Characterization of the Human Cytochrome P4502D6 Promoter

A POTENTIAL ROLE FOR ANTAGONISTIC INTERACTIONS BETWEEN MEMBERS OF THE NUCLEAR RECEPTOR FAMILY*

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The functional mapping of the human cytochrome P4502D6 (CYP2D6) promoter in HepG2 cells revealed the presence of both positive and negative regulatory elements. One of these regulatory elements overlapped a sequence that is highly conserved in most members of the CYP2 family. This element, which consists of a degenerate AGGTCA direct repeat spaced by 1 base pair (DR1) and is known to be a target for members of the steroid receptor superfamily, was found to bind in vitro translated hepatocyte nuclear factor 4 (HNF4) in gel retardation analysis. Using HepG2 nuclear extracts, three protein-DNA complexes were formed on the DR1 element, one of which was confirmed to be dependent on the binding of HNF4. The other DR1 complexes were shown to be due to the interaction of the orphan receptor chicken ovalbumin upstream promoter transcription factor I (COUP-TFI). Experiments in COS-7 cells showed that HNF4 could activate the CYP2D6 promoter 30-fold. Surprisingly, mutation of the DR1 element produced a relatively minor 23% decrease in activity in HepG2 cells. Additionally, COUP-TFI was shown to inhibit HNF4 stimulation of the CYP2D6 promoter in COS-7 cells, suggesting that COUP-TFI could attenuate the effect of HNF4 in HepG2 cells. However, when HNF4 levels were increased in HepG2 cells by co-transfection, it resulted in the enhancement of CYP2D6 promoter activity, indicating that HNF4 could overcome the repressive effect of COUP-TFI. Therefore, the contribution of the DR1 element in controlling the transcription of the CYP2D6 gene depends on the balance between positively and negatively acting transcription factors.

Certain hepatic P450s are constitutively expressed, while others are known to be induced by various foreign chemicals including phenobarbital, polycyclic aromatic hydrocarbons, and peroxisome proliferators (3). The latter two classes of chemicals act through the aryl hydrocarbon receptor (4) and peroxisome proliferator-activated receptor (5), respectively, while the exact mechanism(s) responsible for transducing the response to phenobarbital have yet to be elucidated (3). In addition, the expression of some P450 enzymes can be modulated by endogenous steroid and peptide hormones (3, 6). In rodents for example, the sexually dimorphic expression of certain P450s is controlled by the sex-specific pattern of growth hormone secretion (6). Most of the regulatory effects on P450 expression are at the transcriptional level. However in some instances, such as the induction of cytochrome P4502E1 (CYP2E1) by ethanol, post-transcriptional mechanisms are also involved (7).

Within the P450 superfamily, the CYP2 family is the largest and most diverse (1). This family, whose members are mainly expressed in the liver, contains many of the drug-metabolizing isoforms and also some of the enzymes involved in the metabolism of endogenous substrates (for review see Ref. 8). In addition to the constitutively expressed CYP2 members, this family also contains isoforms that are regulated by phenobarbital, ethanol, and growth hormone (8). Regarding the study of CYP2 gene regulatory DNA elements and their corresponding transacting transcription factors, relatively little is known in comparison to members of the CYP1 family or some of the steroid metabolizing P450s (2). Research in this area has been hampered by the difficulty in maintaining the expression of, or the ability to induce, P450s in isolated hepatocytes or liver-derived cell lines. As a result, most of the data generated to date comes from studies using transient transfection of promoter constructs into various hepatoma cell lines. Nevertheless, using this approach some information has been obtained about the transcriptional control of the CYP2 genes. The transcription of the CYP2E1 gene was reported to be partly controlled by HNF1α (9), and that of CYP2C6 by DBP (10), both of these transcription factors being hepatocyte-enriched. A phenobarbital-responsive region was identified in the chicken CYP2H1 gene using transient transfection of primary chicken hepatocytes (11), and a functional glucocorticoid response element was identified in the rat CYP2B2

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1 The abbreviations used are; CYPs, cytochrome P450s (eg. CYP2E1, cytochrome P4502E1); RXR, retinoid X receptor; COUP-TFI, chicken ovalbumin upstream promoter transcription factor I; HNF, hepatocyte nuclear factor; CAT, chloramphenicol acetyltransferase; CTE, common transcription element; ARF1, apoA1-regulatory protein 1; EAR3, v-erbA related protein 3; PPAR, peroxisome proliferator-activated receptor; DBP, D-box binding protein; C/EBP, CCAAT-box enhancer binding protein; WCE, whole cell extracts; CMV, cytomegalovirus; DR1, degenerate AGGTCA direct repeat spaced by 1 base pair.
gene promoter (12). Analysis of the rabbit CYP2C1 and CYP2C2 promoters in HepG2 cells revealed the presence of a regulatory element, which was shown to be a target for HNF4 (13), a member of the steroid receptor superfamily (14). Co-expression of HNF4 in COS-1 cells resulted in the induction of the CYP2C2 promoter (13). Furthermore, mutation of the CYP2C2 HNF4 element resulted in a marked decrease in promoter activity in HepG2 cells (13). This HNF4 element has been reported to be conserved in other members of the CYP2 family, and it was proposed to be of importance in the transcriptional control of other CYP2 genes (15). Additional studies have also demonstrated a role for HNF4 in the transcriptional control of the human CYP2C9 gene (15). However, in contrast, studies of the rat CYP2C genes (CYP2C7, CYP2C11, CYP2C12, and CYP2C13) demonstrated that co-expressed HNF4 gave only a maximal 3-fold induction of promoter activity in COS-7 cells (16). Furthermore, mutation of the HNF4 binding site in the respective promoters had no effect on the activity of the 2C7 or 2C11 promoters in HepG2 cells, while it caused decreases to 60 and 80% in the activity of CYP2C13 and CYP12 promoters, respectively (16).

Regarding the CYP2D subfamily, using in vitro transcription analysis and transient transfections into HepG2 cells, it was possible to identify basal and sex-specific regulatory elements in the mouse CYP2D genes (17, 18). Another CYP2D promoter that has been analyzed is that of the rat CYP2D5 gene, where it was reported that C/EBP and Sp1 cooperate in controlling its transcriptional activity (19). There was no evidence presented in support of a role for the HNF4 binding site in the modulation of CYP2D5 expression (19).

Human CYP2D6 is known to play a major role in the metabolism of a wide range of clinically important drugs (20). It is also polymorphic, with 5–10% of the Caucasian population classified as poor metabolizers of CYP2D6 substrates (21). This is caused by mutations within the gene resulting in the absence of CYP2D6 protein (22). This polymorphism was subsequently reported to be associated with the incidence of various forms of cancer (23) and the susceptibility to Parkinson’s disease (24). The CYP2D6 protein was also reported to be absent until the first week after birth (25), suggesting that its expression might be repressed by maternal hormones. Given that the levels of CYP2D6 expression may be critical in the responsiveness to certain clinically used drugs and in disease susceptibility, it is important to understand how CYP2D6 expression is controlled at the transcriptional level. Therefore, in this paper, we have performed the functional analysis of the CYP2D6 promoter and investigated what role the HNF4 binding site plays in controlling transcription of the CYP2D6 gene. First, the results indicate that both positive and negative regulatory elements contribute toward promoter activity. Second, although the HNF4 binding site alone appears to play a relatively minor role in HepG2 cells, the findings indicate that the balance between HNF4 and negatively acting transcription factors is an important factor.

**Experimental Procedures**

**Cell Culture**—HepG2 and COS-7 cells were grown in monolayer and cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies, Inc.) at 37°C in 5% CO₂.

**Transient Transfection Analysis and Expression Constructs**—DNA transfections were carried out by the calcium phosphate method (26) as described by Gorman (27) with the exception that the glycerol step was omitted. Cells were harvested 24–36 h after transfection, extracts were prepared, and chloramphenicol acetyltransferase (CAT) activity was assayed as described by Gorman et al. (28). Cell extracts were assayed for protein content (29). All CAT assays were performed such that the rate of acetylation was in the linear range. In all experiments, the values given ± S.E. of at least three experiments. A minimum of two plasmid preparations were used for each construct. Cells were also co-transfected with pSVgal (Promega) to assay for β-galactosidase activity as a control for transfection efficiency. Using this technique, transcription efficiency was found to vary by less than 10%. The expression plasmid pCMVHNF4 was kindly provided by Prof. B. Kemper (13), and the pMTHNF4 and pMTEAR3 constructs were gifts from Dr. J. Ladis (30). For the expression of HNF4 and COUP-TF1 in COS-7 cells, 75-cm² tissue culture flasks were transfected with 10 μg of pMTHNF4 or pMTEAR3, respectively, as described above. After 36 h, cells were harvested, and whole cell extracts were prepared by three cycles of freeze-thawing in 0.4 mM KCl, 20 mM Tris-HCl, pH 8, 2 mM diethiothreitol, and 20% (w/v) glycerol. Extracts were centrifuged at 100,000 × g for 15 min at 4°C, and the supernatant and dimethylsulfoxide were added to a final concentration of 1 mM.

**Promoter Deletion Constructs**—The CYP2D6 promoter was isolated by polymerase chain reaction from human genomic DNA using the upstream oligonucleotide 5’-CAGTAAACCTGCAGAAGCTACCT-3’ (with HindIII site) and downstream oligonucleotide 5’-GGGTCCTCTTCTAGACACCCCTCCAC-3’ (with XbaI site). The resulting promoter fragment (~392 to +56) was subcloned between the HindIII and XbaI sites in pCATbasic (Promega) and checked by sequencing. Deletion fragments of the CYP2D6 promoter were generated using the Erase-a-Base system following the manufacturer’s protocol (Promega). After confirming the sequence of the various promoter fragments, they were subcloned into the HindIII and XbaI sites of pCATbasic (Promega) after deletion of a HindIII linker at the 5′ end. The MTMUT construct was prepared by polymerase chain reaction using the 5′-mutated oligonucleotide with HindIII linker 5′-TTGGAGAAGTTTCTACTC-AACGACATTTAATACCTGACGTCGC-3′ and the 3′ oligonucleotide with XbaI linker 5′-AACAATCTAGACACACCTTGCCACCCA-CCC-3′. After sequencing, the mutated fragment was subcloned into the HindIII and XbaI sites of pCATbasic (Promega).

**Gel Retardation Analysis**—Nuclear extracts from HepG2 cells were prepared as described by Dignam et al. (31). Radiolabeled probe for DNA-binding reactions was prepared by isolating the promoter fragment (~69 to +56 from -69CAT, dephosphorylating the DNA with alkaline phosphatase (Boehringer Mannheim), and phosphorylating with [γ-32P]ATP (Amersham Corp.), 500 Ci/mmol) and T4 polynucleotide kinase (Promega). Binding reactions of 20 μl were carried out in buffer containing 10 mM Heps, pH 7.5, 2.5 mM MgCl₂, 10% (w/v) glycerol, 0.1 mM EDTA, 2 μg of poly(dI-dC) (Pharmacia Biotech Inc.), 50 mM KCl, 0.1–0.3 ng of radiolabeled probe, and the indicated amount of protein. Reactions were incubated at 20°C for 1 h. After migration, DNA-protein complexes were detected by autoradiography of a dried gel.

**In Vitro Translation of HNF4**—In order to determine potential regulatory elements in the CYP2D6 promoter, progressive 5′ deletions were generated. The various promoter fragments were then fused to the CAT gene in pCAT-basic and transiently transfected into HepG2 cells to assay for promoter activity. As can be seen in Fig. 1, the fusion of CYP2D6 promoter sequences from -392 to +56 upstream of...
the CAT gene resulted in the 30-fold induction of CAT activity when compared with pCATbasic. No activity was observed when the same construct was transfected into HeLa cells (data not shown). The deletion of sequences from −392 to −308 resulted in a 28% drop in activity in HepG2 cells, and further deletion to −242 had no additional effect. However, removal of sequences down to −156 produced an additional 2-fold decrease in activity. The presence of a negative regulatory element between −128 and −90 was indicated by the approximately 2-fold increase in CAT activity. Further deletions to −69 and −18 produced additional 2.5- and 3.5-fold decreases in activity, respectively. Therefore, the deletion analysis revealed the presence of four positively acting regulatory regions (−392/−308, −242/−156, −90/−69, and −69/−18), and one negative element (−128/−90). It is noteworthy that none of the deletions produced a difference in activity in excess of 2–3-fold, despite that fact that overall promoter activity was 20–30-fold higher than pCATBasic. This would suggest that these regulatory elements work together to control the activity of the CYP2D6 promoter.

Analysis of Orphan Receptors Binding to the CYP2D6 Promoter—The deletion of sequences between −69 and −18 produced the largest change in CAT activity (Fig. 1). This region contains, in addition to the TATA-box, a sequence that is highly conserved within the CYP2 family (13) (see Fig. 2A). This element consists of a DR1. In addition to the high degree of conservation, perhaps the most striking feature is the fact that the nucleotide at the fourth position in both half-sites of every element is nonconserved with respect to the consensus DR1. Whether this characteristic has any functional significance is unclear at present.

The DR1 elements of other CYP2 genes have been reported to bind HNF4 (13, 15, 16). Therefore, we examined if the CYP2D6 DR1 was a target for in vitro translated HNF4 in gel retardation analysis. The addition of in vitro translated HNF4 to the radiolabeled −69/+56 promoter fragment containing the DR1 element resulted in the formation of one major (complex A) and one minor (complex b) protein-DNA complex (Fig. 2B, lane 2). Complex A was shown to be specific, since it was competed out by the addition of an oligonucleotide (CTE) spanning the DR1 site (lane 3) but not by an unrelated oligonucleotide (Sp1) corresponding to the Sp1 consensus sequence from the SV40 early promoter (lane 4). Complex b was observed to be nonspecific, since its formation was not abolished by the addition of the CTE oligonucleotide (lane 2) or the unlabeled −69/+56 fragment (data not shown). Indeed, this complex was observed to be due to the reticulocyte lysate itself, since it was also seen using nonprogrammed lysate (lane 5). The identity of complex A was confirmed as being due to HNF4 by the addition of anti-HNF4 antisera, which produced a supershift of the protein-DNA complex (lane 7), which was not seen with the nonimmune serum (lane 8).

In order to test which nuclear proteins from HepG2 cells could bind to the CYP2D6 DR1 element, gel retardation analysis was performed using nuclear extracts. As shown in Fig. 2C (lane 1), the addition of HepG2 nuclear protein resulted in the formation of three protein-DNA complexes (complexes 1–3) on the −69/+56 promoter fragment. All of these complexes were observed to bind specifically to the DR1 sequence, since their formation was abolished by the addition of CTE oligonucleotide (lane 2) but not by Sp1 oligonucleotide (lane 3). Since HNF4 was previously shown to be capable of binding to this DR1 element Fig. 2B), we examined if any of the complexes were due to the interaction of HNF4. Subsequently, complex 2 was shown to be dependent on the interaction of HNF4 as it was supershifted by the inclusion of anti-HNF4 antisera in the DNA-binding reaction (Fig. 2C, lane 5). This supershift was observed to be specific, since the antisera had no effect on the mobility of the other two complexes, and the addition of nonimmune serum to the DNA-binding reactions had no effect (lane 6). It was noted that when HNF4 was effectively removed from the DNA-binding reaction by its antisera, the intensity of complex 1 increased (lane 5), suggesting that HNF4 and the factor responsible for the formation of complex 1 may bind to the DR1 element in a mutually exclusive manner.

It was previously demonstrated that DR1 elements from other genes could be recognized by not only HNF4, but also by other members of the steroid receptor superfamily, including COUP-TFI (EAR3), ARP1 (COUP-TFII), EAR2, peroxisome proliferator-activated receptor (PPAR) and retinoid X receptor (RXR), (30, 34, 35). Therefore, we tested if any of the unidentified complexes formed by HepG2 nuclear extracts on the CYP2D6 DR1 element were due to the interaction of other members of the steroid receptor superfamily. Based on what was already reported in the literature, the most obvious candidates were COUP-TFI/ARP1. These two proteins are highly homologous...
members of the steroid receptor family (36), and ARP1 has also been termed COUP-TFII. As Fig. 2D demonstrates, the addition of HepG2 nuclear extracts to radiolabeled −69/+56 promoter fragment resulted in the formation of the same three complexes (lane 2). The inclusion of anti-COUP antiserum (which recognizes both COUP-TFI and ARP1 (37)) in the DNA-binding reactions, resulted in the inhibition of complex 1 and the disappearance of complex 3, with the concomitant appearance of supershifted complexes (lane 3). None of the complexes were affected by the addition of nonimmune serum (lane 4). The identification of COUP-TFI/ARP1 as being responsible for the formation of complex 1 was in agreement with the observed relative mobilities of HNF4 and COUP-TFI/ARP1 reported by other groups (37). The observation that complex 3 was also supershifted by anti-COUP antiserum suggests that its formation was also dependent on COUP-TFI/ARP1. Alternatively, the factor(s) responsible could be antigenically related to COUP-TFs. However, results from gel retardation analysis using whole cell extracts from COS-7 cells transfected with a COUP-TFI expression vector support the former (see Fig. 5A), where the addition of COUP-TFI resulted in the formation of two protein-DNA complexes similar in mobility to complexes 1 and 3.

**HNF4 Effect on the Activity of the CYP2D6 Promoter**—In order to investigate if HNF4 was capable of activating the CYP2D6 promoter, co-transfection experiments were performed in COS-7 cells (see Fig. 3). In contrast to the results obtained from HepG2 cells, the transfection of −382CAT into COS-7 cells did not result in any enhancement of CAT activity above that observed with pCATbasic. Co-transfection with the mammalian HNF4 expression vector CMVHNF4 produced an approximately 30-fold induction of CAT activity from
−392CAT, which was not seen with the empty expression vector CMV. Therefore, HNF4 was capable of activating the CYP2D6 promoter.

**Mutational Analysis of the CYP2D6 DR1 Element**—To assess the functional importance of the CYP2D6 DR1 element, it was mutated in both repeats in order to abolish any interaction with nuclear factors. Gel retardation analysis using HepG2 nuclear extracts was performed to check that the mutations had indeed abolished the ability of nuclear factors to interact with the DR1 element. As shown in Fig. 4A, competition with the wild-type −69/+56 fragment abolished the formation of all three complexes (compare lanes 2 and 3). The addition of the mutated −69/+56 fragment, however, had no effect on the formation of any of the complexes (lane 4), indicating that the mutations had abolished the protein-DNA interactions. Furthermore, gel retardation analysis using the mutated fragment as a probe for HepG2 nuclear extracts did not result in the formation of any protein-DNA complexes (data not shown).

To examine the functional effect of the DR1 mutation, the mutated fragment was subcloned upstream of the CAT gene in pCATBasic, and its activity was compared with that of the wild-type fragment (−69CAT). Fig. 4B shows that the −69CAT construct gave approximately 7-fold higher CAT activity than that observed with pCATBasic. However, mutation of the DR1 element (−69CATMUT), resulted in only a 23% decrease in CAT activity. This result indicated that first, the DR1 element alone appears to play a relatively minor role in controlling the expression of CYP2D6 promoter in HepG2 cells, and second, that the observed difference in activity between the −69CAT and −18CAT constructs in HepG2 cells is probably due to the presence of the TATA-box in the longer construct, although at present we cannot rule out the possibility of an as yet unidentified transcription factor binding to this region.

**The Effect of COUP-TFI on Transactivation by HNF4**—It was reported that COUP-TFI could antagonize HNF4 activity on DR1 elements present in other genes, mediated by competition for the same DNA-binding site (35, 37). One possible reason for the relatively minor effect of the DR1 element mutation is that in HepG2 cells, the stimulatory effect of HNF4 was being repressed by COUP-TFI. Therefore, the mutation would abolish both a positive and negative response. This was investigated *in vitro* using gel retardation analysis and *in vivo* by co-transfection experiments in COS-7 cells. First, COS-7 cells were transfected with expression vectors for HNF4 or COUP-TFI, and whole cell extracts (WCE) were prepared. When analyzed by gel retardation analysis, the addition of increasing amounts of WCE containing HNF4 or COUP-TFI resulted in the formation of their respective protein-DNA complexes (Fig. 5A). Interestingly, the addition of the higher concentrations of COUP-TFI resulted in the appearance of major and minor complexes, similar to the two COUP-TFI-dependent complexes observed with HepG2 nuclear extracts (Fig. 2D). Both HNF4- and COUP-TFI-dependent complexes were shown to be specific, since they were abolished by the addition of a 100-fold molar excess of CTE oligonucleotide (Fig. 5B, lanes 3 and 6) but not by the unrelated Sp1 oligonucleotide (Fig. 5B, lanes 4 and 7). In addition, none of the complexes were observed with untransfected COS-7 WCE (Fig. 5B, lane 1), and they could be supershifted with their respective antisera (data not shown). Using the COS-7 WCE, we then tested if COUP-TFI could antagonize HNF4 binding to the DR1 element. The
Transcriptional Control of the CYP2D6 Promoter

In this paper, the deletion analysis performed on the CYP2D6 promoter revealed that its overall activity was controlled by several regulatory elements, both positive and negative in nature. Although the progressive deletion of each of the regulatory elements did not result in any dramatic changes in promoter activity, their combined effect still resulted in the 30-fold stimulation of transcription (see Fig. 1). At present we do not know if each of these elements is acting individually to modulate promoter activity or if there are any cooperative/antagonistic interactions between the various factors. In addition, it is possible that other DNA elements that are situated upstream of −392 base pairs may also contribute to the activity of the CYP2D6 promoter.

Regarding the CYP2 family, there have been various studies that investigated the functional role of the conserved DR1 element. In the case of the CYP2D6 promoter, it was clear that HNF4 could significantly stimulate its activity in COS-7 cells; in HepG2 cells, the activity of this mutated construct was repressed by COUP-TFI/ARP1. As shown in Fig. 6, the co-transfection of HNF4 into HepG2 cells resulted in the marked enhancement in the activity of both −392CAT and −69CAT promoter constructs, but not the −69CATMUT construct containing the mutated DR1 element. In fact, the activity of this mutated construct was repressed by the presence of excess HNF4, probably caused by the squelching of promoter activity in a DNA binding-independent manner. One important conclusion from these results is that the stimulatory effect of HNF4 is still observed with the longer construct, despite the fact that its expression in HepG2 cells was initially relatively high. This experiment demonstrates that altering the balance between the two transcription factors could modulate the CYP2D6 promoter activity.

**DISCUSSION**

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COS-7 cells were transfected as described under "Experimental Procedures," with 2.5 μg of −392CAT alone (NONE) or cotransfected along with 0.25 μg of pCMV, pCMVHNF4, pCMVHNF4 and pMT2, pCMVHNF4 and pMTEAR3, pMTEAR3. CAT activity was calculated and the data were expressed as described in the legend to Fig. 1.

**Fig. 5.** The effect of COUP-TFI on the activity of HNF4 in vitro and in vivo. A, WCE from COS-7 cells transfected with HNF4 or COUP-TFI were tested for DNA-binding activity on the −69/+56 promoter fragment by gel retardation analysis as described under "Experimental Procedures." Radiolabeled probe was incubated in the absence of any WCE (lanes 1 and 5), in the presence of 0.5, 1, and 3 μl of HNF4-WCE (lanes 2-4) or COUP-TFI-WCE (lanes 6-8). Complexes formed by HNF4 and COUP-TFI are indicated by arrows. B, gel retardation was carried out as described under "Experimental Procedures." Radiolabeled −69/+56 promoter fragment was incubated in the presence of 1 μl of COS-7 WCE from untransfected cells (lane 1), 1 μl of COUP-TFI-WCE (lanes 2-4), or 1 μl of HNF4-WCE (lanes 5-7), alone (lanes 2 and 5) or in the presence of a 100-fold molar excess of either CTE oligonucleotide (lanes 3 and 6) or Sp1 oligonucleotide (lanes 4 and 7). 1 μl of HNF4-WCE was incubated together with 1 μl of COUP-TFI-WCE (lane 8) or with 1 μl of WCE from untransfected cells (lane 9). C, inclusion of COUP-TFI in the HNF4 DNA-binding reactions resulted in a decrease in the amount of HNF4-dependent complex with a concomitant appearance of the COUP-TFI-dependent complexes (Fig. 5B, compare lanes 5 and 8), while the addition of untransfected COS-7 WCE had no effect on the activity of HNF4 (Fig. 5B, lane 9). This experiment demonstrated that HNF4 and COUP-TFI were competing for the same DNA element.

To examine the effect of COUP-TFI in vivo, co-transfection experiments were performed in COS-7 cells. Fig. 5C demonstrates that co-transfection with pCMVHNF4 produced a 12-fold induction of CYP2D6 promoter activity, as observed previously (Fig. 3). However, in the additional presence of COUP-TFI/EAR3 (from the expression of pMTEAR3), this activity was totally abolished, while no effect was observed with the empty expression vector pMT. The expression of COUP-TFI/EAR3 in the absence of HNF4 had no effect on the activity of the promoter, indicating that COUP-TFI itself lacked any transcription capability when bound to this element. This experiment demonstrated that the induction of the CYP2D6 promoter by HNF4 can be inhibited by COUP-TFI and suggested that the presence of COUP-TFI in HepG2 cells attenuated the effect of HNF4.

The Effect of Increasing HNF4 Levels in HepG2 Cells—The co-expression of COUP-TFI in COS-7 cells resulted in the complete inhibition of HNF4 activity on the CYP2D6 promoter, suggesting that it was the dominant factor. Therefore, we tested if increasing the concentration of HNF4 in HepG2 cells could overcome the repressive effect of COUP-TFI/EAR1. As shown in Fig. 6, the co-transfection of HNF4 into HepG2 cells resulted in the marked enhancement in the activity of both −392CAT and −69CAT promoter constructs, but not the −69CATMUT construct containing the mutated DR1 element. In fact, the activity of this mutated construct was repressed by the presence of excess HNF4, probably caused by the squelching of promoter activity in a DNA binding-independent manner. One important conclusion from these results is that the stimulatory effect of HNF4 is still observed with the longer construct, despite the fact that its expression in HepG2 cells was initially relatively high. This experiment demonstrates that altering the balance between the two transcription factors could modulate the CYP2D6 promoter activity.

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CAT activity was calculated and the data were expressed as described under "Experimental Procedures," with 2.5 μg of pCATbasic, −392CAT, −69CAT, or −69CATMUT. The latter two constructs were co-transfected along with 0.25 μg of either pMT2HNF4 or pMT2. CAT activity was calculated and the data were expressed as described in the legend to Fig. 1.

Despite the fact that HNF4 could significantly activate the CYP2D6 promoter when expressed in COS-7 cells, mutation of the DR1 element resulted in a relatively minor (23%) decrease in promoter activity in HepG2 cells (Fig. 4B). We believe that this is caused by the additional presence of COUP-TFI/ARP1 in HepG2 cells, which counteracts the stimulatory potential of HNF4 on the wild-type element, by competing with it at the DNA binding level. It is also possible that COUP-TFI may actively repress minimal promoter activity via an interaction with corepressor molecules, and in fact evidence for such a mechanism has recently been presented (38). If this were the case, then abolishing the interaction of COUP-TFI with its DNA target would also result in the relief of active transcriptional repression. However, at present this activity of COUP-TFI on the CYP2D6 promoter has not yet been investigated.

The results from the mutational analysis of the CYP2D6 DR1 element are analogous to the findings reported for several rat CYP2C genes, where the mutation of their respective DR1 elements had either no effect or caused similar minor decreases in promoter activity (16). The minor effects upon mutating the DR1 elements in the CYP2D6 and several rat CYP2C genes are in contrast to the rabbit CYP2C2 gene, where mutation of its DR1 element produced a marked decrease in promoter activity (13). Importantly, however, in contrast to the rat CYP2C2 genes (16), the CYP2D6 DR1 element retained the potential to respond to HNF4 as observed by the significant inductions upon co-expression of HNF4 in both COS-7 and HepG2 cells (Figs. 3 and 6).

The reason for the above differences in the activity of the conserved DR1 element between different CYP2 genes is unclear at present, since the respective studies were all performed in similar cell lines. Therefore, it cannot be due to any cell specific differences, for example in the expression of co-activators. It is possible that small changes in the sequence of the respective DR1 elements may determine the stimulatory capability of HNF4 once it is bound to the DNA. The same sequence differences may alter the relative affinity of the element for HNF4 and COUP-TFI/ARP1 in HepG2 cells, thereby affecting its capacity to stimulate transcription. In fact, DR1 elements appear to fall into two classes, those that bind HNF4 but not COUP-TFI and those that bind both (39). Alternatively, the relative positioning of the DR1 element with respect to the TATA-box or differences in the composition of the basal transcription machinery may be critical.

Despite the fact that the mutation of the CYP2D6 DR1 element had a relatively minor effect on promoter activity, it could be predicted that this sequence will still have an important role to play in regulating CYP2D6 expression, with its activity being controlled by the relative concentrations of HNF4 and COUP-TFI in any given cell type. Studies on the human coagulation factor VII promoter revealed that a mutation that inhibited HNF4 binding to its DNA element only produced a 20–50% drop in activity, but significantly, this same mutation when it occurs in a similar site of the factor IX promoter causes a severe bleeding disorder (Ref. 40 and references therein). Therefore, relatively small changes in promoter activity can have rather drastic consequences. It is noteworthy that two tissues where the rat CYP2D1 enzyme is highly expressed, namely liver and kidney (2), coincide with two of the major sites of HNF4 expression (14). HNF4 has also been reported to cooperate with other transcription factors in regulating the expression of certain genes (41). Therefore, it is possible that additional upstream elements in the CYP2D6 promoter may require HNF4 for their stimulatory effect. In fact they may even influence the occupancy of the DR1 element, such that it is in favor of HNF4 rather than COUP-TFI/ARP1. Since these studies were performed by transient transfection analysis, it remains possible that regarding the endogenous gene, the presence of chromatin may also influence the relative affinities of HNF4 and COUP-TFI/ARP1 for the DR1 element. Indeed, the presence or absence of chromatin has been reported to influence transcriptional activation by other transcription factors (42).

One interesting question that arises from this and other studies is why HNF4 and COUP-TFI differ in their transcriptional capabilities on several DR1 elements. Since COUP-TFI is able to stimulate the transcription of certain genes (43), it cannot be due to its lack of a transcriptional activation domain. It is possible that the ability of COUP-TFI to stimulate transcription depends upon the promoter context, with HNF4 and COUP-TFI differing in their abilities to interact with other transcription factors or co-activators. Alternatively, the exact sequence of the DNA binding site may be more critical for COUP-TFI than it is for HNF4 to function in a positive manner.

Regarding the functional importance of the CYP2D6 DR1 element, the experiments performed in this work do not take into account natural situations where the relative concentrations of HNF4 and COUP-TFI might vary. For example, HNF4 levels are regulated during development (44), and its concentration has been reported to vary in rat hepatocellular carcinomas (45). In addition, HNF4 is expressed in a tissue-specific manner (14, 44). Therefore, we believe that the CYP2D6 DR1 element is not functionally redundant but has the potential to respond to changes in the balance between positive and negative regulators. Interestingly, DR1 elements present in other genes have been demonstrated to exhibit differential occupancy by various transcription factors in a tissue-specific manner (46).

The activity of the DR1 element may also be modulated by

**Fig. 6. The effect of increasing HNF4 levels in HepG2 cells on the activity of the CYP2D6 promoter.** HepG2 cells were transfected as described under “Experimental Procedures,” with 2.5 μg of pCATbasic, −392CAT, −69CAT, or −69CATMUT. The latter two constructs were co-transfected along with 0.25 μg of either pMT2HNF4 or pMT2. CAT activity was calculated and the data were expressed as described in the legend to Fig. 1.
extracellular signals. In fact, HNF4 has recently been reported to be regulated by a phosphorylation signal-dependent pathway (47), and dopamine has been demonstrated to activate COUP-TFI (48). Furthermore, preliminary evidence indicates that RXR homodimers and PPAR-RXR heterodimers are capable of interacting with the CYP2D6 DR1 element, suggestive of a possible effect of peroxisome proliferators/fatty acids or 9-cis-retinoic acid on CYP2D6 expression. Both COUP-TFI (49) and CYP2D6 are expressed in the brain; therefore, given the involvement of CYP2D6 in the susceptibility to Parkinson’s Disease (24), it is tempting to speculate that dopamine may alter CYP2D6 levels in the brain via its effects on the activity of COUP-TFI. Interestingly, CYP2D6 expression in primary human hepatocytes has recently been reported to be modulated by extracellular matrix proteins (50), although the exact mechanism behind this effect was not investigated.

In summary, several DNA elements are responsible for controlling the transcriptional activity of the CYP2D6 promoter, while the conserved DR1 element has the potential to modulate CYP2D6 expression in response to temporal, spatial, and hormonal signals via changes in the balance between positive and negative transcription factors.

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