Transcripational Regulation of the Human CYP2A6 Gene

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ABSTRACT

Nicotine C-oxidation is primarily catalyzed by CYP2A6 in humans. This enzymatic activity exhibits a large interindividual variability, which to a great extent is caused by genetic polymorphisms in the CYP2A6 gene. There are large interindividual differences in CYP2A6 mRNA and protein levels, but little is known about the transcriptional regulation of CYP2A6, which can, e.g., explain such differences. Using transient transfection of the human hepatoma B16A2 cells, we show that maximal promoter activity was harbored in the sequence spanning from −112 to −61. Putative response elements for the transcription factors hepatocyte nuclear factor-4 (HNF-4α), CCAAT-box/enhancer binding protein (C/EBPα), C/EBPβ, and octamer transcription factor-1 (Oct-1) were identified in this region, and electrophoretic mobility shift assays showed that these transcription factors bind to the predicted elements. To determine the relevance of these sites, expression vectors for these transcription factors were cotransfected with CYP2A6 promoter constructs in HepG2 cells. HNF-4α, C/EBPα, and Oct-1 exerted an activating effect, whereas overexpression of C/EBPβ reduced CYP2A6 promoter activity. To confirm the importance of these sites in vivo, mutated CYP2A6 reporter constructs were injected into mouse liver. Mutation of either HNF-4 or C/EBP-Oct-1 motifs significantly decreased promoter activity, 52 and 26% of wild-type, respectively, whereas when both motifs were mutated the activity in mice decreased to 14% of wild type. In conclusion, the data indicate that the constitutive hepatic expression of CYP2A6 is governed by an interplay between the transcription factors HNF-4α, C/EBPα, C/EBPβ, and Oct-1. These results will be important for the identification of new polymorphisms affecting CYP2A6 gene expression.

The cytochromes P450 (P450) mediate the oxidative metabolism of numerous structurally diverse exogenous and endogenous compounds. More than 50 different P450 genes have been identified in the human genome, and based on their sequence identities, they are classified into different families and subfamilies. The cluster of CYFl family genes on human chromosome 19 comprises about 350 kilobases and is located at 19q13.2 (Fernandez-Salguero and Gonzalez, 1995; Hoffman et al., 1995). A considerable number of genes belonging to subfamilies CYP2A, CYP2B, CYP2F, CYP2G, CYP2S, and CYP2T are present in this locus (Hoffman et al., 2001). In particular, the CYP2A subfamily comprises four full-length genes: CYP2A6, CYP2A7, CYP2A13, and CYP2A18P.

CYP2A6 plays a major role in the metabolism of nicotine and coumarin, and it is involved in the clearance of certain pharmaceuticals and the activation of some tobacco-related nitrosamines (Oscarson et al., 1998; Pelkonen et al., 2000; Raunio et al., 2001, and references therein). CYP2A6 is predominantly expressed in the liver with a large interindividual variability. Indeed, more than a 100-fold variability in both CYP2A6 mRNA and protein levels has been reported (Yun et al., 1991; Shimada et al., 1994; Pelkonen et al., 2000; Rodriguez-Antona et al., 2001). Although part of these interindividual differences in CYP2A6 activity might be attributed to physiological factors (Ujim et al., 2002), disease (Pasanen et al., 1997; Raunio et al., 1998), and exposure to clinically used drugs (Dale-Beluche et al., 1992; Sotaniemi et al., 1995; Donato et al., 2000), a major part of the inter-

ABBREVIATIONS: P450, cytochrome P450; SNP, single nucleotide polymorphism; HNF, hepatocyte nuclear factor; EMSA, electrophoretic mobility shift assay; C/EBP, CCAAT-box/enhancer binding protein; DR, direct repeat; Oct-1, octamer transcription factor-1; CAR, constitutive androstane receptor; DBP, D-box binding protein; RXR, retinoid-X-receptor; LAP, liver-enriched transcriptional activating protein; LIP, liver-enriched transcriptional inhibitory protein; NF-1, nuclear factor 1; PCR, polymerase chain reaction.
Regulation of the CYP2A6 Promoter

Materials and Methods

Materials. Restriction and DNA modifying enzymes, T4 polynucleotide kinase, elongase enzyme mix, deoxynucleotide triphosphates, polymerase chain reaction primers, the GeneTailor site-directed mutagenesis system and all oligonucleotides used in this study as well as all reagents for cell culture were purchased from Invitrogen (Carlsbad, CA). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). The endotoxin-free maxiprep kit was purchased from QIAGEN (Valencia, CA). The dual luciferase reporter assay system, the pGL3-Basic firefly luciferase reporter vector, the pRL-SV40 Renilla plasmid, and TfX-20 were purchased from Promega (Madison, WI). The TransIT in vivo gene delivery system kit was from Mirus (Madison, WI). [32P]dATP was purchased from Amersham Biosciences Inc. (Piscataway, NJ). Polydeoxyinosinose:deoxycytosine was purchased from Pfizer, Inc. (New York, NY). The expression vectors used in this study were pCMV5-HNF-4 (a kind gift from Dr. B. Kemper, University of Illinois at Urbana-Champaign, Urbana, IL); pAC-C/EBPα, pBS-DBP, pBS-LAP, and pBS-LIP (kindly provided by Dr. U. Schibler, University of Geneva, Geneva, Switzerland); and pCG-Oct-1 (a kind donation from Dr. W. Herr, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The anti-HNF-4 (sc-6557 X), anti-C/EBPα (sc-9315 X), anti-C/EBPβ (sc-150 X), anti-Oct-1 (sc-232 X), and anti-RXRα (sc-774 X) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Dr. M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC) kindly provided the anti-mouse CAR antibody.

Human CYP2A6 Promoter Constructs. Oligonucleotide primers were designed based on the CYP2A6 sequence (GenBank accession no. NG_000008.5) and the elongase enzyme mix was used to amplify several fragments of the human CYP2A6 promoter. The longest fragment was obtained using the upstream primer 2A6-841F (with a XhoI site) and downstream primer 2A6-1R (with a HindIII site). The resulting product (−841/1) was cloned into the XhoI/HindIII site of the promoterless pGL3-Basic firefly luciferase reporter vector to generate the −841LUC construct. Position +1 refers to the translational start site (ATG codon). Using this plasmid as template and the appropriate upstream primers, additional 5′-promoter deletion constructs were made: −693LUC, −500LUC, −450LUC, −387LUC, −355LUC, −265LUC, −200LUC, −135LUC, −112LUC, −95LUC, −87LUC, −61LUC, and −40LUC. By means of the GeneTailor site-directed mutagenesis system and the corresponding primer pairs, the −200LUC construct was mutated to generate −200LUC/105-103mut (primers 5′-GGGGCATGATGGTGAGGATGGAATGTGGATTAA-3′ and 5′-TCCCAACTACTGCGCCCACTGATCCTC-3′), −200LUC/90-88mut (primers 5′-GAGGGTGAATGGAGGATGGAATGTGGATTAA-3′ and 5′-TTCACTCATTTCACCCTCCAAACCAGGTTCA-3′), and the −200LUC/HNF-4mut4 construct (primers 5′-GTAATTTAGTAATTCGAGCAATGTGCGCTTTC-3′ and 5′-TGGCTGATTACATGAACTTTCCTGATCTTTCC-3′), where mutated nucleotides are underlined and in lowercase. The −200LUC/90-88HNF-4mut plasmid was constructed by mutagenesis of the −200LUC/HNF-4mut construct. After mutagenesis and DNA sequencing confirmation, the −200LUC CYP2A6 promoter regions were XhoI/HindIII cut and recloned in pGL3Basic, to avoid any polymerase chain reaction artifact that could have been introduced in the plasmid during mutagenesis. Mutations in the −112LUC construct were created using the QuikChange site-directed mutagenesis kit and the primers 5′-GGTTAATTAGTAATTCGAGCAATGTGCGCTTTC-3′, 5′-GGGGGATGGAATGGAGGATGGAATGTGGATTAA-3′, and 5′-CTCGAGGATGGAATGGAGGATGGAATGTGGATTAA-3′ (where mutated nucleotides are underlined and in lowercase) together with their corresponding complementary oligonucleotides, yielding the −112LUC/HNF-4mut, −112LUC/90-88mut, and −112LUC/105-103mut constructs, respectively. The sequence of all promoter deletion and mutated constructs was verified by DNA sequencing.

Nuclear Extracts and EMSAs. Nuclear protein extracts from B16A2 and HepG2 cells were prepared according to a previously described method (Dignam et al., 1983). Extracts from rat liver nuclei were prepared according to the procedure of Nakabayashi et al. (1991). Three double-stranded DNA probes were used for EMSA analysis: probe A (5′-GTAATTCGAGCAATGTGCGCTTTC-3′) from 86 to 62 of the CYP2A6 promoter, probe B (5′-AAATGAGGATGGAATGTGGATTAA-3′) from 97 to 76, and probe C (5′-GTAATTCGAGCAATGTGCGCTTTC-3′) from 112 to 90, in which the putative HNF-4, C/EBP, and a NF-Y binding site (745A) group has recently described three single nucleotide polymorphisms (SNPs) disrupting the binding sites of CYP2A6, that could affect transcription, or in the 3′ flanking region, that could influence mRNA stability, could be involved in the observed variation. Furthermore, our knowledge about the genetic factors responsible for the observed interindividual variability in CYP2A6 activity has increased during the past few years, mainly due to the identification of a significant number of new allelic variants that cause altered CYP2A6 activity. However, the great interindividual variability in CYP2A6 mRNA and protein content cannot be solely explained by the identified polymorphisms (for an updated list of CYP2A6 polymorphisms, see http://www.immi.ki.se/CYPalleles). The large interindividual differences in CYP2A6 mRNA content suggest that genetic variations in CYP2A6 promoter, that could affect transcription, or in the 3′ flanking region, that could influence mRNA stability, could be involved in the observed variation. Furthermore, our group has recently described three single nucleotide polymorphisms (SNPs) in the CYP2A6 promoter, one of them disrupting a NF-Y binding site (−745A−G), affecting gene expression levels (Pitarque et al., 2001; Pitarque et al., 2004; von Richter et al., 2004). Therefore, SNPs disrupting the binding sites of transcription factors that are critical for CYP2A6 transcription, would likely result in a reduction of CYP2A6 mRNA and protein content. However, transcriptional regulation of human CYP2A6 mRNA and protein are not well understood, even though the regulation of murine Cyp2a orthologs has been well studied. It has been reported that the hepatocyte nuclear factor-4 (HNF-4) activates hepatic expression of the mouse Cyp2a4 and Cyp2a5 genes (Yokomori et al., 1997; Uvilia et al., 2004). Similarly, human hepatocytes with reduced HNF-4a expression have decreased CYP2A6 mRNA levels, suggesting an important role of this transcription factor in CYP2A6 expression (Jover et al., 2001).

To increase the knowledge about CYP2A6 transcriptional regulation, we carried out luciferase reporter assays with a −841 promoter of CYP2A6 and a series of 5′-deleted constructs. Electrophoretic mobility shift assays (EMSA) were performed to test the binding of transcription factors to regulatory elements predicted by computer algorithms. We subsequently conducted DNA transfection of wild-type and mutated constructs into mouse liver in vivo to evaluate the functional importance of such elements. Finally, to corroborate the results in a human-based system, we cotransfected CYP2A6 promoter constructs with expression vectors encoding relevant transcription factors. By these means, we identified HNF4-α, C/EBPα, C/EBPβ, and Oct-1 as the major transcription factors driving CYP2A6 transcription.
and direct repeat (DR)-4 binding sites were encompassed. Binding reactions were carried out in a buffer containing 20 mM HEPES, pH 7.9, 50 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid, 5% glycerol, 2 μg of polydeoxyinosine:deoxyctosine, 30 fmol of 32P-end-labeled double-stranded probe, and 7 to 8 μg of nuclear protein in a total volume of 25 μl. For competition experiments, a 50-fold excess of the unlabeled oligonucleotides were added before the addition of the radiolabeled probe. The competitors used in this study were as described in Table 1. Reactions were preincubated at 37°C for 20 min, the probe was then added, and the incubation was continued for another 30 min. For supershifts, 2 μg of antibody were subsequently added to the binding reaction, and samples were incubated on ice for 45 min. Free and protein-bound DNA was resolved on 4% nondenaturing polyacrylamide gels running at 4°C and a constant voltage of 200 V in 0.5× 45 mM Tris-borate, 1 mM ethylenediaminetetraacetic acid.

**Cell Culture, Transient Transfection, and Luciferase Assay.** The culture conditions for the human hepatoma cell line B16A2 have been established previously (Le Jossic et al., 1996). After reaching confluence in 12-well plates, B16A2 cells were cultured for an additional 3 weeks before transfection experiments were conducted (Pitarque et al., 2001). HepG2 cells were grown in minimal essential medium, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% Na-pyruvate, and 1% penicillin-streptomycin at 37°C in 5% CO2. A DNA mixture of either 1 μg of pGL3-Basic or the appropriate reporter constructs plus 10 ng of the internal reference Renilla plasmid pRL-SV40 was transfected into HepG2 cells (at 70–80% confluence in 12-well plates) using TransIT, according to the manufacturer’s instructions. Cells were harvested 24 h after transfection, and reporter activities in cell lysates were determined by a dual luciferase reporter assay system. In cotransfection experiments, HepG2 cells were transfected with 500 ng of the promoter construct plus 10 ng pRL-SV40 and a selected combination of expression vectors (50 ng each): pCMV5-HNF-4, pAC-C/EBPα, pBS-DBP, pBS-LAP, pBS-LIP, and pCG-Oct-1 or the corresponding empty expression vectors. Forty-eight hours after transfection, cells were harvested and lysates were used to measure firefly and Renilla luciferase activities.

**In Vivo Mouse Liver Cell Transfection.** For in vivo cell transfection, 20- to 22-g male CD1 mice were transfected through tail injection with 10 μg of the −200/LUC CYP2A6 constructs and 100 ng of pRL-SV40, in volumes of 2.0 to 2.2 ml in less than 8 s using the TransIT in vivo gene delivery system kit. Twenty-four hours later, animals were sacrificed and the liver was removed and homogenized in 2 ml of passive lysis buffer (dual luciferase reporter assay system; Promega). This homogenate was diluted 10 times and used to measure firefly and Renilla luminescence. Two independent experiments were performed, and for each construct, at least six mice were transfected using two plasmid batches obtained from different endotoxin-free maxipreps. The study was approved by the ethical committee for animal experiments at Karolinska Institutet, Stockholm (N182/02).

**Results**

**Functional Mapping of the Human CYP2A6 Promoter.** To identify regulatory elements contributing to CYP2A6 hepatic expression, a series of reporter plasmids containing various CYP2A6 5′-flanking fragments fused to the firefly luciferase gene were transiently transfected into the human hepatoblastoma cell line B16A2. A schematic illustration of the constructs and their promoter activities is shown in Fig. 1. In general, the promoter activity gradually increased with progressive 5′-flanking deletions from −841/LUC to −200/LUC. This pattern of activity suggests that the promoter sequence between −841 and −200 may harbor several negative elements. Further 5′ truncation to −112 was not associated with a significant change in activity. The results highlight that the promoter core is contained within the −112/−1 fragment. The removal of sequences down to −95 caused a 2.8-fold decrease in activity and further deletion to −87 resulted in an additional 2.2-fold reduction of the activity. The deletion of sequences from −87 to −61 caused a slight but significant 29% reduction in activity. Finally, the deletion of the TATA box (−61/LUC to −40 LUC) had no significant effect on activity, thus indicating that this element is not sufficient for the basal promoter activity.

**Using the TRANSFAC computer search tool (Wingender et al., 2000), we identified several putative transcription factors binding sites in the CYP2A6 proximal promoter, which are depicted in Fig. 2. We initially found a direct repeat with one-base pair spacing (DR-1) element (between −81 and −69) that might represent an HNF-4 binding site. This element matches the HNF-4 consensus site KnCWARGKCa (K = G or T, W = A or T, R = A or G) that is highly conserved within the CYP2 family (Corchero et al., 2001). The POU domain factor Oct-1 has a bipartite DNA binding domain (formed by POU-homeo and POU-specific subdomains) that binds an 8-base pair target ATGGAAAT, and with lower affinity variants of this octamer (Herr and Cleary, 1995). One of these putative nonconventional sites (ATTATGTAT), a combination of the sequences individually recognized by the two POU subdomains (Phillips and Luise, 2000), was identified at −91/−82 in the CYP2A6 promoter, overlapping a D-box binding protein (DBP)-responsive element. Two overlapping C/EBP sites were located between −101 and −90 and between −91 and −82 in the antisense and sense orientation, respectively.

**Binding of Transcription Factors to CYP2A6 Promoter.** EMSA experiments were conducted to evaluate binding of transcription factors to the putative binding sites. We first investigated whether HNF-4 could bind to the DR-1-like sequence 3′-CAGCCAAAGTCCA-5′ of the CYP2A6 promoter. As shown in Fig. 3A, a specific protein-DNA complex was formed when the radiolabeled probe A was incubated with rat liver nuclear extracts (lane 2). In this experiment, unlike the unlabeled wt probe A (lane 3), a 50-fold excess of the Amut oligonucleotide, carrying mutations at −75, −70, and −69, did not compete the specific band (lane 4), indicating that these positions were necessary for the binding of the protein. By competition with the well characterized HNF-4 site in the apoCIII gene promoter (lane 5) and by means of an anti-HNF-4α antibody (lane 6), we could unambiguously identify the transcription factor as HNF-4α. No difference in binding was observed when using the unlabeled probes B, Oct-1cons, or GATAcons for competition (lanes 7–9).

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<tr>
<th>Table 1</th>
<th>Oligonucleotides (sense strand) used in EMSA competition experiments</th>
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<tr>
<td>Amut</td>
<td>5′-GTTGAAATCAGCCAAAGTCCA-3′</td>
</tr>
<tr>
<td>B88-87mut</td>
<td>5′-GAGGTATTCGCTGTAATCAGCCA-3′</td>
</tr>
<tr>
<td>B84-83mut</td>
<td>5′-GAGGTTATGGTCCTCCAGCCA-3′</td>
</tr>
<tr>
<td>C105-103mut</td>
<td>5′-GTTGGATATTGGAAATGGTAAAT-3′</td>
</tr>
<tr>
<td>C95-93mut</td>
<td>5′-GGTTCAGGTGAATGATGAAAT-3′</td>
</tr>
<tr>
<td>apoCIII-HNF-4</td>
<td>5′-CGACCGCTG-TGCCAAAGTCTACCTGC-3′</td>
</tr>
<tr>
<td>C/EBPcons</td>
<td>5′-CTAGGCTATTTGCTGTAATGCTAATCTGA-3′</td>
</tr>
<tr>
<td>Oct-1cons</td>
<td>5′-TGTCTGATGCAAATCACTAGAAA-3′</td>
</tr>
<tr>
<td>GATAcons</td>
<td>5′-CAGGTTGATACAGGAAAGTGAATACTACT-3′</td>
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important for the basal expression of the CYP2A6 promoter. To evaluate this hypothesis, EMSAs were carried out using a double-stranded oligonucleotide corresponding to the −97/−76 promoter sequence (probe B). As shown in Fig. 3B, a complex of proteins from rat liver nuclear extracts bound to this labeled probe (lane 2). The specific binding was entirely competed by a 50-fold molar excess of the same unlabeled oligomer (lane 3), whereas mutations at either −88/−87 or −84/−83 resulted in a less pronounced competition (lanes 4 and 5). An oligomer containing a C/EBP consensus site strongly competed for the binding of the protein (lane 6). Finally, using polyclonal antibodies for supershifts, both C/EBPα and C/EBPβ were identified as components in these protein complexes (lanes 7 and 8). Moreover, addition of an Oct-1 consensus competitor to the binding reaction resulted in the complete disappearance of the DNA-protein complex (lane 9), suggesting that Oct-1 is also part of the specific complex. The participation of Oct-1 in probe B-protein complex was further investigated in experiments with B16A2 nuclear extracts (Fig. 3C). The specific binding was greatly reduced when competed with an Oct-1 consensus probe (lane 4) and the inclusion of a specific anti-Oct-1-antibody decreased the mobility of the protein complex (lane 5). An unrelated oligonucleotide, GATA consensus, did not compete for binding (lane 6). These results, therefore, indicate that Oct-1 and C/EBP are able to simultaneously bind to their overlapping sites in the proximal promoter of CYP2A6.

In CYP2A6 promoter, we also identified two AGGTCA-related hexamers separated by 4 nucleotides (−112AGGT-GAAATGAGGTAA−91) that are reminiscent of the DR-4 motifs present in the CYP2B promoters to which CAR/RXR heterodimers bind. When using radiolabeled probe C (Fig. 2) and B16A2 extracts, EMSA analysis showed a specific complex that was supershifted with RXRα and CAR but not pregnane X receptor antibodies. These supershifts were however very weak, suggesting a minor binding of these nuclear receptors (data not shown).

**HNF-4α, C/EBPα, and Oct-1 Cooperatively Transactivate CYP2A6 Promoter.** To evaluate the functional relevance of the transcription factors binding sites that we identified by EMSA and to be able to study interactions between them, we cotransfected different combinations of expression plasmids and 2A6-luciferase constructs into human hepatocarcinoma HepG2 cells. In agreement with the in vivo transfections data, we found that HNF-4α, C/EBPα, and Oct-1 had an activating effect of CYP2A6 activity when the −112LUC reporter construct, which contains intact elements for these transcription factors, was used (1.6-, 1.4-, and 1.5-fold induction with respect transfection of the corresponding empty vector; Fig. 4). Interestingly, when HNF-4α was cotransfected with either C/EBPα or Oct-1 expression vectors, there was a stronger activation of −112LUC (2.3- and 2.7-fold, respectively), showing that the proximity of the of these transcription factors elements do not hinder the binding. According to these findings, mutation of three important nucleotides of the HNF-4 recognition site on the −52 promoter sequence (probe B) was enough to decrease the HNF-4α stimulatory effect, to abolish its cooperativity with C/EBPα, and to reduce that with Oct-1. We can speculate that some binding of HNF-4α to the mutated construct remains, and it this is
sufficient to moderately interact with Oct-1. As shown in Fig. 2, we found two putative C/EBP binding sites in -112/-1 promoter (-101/-90 and -91/-82). Transfection of the -95LUC construct, where the distal C/EBP binding site is truncated, shows no reduction in C/EBP effect compared with -112LUC, and the -101/-90 C/EBP site therefore seems to be nonfunctional. The functionality of the Oct-1 binding site was confirmed by cotransfections using -87LUC, where the Oct-1 site is truncated, and as a result of that, the Oct-1 effect was lost.

**C/EBPβ Plays an Inhibitory Role on the HNF-4α-Mediated Activation of the CYP2A6 Promoter.** In addition to C/EBPs, another protein from the same family of transcription factors, C/EBPβ, also known as liver-enriched activating protein (LAP), is highly expressed in liver and binds to the same DNA motifs as C/EBPs but has a different transactivation potential. Moreover, the truncated inhibitory form of C/EBPβ (LIP) is able to bind DNA but is devoid of the transactivation domain (Descombes and Schibler, 1991; Ossipow et al., 1993). To investigate the role of these proteins in CYP2A6 transcription, we cotransfected 2A6-luciferase constructs and expression vectors for HNF-4α, LAP, and LIP. As shown in Fig. 5, when -112LUC was cotransfected with a preponderance of LAP protein (LAP/LIP ratio 4:1), a reduction to 60% of the control luciferase activity was found. This reduction was greater when the inhibitory protein LIP was overexpressed (LAP/LIP ratio 1:4), reaching 34% of the basal activity. HNF-4α was able to abolish the LAP down-regulatory effect but not to counteract the stronger LIP inhibitory effect. HNF-4α effect was lost in a HNF-4 mutated construct. Mutation or deletion of the nonfunctional C/EBP binding site at -101/-90 (-112LUC -95/-93 mut and -95LUC constructs, respectively) did not have any effect on LAP/LIP action, but partial deletion of the functional C/EBP binding site at -91/-82 (-87LUC construct), resulted in the loss of LAP inhibitory effect and a reduction of LIP effect. The fact that LIP down-regulating effect is also observed on -87LUC, suggests that LIP is able to bind to a more proximal C/EBP site. Overall, these data further confirm the functional importance of the C/EBP site comprised between nucleotides -91 and -82.

**HNF-4α and C/EBPα-Oct-1 Binding Sites Are Required for Full Activity of CYP2A6 Promoter in Vivo.** To evaluate the in vivo relevance of the transcription factors identified by in vitro transfections, reporter constructs with wild-type or mutated binding sites for HNF-4α, C/EBP, and Oct-1 were injected into mice. For the mouse DNA injections, we selected the -200LUC CYP2A6 construct and by site-directed mutagenesis, we introduced mutations in critical sites for the HNF-4α, C/EBP, Oct-1, and DR-4 elements (see Fig. 2 and schematically depicted constructs in Fig. 6). Mutation of the DR-4 site caused a nonsignificant, slight increase of the promoter activity, whereas the other mutations significantly decreased this activity. In agreement with the reporter assay in vitro data, mutation of the HNF-4α site reduced CYP2A6 promoter activity to 52% of the wild-type promoter activity, and mutation of the C/EBP-Oct-1 overlapping sites reduced it to 26% (Fig. 6). When both HNF-4α and
C/EBP-Oct-1 sites were simultaneously mutated, the promoter activity reached only 14% of the wild-type levels. These data further show that C/EBP, Oct-1, and HNF-4 are critical for CYP2A6 transcription.

Discussion

The present study constitutes the first comprehensive functional analysis of the human CYP2A6 gene promoter. By means of a CYP2A6 promoter deletion analysis in B16A2 cells and by EMSA experiments, we have identified the regulatory elements and transcription factors involved in the basal expression of the CYP2A6 gene. However, it cannot be ruled out that other elements, not contained in the studied region, also could contribute to the gene transcription. The human hepatoma cell line B16A2 was chosen for our deletion assay, because, like the related BC2 cells, it shows higher expression levels of some hepatic specific genes such as CYP2A6, compared with other human hepatic cell lines (Gomez-Lechon et al., 2001; Rodriguez-Antona et al., 2002). Stepwise deletion of the 5′-flanking region of CYP2A6 defined the core promoter as the fragment comprised between −1 (preceding the translational start site) and −112 (Fig. 1). In the region between −95 and −61, we showed by EMSA that the liver-enriched transcription factors HNF-4α, C/EBPα, and C/EBPβ, as well as the ubiquitously expressed Oct-1 factor, bind to their respective cognate binding sites.

Nuclear factor I (NF-I) has been shown to regulate the expression of CYP2A genes and recently a functional NF-I binding site has been described in the proximal promoter of cyp2a5 (Ulvila et al., 2004). This NF-I element is found in CYP2A6 (−158 to −141) with four nucleotide changes, but the similar promoter activity of the −200 and −135 constructs (containing and lacking this NF-I putative element, respectively), suggest that it is not functional (Fig. 1).

To investigate the functional relevance of the binding sites localized by EMSA, human hepatic cells were cotransfected with CYP2A6 promoter plasmids and transcription factor expression vectors. HepG2 cells express low levels of CYP2A6, and they also have an altered expression of hepatic specific transcription factors (Rodriguez-Antona et al., 2002). Therefore, we chose to cotransfect expression vectors for transcription factors together with CYP2A6 promoter constructs in these cells. We found that overexpression of HNF-4α, C/EBPα, and Oct-1 resulted in an activation of CYP2A6 promoter in HepG2 cells. In addition, when HNF-4α was cotransfected with either C/EBPα or Oct-1, there was an increased activity, suggesting a cooperation between these transcription factors. The results do not exclude, however, important enhancers or repressors affecting CYP2A6 transcription located somewhere else in the CYP2A6 gene.

These results are in agreement with previous evidence about the important role of HNF-4α regulating the expression of several P450s: CYP2A6 (Jover et al., 2001), CYP2C9
entire data set from three independent experiments (\(n\))/C/EBP (Jover et al., 1998). We also observed that both CYP2B6 done in triplicate, and the values given represent the mean expression of members of the different reporter constructs (500 ng) plus 10 ng of pRL-SV40 along with HepG2 cells were cotransfected with either the pGL3-Basic plasmid or the corresponding empty cloning vectors. Luciferase activities were measured 48 h after transfection, and the firefly/Renilla ratios were calculated and normalized with regard to the corresponding pGL3-Basic activities; the luciferase activity of the different constructs in basal conditions is shown in brackets. The transcriptional activity of each construct is expressed as its transactivation rate (-fold induction) over the basal condition. For each reporter construct, the transactivation domain of each construct is expressed as its transactivation rate corresponding pGL3-Basic activities; the luciferase activity of the different promoter constructs was compared against the basal situation, and the statistical analysis was performed as described in the legend to Fig. 4. Luciferase activity of the different constructs in basal conditions is shown in brackets. All experiments were done in triplicate, and the values given represent the mean ± S.D. of the entire data set from three independent experiments (\(n = 9\)).

(Ibeanu and Goldstein, 1995), CYP2D6 (Corchero et al., 2001), CYP3A4 and CYP3A5, and to a moderate extent, CYP2B6 (Jover et al., 2001). Furthermore, it was recently shown that an HNF-4α element in the proximity to the binding sites for pregnane X receptor and CAR mediates CYP3A4 induction (Tirona et al., 2003). Similarly, the expression level of dihydriodiol dehydrogenase mRNA is cooperatively regulated by the amounts of HNF-1α, HNF-4α, and HNF-4γ (Ozeki et al., 2003).

The cotransfection experiments showed that interaction between HNF-4α and C/EBPα triggered a cooperative effect on CYP2A6 transcription (Fig. 4). Actually, C/EBPα has previously been shown to up-regulate the expression of other members of the CYP2 family (CYP2B6, CYP2C9, and CYP2D6) (Jover et al., 1998). We also observed that both C/EBPβ isoforms LAP and LIP negatively influenced the expression of CYP2A6 (Fig. 5). C/EBP proteins bind to their DNA binding sites as homo- (α-α, β-β) and hetero-dimers (α-β), and both C/EBPα and C/EBPβ have a different trans-activatory potential. In addition, the transactivation domain of the full-length C/EBPβ protein (LAP) is absent in the short LIP isoform (Descomes and Schibler, 1991; Ossipow et al., 1993). The C/EBPα overexpression experiments clearly showed that the C/EBPα-α homodimers exert an activating effect on the CYP2A6 promoter. An excess of C/EBPβ (LAP) might lead to a displacement of the activating C/EBPα-α homodimers for the less-active C/EBPα-β heterodimers and β-β homodimers, hindering CYP2A6 activation and even exerting a down-regulating effect. Thus, the different abilities of the C/EBP transactivation domains might likely represent a fine-tuning mechanism to regulate CYP2A6 expression. In agreement with these data, overexpression of the truncated C/EBPβ dominant negative form (LIP) caused a greater down-regulation of the CYP2A6 promoter, a transcriptional block that was not even rescued by an excess of HNF-4α.

We have also found a functional synergism between the transcription factors Oct-1 and HNF-4α in the regulation of CYP2A6 expression (Fig. 4). The ubiquitous transcription factor Oct-1 has previously been described to play an important role in the regulation of other genes like the human UDP-glucuronosyltransferase 2B7 (UGT2B7) gene, where there is an interaction between Oct-1 and HNF-1α. In this case, Oct-1 does not bind to the promoter sequence, but its effect on the UGT2B7 expression depends on its recruitment via a direct interaction with HNF-1α bound to the proximal promoter HNF-1 site (Ishii et al., 2000). For some genes, Oct-1 plays a negative role in the transcriptional regulation as has been shown for rat CYP1A1 (Sterling and Bresnick, 1996).

To confirm in an in vivo system the functional relevance of the HNF-4α, C/EBPα, C/EBPβ, and Oct-1 motifs identified in the proximal promoter of CYP2A6, mutated CYP2A6 promoter constructs were injected into mouse liver and their activity was compared with that of the wild-type promoter (Fig. 6). In agreement with the EMSA results, the mutation of the identified DR-4 motif did not significantly affect promoter activity, neither in control nor in phenobarbital-treated mice (data not shown). These findings indicate that the identified CAR element is not involved, to a great extent, in CYP2A6 induction and CYP2A6 basal transcription. In accordance with the in vitro results, the in vivo transfections also showed that HNF-4 and C/EBP-Oct-1 binding sites were functional and required to achieve full CYP2A6 promoter activity. A 1.9- and 3.8-fold reduction in promoter activity
resulted from mutation of HNF-4 and C/EBP-Oct-1 binding sites, respectively, and mutation of both sites resulted in a 7-fold reduction of promoter activity.

Together, our study has uncovered the key transcription factors that in concert regulate the basal activity of CYP2A6 promoter as summarized in Fig. 7. We have provided data that clearly show that the interplay between HNF-4α, C/EBPα, and C/EBPβ, and the ubiquitous Oct-1 factor, contribute to the fine-tuned regulation of CYP2A6 transcription. Interindividual differences in CYP2A6 mRNA and protein levels have been reported previously (Yun et al., 1991; Shimada et al., 1994; Pelkonen et al., 2000; Rodriguez-Antonio et al., 2001), but not too much effort has been dedicated to study the CYP2A6 promoter and its genetic polymorphisms. In fact, only a few allelic variants have been described carrying SNPs in the 5′-flanking region of CYP2A6, which have been shown to affect expression levels, e.g., CYP2A6*9 (Pitarque et al., 2001), CYP2A6*1D (Pitarque et al., 2004), and CYP2A6*1H (von Richter et al., 2004). It is important to identify critical regions for CYP2A6 promoter activity because polymor-
phisms in these regions would probably lead to altered mRNA levels and therefore could contribute to the substan-
tial CYP2A6 activity variability observed within the popula-
tions. The functional characterization of the CYP2A6 pro-
pressor conducted in this study will help us to gain insight into 
the molecular mechanisms modulating CYP2A6 expression 
and should provide a new framework and strategies to search 
for regulatory polymorphisms affecting its expression.

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