0026-895X/95/040677-09$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 47:677–685 (1995).

MOL PHARM

MOLECULAR PHARMACOLOGY

Regulation of the Constitutive Expression of the Human *CYP1A2* Gene: *Cis* Elements and Their Interactions with Proteins

INJAE CHUNG[1] and EDWARD BRESNICK[2]

Department of Pharmacology and Toxicology, Dartmouth Medical School (I.C., E.B.), and Norris Cotton Cancer Center (E.B.), Hanover, New Hampshire 03755–3835

Received October 18, 1994; Accepted January 17, 1995

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

SUMMARY

Cytochrome P4501A2 (CYP1A2) is a member of the cytochrome P450 family that is involved in phase I drug metabolism in vertebrates. To understand how the constitutive expression of the human *CYP1A2* gene is regulated, its 5′ flanking region was analyzed. The promoter activity of a human *CYP1A2* gene sequence [base pairs (bp) −3203 to +58 bp] was measured in transiently transfected HepG2 cells using fusion constructs containing the luciferase reporter gene. Using 5′-end deletion analysis, two functionally important *cis* elements, i.e., a proximal 42-bp DNA from bp −72 to bp −31 and a distal 259-bp DNA from bp −2352 to bp −2094, were identified. The proximal sequence (bp −72 to −31) contained CCAAT and GC boxes, with which well characterized transcription factors such as nuclear factor-1/CCAAT transcription factor and simian virus 40 promoter factor-1 could interact. With regard to the 259-bp fragment (bp −2352 to bp −2094), gel mobility shift analyses with HepG2 nuclear lysates indicated high affinity, specific interactions of several *trans*-acting factors. Three protein binding sites within the 259-bp fragment were identified by DNase I footprinting analysis; these sites contained activator protein-1, nuclear factor-E1.7, and one-half hepatic nuclear factor-1 (HNF-1) binding consensus sequences. Only the region from bp −2124 to bp −2098, in which the HNF-1 binding site was located, was markedly protected by a HepG2 nuclear extract, compared with a MCF7 human breast cancer nuclear extract. These results suggested that the 259-bp DNA fragment contained positive regulator binding sites and HNF-1 could contribute to the liver-specific expression of human *CYP1A2*.

CYPs are a family of monooxygenases that catalyze the biotransformation of a variety of substances. This family of isozymes consists of >20 different hemoproteins, some of which are constitutive and others inducible (1, 2). *CYP1A2* is constitutively expressed in hepatic tissue and it is further induced by PAHs and dioxins in a liver-specific manner (3). The PAHs and dioxins also induce expression of another isozyme, CYP1A1, which is not restricted to liver.

CYP1A2 is responsible for the activation of a number of aromatic amines to carcinogenic forms. In particular, CYP1A2 activates the imidazolyl quinolines that have been isolated from food pyrolysates to mutagenic and carcinogenic products (4, 5). CYP1A2 is also involved in the conversion of aflatoxin B_1, a potent hepatocarcinogen in humans, to a less potent metabolite, aflatoxin M_1 (6). Thus, CYP1A2 can catalyze reactions that contribute positively to the carcinogenic potential of certain chemicals and are involved in the detoxification of other carcinogens. Moreover, interindividual variations in *CYP1A2* expression among populations have been reported by several groups, based upon the metabolism of the CYP1A2 substrate caffeine (7–9). Therefore, the level of CYP1A2, the major isozyme of the CYP1A subfamily in human liver (5, 10), may determine individual susceptibility to mutagenesis produced by certain substances. The elevated levels of CYP1A2 might also contribute to a human disease, porphyria cutanea tarda, that can result after exposure to TCDD (11), because CYP1A2 is known to mediate the oxidation of uroporphyrinogen to uroporphyrin (12), which is not a substrate for uroporphyrinogen decarboxylase, an enzyme involved in heme biosynthesis.

This research was supported in part by Grant ES03980 from the National Institutes of Health.

[1] Current address: Department of Hematology, Box 24, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

[2] Current address: Department of Pharmacology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655.

aspet

ABBREVIATIONS: CYP, cytochrome P450; BSA, bovine serum albumin; DRE, dioxin response element; FP, forward primer; 3MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; PCE, positive control element; PCR, polymerase chain reaction; PRA, protected region A; PRB, protected region B; PRC, protected region C; RP, reverse primer; RT, reverse transcription; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; T-318, template 318; T-185, template 185; T-101, template 101; T-231, template 231; TK, thymidine kinase; XRE, xenobiotic response element; AP, activator protein; SP-1, simian virus 40 promoter factor-1; NF, nuclear factor; HNF, hepatic nuclear factor; bp, base pair(s); kb, kilobase(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MOL PHARM

Several studies showed striking variability in CYP1A2 enzyme activity, protein, and mRNA within the human population (9, 13, 14). Recently, Schweikl *et al.* (14) showed that no coding changes existed in the *CYP1A2* gene of an individual who expressed extremely low levels of CYP1A2. The high variability of *CYP1A2* expression between individuals might result from induction by environmental factors or differential expression of the gene. Because of the significance of the functional role of CYP1A2, the mechanism responsible for the interindividual differences in the amounts of this enzyme is of interest.

Dioxins and some PAHs are known to induce *CYP1A1* through the aryl hydrocarbon receptor, which, in turn, acts as a transcription factor by binding to the XREs or DREs present in the 5′ flanking regions of *CYP1A1* (15). Although the occurrence of several XREs or DREs in the 5′ regulatory region of *CYP1A1* is quite common in many species (15), few have been found in the 5′ flanking regions of *CYP1A2*. Indeed, no such motifs have been found in the mouse and rat genes (16), although a XRE is purportedly located in the 5′ regulatory region of the human (17) and rabbit (18) genes. The 5′ regulatory regions derived from the human, mouse, and rabbit genes have been tested in transiently transfected cells, to determine the presence of sequences that are responsible for enhanced transcription after exposure to PAHs. The 5′ regulatory region of the mouse and rabbit genes did not respond to TCDD exposure in hepatoma cells. Elevated reporter gene expression was noted with human 5′ regulatory region-fusion gene constructs after treatment of HepG2 cells with 3MC or TCDD (17–20); however, the same regulatory region of human *CYP1A2* failed to respond to the inducers in stably transfected cells (19). Recently, two XRE-like sequences, termed X1 and X2, that are responsible for 3MC-mediated enhancement of human *CYP1A2* promoter activity in HepG2 cells have been reported (20). No basal transcription element, negative regulatory element, or glucocorticoid response element that could regulate the constitutive expression of *CYP1A2* has been identified.

The lack of an established cell line expressing *CYP1A2* has hampered studies of its regulation. However, the RT-PCR technique has made possible the detection of low levels of CYP1A2 mRNA in HepG2 cells after extensive 3MC treatment (21). Furthermore, by improved RT-PCR, endogenous as well as induced levels of CYP1A2 mRNA have been demonstrated in HepG2 cells after a single 3MC treatment (22).

The present studies were designed to define *cis*-acting elements that could regulate constitutive expression of the human *CYP1A2*. A series of transient transfection experiments with fusion gene constructs that contained the luciferase gene as a reporter and various lengths of the 5′ flanking region of human *CYP1A2* as a promoter were carried out to determine the presence of any regulatory sequences. To assess the occurrence of DNA-protein interactions, gel mobility shift assays and DNase I footprinting analyses were also conducted.

Experimental Procedures

Cloning of *CYP1A2* genomic DNA. A human genomic library generated from lymphocyte DNA and cloned into the *Bam*HI site of λ phage EMBL3 was purchased from Clontech (Palo Alto, CA) and was screened by plaque hybridization. The probe was a ^{32}P-labeled, 206-bp, PCR-generated DNA that represented the sequence from bp −2666 to bp −2461 of the 5′ flanking region of human *CYP1A2* (17). FP and RP represent the FP derived from the sense strand and the RP derived from the antisense strand, respectively. The FP sequence for the 206-bp probe was 5′-AGTCTGTCATACAGGAGAAT-3′, and the RP was 5′-GGATGTTGACCTTGATCACC-3′. A genomic clone, H1A2–4-10, was isolated and compared with that reported previously (17) by restriction endonuclease mapping, Southern blot analysis, and partial sequencing.

Plasmid DNA constructs. Human *CYP1A2*-luciferase fusion gene plasmids were constructed as follows. A 3261-bp *Kpn*I fragment of *CYP1A2*, from bp −3203 to bp +58, relative to the transcription start site, was isolated from H1A2–4-10 and subcloned into the *Kpn*I site of the luciferase expression vector pXP1 (23), to generate pICluc. A series of 5′-end unidirectional deletions from the 3261-bp fragment were also prepared. The 3261-bp *Kpn*I fragment from *CYP1A2* (see above) was subcloned into pUC19, followed by *Xba*I digestion, filling in with α-phosphorothiodeoxynucleoside triphosphates by Klenow polymerase, *Sma*I digestion, timed exonuclease III, S1 nuclease, and T4 DNA ligase digestion, and sequencing with a reverse sequencing primer (no. 1201; New England Biolabs, Beverly, MA). *Kpn*I/*Hin*dIII fragments of different lengths were cloned back into the *Kpn*I/*Hin*dIII site of pXP2 (48). To generate 259tkluc, the 259-bp DNA from bp −2352 to bp −2094 was prepared by PCR. The FP and RP for the 259-bp DNA fragment were designed to have *Bam*HI and *Hin*dIII restriction endonuclease recognition sequences, respectively, at the 5′ ends, in addition to the DNA sequence from the *CYP1A2* gene. The FP for the 259-bp DNA fragment was from bp −2352 to bp −2341 (5′-CGGGATCCGAGAAGAAAAAC-3′) and the RP was from bp −2094 to bp −2114 (5′-CCAAGCTTCTGTCTGTCTGTCTCTCTAA-3′). The *Bam*HI- and *Hin*dIII-digested 259-bp DNA fragment was subcloned into the *Bam*HI/*Hin*dIII sites of pT81luc vector (23) to generate 259tkluc. The correct orientation of all constructs was verified by restriction endonuclease mapping and sequence analysis.

Cell culture. Human hepatoma HepG2 cells were maintained in a humidified atmosphere of 5% CO_2/95% O_2 at 37°, in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 1 μg/ml gentamycin.

Transfection and reporter gene assays. Transient DNA transfections were performed by the calcium phosphate precipitation method. Cells were plated on 6-cm dishes at an initial concentration of 0.3×10^6 cells/dish, and the medium was changed 20 hr later. Four hours later the DNA, which had been co-precipitated with calcium phosphate, was introduced into the cells. Each dish received 10 μg of test plasmid pSluc (23) (as a positive control) or pXP2 and 2 μg of the β-galactosidase expression vector pCH110 (Pharmacia Biotechnology, Piscataway, NJ). The latter served as a means for normalizing transfection efficiency. The cells that were exposed to the calcium phosphate-DNA precipitate for 24 hr were subjected to a 15% glycerol shock for 2 min. After an additional 48 hr, the cells were harvested and assayed for the reporter enzyme activities. The luciferase (24) and β-galactosidase (25) assays were performed as described. All plasmids were prepared by equilibrium sedimentation in CsCl gradients. All transfection experiments were performed a minimum of three times, and at least two different preparations of plasmid DNA were tested. Within each experiment, each construct was studied in triplicate. To correct for differences in transfection efficiencies between dishes within a given experiment, the luciferase activities in the cell extracts were normalized to the β-galactosidase activity and protein concentration.

Preparation of DNA templates for gel mobility shift assays and DNase I footprinting analysis. All template DNAs were prepared by PCR technology with appropriate primers. The primer sets were as follows. In a few instances, a part of the vector plasmid DNA sequence was used. For T-318, the primers were 5′-CCAAGCTTGCATGCCTGCA-3′ (FP) and 5′-CGGAATTCTAGAAAATGGACTCTTTC-3′ (RP). For T-185, the primers were 5′-CGGGATCCGAGAAGAAAAAC-3′ (FP) and 5′-CCAAGCTTCCC-

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

ATTGGATAT-3′ (RP). For T-101, the primers were 5′-CGGGATC-CTGGAACAGGAAAAGGACATT-3′ (FP) and 5′-CCAAGCTTCTG-TCTGTCTGTCTCTCTAA-3′ (RP). T-231 represented the larger fragment from *Sca*I-digested T-318. The DNA sequences of *CYP1A2* encompassed by the various templates were as follows: T-318. bp −2362 to −2069; T-185, bp −2352 to −2186; T-101, bp −2185 to −2094; T-231, bp −2294 to −2069.

Preparation of nuclear extracts. The nuclear extracts of HepG2 and MCF7 cells were prepared from adherent tissue culture cells by the method of Shapiro *et al.* (26), with modifications. All procedures were performed at 4°. The isolated nuclei were resuspended in a minimal volume (approximately 2 ml/1 × 10^9 cells) of nuclear resuspension buffer (20 mM HEPES, 20% glycerol, 600 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.0 μg/ml pepstatin, 0.5 μg/ml leupeptin). The nuclear suspension was homogenized once with a loose Dounce pestle, placed on ice for 30 min with stirring, and centrifuged at 18,000 rpm for 30 min at 4°, using a Beckman JA 20 rotor. The nuclear supernatant was saved and dialyzed twice, for 60 min each time, against 50 volumes of dialysis buffer (1× buffer D) (20 mM HEPES, pH 7.8, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.0 μg/ml pepstatin A, 0.5 μg/ml leupeptin). The dialyzed nuclear lysate was centrifuged at 18,000 rpm for 20 min at 4°. The clear supernatant (in small aliquots) was quick-frozen in liquid nitrogen and stored at −80°.

Gel mobility shift assays. The specific binding of proteins to the various DNA sequences of the 5′ flanking region of *CYP1A2* was determined by gel mobility shift assays (27). The binding reaction mixtures contained 1.5–1.8 fmol (representing approximately 20,000 cpm) of ^{32}P-labeled probe, nuclear extract (1–3 μg of protein), poly(dI·dC) at 10,000- or 20,000-fold mass excess (compared with the probe), and buffer D, in a total volume of 12.5 μl. To demonstrate the sequence specificity of the protein-DNA complex, an additional unlabeled DNA was included in some of the binding reaction mixtures. After a 20-min incubation at room temperature, the reaction mixtures were fractionated by electrophoresis through a 6% nondenaturing polyacrylamide gel, and the gels were dried *in vacuo* and autoradiographed.

DNase I footprinting analysis. DNase I footprinting analysis was conducted according to the method of Galas and Schmitz (28). The reaction mixture contained 2% polyvinyl alcohol, 0.8 mg/ml BSA, 0.5× buffer D, 5 fmol of ^{32}P-labeled probe, and various amounts of the nuclear extracts, in a total volume of 50 μl. The reaction mixtures were incubated on ice for 1 hr, and the DNA was digested for 1 min with 8–500 ng of DNase I (DOFF; Worthington Biochemicals, Freehold, NJ), in the presence of 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. The reactions were then terminated by the addition of 90 μl of DNase I stop buffer (20 mM EDTA, pH 8.0, 1% sodium dodecyl sulfate, 0.2 M NaCl, 250 μg/ml yeast total RNA). The reaction mixture was extracted with phenol-chloroform, and the samples were electrophoresed on 6–8% polyacrylamide-7.8 M urea sequencing gels, along with chemical sequencing tracks.

Results

Isolation of 5′ flanking regions of *CYP1A2*. A human genomic library was screened for 5′ flanking regions of *CYP1A2*, using a 206-bp DNA probe (bp −2666 to −2460). A genomic clone was isolated by plaque hybridization and its identity as a portion of *CYP1A2* was verified by restriction enzyme mapping and Southern blot analysis (data not shown). This genomic clone included 8 kb of the 5′ flanking region, 7.65 kb of the region containing seven exons and six introns, and some 3′ flanking region. A 3.2-kb *Kpn*I fragment was size-selected by agarose gel electrophoresis and was cloned into pXP1, to generate the parent chimeric construct pICluc (Fig. 1). This clone contained 5′ sequences of *CYP1A2* from bp −3203 to bp +58, with respect to the transcription start site.

Constitutive reporter gene activity as affected by various deletion mutations. Sequence analysis of the human *CYP1A2* 5′ flanking region revealed regions of homology to characterized *cis*-acting elements such as AP-1 and AP-2 and to some elements that endowed liver-specific expression (29), i.e., cAMP response element-binding protein consensus sequence [T(T/G)NNG(T/C)AA(T/G)] at bp −1632 and HNF-5 consensus sequences [(A/G)CAAA(T/C)A] at bp −1415 and bp −1185. Because degenerate DNA consensus sequences can still bind *trans*-acting factors, regions that had only one mismatched nucleotide were also searched. For example, *CYP1A2* contains 13 regions distributed throughout the 5′ flanking region (up to bp −3202) that have only one mismatched nucleotide, compared with liver-specific cAMP response element-binding protein consensus sequences ATT-NNGNAAT (29) or TCNTACTC (30). To find the major elements that might regulate the constitutive expression of *CYP1A2*, 5′-end deletion mutant constructs were prepared (Fig. 1). The results of transient transfection experiments with those deletion constructs in HepG2 cells are shown in Fig. 2. A deletion of up to 850 bp from the 5′ end of the parent vector pICluc had little effect upon promoter activity in HepG2 cells. However, when a 259-bp fragment from bp −2352 to bp −2094 was further deleted, a statistically significant ($p < 0.0005$) 50% diminution in promoter activity was observed. Consequently, these experiments indicated that the 259-bp DNA from bp −2352 to bp −2094 of *CYP1A2* was necessary for full promoter activity. Interestingly, the construct that contained *CYP1A2* sequences only up to bp −72 from the transcription start site, i.e., pICluc∆3130, re-

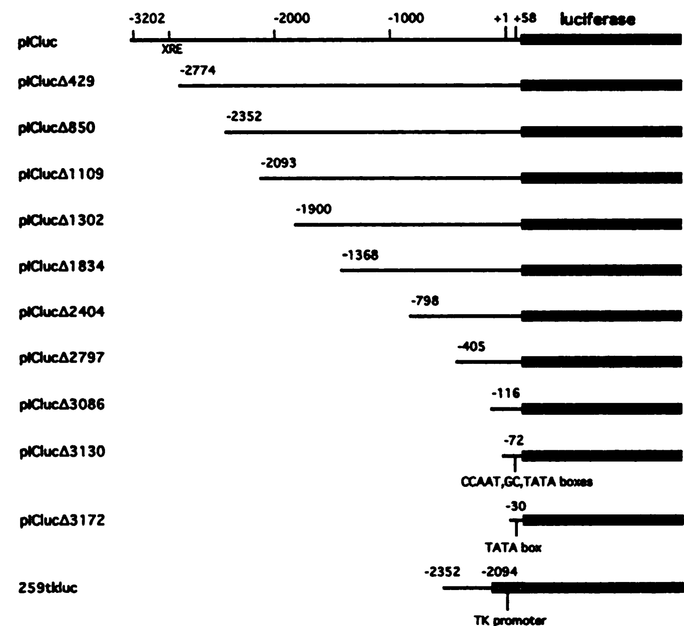

Fig. 1. Schematic presentation of the human *CYP1A2* plasmid constructs. Constructs are presented in the sense orientation, relative to the reporter genes. *Thin lines*, *CYP1A2* promoter; *thick lines*, reporter gene (luciferase). *Numbers*, positions of the nucleotides relative to the transcription start site. The 5′ end of the plasmid is on the *left*. ∆, deletion mutants; *numbers after ∆ symbol*, extent of the deletion (in bp), e.g., pICluc∆850 indicates a deletion of 850 bp.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

tained 70% of the full length promoter activity in HepG2 cells. Located within this region are a TATA sequence for transcription factor IID binding, a SP-1 binding site (GC box), and a CCAAT box. An additional deletion to bp −30 (pICluc∆3172) almost completely eliminated reporter gene activity. These results indicate that the 42-bp sequence from bp −72 to bp −31 is essential for constitutive expression.

Because the first discovered property of enhancers was their lack of promoter specificity, the 259-bp PCE was tested for its ability to enhance transcription from a heterologous promoter. To address this question, the 259-bp (bp −2352 to −2094) DNA was ligated to the upstream region of the TK promoter in the pT81luc vector plasmid to generate a chimeric plasmid, 259tkluc. The ability of the 259-bp PCE to activate TK promoter activity was assessed in HepG2 cells. As shown in Fig. 3, the 259-bp DNA fragment was able to enhance transcription by 7-fold in HepG2 cells, compared with vector (pT81luc)-transfected cells. These results showed that this element displayed at least one characteristic of an enhancer.

PCE-protein interactions determined by gel mobility shift assays. Gel mobility shift assays were conducted to determine whether the 259-bp PCE from bp −2352 to bp −2094 could interact with proteins in HepG2 nuclear extracts. Three different DNA templates used in these studies

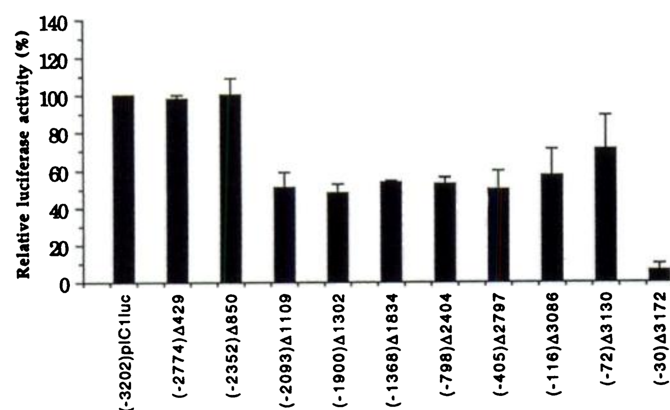

Fig. 2. Effects of progressive deletion of DNA sequences in the 5′ flanking region of *CYP1A2* on the expression of luciferase in HepG2 cells. *Numbers in parentheses*, 5′ end (in bp) of the particular construct; *numbers after* ∆ *symbol*, numbers of deleted bases. The luciferase activities of cells transfected with the constructs are given relative to that of the parent construct, pICluc, which is expressed as 100%. The luciferase activities are given as means ± standard deviations of three to five experiments.

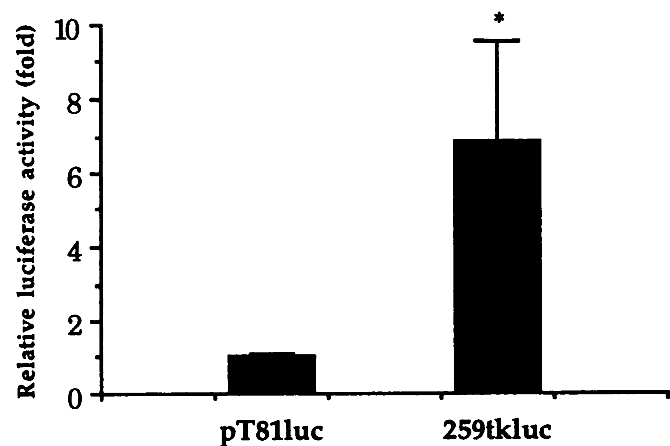

Fig. 3. Enhancement of luciferase activities from a heterologous promoter by the 259-bp PCE. The enhanced luciferase activities are given relative to the activity of the corresponding enhancerless control plasmid pT81luc (defined as 1). The 259tkluc construct contains a 259-bp segment (bp −2352 to −2094) ligated to the upstream region of a minimal TK promoter in pT81luc, in the sense orientation. *, Statistically significant fold difference ($p < 0.0005$) in promoter activity between the two constructs.

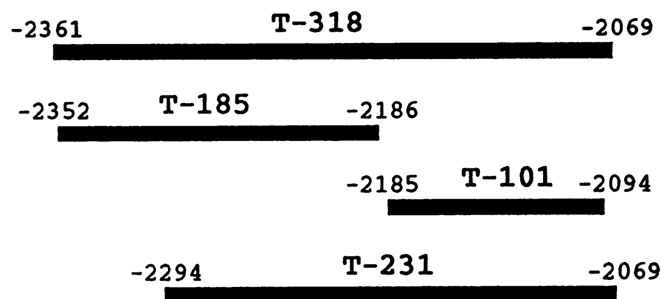

Fig. 4. Schematic presentation of DNA templates for the gel mobility shift assay and DNase I footprinting analysis. All template DNAs were prepared by PCR with appropriate primers. The DNA sequences of *CYP1A2* encompassed by the various templates were as follows: T-318, bp −2362 to −2069; T-185, bp −2352 to −2186; T-101, bp −2185 to −2094; T-231, bp −2294 to −2069.

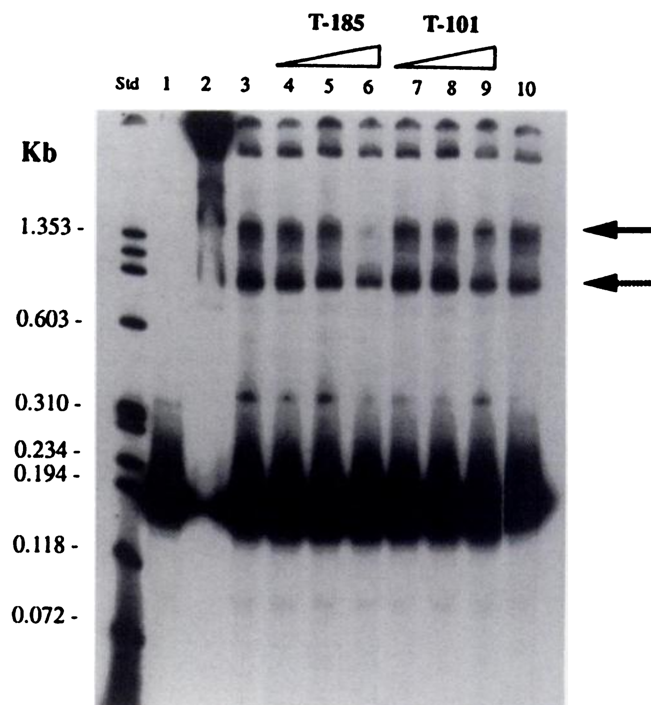

Fig. 5. *Trans* factors in HepG2 nuclear extracts that interact with T-185 and T-101. HepG2 nuclear extract (3 μg of protein) was incubated with labeled T-185 and interactions were determined by gel mobility shift assays. *Std*, standards. *Lane 1*, probe only; *lane 2*, probe and protein; *lane 3*, same as *lane 2* but with the addition of a 20,000-fold mass excess of poly(dI·dC); *lanes 4*, *5*, and *6*, same as *lane 3* but with the addition of a 2-, 20-, or 200-fold molar excess, respectively, of unlabeled T-185; *lanes 7*, *8*, and *9*, same as *lane 3* but with the addition of a 2-, 20-, or 200-fold molar excess, respectively, of T-101; *lane 10*, same as *lane 3* but with the addition of a 200-fold molar excess of a 104-bp DNA fragment from the rat *CYP1A1* promoter region (negative control). *Solid* and *dashed arrows*, two retarded regions.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

are shown in Fig. 4, i.e., T-318, which contained 318 bp, including all of the 259-bp sequence that had been demonstrated to be important in the promoter-reporter fusion gene experiments (bp −2362 to −2069); T-185, which contained the sequence from bp −2352 to bp −2186; and T-101, which included the DNA sequence from bp −2185 to bp −2094. Gel mobility shift assays with T-318 suggested that the HepG2 nuclear extract contained proteins that could bind with high affinity and specificity to the 259-bp PCE (data not shown).

To localize the protein binding sites within the 259-bp DNA sequence, gel mobility shift assays were conducted with T-185 and T-101. These results are presented in Figs. 5 and 6. Several major shifted bands were observed upon incubation of T-185 with HepG2 nuclear extracts (Fig. 5, *lane 3*). A more slowly migrating band was almost competed out by a 200-fold molar excess of unlabeled T-185 (Fig. 5, *lane 6*, *solid arrow*). The intensity of a faster migrating band was also diminished, but to a lesser extent (Fig. 5, *lane 6*, *dashed arrow*). None of the other bands was consistently diminished by a 200-fold excess of T-185. A 200-fold molar excess of unlabeled T-101 partially competed out the more slowly migrating T-185 band but did not affect the intensity of the faster migrating band (Fig. 5, *lane 9*). No change in the intensity of the shifted areas was observed with a 200-fold molar excess of a 104-bp DNA fragment from the rat *CYP1A1* promoter (Fig. 5, *lane 10*).

The interaction between HepG2 nuclear proteins and T-101 is shown in Fig. 6. Two major shifted regions were observed (Fig. 6, *lane 3*). A diminution in the intensity of this major shifted band was seen after the addition of unlabeled T101, with the faster moving band exhibiting the greater reduction (Fig. 6, *lanes 4* and *5*). The addition of an excess of unlabeled T-185 in the reaction also significantly reduced the signal of the shifted bands (Fig. 6, *lanes 7-9*). Addition of the negative control sequence from the *CYP1A1* promoter was

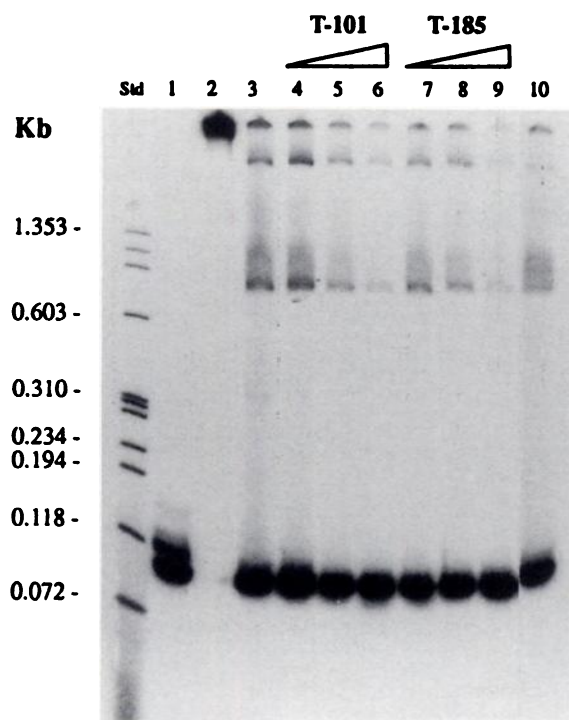

Fig. 6. Interaction between HepG2 nuclear proteins and T-101. Labeled T-101 was incubated with 3 μg of HepG2 nuclear protein as indicated in the text. *Std*, standards. *Lane 1*, probe alone; *lane 2*, probe and HepG2 nuclear extract; *lane 3*, same as *lane 2* except for the addition of a 20,000-fold mass excess of poly(dI·dC); *lanes 4*, *5*, and *6*, same as *lane 3* but with the addition of a 2-, 20-, or 200-fold molar excess, respectively, of unlabeled T-101; *lanes 7*, *8*, and *9*, same as *lane 3* but with the addition of a 2-, 20-, or 200-fold molar excess, respectively, of T-185; *lane 10*, same as *lane 3* but with the addition of a 200-fold molar excess of a 104-bp DNA fragment from the rat *CYP1A1* promoter (a negative control).

Fig. 7. DNase I footprinting analysis of T-318 with HepG2 nuclear proteins. The 5′ strand of T-318 was labeled for probe in the DNase I footprinting assay. *A+G* and *T+C*, chemical sequencing tracks. *Lanes 1-3*, control reactions in which 20 μg of BSA were incubated with the probe before digestion with 10, 20, or 30 ng of DNase I, respectively; *lanes 4-6*, incubation of the probe with 5 μg of HepG2 nuclear extract before digestion with 50, 100, or 150 ng of DNase I, respectively. *Lines on the right*, positions of the bound regions of *CYP1A2*; *thick lines*, marked interaction; *dashed line*, partial interaction.

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

aspet

also without effect upon the signal in the shifted region (Fig. 6, *lane 10*). The larger fragment, i.e., T-185, was able to interfere with the formation of T-101 protein(s) but the reverse did not occur. These results suggest but do not prove that T-185 and T-101 may interact through a protein-protein interaction.

In summary, the larger fragment, i.e., T-185, was able to interfere with the formation of T-101 proteins. Some of the proteins interacting with T-185 could also bind to T-101 with lower affinity, compared with T-185, whereas the other proteins recognized only T-185.

DNase I footprinting analysis of the distal 259-bp PCE. The nucleotide sequence of the distal 259-bp PCE that binds to HepG2 nuclear proteins was determined by *in vitro* DNase I footprinting analysis (Fig. 7). Three regions were protected from DNase I digestion when T-318, with the 5′ strand labeled, was incubated with the HepG2 nuclear extract. The protected areas were bp −2282 to −2243, bp −2218 to −2187, and bp −2124 to −2107. Because the large size of the probe interfered with the resolution of some of the bands, the smaller T-185 and T-101 were also subjected to DNase I footprinting analysis. MCF7 nuclear extracts were used to determine whether any protected regions were hepatocyte specific. A protein concentration-dependent footprint (bp −2218 to −2187) was observed upon incubation of the 5′ strand-labeled T-185 with both HepG2 and MCF7 nuclear extracts (Fig. 8A). Although each extract gave the same pattern of protection (bp −2124 to −2098) with labeled T-101, the nuclear proteins appeared more abundant in the hepatoma cells (Fig. 8B). One half of a HNF-1 consensus sequence (GTTAATNATTAAC) (31) is located within this region, at bp −2115 (GTTAAT).

Similar experiments were carried out with the T-231 DNA (see Fig. 4), i.e., bp −2294 to −2069, with the 3′ strand labeled. The results are shown in Fig. 9. Two protected regions were detected, between bp −2283 and bp −2155 and between bp −2123 and bp −2093. The bp −2123 to −2093 region was found exclusively in the reactions that contained the HepG2 nuclear extract. It is also in this region that the one-half HNF-1 consensus sequence is located.

The DNase I footprinting studies are summarized in Fig. 10. PRA covered 41 bp, from bp −2283 to bp −2243, PRB from bp −2218 to bp −2187, and PRC from bp −2124 to bp −2098. PRA and PRC were detected in both DNA strands, whereas PRB was observed only with the 5′ strand. Whereas the 3′ strand of PRC was detected exclusively with the HepG2 nuclear extract, appearing to be liver specific, the 5′ strand was protected by extracts from both the hepatoma and mammary carcinoma cells. Regions of the 259-bp PCE homologous to some known consensus sequences are also indicated in Fig. 10. An AP-1 binding sequence (TGANTNA) (32) and a NF-E1.7 binding sequence (TCATCAC) (33) were found in the partially protected region of PRB. The most conserved

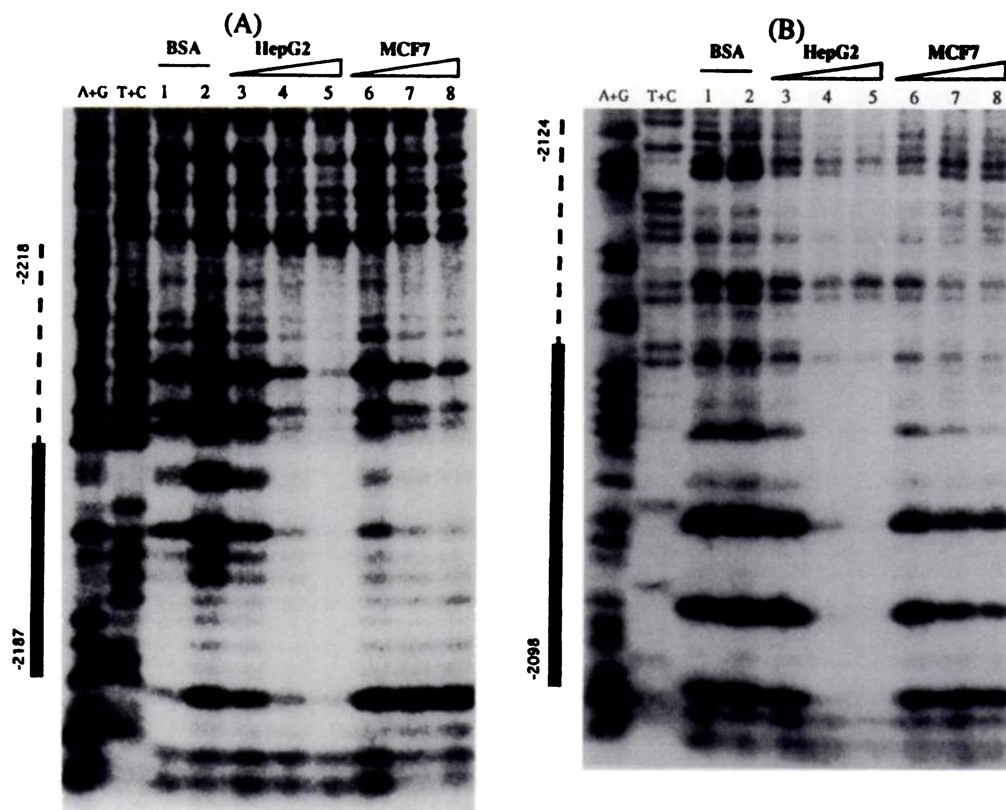

Fig. 8. DNase I footprinting analysis, with T-185 (A) and T-101 (B), of nuclear proteins from HepG2 or MCF7 cells. The 5′ strand of the DNA fragment was labeled. *A+G* and *T+C*, chemical sequencing tracks. *Lanes 1* and *2*, control reactions in which 20 μg of BSA were incubated with the probe before digestion with 8 or 25 ng of DNase I, respectively; *lanes 3-5*, incubation of the probe with 5, 25, or 50 μg of HepG2 nuclear extract before digestion with 40, 200, or 400 ng of DNase I, respectively; *lanes 6-8*, incubation of the probe with 5, 25, or 50 μg of MCF7 nuclear extract before digestion with 40, 200, or 400 ng of DNase I. *Dashed lines*, partially protected regions; *thick lines*, completely protected regions. *Numbers adjacent to the autoradiograph*, positions of protection.

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOLECULAR PHARMACOLOGY

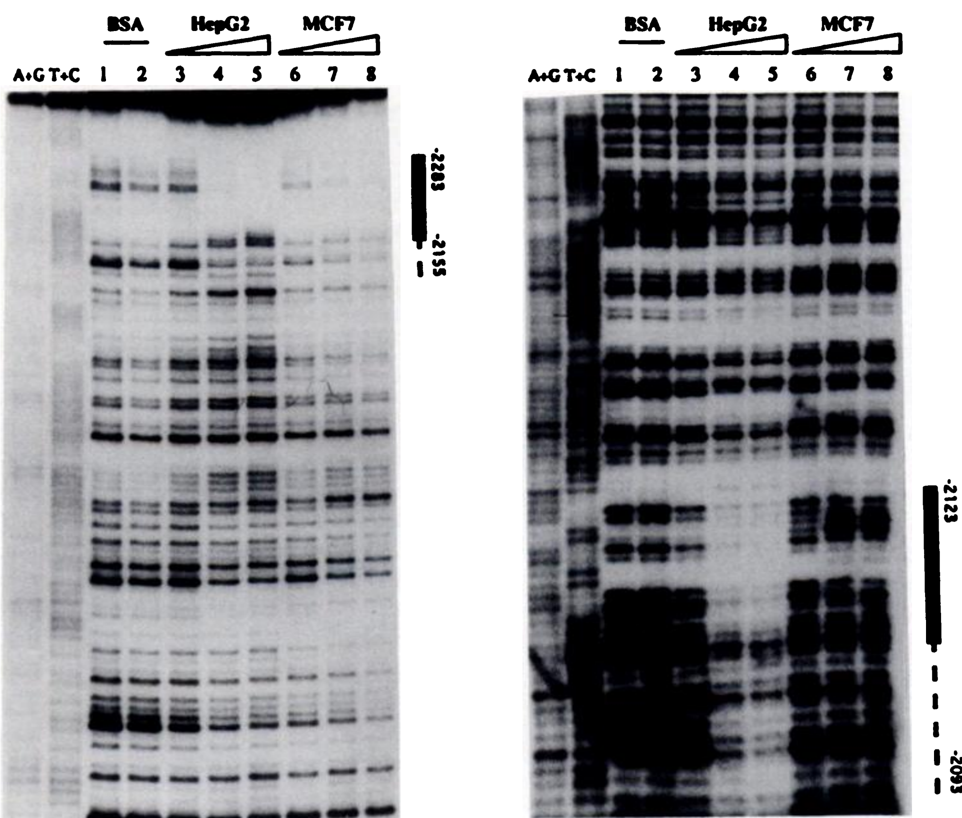

Fig. 9. DNase I footprinting analysis with T-231 and HepG2 or MCF7 nuclear extracts. The 3′ strand of T-231 (bp −2294 to −2069) was labeled. *A+G* and *T+C*, chemical sequencing tracks. *Lanes 1* and *2*, control reactions in which 20 μg BSA were incubated with the probe before digestion with 8 or 16 ng of DNase I, respectively; *lanes 3-5*, incubation of the probe with 5, 25, or 50 μg of HepG2 nuclear extract before digestion with 40, 250, or 500 ng of DNase I, respectively; *lanes 6-8*, incubation of the probe with 5, 25, or 50 μg of MCF7 nuclear extract before digestion with 40, 250, or 500 ng of DNase I, respectively. *Thick lines*, completely protected regions; *dashed lines*, partially protected regions.

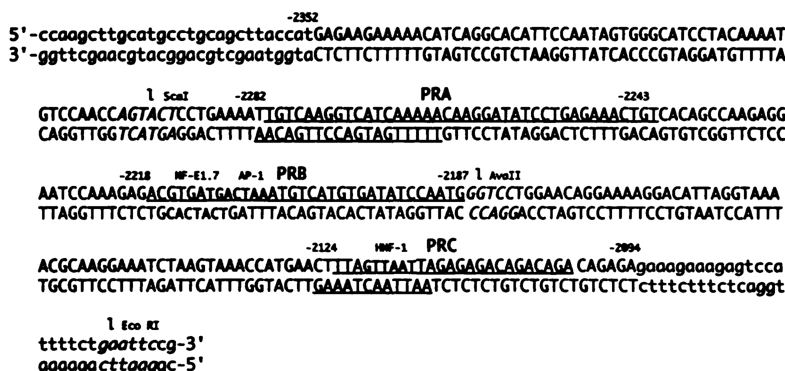

Fig. 10. Nucleotide sequence of the 259-bp PCE (bp −2352 to −2094). The protected regions demonstrated by the DNase I footprinting analysis with the HepG2 nuclear extract are *underlined*. The positions of AP-1, NF-E1.7, and one half of the HNF-1 consensus sequences are also shown.

part of the HNF-1 consensus sequence, i.e., GTTAAT, is located within the PRC.

Discussion

Systematic mutational analysis has revealed that many genes exhibit a combination of positive and negative *cis* elements, whose arrangements confer unique spatial and temporal programs of transcription. An example of such a gene is *CYP1A1*, which is regulated by PAHs through their interaction, via receptors, with *cis*-acting elements of responsive genes (e.g., the XRE or DRE); this contributes to the induction of *CYP1A1* by dioxins (15). Besides XREs and DREs, several *cis* elements were found to regulate the constitutive expression of *CYP1A1*. Studies from this laboratory showed that HepG2 cells retain constitutive expression of CYP1A2 and are inducible by 3MC, as demonstrated by RT-PCR (22). Those observations provided the basis for the examination of the 5′ flanking region of human *CYP1A2* in HepG2 cells.

The present study identified two functionally important *cis* elements in the 5′ flanking region that regulate the constitutive expression of human CYP1A2, i.e., the proximal 42-bp sequence and the distal 259-bp DNA (Fig. 2). The 42-bp sequence from bp −72 to bp −31 is crucial for the constitutive

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

promoter activity. This proximal sequence contains CCAAT and GC boxes, with which well characterized transcription factors such as NF-1/CCAAT transcription factor (34, 35) and SP-1 (36) can interact. These sites of interaction may be essential for *CYP1A2* promoter activity, as shown with many other eukaryotic promoters. An enhancer-like 259-bp DNA spanning bp −2352 to bp −2094 was necessary for full promoter activity of *CYP1A2*. This 259-bp PCE was also operative in the heterologous promoter (Fig. 3). Quattrochi *et al.* (20) demonstrated that two *cis* elements, X1 and X2, are responsible for 3MC-mediated enhancement of *CYP1A2* promoter activity. Interestingly, X2, spanning bp −2160 to bp −2142, is in the distal 259-bp PCE and X1, spanning bp −2505 to bp −2487, is located close to the 259-bp PCE.

Gel mobility shift assays indicated that HepG2 nuclear extracts contain *trans*-acting factors that bind to the 259-bp PCE with high affinity and specificity. In addition, the results from the cross-competition experiments suggested the occurrence of protein-protein interactions within this 259-bp segment (Figs. 5 and 6). There could be many explanations for these observations. One could speculate that the more slowly migrating band of T-185 consists of at least two proteins, called A and B, and only protein A is directly bound to T-185. The faster migrating T-185 band could be due to another protein, C, which interacts with less specificity than protein A. Protein B would complex with T-101 and would also indirectly interact with T-185 through protein A. T-185-bound protein A would have greater affinity for protein B than would T-101. This model is based on the possibility that interplay occurs between T-185 and T-101 through a protein-protein interaction. Alternatively, the same protein that binds T-101 could directly interact with T-185. In this case, the protein would have greater affinity for T-185 than for T-101. The interactions among proteins that bind to T-185 and T-101 are highly likely to be specific, because the addition of a 104-bp DNA from the rat *CYP1A1* promoter region did not affect any of those interactions.

HNF-1 has been reported to be necessary for the liver specificity of other genes. Comparison of the sequences of different HNF-1 binding sites yielded a consensus sequence, GTTAATNNNATTAAC, in which one moiety of the sequence is more conserved than the other (31, 37). One protected region of the 259-bp DNA fragment, PRC (bp −2124 to −2098), contained the sequence GTTAAT, which is the most conserved part of the HNF-1 binding site. Protein bound to the PRC sequence was more abundant in the nuclear extracts prepared from HepG2 cells, compared with MCF7 cells (Figs. 8B and 9). Hence, the PRC could be one of the *cis* elements that contribute to the liver-specific expression of the *CYP1A2* gene, because tissue-specific gene expression occurs through the interactions of several *cis*-acting elements (organized in various combinations) with a set of transcription factors, some of which are tissue specific and some of which are ubiquitous (38).

In this study, the 5′ flanking region of the human *CYP1A2* gene was analyzed in the immortalized HepG2 cell line, which expresses CYP1A2 mRNA at a low level detectable by RT-PCR (22). This, in turn, suggests that HepG2 cells might express some of the positive regulators, including those interacting with the 259-bp PCE, at very reduced levels. Hence, the effects of those transcription factors might have been underestimated in the present study. It would be interesting to carry out transfection experiments in a system in which more abundant mRNA, protein, and enzyme activity for CYP1A2 are measured, to determine the relevance of the observations made in HepG2 cells. Those systems would be human or rodent primary cell cultures or human hepatocyte lines (e.g., see Ref. 39), which have been shown to retain CYP1A2 activity and be inducible by PAHs.

It has been generally observed that the 5′ flanking region of liver-specific genes can regulate reporter gene activity in hepatoma cells but not in cells that are derived from nonhepatic sources. Therefore, hepatic and nonhepatic cells have proven useful for defining the existence of *cis* elements that can direct liver specificity of genes. Because the *CYP1A2* gene is also constitutively expressed in a liver-specific manner, we performed transfection experiments in MCF7 cells to determine whether such a sequence existed in the *CYP1A2* 5′ flanking region. Although much less reporter gene activity was measured from pIcluc-transfected MCF7 cells, compared with HepG2 cells, this was largely due to the inefficient transfection of DNA in the former cell line, as judged by expression of the co-transfected β-galactosidase gene, which was under the control of the simian virus 40 early promoter.[3] Alternatively, the different levels of β-galactosidase activity might result from the different levels of simian virus 40 promoter activity between cell lines, which makes it difficult to normalize transfection efficiency. It was also problematical to use cell lines of nonhepatic origin to study the basis of liver specificity of *CYP1A2* gene expression without knowledge of the relevant levels of CYP1A2 mRNA, compared with those in HepG2 cells (which express CYP1A2 mRNA at low levels).

We envision that the 259-bp PCE-bound transcription factors enhance transcription of human *CYP1A2* by stabilizing the basic transcription assembly via coactivators that are associated with the TATA binding protein (40). Additional studies are required to characterize the transcription factors that are responsible for causing the 259-bp DNA fragment to act as a PCE. We are currently examining the functional significance of the three regions that have been shown to be protein binding sites.

References

1. Waterman, M. R., and R. W. Estabrook. The induction of microsomal electron transport enzymes. *Mol. Cell. Biochem.* **53/54:**267–278 (1983).
2. Nelson, D. R., T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. W. Nebert. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12:**1–51 (1993).
3. Okey, A. B. Enzyme induction in the cytochrome P-450 system. *Pharmacol. Ther.* **45:**241–298 (1990).
4. Battula, N., G. K. Townsend, E. G. Snyderwine, H. A. Schut, and S. S. Thorgeirsson. Preferential activation and genotoxicity of food derived heterocyclic amine mutagens by recombinant cytochrome P4501A2. *Prog. Clin. Biol. Res.* **347:**55–69 (1990).
5. McManus, M. E., W. M. Burgess, M. E. Veronese, A. Huggett, L. C. Quattrochi, and R. H. Tukey. Metabolism of 2-acetylaminofluorene and benzo(*a*)pyrene and activation of food-derived heterocyclic amine mutagens by human cytochromes P-450. *Cancer Res.* **50:**3367–3376 (1990).
6. Faletto, M. B., P. L. Koser, N. Battula, G. K. Townsend, A. E. Maccubbin, H. V. Gelboin, and H. L. Gurtoo. Cytochrome P_3-450 cDNA encodes aflatoxin B_1-4-hydroxylase. *J. Biol. Chem.* **263:**12187–12189 (1988).
7. Kadlubar, F. F., M. A. Butler, K. R. Kaderlik, H. C. Chou, and N. P. Lang. Polymorphisms for aromatic amine metabolism in humans: relevance for human carcinogenesis. *Environ. Health Perspect.* **98:**69–74 (1992).
8. Relling, M. V., J. S. Lin, G. D. Ayers, and W. E. Evans. Racial and gender differences in *N*-acetyltransferase, xanthine oxidase, and CYP1A2 activities. *Clin. Pharmacol. Ther.* **52:**643–658 (1992).

[3] I. Chung and E. Bresnick, unpublished observations.

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

aspet

MOL PHARM

9. Kalow, W., and B. K. Tang. Use of caffeine metabolite ratio to explore CYP1A2 and xanthine oxidase activities. *Clin. Pharmacol. Ther.* **50:**508–519 (1991).
10. Murray, B. P., R. J. Edwards, S. Murray, A. M. Singleton, D. S. Davies, and A. R. Boobis. Human hepatic CYP1A1 and CYP1A2 content, determined with specific anti-peptide antibodies, correlates with the mutagenic activation of PhIP. *Carcinogenesis (Lond.)* **14:**585–592 (1993).
11. Bleiberg, J., M. Wallen, R. Bradkin, and I. Applebaum. Industrially acquired porphyria. *Arch. Dermatol.* **89:**793–797 (1964).
12. Jacobs, J. M., P. R. Sinclair, W. J. Bement, R. W. Lambrecht, J. F. Sinclair, and J. A. Goldstein. Oxidation of uroporphyrinogen by methylcholanthrene-induced cytochrome P-450: essential role of cytochrome P-450d. *Biochem. J.* **258:**247–253 (1989).
13. Butler, M. A., N. P. Lang, J. F. Young, N. E. Caporaso, P. Vineis, R. B. Hayes, C. H. Teitel, J. P. Massengill, M. F. Lawsen, and F. F. Kadlubar. Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. *Pharmacogenetics* **2:**116–127 (1992).
14. Schweikl, H., J. A. Taylor, T. S. Kitareewan, P. Linko, D. Nagorney, and J. A. Goldstein. Expression of *CYP1A1* and *CYP1A2* genes in human liver. *Pharmacogenetics* **3:**239–249 (1993).
15. Nebert, D. W., and J. E. Jones. Regulation of the mammalian cytochrome P_1-450 (*CYP1A1*) gene. *Int. J. Biochem.* **21:**243–252 (1989).
16. Ikeya, K., A. K. Jaiswal, R. A. Owens, J. E. Jones, D. W. Nebert, and S. Kimura. Human *CYP1A2*: sequence, gene structure, comparison with the mouse and rat orthologous gene, and genetic differences in liver 1A2 mRNA concentrations. *Mol. Endocrinol.* **3:**1399–1408 (1989).
17. Quattrochi, L. C., and R. H. Tukey. The human cytochrome *CYP1A2* gene contains regulatory elements responsive to 3-methylcholanthrene. *Mol. Pharmacol.* **36:**66–71 (1989).
18. Strome, D. K., H. Postlind, and R. H. Tukey. Characterization of the rabbit *CYP1A1* and *CYP1A2* genes: developmental and dioxin-inducible expression of rabbit liver P4501A1 and P4501A2. *Arch. Biochem. Biophys.* **294:**707–716 (1992).
19. Postlind, H., T. P. Vu, R. H. Tukey, and L. C. Quattrochi. Response of human CYP1-luciferase plasmids to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* **118:**255–262 (1993).
20. Quattrochi, L. C., T. Vu, and R. H. Tukey. The human *CYP1A2* gene and induction by 3-methylcholanthrene. *J. Biol. Chem.* **269:**6949–6954 (1994).
21. Fukuda, Y., N. Ishida, T. Noguchi, A. Kappas, and S. Sassa. Interleukin-6 down-regulates the expression of transcripts encoding cytochrome P450 IA1, IA2 and IIIA3 in human hepatoma cells. *Biochem. Biophys. Res. Commun.* **184:**960–965 (1992).
22. Chung, I., and E. Bresnick. 3-Methylcholanthrene-mediated induction of cytochrome P4501A2 in human hepatoma HepG2 cells as quantified by the reverse transcription-polymerase chain reaction. *Arch. Biochem. Biophys.* **314:**75–81 (1994).
23. Nordeen, S. K. Luciferase reporter gene vectors for analysis of promoters and enhancers. *BioTechniques* **6:**454–457 (1988).
24. Brasier, A. R., J. E. Tate, and J. F. Habener. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *BioTechniques* **7:**1116–1122 (1989).
25. Norton, P. A., and J. M. Coffin. Bacterial β-galactosidase as a marker of Rous sarcoma virus gene expression and replication. *Mol. Cell. Biol.* **5:**281–290 (1985).
26. Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller. Laboratory methods: a high-efficiency HeLa cell nuclear transcription extract. *DNA* **7:**47–55 (1988).
27. Singh, H., R. Sen, D. Baltimore, and P. A. Sharp. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature (Lond.)* **319:**144–158 (1986).
28. Galas, D., and A. Schmitz. DNase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5:**3157–3170 (1978).
29. Fleming, R. E., and J. D. Gitlin. Structural and functional analysis of the 5′ flanking region of the rat ceruloplasmin gene. *J. Biol. Chem.* **267:**479–486 (1992).
30. Costa, R. H., D. R. Grayson, K. G. Xanthopoulos, and J. E. Darnell, Jr. A liver-specific DNA-binding protein recognizes multiple nucleotide sties in regulatory regions of transthyretin, α_1-antitrypsin, albumin, and simian virus 40 genes. *Proc. Natl. Acad. Sci. USA* **85:**3840–3844 (1988).
31. Mendel, D. B., and G. R. Crabtree. HNF-1, a member of a novel class of dimerizing homeodomain proteins. *J. Biol. Chem.* **266:**677–680 (1991).
32. Lee, W., P. J. Mitchell, and R. Tjian. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49:**741–752 (1987).
33. Wall, L., E. deBoer, and F. Grosveld. The human β-globin gene 3′ enhancer contains multiple binding sites for an erythroid-specific protein. *Genes Dev.* **2:**1089–1100 (1988).
34. Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian. A cellular DNA binding protein that activates eukaryotic transcription and DNA replication. *Cell* **48:**79–89 (1987).
35. Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. *Nature (Lond.)* **334:**218–224 (1988).
36. Kadonaga, J. T., K. A. Jones, and R. Tjian. Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci.* **11:**20–23 (1986).
37. Tronche, F., and M. Yaniv. HNF-1, a homeoprotein member of the hepatic transcription regulatory network. *Bioessays* **14:**579–587 (1992).
38. Maniatis, T., S. Goodbourn, and J. A. Fischer. Regulation of inducible and tissue-specific gene expression. *Science (Washington D. C.)* **236:**1237–1245 (1987).
39. Roberts, E. A., Z. W. Xie, S. Yang, and J. Lipa. Inducibility of enzyme activities associated with the cytochrome P-450 1A family, ethoxyresorufin *O*-deethylase, and methoxyresorufin *O*-demethylase in human hepatocyte lines derived from normal liver tissue. *Drug Metab. Dispos.* **21:**56–61 (1993).
40. Pugh, B. F., and R. Tjian. Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. *J. Biol. Chem.* **267:**679–682 (1992).

Send reprint requests to: Edward Bresnick, Office of Research, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655.

Downloaded from molpharm.aspetjournals.org at Zhejiang University on December 1, 2012

MOLECULAR PHARMACOLOGY

aspet