequence are able to knock down the HNF4α-dependent activation of UGT1A7, UGT1A8 and UGT1A10 promoters, the 1A9p0.5mt to 1A8 and 1A8o.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) (Figure 6b). These constructs were subsequently transfected into HepG2 cells in the same conditions as above (Figure 6d). Interestingly, the luciferase activity of the mutated 1A9p0.5mt to 1A8 plasmid was drastically reduced, with only a 1.4-fold activation when co-transfected with HNF4α. By contrast, the 1A8p0.6mt to 1A9 displayed a 2.5-fold activation in presence of HNF4α (30 ng), instead of a down-regulation as observed with the wild type promoter. The drastic reduction of HNF4α-dependent activation of the mutated UGT1A9 promoter demonstrates the importance of the HNF4α RE located at position -372 to -360. The 2.5-fold activation of the mutated UGT1A8 promoter construct observed in presence of HNF4α indicate that introducing the HNF4α RE from the UGT1A9 allows an activation of the promoter. However, the lower HNF4α-dependent induction of the mutant UGT1A8 promoter when compared to the wild type UGT1A9 (2.5-fold and 7.8-fold, respectively) indicates that the HNF4α RE is insufficient to drive hepatic expression of UGT enzymes under the control of HFN4α.

Overall, these data demonstrate that HNF4α binds and activates specifically the UGT1A9 promoter, while the UGT1A7, UGT1A8 and UGT1A10 promoters are either unaffected or inhibited by this transcription factor.

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Discussion

In the present study, we identify UGT1A9 as a hepatic target gene of HNF4α. Whereas Odom D.T. *et al.* (Odom et al., 2004) recently reported the presence of HNF4α RE in promoter regions of UGT2B11 and UGT2B15 genes, the present study is the first demonstration that HNF4α regulates the expression of an UGT enzyme in the liver. However, the similar patterns of tissue-specific expression shared by UGT1A9 and HNF4α (Albert et al., 1999; Xanthopoulos et al., 1991), suggested that UGT1A9 could be a HNF4α target gene. On the other hand, Metz R. *et al.* (Metz et al., 2000) previously reported that HNF4α does not regulate UGT1A7 expression in rat hepatocytes. Consistently with this result, we observed that HNF4α 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 PPAR 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). While it was previously shown that HNF4α binds to DR1 sequences (Fraser et al., 1998; Odom et al., 2004), we found that the PPRE is not involved in the HNF4α-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 HNF4α 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 UGT1A9 by HNF4α (Barbier et al., 2003). This observation demonstrates that few base pair changes in the HNF4α 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 HNF4α RE of

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the UGT1A9 promoter and the corresponding regions in the UGT1A7, UGT1A8 and UGT1A10 promoters abolish the binding of HNF4α.

The human UGT1A7, UGT1A8, UGT1A9 and UGT1A10 genes possess highly conserved sequences in both coding and promoter regions (Cheng et al., 1998; Gregory et al., 2003; King et al., 2000). 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 (Albert et al., 1999; King et al., 2000; Strassburg et al., 1998; Strassburg et al., 1999). Results presented here suggest that HNF4α 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 due to the absence of HNF4α-dependent activation of these genes. However, the residual expression of UGT1A9 in livers from hepatic HNF4α-deficient mice and the low HNF4α-dependent activation of the mutated UGT1A8 promoter construct suggest that other transcription factors may participate, with HNF4α, to the differential control of UGT1A9 expression in this tissue. Consequently, it could not be excluded that UGT1A7, UGT1A8 and UGT1A10 promoters also lack response elements for such transcription factors. Such hypothesis is supported by the observation that introducing the HNF4α responding region of the UGT1A9 gene in the UGT1A8 promoter context only allows a slight induction of its activity in presence of HNF4α (Figure 6d). Furthermore, despite that HNF4α does not bind nor activate the examined UGT1A7, UGT1A8 and UGT1A10 promoters, we cannot exclude that response elements for HNF4α 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 presence of HNF4α suggesting that the receptor may negatively regulate the expression of these genes. To

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the best of our knowledge, this is the first observation which suggests that HNF4α can negatively regulate gene promoter activity. However, we cannot exclude that this observation is due to an artifact of the transient transfection assay, such as the squelching of transcription machinery proteins by over-expressed HNF4α protein. Interestingly, the expression of the medium chain acyl-CoA dehydrogenase (MCAD) is increased in livers from HNF4α-null mice compared to wild-type animals, suggesting that HNF4α 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.

Interestingly, Gregory *et al*. recently reported that the UGT1A8 and UGT1A10 promoters possess an 8-fold higher basal activity compared to the UGT1A9 promoter when transfected into intestinal Caco2 cells, and that this difference is due to two base pair differences in a Sp1 binding site in the UGT1A9 promoter sequence (Gregory et al., 2003). The same authors also demonstrated that the intestine-specific transcription factor, caudal-related homeodomain protein 2 (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 HNF1α 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 (Gagné et al., 2002; Guillemette, 2003; Tukey and Strassburg, 2000; Yamanaka et al., 2004); thus it is tempting to speculate that SNPs located in the HNF4α 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). Whereas this hypothesis remains to be clearly established, it is supported by the recent identification of a SNP in the AT-rich region of the UGT1A9 promoter which

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provokes a 2.6-fold higher luciferase activity of the promoter in HepG2 cells (Yamanaka et al., 2004). On the opposite, 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, HNF4α 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, HNF4α 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 HNF4α-null livers following treatment with PCN (Tirona et al., 2003). Interestingly, 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 CYP- 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 HNF4α target gene and demonstrates a role for this transcription factor in the hepatic expression of UGT1A9. Since 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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Footnotes

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Legends of figures

Figure 1. HNF4α gene disruption decreases basal UGT1A9 expression in mouse liver.

UGT1A9 mRNA levels in livers from hepatic HNF4α-null (HNF4α -/-) mice were analyzed by real-time RT-PCR and compared with those from wild-type (FLOX) mice (n=3 per group). Values are expressed as means ± SD, relative to the control group set as 1. Statistically significant differences between the different groups are indicated by asterisks (Mann-Whitney test: ***, $p < 0.001$).

Figure 2. HNF4α 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-HNF4α 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, normalized to internal β-galactosidase activity as described in experimental procedures. Values represent the means ± SD.

Figure 3. HNF4α 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 (Luc) reporter plasmids (100 ng) in the absence or presence of pSG5-HNF4α (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, normalized to internal β-galactosidase activity as described in experimental procedures. Values represent the means ± SD.

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Figure 4. HNF4α binds to the HNF4α RE in the UGT1A9 promoter.

Electrophoretic Mobility Shift Assays (EMSA) were performed with end-labeled wild-type or mutated 1A9HNF4α RE or apo CIII probes in the presence of unprogrammed reticulocyte lysate or HNF4α proteins as indicated.

(**a**) Supershift experiments with 1A9HNF4α RE or apo CIII probes were carried out using an anti-HNF4α antibody (0.2 µg).

(**b**) An equal amount of wild type or mutated 1A9HNF4α RE probes were assayed for binding with unprogrammed reticulocyte lysate or HNF4α as described in experimental procedures.

(**c**) Competition EMSA on radiolabeled 1A9HNF4α RE probe were performed by adding 1-, 10-, 50- or 100-fold molar excess of the indicated cold apo CIII, 1A9HNF4α REwt, or 1A9HNF4α REmt oligonucleotides in EMSA with unprogrammed reticulocyte lysate or HNF4α.

Figure 5. HNF4α binds to the HNF4α RE in the UGT1A9 promoter in cells.

Soluble chromatin was prepared from HepG2 cells as described in experimental procedures, and immunoprecipitated with anti-HNF4α (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 HNF4α RE (Panel A), a distal region of the UGT1A9 promoter (Panel B) or the β-actin gene (Panel C) as negative control.

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Figure 6. HNF4α 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-HNF4α (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, normalized to internal β-galactosidase activity as described in experimental procedures. Values represent the means ± SD.

(b) Sequences 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 HNF4α RE, whereas boxes correspond to bases governing the binding by HNF4α.

(c) EMSA were performed with end-labeled wild-type or mutated 1A9HNF4α RE, the corresponding 1A7-368/-355, 1A8 –384/-371 and 1A10 –369/-356 probes in the presence of unprogrammed reticulocyte lysate or HNF4α proteins as indicated.

(d) HepG2 cells were transfected with the indicated luciferase reporter plasmids (100 ng) in the absence or presence of pSG5-HNF4α (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, normalized to internal β-galactosidase activity as described in experimental procedures. Values represent the means ± SD. Values indicated represent the HNF4α-dependent activation of each reporter construct.

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Tables and Figures

Table 1. Oligonucleotides used in this study.

These oligonucleotides were used to amplify UGT1A7, UGT1A8 or UGT1A10 promoters, to mutate the HNF4α 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 bold correspond to the HNF4α response element and nucleotides in italic correspond to mutated bases. The ApoCIII probe derives from (Mietus-Snyder et al., 1992).

Promoter cloning	
UGT1A7p(Xho I) Sense	5’-CTAGCACTCGAGCGAGACCAGCCTGG
UGT1A7p(HindIII) Antisense	5’-CTAGCTGAAGCTTATCAGAGAACTTCAGCCC
UGT1A8/10p(XhoI) Sense	5’-CTAGCACTCGAGCAGGGTTGTCAATGTCATTTC
UGT1A8p(HindIII) Antisense	5’-CTAGCTGAAGCTTATGAGAGAACTGCAGCCC
UGT1A10p(HindIII) Antisense	5’-CTAGCTGAAGCTTATGAGAGAACTGCAGCCC
Site directed mutagenesis	
1A9p1 mt and 1A9p0.5 mt	5’-TTTGCTCT**GGGAC*GGGCCTTG*AA**AAAAATTAG
1A9p mt to 1A8	5’-TTGCTCT**GGGA*TG*AATTCCAA**AAAA
1A8p mt to 1A9	5’-TTGCTTTGGGA*CA*AATTCCAAAAAT
EMSA	
1A9HNF4α REwt	5’-TTTGCTCT**GGGACAAATTCCAA**AAAAAATTAG
1A9HNF4α REmt	5’-TTTGCTCT**GGGAC*GGGCCTTG*A**AAAAAATTAG
1A7 –368/-355	5’-TTTTCTTTGTGACAAATTCCAAAATTATTAGG
1A8 –364/-371	5’-TTTGCTTTGGGATGAATTCCAAAAATATTAGC
1A10 –369/-356	5’-TTTGCTTTGGGATAAATTCCAAAAATATTAGC
Apo CIII	5’-CAGCAGGTGACCTTTGCCCAGCGCCC
ChIP	
UGT1A9 –386 (sense)	5’-TGAGTTGCCATCTTCTCTGG
UGT1A9 –241 (antisense)	5’-ATGCTTTTGGACCTTGAAGGT
UGT1A9 –2050 (Sense)	5’-GATTACAGGCATGCACCACCACCT
UGT1A9-1890 (antisense)	5’-CTCACACCTGTAGTCCCAGCAC
β-actin forward	5’-CGAGCCATAAAAGGCAACTTTCG
β-actin reverse	5’-AGGAAGAGGAGGAGGGAGAGTTT

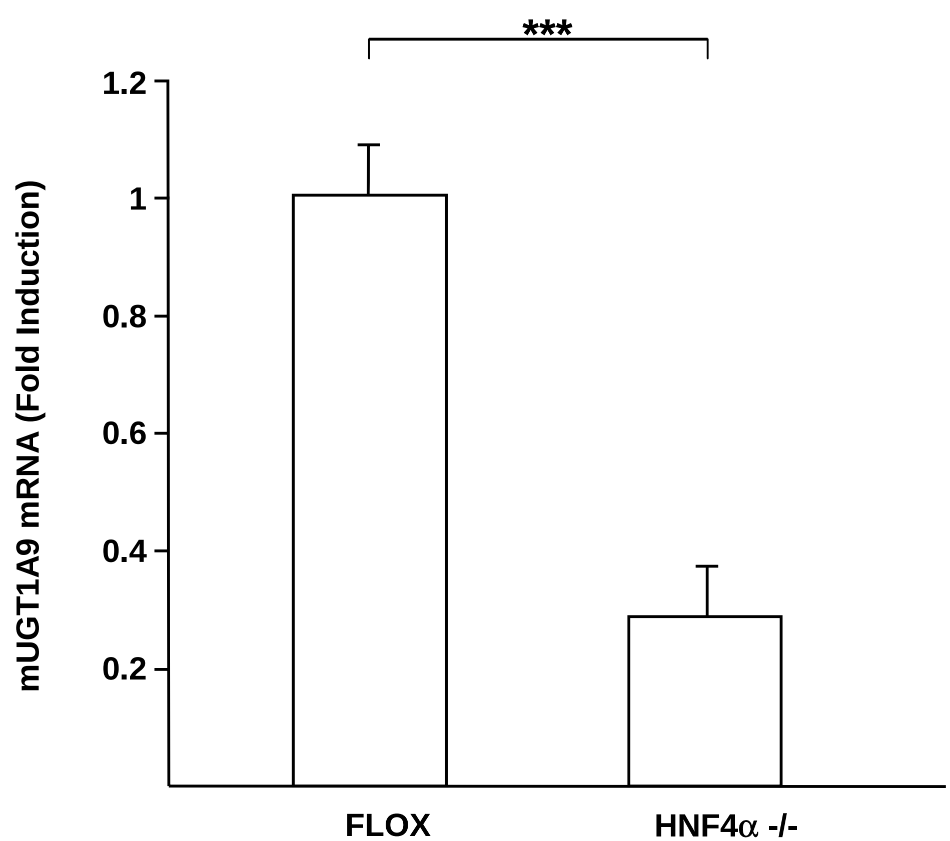
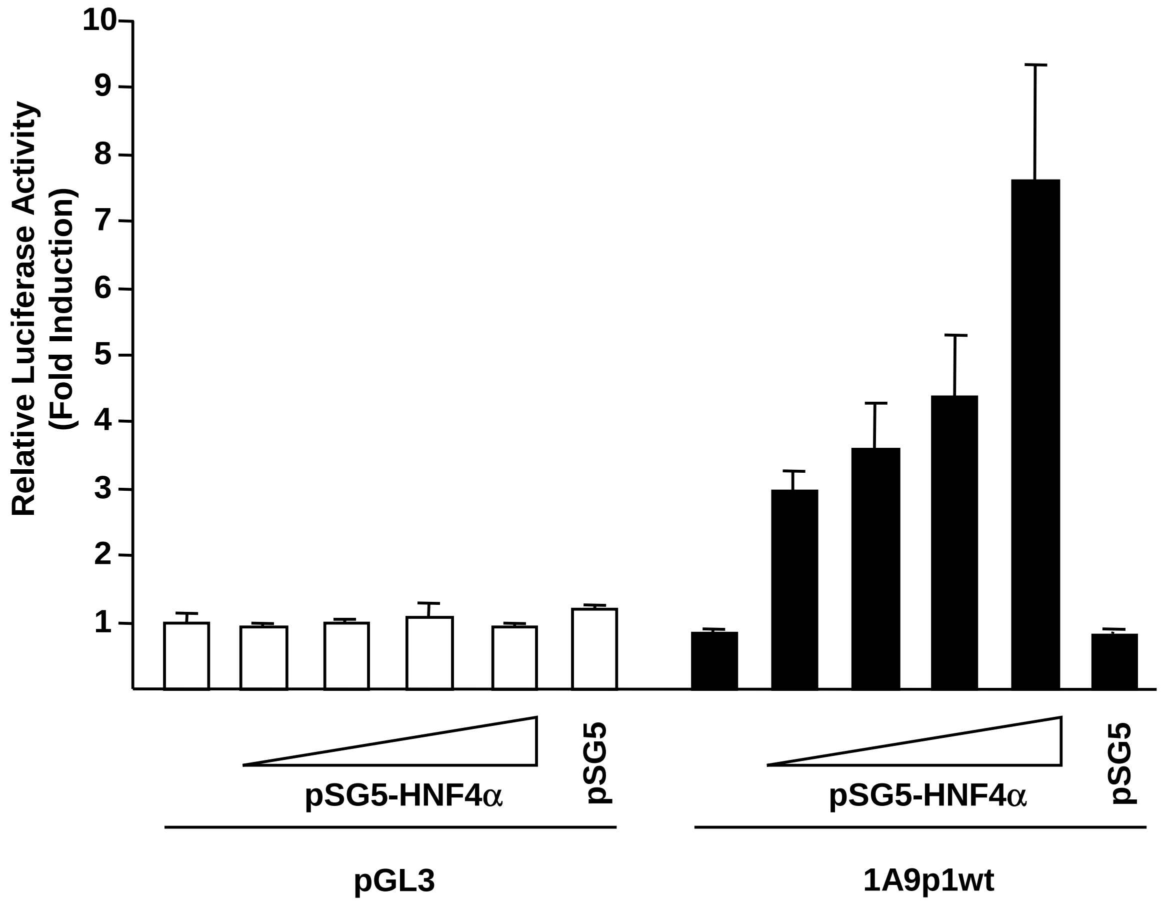
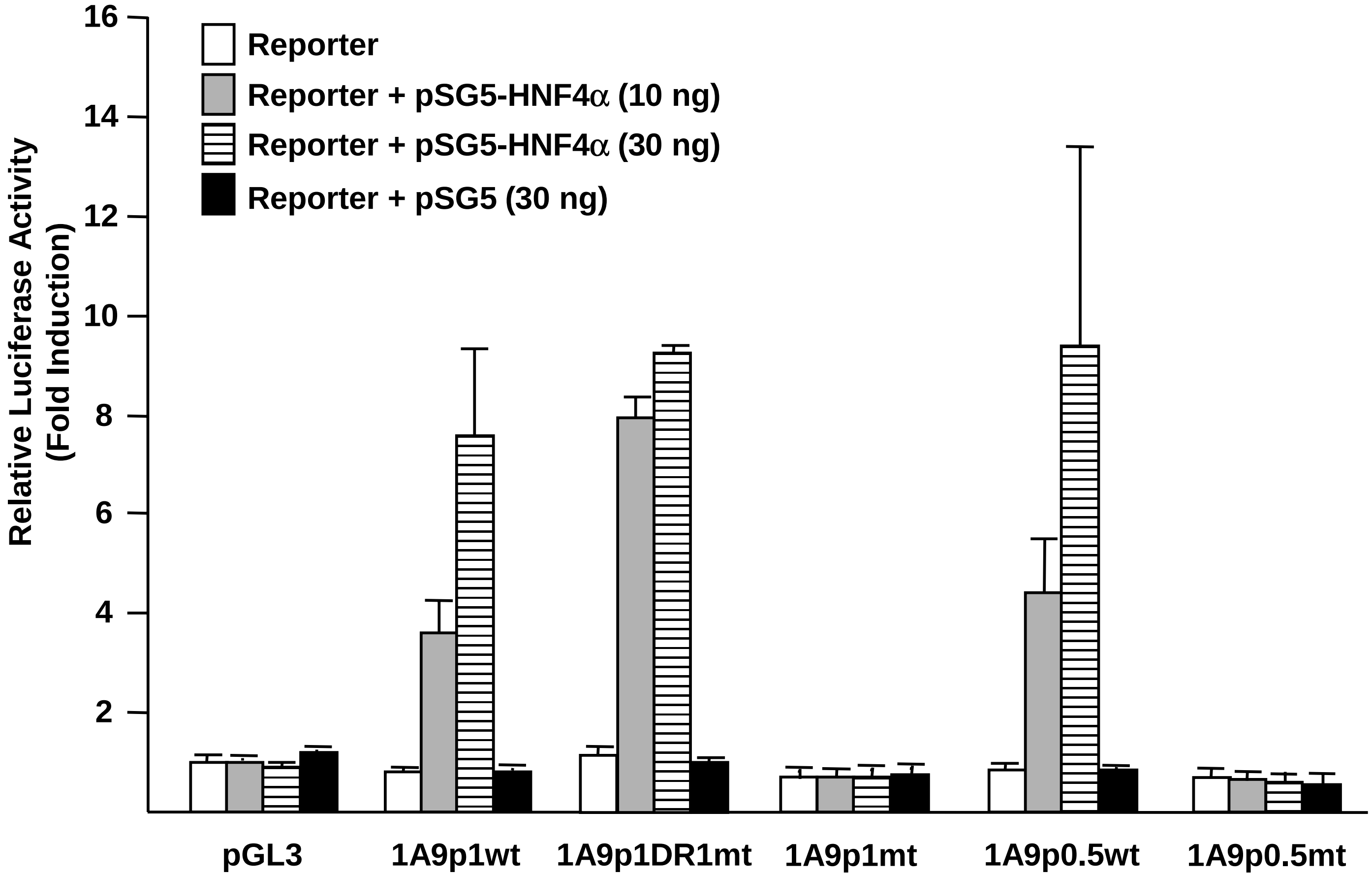


Figure 1



10
9
8
7
6
5
4
3
2
1
Relative Luciferase Activity
(Fold Induction)
pSG5-HNF4α
pSG5
pSG5-HNF4α
pSG5
pGL3
1A9p1wt

Figure 2



16
14
12
10
8
6
4
2
Relative Luciferase Activity (Fold Induction)
Reporter
Reporter + pSG5-HNF4α (10 ng)
Reporter + pSG5-HNF4α (30 ng)
Reporter + pSG5 (30 ng)
pGL3
1A9p1wt
1A9p1DR1mt
1A9p1mt
1A9p0.5wt
1A9p0.5mt

Figure 3

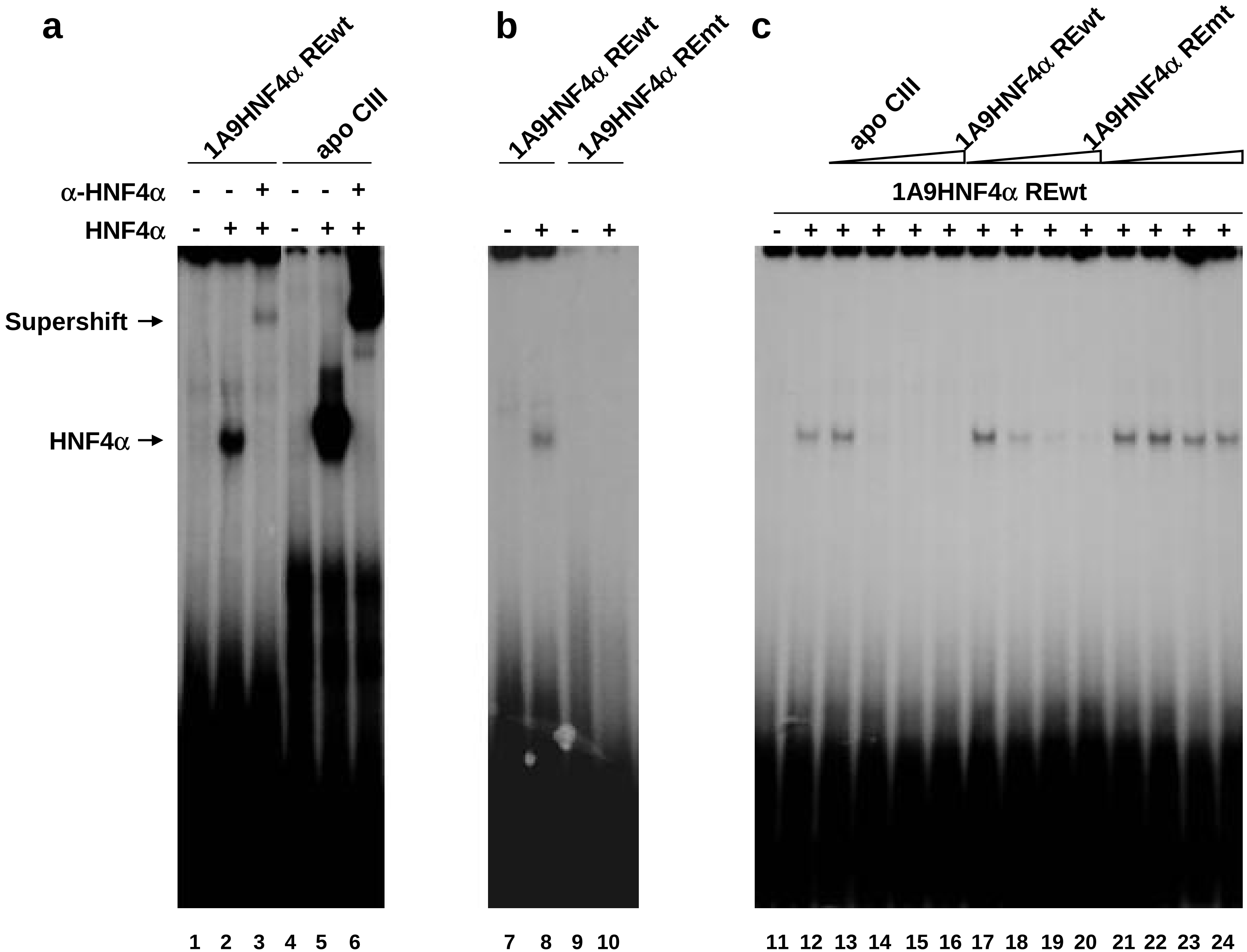

a
1A9HNF4α REwt
apo CIII
α-HNF4α - - + - - +
HNF4α - + + - + +
Supershift
HNF4α
1 2 3 4 5 6
b
1A9HNF4α REwt
1A9HNF4α REmt
- + - +
7 8 9 10
c
apo CIII
1A9HNF4α REwt
1A9HNF4α REmt
1A9HNF4α REwt
- + + + + + + + + + + + + +
11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 4

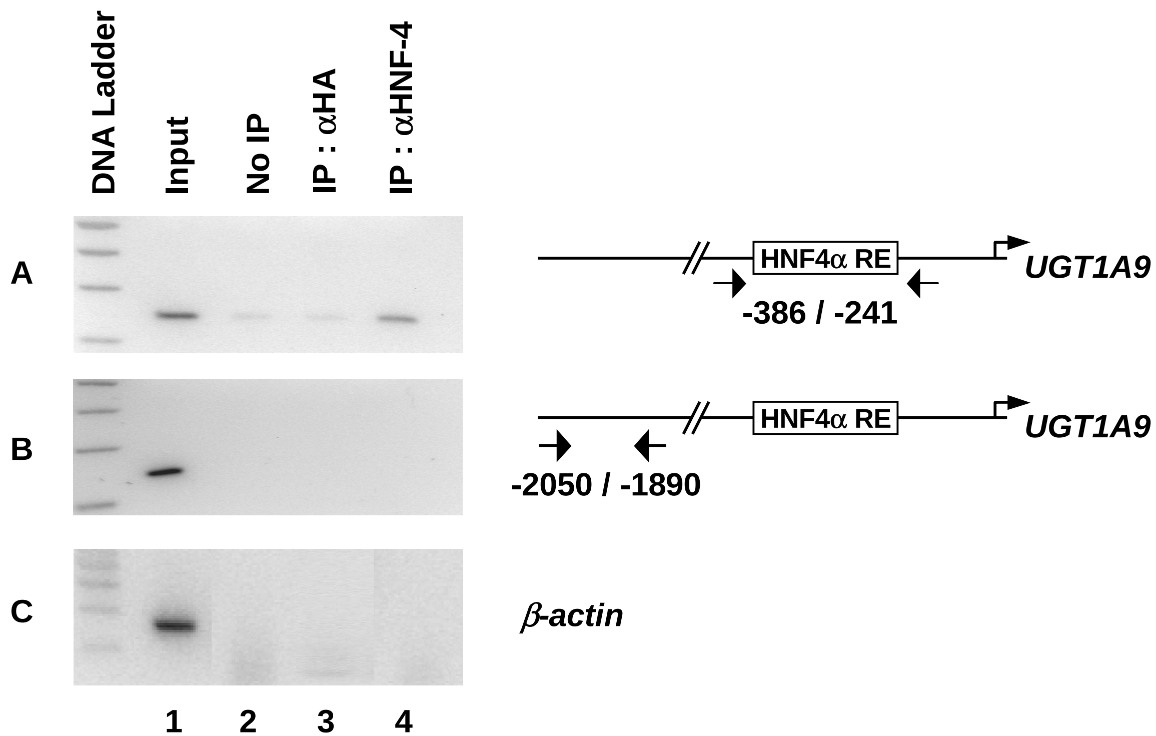
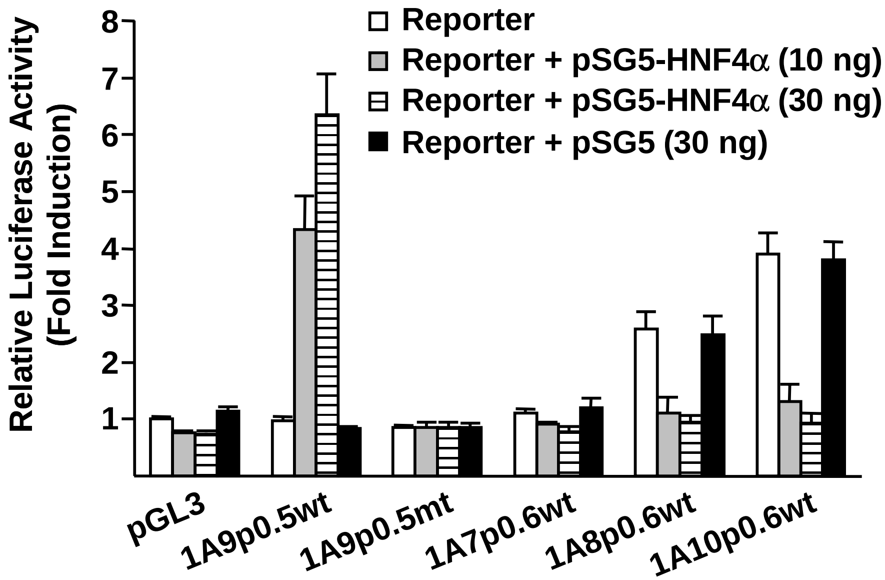

DNA Ladder
Input
No IP
IP : αHA
IP : αHNF-4
A
B
C
HNF4α RE
UGT1A9
-386 / -241
HNF4α RE
UGT1A9
-2050 / -1890
β-actin
1
2
3
4

Figure 5

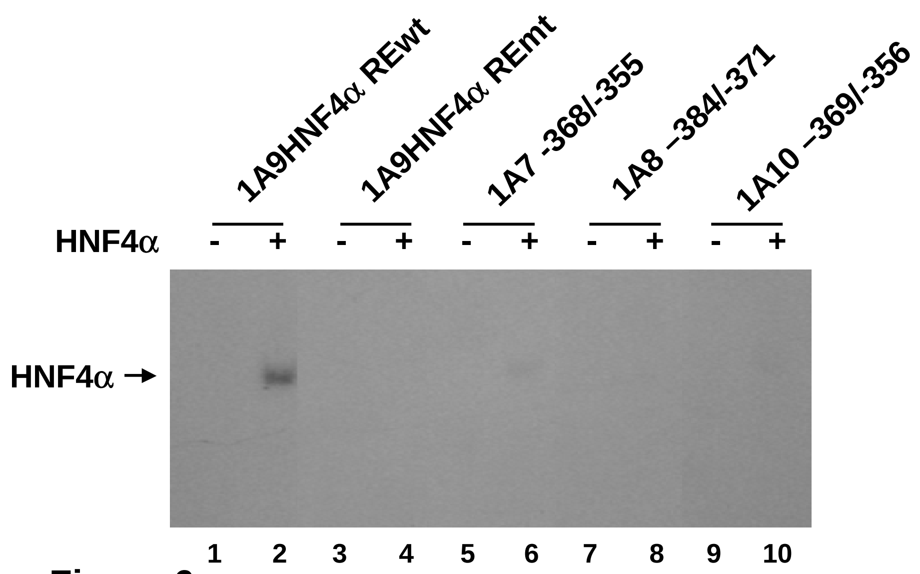
a



b

	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6
Consensus	G/A	G	G	N	C	A	A	A	G	G/T	C/T	C	A/G
UGT1A9wt	G	G	G	A	C	A	A	A	T	T	C	C	A
UGT1A8	G	G	G	A	T	G	A	A	T	T	C	C	A
UGT1A7	G	T	G	A	C	A	A	A	T	T	C	C	A
UGT1A10	G	G	G	A	T	A	A	A	T	T	C	C	A

c



d

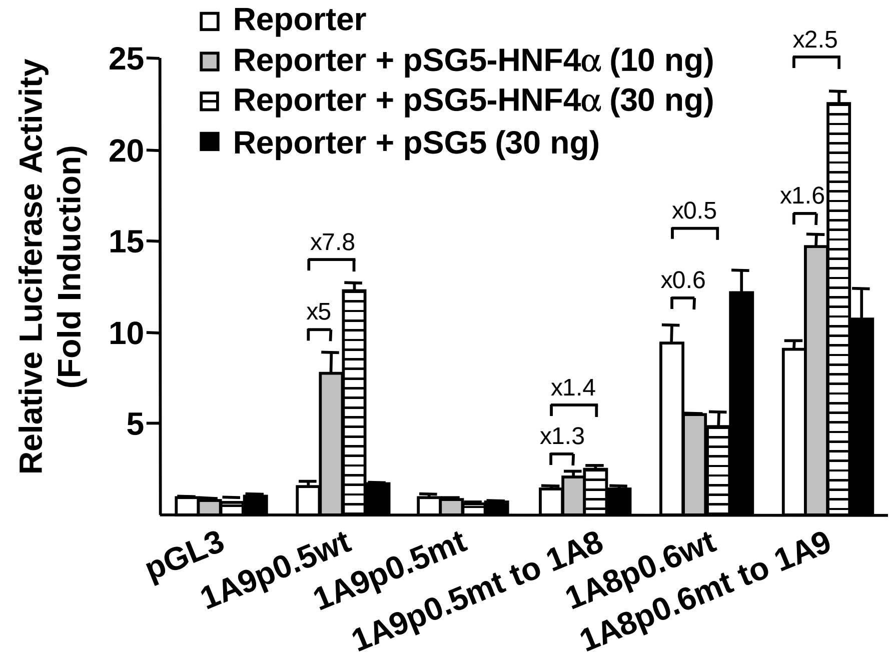


Figure 6