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Involvement of Hepatocyte Nuclear Factor 1 in the Regulation of the UDP-Glucuronosyltransferase 1A7 (UGT1A7) Gene in Rat Hepatocytes[1]

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ABSTRACT

UDP-glucuronosyltransferase 1A7 (UGT1A7) is a major UGT contributing to the glucuronidation of xenobiotic phenols in rats. Its expression in rat liver is tightly regulated, with low constitutive and high inducible expression in response to aryl hydrocarbon receptor ligands and oltipraz. Previously, we reported the absence of 3-methylcholanthrene- or oltipraz-responsive elements in the 1.6-kbp region flanking the *UGT1A7* promoter. However, potential binding sites were noted for several liver-enriched transcription factors. Here we show that deletion of the hepatic nuclear factor (HNF)3, HNF4, and CCAAT-enhancer binding protein-like binding sites had no effect on the expression of a *UGT1A7* reporter plasmid, p(−965/+56)1A7-Luc, in primary rat hepatocytes. The full activity of the promoter was contained in the region between bases −157 and +76. Two sites of binding by rat liver nuclear proteins were detected in this region by DNase footprinting. PR-1 corresponded to the HNF1-like binding site between bases −52 and −38, whereas PR-2 was located between −30 to −6. Gel retardation studies supported the presence of HNF1α in the PR-1 DNA-liver nuclear protein complex. Mutation of PR-1 inhibited binding in the gel shift assay, prevented activation by overexpressed HNF1 in human embryonic kidney cells, and reduced by >80% the maximal luciferase activities expressed from basal and 3-methylcholanthrene-responsive UGT1A7 gene reporter constructs in primary rat hepatocytes. These data provide evidence for an important stimulatory role of HNF1 in promoting UGT1A7 gene expression in rat liver.

Conjugation with glucuronic acid represents one of the major phase 2 detoxification pathways in mammals, and the liver represents one of the primary sites of this reaction. This most likely is due to differential expression within hepatocytes of the enzymes that catalyze glucuronidation, the UDP-glucuronosyltransferases (UGTs). Two related families of UGTs are known, UGT1 and UGT2 (Mackenzie et al., 1997); many from each family are expressed in liver. Although the mechanisms underlying their predominant expression in liver remain poorly understood, recent studies investigating the mechanisms of regulation of the UGT1A1 and UGT2B1 genes suggest the involvement of two different liver-enriched transcription factors: hepatic nuclear factor-1 (HNF1) and CCAAT-enhancer binding protein-α (C/EBPα; Hansen et al., 1997, 1998; Bernard et al., 1999).

In the rat UGT family, one of the major UGTs expressed in liver for the glucuronidation of bulky planar substrates is UGT1A7. The 1A7 isoform is active toward several metabolites of benzo(a)pyrene, a well-established environmental carcinogen. These include the major phase 1 metabolites, 3-hydroxy-, 9-hydroxy-, and 3,6-dihydroxy-benzo(a)pyrene, and the toxicologically significant proximate carcinogen 7,8-dihydrodiol (Grove et al., 1997). UGT1A7 appears unique among UGTs in its ability to form diglucuronides of both chrysene and benzo(a)pyrene-3,6-diphenol (Bock et al., 1999). UGT1A7 from other species has been shown to catalyze the glucuronidation of dietary phenols such as octyl gallate (Bruck et al., 1997), steroid hormone metabolites such as catechol estrogens and 4-hydroxyestrone, tertiary amine drugs such as imipramine (Bruck et al., 1997), and the environmental carcinogen, 2-hydroxyamino-1-methyl-6-phenylimidazo-(4,5β)-pyridine (Strassburg et al., 1999). UGT1A7 also catalyzes the glucuronidation of 7-ethyl-10-hydroxy-camptothecin (SN-38; Ciotti et al., 1999), the active metabolite of the anticancer drug irinotecan, which is implicated in the severe gastrointestinal toxicity observed in some irinotecan-treated patients.

In adult rats, the expression of *UGT1A7* in liver appears to

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HNF, hepatic nuclear factor; C/EBP, CCAAT-enhancer binding protein; HEK, human embryonic kidney; AHR, aryl hydrocarbon receptor; 3-MC, 3-methylcholanthrene; EMSA, electrophoretic mobility shift assay; PAS, period/aryl hydrocarbon receptor nuclear translocator/aryl hydrocarbon receptor/single-minded; kbp, kilobase pair; bp, base pair(s).

be tightly controlled. Control rats exhibit low levels of hepatic microsomal UGT1A7 protein levels as well as low glucuronidating activities toward benzo(*a*)pyrene-7,8-dihydrodiol, a marker substrate of UGT1A7. UGT1A7 mRNA is virtually undetectable by standard Northern blot analysis (Grove et al., 1997). However, on treatment with the aryl hydrocarbon receptor (AHR) agonists 3-methylcholanthrene (3-MC) and β-naphthoflavone, UGT1A7 mRNA levels are markedly increased (Emi et al., 1995; Kobayashi, 1998; Metz and Ritter, 1998). UGT1A7 is also highly induced by oltipraz, a candidate chemopreventive agent in the phase 2 detoxifying enzyme inducer subclass (Grove et al., 1997). Interestingly, this pattern of expression is unique to the liver. The two tissues showing the highest constitutive expression of UGT1A7, intestine and kidney, exhibit lower or no inducibility by oltipraz and β-naphthoflavone (Grove et al., 1997; Kobayashi et al., 1998). Other tissues, such as the spleen, lung, and ovary, express UGT1A7 at slightly higher constitutive levels compared with liver and show no inducibility by oltipraz.

Data from nuclear runoff assays support a role for transcriptional activation in the mechanism of the observed increase in liver UGT1A7 mRNA levels by inducing agents (Metz and Ritter, 1998). Although the locations of elements mediating transcriptional activation remain unknown, the existence of a promoter in the region flanking the *UGT1A7* transcription start sites determined by primer extension and RNase protection analysis (Metz and Ritter, 1998) was supported in gene reporter experiments. Primary rat hepatocytes transfected with the *UGT1A7* reporter plasmid p(−965/+56)1A7-Luc exhibited an increase in luciferase expression compared with cells transfected with the promoterless reporter plasmid pGL3-Basic. Sequence analysis of the promoter indicated the possible contribution of several liver-enriched transcription factors, HNF1, HNF3, HNF4, and C/EBP, in the detected basal expression of the *UGT1A7* promoter.

To test the hypothesis that one or more of these factors contribute to the function of the *UGT1A7* promoter, we investigated the effect of 5′-terminal deletions on the activity of the promoter using primary rat hepatocytes transiently transfected with various *UGT1A7* reporter plasmids. Unexpectedly, the full activity of the promoter was found to reside in the region between −157 and +76 (respective to the 5′-most transcriptional start site), suggesting that neither HNF3, HNF4, nor C/EBP is required for the basal function of the promoter. Analysis by DNase footprinting revealed two sites of binding by rat liver nuclear proteins. One of these corresponds to the HNF1-like binding site located between bases −52 and −38, and the other is a region normally associated with TATA protein and RNA polymerase II binding. Evidence is presented that the HNF1-like site is functional and that HNF1 plays a role as a general enhancer of constitutive and induced UGT1A7 gene expression in rat liver.

Experimental Procedures

Materials. Adult male Sprague-Dawley rats used in this study for the preparation of rat liver nuclei and primary hepatocyte cultures were maintained on a standard 12-h light/dark cycle and allowed free access to water and standard laboratory rodent chow. All uses and handling of the animals required for this study were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

β-Naphthoflavone was obtained from Aldrich Chemical Co. (Arlington Heights, IL). Oltipraz was a gift from the National Cancer Institute, National Institutes of Health (Bethesda, MD). [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from DuPont-New England Nuclear (Boston, MA). All restriction endonucleases were purchased from Promega (Madison, WI). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture supplies were obtained from Mediatech (Bethesda, MD). LipoFECTIN cationic liposomes and the Luciferase Assay System were purchased from Life Technologies (Grand Island, NY) and Promega, respectively. The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). All synthetic oligonucleotides used in this study were prepared by Life Technologies.

Plasmids. The construction of p(−965/+56)1A7-Luc and p(+56/−965)1A7-Luc was described previously (Metz and Ritter, 1998). p(−619/+71)1A7-Luc and p(−157/+71)1A7-Luc were generated by polymerase chain reaction amplification using either 5′-CTA AGG GGC ATC ACA GT-3′ or 5′-CAC ATT GTG TGA TTC TG-3′, respectively, as the forward primer, 5′-ACC AAG GCA AGA CAG CTG CTG AGA CTG T-3′ as the common 3′ primer, and p1.4XB (cloned *UGT1A7* genomic DNA; Metz and Ritter, 1998) as template. Each amplicon was initially cloned into pCR2.1, excised with *Eco*RI, and blunt-ended with Klenow DNA polymerase and then cloned into *Sma*I cut pGL3-Basic, forming p(−619/+71)1A7-Luc and p(−157/+71)1A7-Luc. p340XP contains a 340-bp *Xba*I/*Pst*I fragment from p1.4XB corresponding to bases −253 to +96 cloned into the *Xba*I/*Pst*I sites of pBluescript SK$^+$.

p(−965/+56)1A7 mutPR-1-Luc was generated using the QuikChange site-directed mutagenesis kit (Stratagene) and 5′-TTC TCC CAA GTc gcT CAT TGC CAG GAA CTG-3′ and 5′-CAG TTC CTG GCA ATG Agc gAC TTG GGA GAA-3′ as the forward and reverse amplification primers. The sequence contains three nucleotide substitutions (indicated by lowercase letters) in the more conserved 5′-half site of the *UGT1A7* HNF1-like binding site. Sequencing of the *UGT1A7* promoter insert in p(−965/+56)1A7 mutPR1-Luc revealed no other differences. The same mutation in the rat UGT2B1 HNF1 binding site was shown to prevent binding by HNF1 in the electrophoretic mobility shift assay (EMSA; Hansen et al., 1997).

The rat HNF1α expression vector, pRHNF1α, was provided by Dr. Philip Hylemon (Department of Microbiology and Immunology, Virginia Commonwealth University). It contains the coding region of the rat HNF1α cDNA inserted in the correct orientation behind the cytomegalovirus (CMV) promoter of pRc-CMV (Invitrogen, San Diego, CA). pRHNF1β was generated by amplification of the HNF1β coding region from F344 rat kidney cDNA using 5′-C CGT TCT TGG AAA ATG GTG TCC-3′ and 5′-GTG GTT ATG TGG GTA TCA CCA-3′) as primers. After amplification, a product of the predicted size (~1.6 kbp) was ligated with *Eco*RV-cut pSK+ to generate pSK-RHNF1β (clone B9). Restriction endonuclease site mapping with *Sac*I, *Sac*II, and *Pvu*II was consistent with the published sequence of the rat HNF1β sequence. The HNF1β expression vector pCMV-RHNF1β was prepared by excising the HNF1β coding region from pSK-RHNF1β clone B9 with *Eco*RI and *Xho*I, blunt-ending with Klenow, and inserting the fragment into the *Eco*RV site of pcDNA3. pCMV-RHNF1β expresses rat HNF1β under the control of the CMV promoter.

A 3-MC-responsive *UGT1A7* reporter plasmid was constructed by cloning the 3.7-kbp *Bam*HI fragment of rat *UGT1A7* genomic DNA clone λRPT-6 (Metz and Ritter, 1998) into the *Bam*HI site of either p(−965/+56)1A7-Luc or p(−965/+56)1A7 mutPR1-Luc. The latter fragment encompasses the last 414 bp of the UGT1A7 coding region and ~3.3 kbp of 3′-flanking intron sequence and contains 3-MC-responsive element or elements that are currently being characterized in this laboratory. The resulting 3-MC-responsive *UGT1A7* reporter plasmids were designated p(−965/+56)1A7-Luc-3.7B and p(−965/+56)1A7-Luc-3.7B, respectively.

Cell Culture. Primary rat hepatocytes isolated by collagenase-EDTA perfusion were allowed to adhere to gelatin-coated plastic tissue culture plates (0.8×10^6 cells/35-mm dish) at 37°C under a humidified atmosphere containing 5% CO_2. The plating medium was Williams E (Life Technologies) without fetal bovine serum, supplemented with 0.6 μg/ml insulin, 100 μg/ml transferrin, 1 μM dexamethasone, fungizone, and gentamicin.

The continuous cell lines (HepG2, HeLa, and HEK) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

RNA Isolation and Northern Analyses. Total RNA from cultured cells was isolated and analyzed by Northern blotting as described previously (Grove et al., 1997), using rat HNF1 cDNA labeled with ^{32}P-dCTP by random priming as the probe. After exposure of the hybridized blot to x-ray film for 6 days, the blot was rehybridized with radiolabeled rat cyclophilin cDNA to demonstrate similar loading and quality of the RNA samples.

Transient Transfection. Primary rat hepatocytes were transfected 6 h after plating by removing the medium and adding 1.5 ml of fresh Williams E (without supplements) containing freshly mixed LipoFECTIN (3.75 μg/dish) and *UGT1A7* reporter plasmid (1.5 μg). For normalization of interplate differences in transfection efficiency, a small amount (0.5 μg) of control plasmid, pRSV-βGal, was included in each transfection. In the cotransfection studies, the HNF1 expression plasmid pRHNF1α or pRHNF1β was included (0.03–1 μg/dish as indicated). After overnight incubation, the medium was removed and replaced with fresh serum-free medium. At 24 h later, the medium was replaced with fresh medium. Cells were harvested 24 h later in 150 μl of 1× cell culture lysis reagent (Promega), and luciferase activity was measured using the Luciferase Assay kit and a Berthold Lumat LB9501 luminometer. β-Galactosidase activities were determined as described by Sambrook et al. (1989).

For the transfection of HepG2 and HeLa cells, cells (4×10^5 cells/35-mm tissue culture dish) were grown for 24 h, washed twice with serum-free medium, and transfected as described above for primary rat hepatocytes with the exception that the cells were exposed to the LipoFECTIN/DNA mixture for 3 h.

Isolation of Rat Liver Nuclear Extract. The preparation of liver nuclear extract from adult male Sprague-Dawley rats was based on the method of Gorski et al. (1986) with the following modifications. Sodium fluoride was included in the homogenization and nuclear lysis buffers at 10 and 5 mM, respectively. Protease inhibitors in addition to phenylmethylsulfonyl fluoride were added to the nuclear lysis and nuclear dialysis buffers, including pepstatin A, chymostatin, antipain, and leupeptin at 25 μg/ml and aprotinin (Trasylol) at 10 μg/ml (0.1 mM final concentration). The second centrifugation step was modified as follows: the initial nuclear pellet was resuspended in 25 ml of nuclear lysis buffer (containing 100 mM KCl, 10 mM HEPES, pH 7.4, 0.1 mM EDTA, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol in addition to the phenylmethylsulfonyl fluoride, NaF, and protease inhibitors) and immediately recentrifuged at 9000*g* for 10 min at 4°C. For precipitating protein and chromatin material, 5 M NaCl was used in place of 4 M $(NH_4)_2SO_4$ (final NaCl concentration 0.4 M), and the solid salt addition step was omitted. The protein concentrations of the final dialyzed extracts were determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL) and adjusted to a final concentration of ~1 μg/μl with nuclear dialysis buffer. The extracts were stored in 0.1-ml aliquots and stored at −86°C.

DNase I Footprinting Assay. The probe used for DNase I footprinting was a ~390-bp fragment corresponding to bases −253 to +96 of *UGT1A7* (described by Metz and Ritter, 1998). The probe was labeled by digesting p340XP with *Hin*dIII, filling in with dNTPs containing [α^{32}P]dCTP, and then digesting with *Sac*I to release a ~390-bp fragment labeled specifically at the 3′ end of the top (coding) strand. The fragment was purified by electrophoresis through low melting point agarose followed by purification using Promega PCR Wizard. Binding reactions and DNase I digestions were performed as described by von der Ahe (1991). Briefly, a binding reaction containing 1× buffer B (10 mM HEPES, pH 7.9, 0.5 mM EDTA, 1 mM dithiothreitol, and 6% glycerol), 60 μg of nuclear extract, 3×10^4 cpm of the probe, 5 μg of poly(dI/dC), and water to a final volume of 20 μl was incubated for 25 min at 25°C. DNase I was added where applicable to a final concentration of 0.03 μg/ml, and the samples were incubated for 2 min at 25°C. The reactions were stopped by the addition of 75 μl of DNase stop solution (30 mM EDTA, 0.25% SDS, 0.5 mg/ml proteinase K) and incubated for 30 min at 42°C. Samples were phenol-extracted and ethanol-precipitated overnight at −20°C. Pellets were dissolved in 3 μl of formamide loading buffer (95% formamide, 0.5× Tris-Borate-EDTA (TBE), 0.1% xylene cyanol, 0.1% bromophenol blue), denatured at 95°C for 3 min, and run on a 7 M urea-0.5× TBE-6% polyacrylamide sequencing gel.

EMSA. Oligonucleotides corresponding to the top and bottom strands of PR-1, mutPR-1, and the rat albumin HNF1 sequences (Fig. 2B) were annealed by heating to 70°C and slow cooling to room temperature. Oligonucleotide probes (PR-1 or the rat albumin HNF1 sequence as indicated in each figure) were 5′-end-labeled in T4 polynucleotide kinase reactions containing 2 pmol annealed PR-1 oligonucleotide and 20 nmol [γ-^{32}P]ATP. Binding reactions (20 μl total volume) contained 1 μg of nuclear extract protein, 10 fmol (~20,000 cpm) of radiolabeled probe, 2 μg of poly(dI/dC), 1 μmol of KCl, 100 nmol of $MgCl_2$, 0.4 μmol of Tris-HCl, pH 7.9, 4 nmol of EDTA, 800 μg of Ficoll 400, and 50 μmol of dithiothreitol. After a 20-min incubation on ice, 2 μl of 0.02% bromophenol blue tracking dye was added, and the samples were loaded onto a 20 × 25 cm 0.5× TBE-4.5% polyacrylamide slab gel (1.5 mm thick). The samples were electrophoresed at 150 V for 2 h (until the tracking dye migrated two thirds of the way down the length of the gel). The gel was then dried and exposed to film.

For supershift analysis, 1 μl of antisera to HNF1α or HNF1β (Santa Cruz Biotechnology, Santa Cruz, CA) was added to binding reactions containing all components except the probe and incubated on ice for 1.5 h. The probe was then added, and the binding reaction was incubated on ice for an additional 20 min before gel loading and electrophoresis as described earlier.

Results

The transfection of primary rat hepatocytes with the *UGT1A7* promoter-luciferase reporter plasmid p(−965/+56)1A7-Luc resulted in ~5-fold greater luciferase activity compared with cells transfected with the promoterless control plasmid pGL3-Basic (Fig. 1). This activity, as expected,

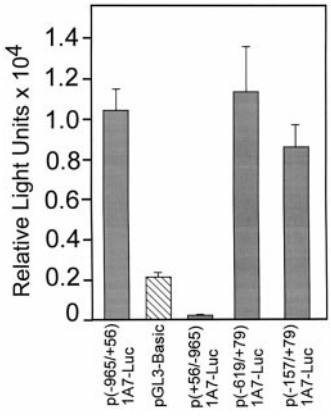


Fig. 1. Effect of 5′ deletions of the *UGT1A7* promoter on expression of luciferase in primary rat hepatocytes transfected with *UGT1A7* reporter constructs. Indicated reporter plasmids were transfected with pRSV-βGal into primary cultures of rat hepatocytes (three wells/group). Cells were harvested at 48 h post-transfection, and luciferase activities of the cellular lysates were determined. Data (mean ± S.E.) were normalized for transfection efficiency by dividing by the relative β-galactosidase activity.

was dependent on the orientation of the *UGT1A7* promoter fragment. Luciferase activity was very low in hepatocytes transiently transfected with p(+56/−965)1A7-Luc, even compared with pGL3-Basic. The promoter activity of p(−965/+56)1A7-Luc appeared weak compared with the SV40 early promoter, which exhibited >100-fold higher activity (data not shown). To define the sequence or sequences contributing to basal expression, the effect of introducing 5′-terminal truncations on the function of the promoter was examined. Hepatocytes transfected with either p(−619/+71)1A7-Luc or p(−157/+71)1A7-Luc exhibited luciferase activities that were similar to that of p(−965/+56)1A7-Luc-transfected cells (Fig. 1). From these observations, the potential regulatory elements for HNF4 (bases −365/−353, 5′-AGGGGCAGGTTCA-3′), HNF3 (bases −248/−238, 5′-AAATGTAGGCT-3′), and C/EBP (bases −181/−171, 5′-TTTCCTCACA-3′) appear not to contribute significantly to the basal *UGT1A7* promoter activity in rat hepatocytes.

To characterize the rat liver nuclear factors recognizing the proximal region of the *UGT1A7* promoter, DNase I footprint analysis was performed using a probe representing bases −253 to +96. Two regions were found to be protected from DNase I cleavage, which were designated PR-1 and PR-2 (Fig. 2). PR-1 extends from −50 to −40 and is centered over the HNF1-like binding site identified previously (Metz and Ritter, 1998). Specific sites protected from DNase cleavage include the cytosine and guanine residues at −40 and −42, respectively. Two other protected sites not visible in the figure corresponding to cytosine −46 and adenine −50 were apparent after longer film exposures. The binding was not dependent on whether the liver nuclear extract was prepared from control or inducer-treated rats. Binding to the PR-1 and PR-2 positions was also apparent when the noncoding strand was used in the analysis (data not shown).

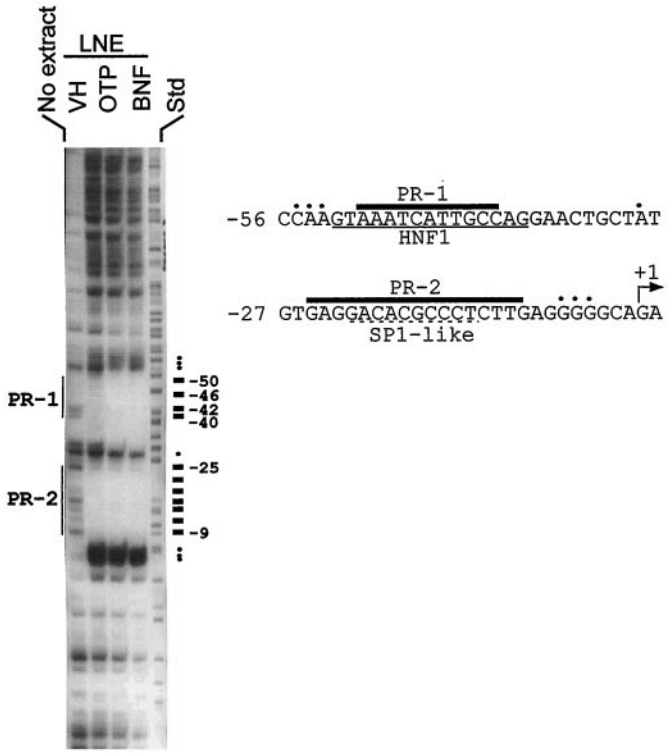


Fig. 2. DNase I footprint analysis of the rat *UGT1A7* promoter. Left, probe corresponding to bases −252 to +96 of UGT1A7 was incubated without liver nuclear extract ("None") or with liver nuclear extracts from oltipraz (OTP)- or β-naphthoflavone (BNF)-treated rats. After limited DNase I digestion, DNA was isolated and analyzed by polyacrylamide gel electrophoresis. Two regions of DNA, PR-1 and PR-2 (indicated by vertical lines), were protected from DNase I digestion in the presence of liver nuclear extract. PR-1 and PR-2 were flanked by sites that were hypersensitive to DNase I digestion in the presence of liver nuclear extracts (indicated by dots). A DNA sequence ladder was used as a standard to determine fragment sizes (STD). Right, sequence of the UGT1A7 gene surrounding PR-1 and PR-2 and their relationship to the 5′-most transcription start site (indicated by the arrow at base +1). The HNF1- and Sp-1-like binding sites are indicated by the solid and dashed underlining, respectively.

PR-2 is located immediately 3′ of PR-1 and features a cluster of protected bands between positions −9 and −25. This region includes a potential TATA element, TATGT, from bases −30 to −26 and a 10-base sequence with 6 of 10 bases matching the consensus Sp-1-binding site sequence 5′-GGGGCGGGGC-3′, located between bases −22 to −13. In addition, several DNase hypersensitive sites were evident in the samples containing rat liver nuclear extract (indicated by the dots in Fig. 2). These were located in positions immediately flanking the protected regions, PR-1 and PR-2, and likely reflect changes in the DNA configuration induced by binding of rat liver nuclear extract, thereby increasing accessibility by DNase I. There were no other sites of binding detected in the region from −252 to +94 by DNase I footprinting.

To further characterize the binding to PR-1 and assess the possibility that it represents a functional HNF1 binding site, EMSA analysis was performed using radiolabeled PR-1 probe corresponding to bases −61 to −30 as shown in Fig. 3. The formation of the DNA-protein complex indicated by the arrow was completely prevented in binding reactions containing a 50-fold excess of cold PR-1 but not mutPR-1 probe (Fig. 3). mutPR-1 contained three base substitutions in the higher conserved 5′ half of the HNF1 binding sequence. The binding was significantly competed using a competitor probe corresponding to the rat albumin HNF1 site (Lichtsteiner et al., 1987).

To determine whether HNF1 is present in the protein complex recognizing PR-1, EMSAs were carried out using specific antibodies to HNF1α and HNF1β (Fig. 4). For comparison, the rat albumin HNF1 site probe (see Alb HNF1 in Fig. 3) was ^{32}P-end-labeled and run in parallel with the PR-1 probe. Both probes yielded similar profiles in the EMSA assay. In the absence of antibody, each probe exhibited a similarly migrating DNA-protein complex. The addition of the HNF1α antibody to the binding reaction retarded the mobility of the complex, whereas the HNF1β antibody had no apparent effect. These results support the presence of HNF1α in the protein complex recognizing PR-1 and are consistent with the dominant expression of HNF1α in liver (Rey-Campos et al., 1991).

To investigate the in vivo significance of the PR-1 site in *UGT1A7* transcription, the effect of mutating PR-1 on the expression of the UGT1A7 gene reporter in transiently transfected primary rat hepatocytes was determined. A mutant version of p(−965/+56)1A7-Luc, p(−965/+56)1A7 mutPR1-Luc, was constructed that contained the same three base substitutions in the PR1 site as the mutPR-1 oligonucleotide (Fig. 3). The mutation was associated with a marked reduction in luciferase expression directed by the UGT1A7 gene reporter in primary rat hepatocytes. The relative luciferase/

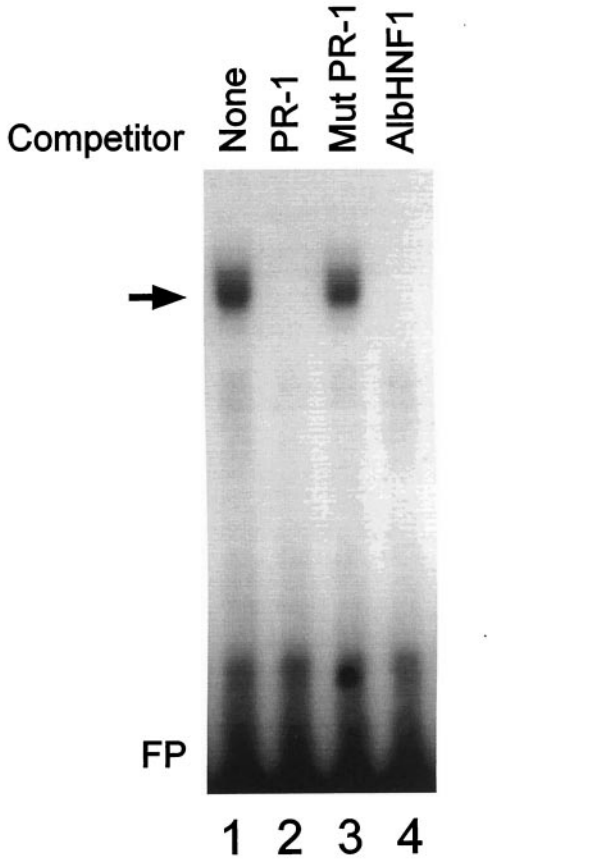
β-galactosidase ratios were 1.00 ± 0.15 for p(−965/+56)1A7-Luc- transfected cells, 0.12 ± 0.024 for p(−965/+56)1A7 mutPR1-Luc, and 0.022 ± 0.006 for pGL3-Basic.

Experiments were conducted to examine the effect of overexpressing the two major HNF1 isoforms, HNF1α and HNF1β, on *UGT1A7* reporter expression. Using primary rat hepatocytes as a cellular model, cotransfection of pRHNF1α produced a marked decrease in luciferase activity (data not shown). This effect was potent (with detectable inhibition only at 0.01 μg of pRHNF1α) and was concentration-dependent and may be due to a transcriptional squelching phenomenon, based on the observation that the expression of cotransfected pRSV-βGal was also highly attenuated.

In HepG2 cells, which were used by Hansen et al. (1997) to demonstrate the responsiveness of the rat UGT2B1 promoter to HNF1 overexpression, cotransfection of pRHNF1α had no apparent effect on p(−965/+56)1A7-Luc-directed expression of luciferase, although pRHNF1β cotransfection produced up to a ~3-fold stimulation (Fig. 5A). Similar effects were observed (Fig. 5A) using a 5′ deleted *UGT1A7* reporter plasmid, p(−157/+79)1A7-Luc, which is consistent with the presence of the HNF1-binding site between bases −52 to −38. For comparison of our data with those of Hansen et al. (1997), a control experiment was also carried out using a rat UGT2B1-luciferase reporter plasmid, p(−303/+13)2B1-Luc. In contrast to the findings of Hansen et al. (1997), we were unable to observe the expected stimulation by pRHNF1α. These findings are likely explained by high endogenous HNF1α expression in HepG2 cells as demonstrated by Northern blot analysis (Fig. 5C).

Experiments were therefore carried out using a model cell line that does not express significant levels of endogenous HNF1, HEK 293 cells (Fig. 5C). The latter were used by Bernard et al. (1999) to demonstrate the responsiveness of the UGT1A1 promoter to coexpressed HNF1. In contrast to HepG2, HEK cells showed strong responsiveness to coexpression of HNF1α or HNF1β (>40-fold maximal increase in each case; Fig. 5B). The positive control UGT2B1 reporter also showed much greater responsiveness in HEK cells. Consistent with the hypothesis that PR-1 mediates the transactivational effect of HNF1α or HNF1β, the stimulatory effect of pRHNF1α or pRHNFβ cotransfection was blocked in HEK cells transfected with p(−965/+56)1A7 mutPR-1 (Fig. 6).

To further investigate the role of the HNF1 site in the function of the UGT1A1 promoter, an experiment was carried out using primary rat hepatocytes transfected with the "basal" *UGT1A7* reporter plasmids [p(−965/+56)1A7-Luc and p(−965/+56)1A7 mutPR1-Luc] and two 3-MC-responsive *UGT1A7* plasmids designated p(−965/+56)1A7-Luc-3.7-B and p(−965/+56)1A7 mutPR1-Luc-3.7B. The latter two



PR-1
5'-TTCTCCCAAGTAAATCATTGCCAGGAACTG-3'
3'-AAGAGGGTTCATTTAGTAACGGTCCTTGAC-5'

mutPR-1
5'-TTCTCCCAAGTcgcTCATTGCCAGGAACTG-3'
3'-AAGAGGGTTCAgcgAGTAACGGTCCTTGAC-5'

AlbHNF
5'-GGTAAGTATGGTTAATGATCTACAGTTA-3'
3'-CCATTCATACCAATTACTAGATGTCAAT-5'

Fig. 3. EMSA analysis of UGT1A7 PR-1 binding site. Liver nuclear extract from a control rat was mixed with radiolabeled double-strand DNA corresponding to PR-1 and incubated in either the absence (None) or presence of a 50-fold excess of cold PR-1 (PR-1) probe, mutant PR-1 probe (Mut PR-1), or rat albumin promoter HNF1 probe (AlbHNF1) (sequences indicated). The arrow indicates the specific complex formed between the probe and rat liver nuclear protein. FP, free probe.

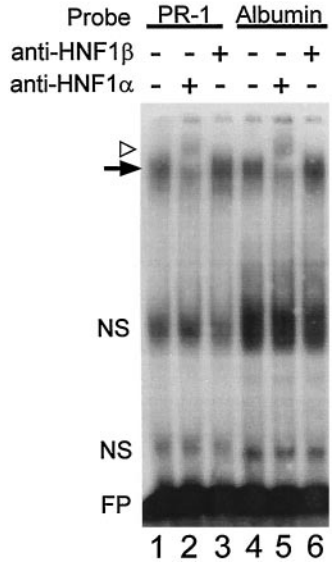


Fig. 4. EMSA using antibodies to rat HNF1α and HNF1β. Liver nuclear extract from a control rat was preincubated in either the absence or presence of antibody to HNF1α (lanes 2 and 5) or HNF1β (lanes 3 and 6) followed by the addition of either radiolabeled PR-1 (lanes 1–3) or rat albumin HNF1 (lanes 4–6) probe. Complexes were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography. The closed arrow and open arrowhead indicate the unshifted and shifted complex, respectively. NS, nonspecific complex. FP, free probe.

plasmids contain a 3.7-kbp *Bam*HI fragment from the UGT1A7 intron (Fig. 7, A and B; Metz and Ritter, 1998), which encompasses a 3-MC-responsive element or elements that may contribute to 3-MC-induced expression of the endogenous UGT1A7 gene. In agreement with the finding that the HNF1 mutation significantly reduces basal expression from the base *UGT1A7* reporter plasmids p(−965/+56)1A7-Luc, a similar effect on basal expression was apparent using the 3.7B-containing reporter plasmids [Fig. 7C, compare vehicle-treated p(−965/+56)1A7-Luc-3.7B- and p(−965/+56)1A7 mutPR1-Luc-3.7B-transfected cells]. The reduced expression was also apparent after induction with 3-MC. Although the fold of induction appeared not to be significantly affected by the HNF1 site mutation [4.7- versus 3.4-fold induction ratio for p(−965/+56)1A7-Luc-3.7B and p(−965/+56)1A7 mutPR1-Luc-3.7B-transfected cells, respectively], the maximal induced activity of the reporter was significantly decreased by this mutation (Fig. 7C, compare luciferase/β-galactosidase activity ratios for the two 3-MC treatment groups). These results are consistent with a general role of HNF1 as an enhancer of UGT1A7 gene transcription in rat hepatocytes under either basal or induced conditions.

Discussion

This report describes evidence that the UGT1A7 gene is under the control of HNF1, a transcription factor associated with hepatocyte-specific gene expression and differentiation. HNF1 occurs in two different forms, HNF1 α and HNF1β, which are expressed in a tissue-specific manner (Rey-Campos et al., 1991). The three cell types recognized as having the highest HNF1 expression are hepatocytes, enterocytes, and renal proximal tubular epithelial cells. Hepatocytes preferentially express the HNF1α form, whereas enterocytes and proximal tubular cells express HNF1α and HNF1β in a more equal ratio. Because the kidney, intestine, and liver correspond to the tissues with high or (in the case of liver) potentially high UGT1A7 mRNA expression, these data suggest a role for HNF1 in tissue-specific expression of UGT1A7.

The binding site for HNF1 in *UGT1A7* is located immediately upstream of the *UGT1A7* transcription start site region between positions −52 to −38. This context for HNF1 binding is typical of liver-expressed genes under HNF1 control. These include the two other known UGT genes controlled by HNF1, *UGT2B1* and *UGT1A1*, which feature HNF1 binding sites at positions −55 to −42 and −75 to −63, respectively (Hansen et al., 1997; Bernard et al., 1999). Furthermore, it appears that a number of additional UGT genes are under the control of HNF1, based on the presence of HNF1-like binding sites in their flanking sequence. Several of these were identified by Tronche et al. (1997), who applied a consensus HNF1 site sequence deduced from 21 known functional HNF1 binding sites [5′-GGTTAAT(A/T)ATTA(A/C)CA-3′]. Proposed HNF1 sites are located in the intronic sequence 3′ of *UGT1A1* exon 1 and 5′ of *UGT1* exon 2, in the promoter region of *UGT1A4*, and in the human and rat *UGT1A6* promoter regions. In addition, the human UGT1A7 gene (GenBank entry HSU39570) contains an almost identical HNF1 site to that of rat (11 of 12 bases match) in its putative proximal promoter region.

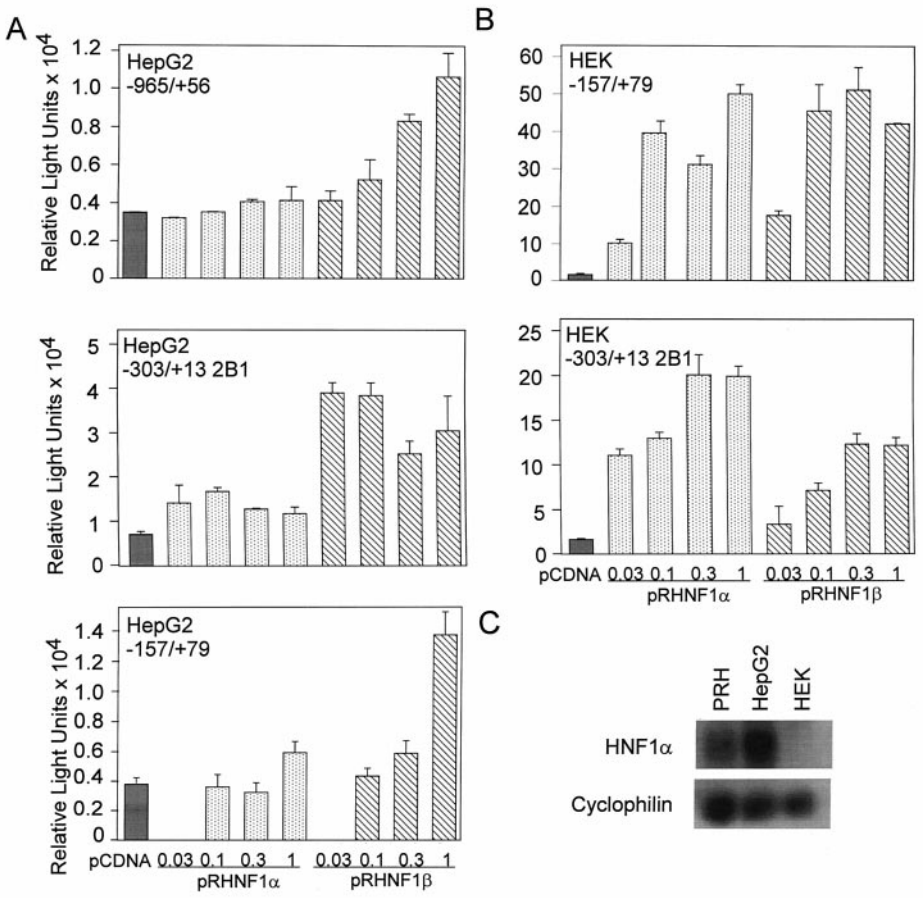


Fig. 5. The effect of overexpressing HNF1α or HNF1β on luciferase activity in various cell lines cotransfected with *UGT1A7* or UGT2B1 reporter plasmids. HepG2 cells (A) or HEK cells (B) were cotransfected with the indicated *UGT1A7*- or *UGT2B1*-luciferase reporter plasmids in the presence of pRSV-βGal and increasing amounts of HNF1α or HNF1β expression plasmid. For controls, cells were cotransfected with the blank expression vector pCDNA 3.1. Cells were harvested for the determination of luciferase and β-galactosidase activities. Data represent the mean ± S.D. of three determinations per group normalized for the relative β-galactosidase activity. C, Northern analysis of the HNF1α mRNA in primary rat hepatocytes, HepG2, and HEK 293 cells. Total RNA (10 μg/lane) was prepared and analyzed as described previously (Grove et al., 1997) using rat HNF1α cDNA excised from pRHNF1α as probe. The blot was reprobed for cyclophilin mRNA using rat cyclophilin cDNA to evaluate the quantity and quality of the RNA.

A unique feature of the UGT1A7 gene compared with other UGT genes under HNF1 control is its weak constitutive expression in livers of untreated rats (Metz and Ritter, 1998). These data appear to contrast with the fact that HNF1 is abundantly expressed and constitutively active in this tissue. One explanation is that the *UGT1A7* promoter is intrinsically weak and that HNF1 alone is not sufficient for high-level expression. In this study, we analyzed the *UGT1A7* promoter function in primary rat hepatocytes, a cell model in which low *UGT1A7* expression is maintained (in contrast to rat hepatoma and SV40-immortalized rat hepatocyte cell lines). The *UGT1A7* promoter activity assessed by the transiently transfected gene reporter method appeared weak in hepatocytes, with luciferase expression only ~5- to 10-fold higher than that in control hepatocytes transfected with a promoterless control reporter (pGL3-Basic). In contrast, the *UGT1A7* reporter was highly expressed in HEK cells driven to overexpress HNF1. This observation suggests that HEK cells contain additional factors not found in hepatocytes or HepG2 cells that act in concert with HNF1 to promote *UGT1A7* promoter expression. The transformation of hepatocytes may also result in the up-regulation of additional factors that result in the overexpression of UGT1A7. Indeed, we have observed high *UGT1A7* reporter expression in RALA tsA25510G LCS-3 cells, an immortalized rat liver-derived cell line that exhibits high UGT1A7 mRNA levels and its associated benzo(*a*)pyrene-7,8-dihydrodiol UGT activity (Grove et al., 1997). These findings support a general correlation between the level of UGT1A7 mRNA expression and the apparent activity of the *UGT1A7* reporter.

This study suggests that the full basal *UGT1A7* promoter activity is contained in a short segment between −157 and +56. This observation, together with the finding that mutation of PR-1 nearly abolishes expression of the promoter in primary rat hepatocytes, supports a principal role of HNF1 in maintaining basal expression of UGT1A7 in primary rat hepatocytes. However, the data suggest that HNF1 binding to a single site is not sufficient in itself for attaining high-level *UGT1A7* expression in liver. Indeed, most liver-expressed genes are controlled by multiple liver-enriched transcription factors or by multiple binding sites for a single factor. The human albumin gene, for example, is under the control of three distinct HNF1 binding sites (Hardon et al., 1988; Frain et al., 1990; Hayashi et al., 1992) in addition to binding sites for C/EBP and several other transcription factors (Friedman et al., 1989). In the case of UGTs, the constitutively expressed UGT2B1 gene has been shown to be regulated by both C/EBP and HNF1, which may act cooperatively to confer high-level expression. For UGT1A7, it is likely that high expression requires HNF1 acting either additively or synergistically with an additional transcription factor or factors. The identities of these factors remain currently unknown; however, one possibility is that one or more of these factors are drug-regulated. The known inducibility of UGT1A7 by polycyclic aromatic hydrocarbons suggests that this factor is the AHR, a ligand-dependent transcription factor in the PAS-basic helix-loop-helix domain transcription factor family (Whitlock, 1999), although this remains to be definitively established. AHR mediates the induction of certain xenobiotic-metabolizing enzymes, including CYP1A1, CYP1A2, and CYP1B1, by various receptor agonists, such as 3-MC. The agonists bind to AHR, triggering its movement from the cytosol to the nucleus, where it dimerizes with AHR nuclear translocator, a second member of the PAS basic he-

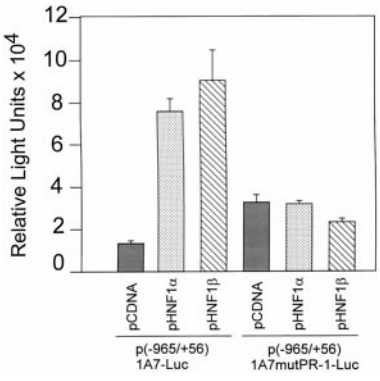


Fig. 6. Mutation of UGT1A7 PR-1 site prevents HNF1α and HNF1β stimulation of luciferase activity in p(−965/+56)1A7-Luc-transfected HEK cells. HEK cells were cotransfected with either p(−965/+56)1A7-Luc or p(−965/+56)1A7 mutPR-1-Luc in the absence (pCDNA) or presence of HNF1 expression vector (as indicated) and pRSV-βGal. At 48 h after transfection, the cells were harvested for the determination of luciferase and β-galactosidase activities. Data represent the mean ± S.D. of three determinations normalized for β-galactosidase activity.

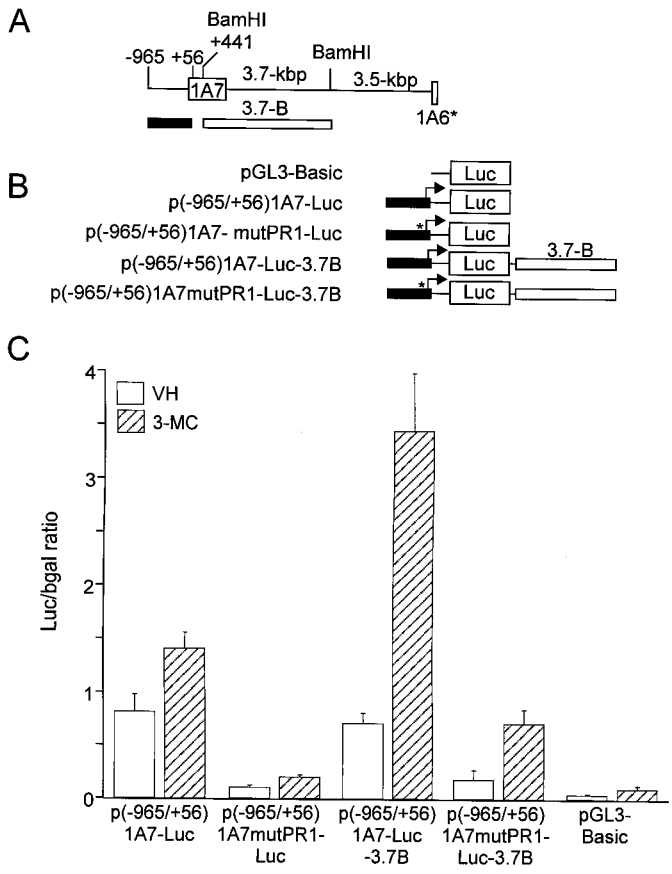


Fig. 7. The effect of mutating the UGT1A7 PR-1 site on basal and 3-MC-induced *UGT1A7* reporter expression in primary rat hepatocytes. Primary rat hepatocytes were transfected with the indicated reporter plasmids and treated for 48 h posttransfection with either 0.1% DMSO (VH) or 2.5 μM 3-MC before harvesting the cells for the determinations of luciferase and β-galactosidase activities. A, structure of the *UGT1A7/UGT1A6* locus and relationship of fragments used for the construction of *UGT1A7* reporter plasmids. B, schematic showing the general structures of the UGT1A7 reporter constructs. C, data represent the mean ± S.D. expressed as the ratio of luciferase to β-galactosidase activity.

lix-loop-helix domain family. The AHR-AHR nuclear translocator complex binds to xenobiotic response elements usually found in the 5′ flanking regions of responsive genes to activate transcription of nearby genes. Although the identities and positions of elements mediating UGT1A7 inducibility by 3-MC in vivo are not yet known, a 3.7-kbp *Bam*HI fragment representing a portion of the UGT1A7 coding exon and intron flanking region has been found to confer a limited extent of 3-MC inducibility on the *UGT1A7* promoter. We therefore tested the impact of mutating the HNF1 site on the extent of 3-MC-inducibility conferred by the 3.7-kbp *Bam*HI fragment. Although our data clearly show that HNF1 is not required for inducibility by 3-MC [measured as fold of induction; Fig. 7C, 4.7-fold induction for p(−965/+56)1A7-Luc-3.7B and 3.4-fold induction for p(−965/+56)1A7-Luc-3.7B], the absolute extent of expression in the 3-MC-treated cells was clearly decreased (Fig. 7C, Luc/βgal ratio of 3.5 for p(−965/+56)1A7-Luc-3.7B versus 0.7 for p(−965/+56)1A7-Luc-3.7B). The latter decrease was roughly in the same proportion (80% decrease) as was observed for the basal *UGT1A7* reporter construct containing the HNF1 mutation (85–90% decrease). These data suggest that HNF1 serves a general role to enhance expression from the *UGT1A7* promoter to comparable extents under basal and induced conditions. A similar conclusion was reached in the case of the phenylalanine hydroxylase gene, which requires functional HNF1 and C/EBP binding sites for basal activity as well as a maximum induction response to glucocorticoids (Faust et al., 1996).

It is interesting to speculate about the role of HNF1 in the species-specific pattern of hepatic *UGT1A7* expression. Similar to in the rat, the human liver expresses very low levels of UGT1A7 (Strassburg et al., 1997), but in contrast, the level of UGT1A7 mRNA is not significantly increased by 3-MC or oltipraz in cultured primary human hepatocytes (J. K. Ritter, F. K. Kessler, and R. A. Fisher, unpublished observations). Because the human UGT1A7 gene shows conservation of the HNF1 binding sequence, it may be hypothesized that the difference between the human and rat UGT1A7 gene is the absence of drug-regulated responsive elements in the human gene. In contrast to human and rat, the mouse and rabbit express high basal UGT1A7 mRNA levels in liver and do not show significant inducibility by AHR agonists (Bruck et al., 1997). The mechanism of these differences remains to be established.

The identity of the protein(s) recognizing the second *UGT1A7* promoter site (i.e., PR-2) currently remains unknown. However, the general position of binding suggests that PR-2 may correspond to a binding site for factors involved in the recruitment of a functional RNA polymerase II initiation complex. We have previously described the rat UGT1A7 gene as a TATA-independent gene (Metz and Ritter, 1998), based on the utilization of >25 distinct transcription start sites, one hallmark of TATA-less promoters (Smale, 1997). However, it is interesting that PR-2 overlaps a potential TATA element, 5′-TATGT-3′, which is located in an appropriate position (−30 to −26) for initiation from the 5′ most transcription start site or sites. This could indicate that TATA-binding factors represent the proteins recognizing PR-2 and that transcription of UGT1A7 represents a combination of TATA-dependent and TATA-independent mechanisms. An additional possibility is that PR-2 represents a low-affinity binding site for Sp-1. The core region of PR-2 includes a 10-bp sequence with 6 of 10 bases matching the consensus Sp-1 binding site, 5′-GGGGCGCCCC-3′.

In summary, this study demonstrates an enhancer function for HNF1 in basal regulation of UGT1A7 in rat hepatocytes. UGT1A7 represents a third UGT gene demonstrated to be under the control of a functional HNF1 binding site. HNF1 is proposed to be required to obtain maximum UGT1A7 induction responses to various inducing agents, such as the polycyclic aromatic hydrocarbons and oltipraz. Furthermore, our data suggest that HNF1 is likely to contribute to the high expression of UGT1A7 observed in other HNF1-enriched tissues, such as intestine and kidney.

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