MOLECULAR MECHANISMS REGULATING HUMAN CYP4B1 LUNG-SELECTIVE EXPRESSION

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ABSTRACT:

Lung-selective cytochrome P450 expression is well recognized; however, little is known regarding regulatory mechanisms. To address this knowledge gap, transient expression of CYP4B1/luciferase constructs was used to identify a proximal, positively acting regulatory element, position −139 to −45, that functioned in all cells examined; a negatively acting element, position −457 to −216, that only functioned in HepG2 hepatoblastoma cells; and a distal, positively acting element, position −1087 to −1006, that functioned in A549 or BEAS-2B lung-derived cells but not HepG2 cells or 293 kidney-derived cells. Competitive electrophoretic mobility shift assays further localized specific A549, but not HepG2, nuclear protein binding to two sites within the distal element, CYP4B1 position −1052 to −1042 and −1026 to −1006. Several potential lung-selective transcription factor recognition sequences were identified within these elements. However, attempts to identify specific factor(s) were unsuccessful. In contrast, in vitro DNA/protein binding assays combined with transient expression and mutagenesis studies identified two functional Sephadex protein/Krüppel-like factor families of transcription factor sites within the proximal element (position −118 to −114 and position −77 to −73) that bound both Sephadex protein 1 (Sp1) and Sephadex protein 3 (Sp3) in vitro. Furthermore, Sp1-dependent synergistic regulation was observed in A549 cells involving the proximal and distal regulatory elements. Chromatin immunoprecipitation assays demonstrated binding of neither Sp1 nor Sp3 to the CYP4B1 proximal element in human liver tissue, whereas selective Sp1 binding was observed in human lung tissue. Thus, the composite findings are consistent with both the proximal Sp1 elements and the distal regulatory element acting to synergistically control CYP4B1 lung-selective expression.

The cytochrome P450-dependent monooxygenases consist of a large family of heme proteins that catalyze the oxidative metabolism of both endogenous and exogenous compounds, often facilitating their elimination from the organism. Depending on the substrate, however, these same reactions can lead to reactive intermediates and contribute to a pathological response (Guengerich, 1993; Nebert and Russell, 2002). Although the liver possesses the highest amount of cytochrome P450 metabolic activity, the lung represents a primary portal to the body and as such, is an important exposure site to many inhaled environmental protoxicants and procarcinogens (Dahl and Lewis, 1993). As one might expect, lung tissue also can activate several procarcinogens and protoxicants, contributing to the etiology of cancer and other toxic outcomes (Ding and Kaminsky, 2003). Gaining a better understanding of mechanisms controlling the selective expression of pulmonary cytochrome P450 enzymes will be critical to fully appreciating their role in pulmonary disease.

Several cytochrome P450 enzymes are selectively expressed in the lung, including CYP2A13, CYP2F1, CYP2S1, CYP3A5, and CYP4B1. Recent studies have begun to reveal mechanisms controlling the induction of pulmonary CYP3A5 by glucocorticoids (Hukken et al., 2003) and CYP2S1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (Rivera et al., 2002). However, there is a paucity of knowledge regarding constitutive lung-selective regulatory mechanisms.

In animal models, CYP4B1 bioactivates several protoxicants and procarcinogens, including 2-aminoanthracene (Smith et al., 1995), 2-aminoisfluorene (Vanderslice et al., 1985), and valproic acid (Rettie et al., 1995). Combined with its lung-selective expression pattern, such observations resulted in considerable interest in CYP4B1. Yet, the activity and function of human CYP4B1 is controversial. Individual differences in CYP4B1 expression have been associated with bladder cancer susceptibility (Imaoka et al., 2000), and elevated CYP4B1 mRNA was demonstrated in lung carcinoma versus normal lung tissue (Czerwinski et al., 1994). Imaoka et al. (2001) also demonstrated elevated lauric acid ω-hydroxylase and 2-aminoflurorene-dependent umu gene expression using microsomes from human CYP4B1 transgenic mice that were inhibited by a CYP4B1 antibody. Similar activities also were observed using a CYP4B1-NADPH cytochrome P450 oxidoreductase fusion protein expressed in yeast. In contrast, Zheng et al. (2003) reported that human CYP4B1 does not appear to be an active enzyme due to a unique amino acid substitution in the meander region. Irrespective of this controversy, CYP4B1 mRNA is detectable in lung. Thus, this system remains a viable model to better understand molecular mechanisms controlling cytochrome P450 lung-selective expression and was the focus of this study.

ABBREVIATIONS: Sp1, Sephadex protein 1; Sp3, Sephadex protein 3; AP-4, activator protein-4; C/EBP, CCAAT/enhancer binding protein; Elk-1, member of ETS family of transcription factors; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; Sp/XKLF, Sephadex protein/Krüppel-like factor family of transcription factors; HNF3, hepatocyte nuclear factor 3.
Materials and Methods

Materials. A549 lung epithelial carcinoma cells, BEAS-2B-immortalized human bronchial epithelial cells, and 293 adenovirus 5-transformed human kidney epithelial cells were obtained from the American Type Culture Collection (Manassas, VA). HepG2 hepatoblastoma cells were a gift from Dr. Barbara Knowles (Jackson Laboratories, Bar Harbor, ME). The plasmid p14-2 (Yokotani et al., 1990), containing the human CYP4B1 gene, was provided by Dr. Y. Fujii-Kuriyama (Osaka Prefectural Institute of Public Health, Osaka, Japan). Sp1 and Sp3 expression vectors were generously provided by Dr. Guntram Suske (Institut für Molekularbiologie und Tumorforschung, Philipps-Universität, Marburg, Germany), an AP-4 expression vector was obtained from Dr. Michael Lehman (University of Arkansas, Fayetteville, AR), and C/EBPα and β expression vectors were obtained from Dr. Peter Johnson (National Cancer Institute, Bethesda, MD). The luciferase reporter plasmid (pGL3Basic) and luciferase reporter assay kit were purchased through Promega (Madison, WI), and the luminescent β-galactosidase assay kit was obtained from BD Biosciences Clontech (Palo Alto, CA). Oligonucleotides were synthesized by MWG Biotech (High Point, NC). The [α-32P]dCTP radioligase (3000 Ci/ mmol) was acquired from PerkinElmer Life and Analytical Sciences (Boston, MA). The Micro BCA kit for protein determination using the method of Smith et al. (1985) was purchased from Pierce (Rockford, IL). Sp1, Sp3, Elk-1, and C/EBP (α, β, γ, and δ forms) antibodies were purchased from Santa Cruz Biotechnology, Inc., whereas the AP-4 antibody was provided by Dr. Michael Lehmann (Institut für Genetik der Freien Universität Berlin, Berlin, Germany). Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). Cell culture reagents and routine chem- icals were supplied by Sigma-Aldrich (St. Louis, MO). High-purity plasmid purification kits were procured from Marligen (Ijamsville, MD). Human lung tissue was obtained from Rocky Mountain Donor Services (Salt Lake City, UT), whereas human liver tissue was the generous gift of Dr. Michael Franklin (University of Utah, Salt Lake City, UT). Lipofectamine 2000 and Platinum TaqDNA polymerase were purchased from Invitrogen (Carlsbad, CA). All other reagents were obtained from commercial sources at the purest grade available.

 Luciferase Reporter Plasmid Constructs. CYP4B1 gene coordinates are based on human chromosomal 1 contig NT_032977.7 (build 35.1) (http:// www.ncbi.nlm.nih.gov). The transcription start site, 36 bp upstream from the ATG start codon in exon 1, was determined by 5′-rapid amplification of cDNA ends (data not shown) and is in agreement with the major transcription start site reported in the Database of Transcriptional Start Sites (http://dbts.bsc.hgc.jp). A BglII/NcoI fragment representing human CYP4B1 position 2183 to 35 was isolated from p14-2 and cloned into the unique BglII/NcoI sites in pGL3Basic. The CYP4B1 fragments in this plasmid, designated pRNAH867, were verified by DNA sequence analysis (Sanger et al., 1977). Two approaches were used to prepare nested deletions of the CYP4B1 upstream region. First, pRNH684 was digested with XhoI, which cleaved at a unique site within the multiple cloning site immediately upstream of the CYP4B1 insert, along with a second enzyme that cleaved at a unique site within the insert. The released CYP4B1 fragment was discarded, the remaining DNA treated with T4 DNA polymerase to create flush-ends, and then self-ligated using T4 DNA ligase. Thus, digestion of pRNH684 with XhoI/SpeI yielded pRNH686 (CYP4B1 position −1087 to +35 directing luciferase expression), whereas digestion with XhoI/HindIII yielded pRNH683 (CYP4B1 position −457 to +35 directing luciferase expression). In a second approach, high-fidelity polymerase chain reaction (PCR) DNA amplification was performed using BamHI-linearized pRNH684 or pRNH684 as a template. One of multiple unique sense primers that included a nonhomologous 5′ KpnI site was paired with a 3′ antisense primer that annealed immediately downstream of the unique HindIII site at CYP4B1 position 457 (pRNH684 as template) or the unique NcoI site at CYP4B1 position 35 (pRNH683 as template). PCR products were digested with KpnI/HindIII or KpnI/NcoI, respectively, and used to replace the KpnI/ HindIII or KpnI/NcoI fragments in pRNH684, yielding pRNH833 (CYP4B1 position −1006 to +35 directing luciferase expression), pRNH834 (CYP4B1 position −890 to +35 directing luciferase expression), pRNH835 (CYP4B1 position −960 to +35 directing luciferase expression), pRNH836 (CYP4B1 position −660 to +35 directing luciferase expression), pRNH699 (CYP4B1 position −216 to +35 directing luciferase expression), pRNH698 (CYP4B1 position −139 to +35 directing luciferase expression), and pRNH697 (CYP4B1 position −45 to +35 directing luciferase expression). Similarly, an internal deletion was prepared using a sense primer that annealed at CYP4B1 position −217 and contained a nonhomologous 5′ HindIII site and the antisense primer described above that annealed downstream of the NcoI site at +35. The resulting ampiclon was digested with HindIII/NcoI and cloned into pRNH686 that had been digested with HindIII/NcoI, deleting a 492-bp fragment representing CYP4B1 position −457 to +35. The resulting plasmid, pRNH922, contains CYP4B1 position −1087 to −457 spliced to position −217 to +35 directing luciferase expression.

Cell Culture and Transient Expression. A549 cell lung carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 containing 10% fetal bovine serum and the antibiotics penicillin (5 U/ml) and streptomycin (50 μg/ml). HepG2 hepatoblastoma and 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics as described by Boucher et al. (1993). BEAS-2B cells were cultured in LHC-9 medium (Biofluids, Rockville, MD) using fibronectin/collagen-precoated flasks per the recommendations of the Ameri- can Type Culture Collection. For transfection studies, 1 × 105 cells were subcultured in each well of a 24-well plate. Twenty-four hours later, the cells were at approximately 80% confluence and were transfected with reporter constructs and, if appropriate, expression vectors, using a mixture of 3 μg of Lipofectamine 2000, 0.8 μg of test plasmid(s), and 0.2 μg of pCMV/βgal. After incubation for 16 h at 37°C, transfection medium (OptiMEM medium (Optimem with 5% fetal bovine serum) was replaced with growth medium and the cells were incubated an additional 48 h. The cells were processed and luciferase assays performed following the manufacturer’s recommended instructions. Luciferase activity was normalized to β-galactosidase activity to correct for transfection effi- ciency. At least two separate reporter plasmid preparations were used in two independent transfection assays with each experiment performed in triplicate.

Electrophoretic Mobility Shift Assay (EMSA). HepG2 and A549 nuclear protein extracts were prepared as described by Chodosh (1988) using optimized final KCl extraction concentrations of 400 and 600 mM, respectively. Nuclear protein extracts were assayed for protein content, divided into aliquots, and stored at −80°C until used. Based on transient expression assays with nested deletion mutants, EMSA probes were prepared using specific PCR primer pairs containing nonhomologous BamHI recognition sequences on their 5′-end. Amplified CYP4B1 sequences included positions −1087 to −1008, −1052 to −1008, −216 to −138, and −138 to −46. After digestion of the PCR products with BamHI, the probes were radiolabeled using Klenow DNA polymerase (exonuclease−), unlabeled deoxynucleotides and [α-32P]dCTP. EMSA was performed as described by Boucher et al. (1993), with the exception that DNA/protein binding was performed in 10 mM Hepes-HCl pH 7.9, 75 mM KCl, 0.2 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, and 0.24 μg/ml bovine serum albumin. Each 30-μl binding reaction contained 10 μg of nuclear protein, 1.5 μg poly[d(I-C)], and 0.2 ng (approx- imates 10 5 molecules) of radiolabeled probe. To test for competition with homologous DNA elements within the proximal CYP4B1 promoter, double-stranded oligonucleotides were prepared representing CYP4B1 positions −142 to −119, −128 to −105, −112 to −71, and −88 to −61 and used in competitive EMSA at a 25- to 300-fold molar excess over the CYP4B1 position −138 to −46 probe. Somewhat similarly, double-stranded oligonucleotides representing CYP4B1 sequences from position −1068 to −1008, −1052 to −1008, −1052 to −1026, and −1042 to −1008 were prepared and used in competitive EMSA at a 10- to 100-fold molar excess over the CYP4B1 position −1052 to −1008 probe. Finally, double-stranded oligonucleotides representing known or consensus binding sites for the Sp1/2KLF family, Sp-4, Elk-1, HNF3, and C/EBP (Table 1) were prepared and used in competitive EMSA at a 150-molar fold excess over the CYP4B1 position −1052 to −1008 probe. EMSA supershift experiments were performed using Transcruz antibodies (Santa Cruz, CA) or antibody preparations generously donated by other investigators (see above). A549 nuclear extract was incubated for 15 min at 4°C with 0.5 to 1.0 μg of antibody either prior to or after the addition of the labeled probe.

Site-Directed Mutagenesis. Mutagenesis was achieved using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) as directed by the manufacturer. The mutagenic primers (targeted nucleotides are lower case) were designed as:

5′-GGG GGT TGCT GTGC TCT CTC GT TGA CACC TCA GC-3′ and
5′-TGC TGG CTGT CAT GAG TGTT GAG GGG-3′ respectively. These were used with pRNH686 as...
Results

Localization of CYP4B1 Tissue-Selective Regulatory Domains.
To identify functional CYP4B1 regulatory domains, a series of reporter constructs were prepared in which CYP4B1 sequences from position −2183 to +35, −1087 to +35, −457 to +35, −216 to +35, −139 to +35, and −45 to +35 directed luciferase expression. Transient expression of these constructs in A549 lung carcinoma cells (Fig. 1) failed to reveal an increase in promoter activity over background (pGL3Basic with the −45 to +35 construct. However, inclusion of CYP4B1 sequences from position −139 to +35 resulted in a 4-fold increase in reporter activity, suggesting the presence of a proximal positive regulatory domain. An additional 2-fold increase in reporter activity was observed when a more distal domain, CYP4B1 positions −1087 to −457, was included in the reporter construct, whereas a significant decrease in activity was seen by extending the included CYP4B1 sequences to position −2183.

To identify CYP4B1 domains selectively active in lung-derived cells, the same reporter constructs were used to transiently transfect both A549 lung carcinoma and HepG2 hepatocellular carcinoma cells (Fig. 2), as well as BEAS-2B SV40 large T antigen-immortalized bronchial epithelial and 293 adenovirus 5-transformed kidney cells (data not shown). Consistent with what was observed in Fig. 1, transient expression of the construct containing CYP4B1 sequences from position −139 to +35 resulted in a 4-fold increase in A549 cell luciferase activity (Fig. 2B), whereas a 3-fold increase was observed in HepG2 cells (Fig. 2A). Qualitatively similar results were observed in both BEAS-2B and 293 cells (data not shown). Although no further change in luciferase activity was observed in any of the cell lines by including sequences up to position −216, extending the CYP4B1 sequences to position −457 resulted in a significant decrease in HepG2 luciferase activity (Fig. 2A) but no change in A549 (Fig. 2B), BEAS-2B, or 293 cells (data not shown). Finally, an additional 2-fold increase in reporter activity was observed when a more distal domain, CYP4B1 position −1087 to −457, was included in the reporter construct and used to transfect A549 cells (Fig. 2B), but no change was observed in HepG2 cells (Fig. 2A). Results qualitatively similar to those seen with A549 cells were observed when these same transient expression assays were performed using the human bronchial epithelial cells, BEAS-2B (data not shown). In contrast, when the experiments were repeated in the kidney-derived 293 cells, up-regulation was not observed with the CYP4B1 position −1087 to −457 domain (data not shown). These results are consistent with a ubiquitous, proximal positively acting domain between CYP4B1 position −139 and −45, a liver-selective negatively acting domain between CYP4B1 position −457 and −216, and a lung-selective positively acting domain between CYP4B1 position −1087 and −457.

A series of reporter constructs in which CYP4B1 sequences from position −1087 to +35, −1006 to +35, −890 to +35, −749 to +35, −660 to +35, and −457 to +35 directed luciferase expression were utilized for transient expression assays in A549 cells to further define the location of the distal, lung-selective CYP4B1 regulatory element.

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**Table 1**

Oligonucleotides representing consensus and mutated consensus transcription factor elements used for competitive EMSA

<table>
<thead>
<tr>
<th>Sequence (Sense Strand)*</th>
<th>Transcription Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ GATGACTGAGTCAGCTCAAGGACTGACATGCC 3′</td>
<td>AP-4</td>
<td>Boulanger et al., 2000</td>
</tr>
<tr>
<td>5′ CGACCGCGCGCGCGAACTGACGGGC 3′</td>
<td>Sp1 (Sp/XKLF)</td>
<td>Kriwacki et al., 1992</td>
</tr>
<tr>
<td>5′ CGATCTTCGAGGAAGTTATAAG 3′</td>
<td>Elk1</td>
<td>Xin et al., 1992; Li et al., 2000</td>
</tr>
<tr>
<td>5′ TGCAGATGGCGGAATCTGCA 3′</td>
<td>C/EBP</td>
<td>Mahoney et al., 1992</td>
</tr>
<tr>
<td>5′ GGTGACTAAGTCAAATGCAGAAT 3′</td>
<td>HNF3</td>
<td>Roesler et al., 1988; Overdier et al., 1994</td>
</tr>
</tbody>
</table>

* Core binding elements are shown in bold type.
No increase in reporter gene activity over that seen with the proximal regulatory element was observed with constructs that included CYP4B1 sequences up to position -H110021008 (pRNH833) (Fig. 3). However, a significant increase was observed when sequences up to position -H110021087 were included. These data indicated the distal regulatory element resides between CYP4B1 position -H110021087 to -H110021008.

**Regulation by the Proximal CYP4B1 Positive Regulatory Domain Involves Sp/XKLF Factors.** EMSA was used to assess whether or not specific DNA/protein interactions were possible within the proximal CYP4B1 positive regulatory domain (position -138 to -46). Using A549 cell nuclear protein extract, five DNA/protein complexes were observed, three of which represented specific DNA/protein interactions based on competition with unlabeled probe DNA (Fig. 4, complexes A, B, and C, compare lanes 2 and 3). Analysis of the CYP4B1 sequences from position -138 to -46 using the Match program in conjunction with the TRANSFAC V 8.3 Professional database revealed six strong matches to the consensus binding site for the Sp/XKLF family of transcription factors (Fig. 5). Consistent with this observation, competitive EMSA using a 100-fold molar excess of a consensus Sp/XKLF sequence (Table 1) resulted in the elimination of all three specific DNA/protein complexes (Fig. 4, complexes A, B, and C, compare lanes 2 and 4). Supershift EMSA was used to determine which of the Sp/XKLF factors might be involved in specifically binding the CYP4B1 proximal regulatory element. Inclusion of an Sp1-monospecific antibody in the DNA/protein binding reaction resulted in a supershift of complex B (Fig. 4, lane 5), whereas the addition of an Sp3-monospecific antibody resulted in a supershift of both complexes A and C (Fig. 4, lane 6). Addition of both antibodies resulted in a supershift of all three specific DNA/protein complexes (Fig. 4, lane 7). Thus, these data strongly implicate a role for Sp1 and/or Sp3 in regulating CYP4B1 expression through the proximal regulatory element.

Cotransfection of A549 cells with both the CYP4B1-directed reporter constructs and Sp1 or Sp3 expression vectors was used to
assess the functional role of the identified Sp/XKLF sites (Fig. 6). Neither Sp1 nor Sp3 had any impact on the background reporter activity observed with the promoterless pGL3 basic reporter vector. Cotransfection with the pRNH686 reporter construct (CYP4B1 position −138 to +30 directing luciferase expression) and either an Sp1 or Sp3 expression vector resulted in a 3-fold increase in promoter activity relative to that observed with a blank expression vector (Fig. 6A). Cotransfection with the pRNH686 reporter construct (CYP4B1 position −1087 to +30 directing luciferase expression) and the Sp1 expression vector resulted in a 5-fold increase in reporter activity. In contrast, cotransfection with pRNH686 and the Sp3 expression vector had no effect on luciferase activity (Fig. 6B). Similar cotransfection experiments in HepG2 cells also resulted in a 5-fold increase in activity with the pRNH686 reporter construct (data not shown).

Sp1/Sp3 binding to the proximal regulatory domain in vivo was determined using ChIP analysis along with the same cells lines in which transient expression assays were performed (A549, HepG2, and BEAS-2B cells) and autopsy samples of liver and lung tissue. The CYP4B1 proximal regulatory domain was bound by Sp1, but not Sp3, in HepG2 cells (Fig. 7A). In contrast, both Sp1 and Sp3 were observed to bind the proximal regulatory domain in A549 cells with Sp1 in excess of Sp3, whereas in BEAS-2B cells, both Sp1 and Sp3 bound, but Sp3 was in excess of Sp1. Binding appeared much less robust in the latter cell line. A different situation was observed in the lung and liver tissue samples. The CYP4B1 proximal regulatory domain was bound by Sp1 in lung tissue, but not liver (Fig. 7B), whereas Sp3 binding was not observed in either tissue. These latter results contrast to what was observed with the CYP2F1 basal promoter using these same antibodies and tissue samples in which Sp1 binding was observed in lung, but not liver, whereas Sp3 binding was observed in liver, but not lung (Wan et al., 2005).

Given that six putative Sp/XKLF sites were identified in the proximal element (Fig. 5), competitive EMSA was used to determine which of these sites might be responsible for the observed specific DNA/protein interactions and functional impact on the CYP4B1 promoter. Double-stranded oligonucleotides incorporating site 1, sites 3 and 4, and site 6 failed to compete at even a 300-fold molar excess (Fig. 8, lanes 11–13). Consistent with these results, mutagenesis of sites 2 and 5 from 5′-CCGCC-3′ to 5′-TTGCC-3′ within the CYP4B1 position −138 to −46 fragment, although leaving sites 1, 3, and 4, and 6 intact, eliminated the ability of this fragment to compete for specific DNA/protein binding (Fig. 8, lanes 14 and 15).

To validate the activity of Sp/XKLF sites 2 and 5 identified by competitive EMSA, site-directed mutagenesis was used to eliminate
FIG. 5. Identification of putative Sp/XKLF binding sites within the CYP4B1 position −139 to −45 proximal regulatory domain. A computer-assisted search of CYP4B1 sequences from position −139 to −45 using the Match program and the TRANSFAC V8.3 database revealed six putative binding sites for members of the Sp/XKLF transcription factor family. The matches to the core Sp/XKLF binding sites are shown by the shaded boxes. Bases matching the consensus matrix are in bold typeface. Arrows indicate the orientation of the putative Sp/XKLF sites.

FIG. 6. Sp1 and Sp3 positively regulate the CYP4B1 promoter. Cotransfection, transient expression studies were performed in A549 cells with CYP4B1/luciferase constructs containing (A) only the proximal regulatory domain (pRNH698) or (B) both the proximal and distal regulatory domains (pRNH686), along with Sp1 or Sp3 expression vectors. Salient features of each CYP4B1/luciferase construct are shown to the left of the bar graph. Individual numbers refer to the specific reporter construct used for the transfection (see Materials and Methods). The reported activities have been normalized for transfection efficiency and protein content and represent means ± S.D. from triplicate experiments. ***, p < 0.001 indicate a significant difference in activity relative to the CYP4B1/luciferase construct in the absence of the Sp1 or Sp3 expression vector.
Sp1/Sp3 binding at these elements within the CYP4B1 reporter constructs. The same base changes made within Sp/XKLF site 2 (CYP4B1 position −118 to −114) and/or Sp/XKLF site 5 (CYP4B1 position −77 to −73) that eliminated specific DNA/protein binding (Fig. 8) were introduced into pRNH698 (CYP4B1 position −139 to +35 directing luciferase expression) and pRNH686 (CYP4B1 position −1087 to +35 directing luciferase expression) resulting in pRNH844 and 838 (site 2 mutagenized), pRNH845 and 842 (site 5 mutagenized), and pRNH846 and 843 (sites 2 and 5 mutagenized), respectively. The impact of these changes on CYP4B1-directed luciferase expression was examined after transient transfection into A549 cells (Fig. 9). When examined within the context of the proximal regulatory element alone (CYP4B1 position −139 to −45), an approximate 2-fold loss in promoter activity was observed with the elimination of site 2 (pRNH844 versus 698, p < 0.05) or site 5 (pRNH845 versus 698, p < 0.001) alone. However, mutagenesis of both sites 2 and 5 resulted in a complete loss of promoter activity (pRNH846 versus 698, p < 0.001; pRNH846 versus pGL3basic, p > 0.05).

Loss of Sp1/Sp3 binding at the proximal regulatory element also had a substantial impact on the function of the lung-selective distal regulatory element (CYP4B1 position −1087 to −1008) (Fig. 8). Elimination of either Sp/XKLF site 2 or site 5 by itself within the context of both the proximal and distal elements decreased CYP4B1 promoter activity by 1.5- (pRNH838 versus 686, p < 0.01) or 3-fold (pRNH842 versus 686, p < 0.001), respectively. The reporter vector pRNH846 with both Sp/XKLF sites 2 and 5 mutagenized decreased the reporter activity 5-fold (pRNH843 versus 686, p < 0.001) but to a level that remained 2-fold above that observed with the promoterless vector (pRNH843 versus pGL3Basic, p < 0.001).

**Further Characterization of the Distal CYP4B1 Regulatory Element.** To evaluate specific protein binding within the CYP4B1 distal, lung-selective regulatory element, EMSAs were performed using CYP4B1 sequences from position −1087 to −1008 as a probe along with nuclear protein extract prepared from A549 cells. A single specific DNA/protein complex was observed, as demonstrated by the ability of a 50-fold molar excess of unlabeled DNA probe to eliminate binding to the radiolabeled DNA probe. Competition with two overlapping double-stranded oligonucleotides further localized the domain responsible for specific binding to CYP4B1 sequences position −1052 to −1008 (data not shown). Using CYP4B1 sequences from −1052 to −1008 as an EMSA probe, two major and one minor specific DNA/protein complexes were observed (Fig. 10, complexes A, B, and C; compare lane 2 with lanes 3 and 4). Competition with a 100-fold molar excess of an oligonucleotide representing CYP4B1 position −1052 to −1026 eliminated specific DNA/protein complex C, but not A or B (Fig. 10, lane 5). In contrast, an oligonucleotide representing CYP4B1 position −1042 to −1008 failed to compete for specific binding (Fig. 10, lane 6). When repeated using HepG2 nuclear extract, specific DNA protein interactions were not observed with the CYP4B1 position −1052 to −1008 probe. These observations are consistent with two binding domains between position CYP4B1 position −1052 and −1042 and position −1026 and −1008 in which binding to the upstream site is obligatory for subsequent binding to the downstream site. Furthermore, these DNA/protein interactions appear lung-selective.

Analysis of the CYP4B1 distal regulatory element position −1052 to −1008 using the Match program in conjunction with the TRANSFAC V 8.3 Professional database revealed strong matches for putative AP-4, Elk-1, C/EBP, and HNF3 sites. Competitive EMSA was used to identify which, if any, of the identified factors were capable of specifically binding the CYP4B1 lung-selective distal element. However, competition was not observed using a 150-fold molar excess of known consensus binding sequences for AP-4 (Fig. 10, lane 7), C/EBP (Fig. 10, lane 8), Elk-1 (Fig. 10, lane 9), or HNF3 (Fig. 10, lane 10). In addition, antibodies for AP-4, Elk-1, C/EBPα, C/EBPβ, C/EBPδ, and C/EBPγ failed to shift the mobility of any of the specific DNA/protein complexes observed with the CYP4B1 position −1052 to −1008 probe (data not shown). Finally, cotransfection with pRNH886 and AP-4, C/EBPα, or C/EBPβ expression vectors failed to alter reporter activity (data not shown).

**Discussion**

This investigation used CYP4B1 as a model system to examine lung-selective mechanisms controlling cytochrome P450 expression. Three major regulatory elements were identified, a proximal positive
regulatory element located between CYP4B1 position -118 and -73 that involves Sp1 and/or Sp3 binding at two sites, a liver-selective negative regulatory element located between CYP4B1 position -457 and -216, and a distal, lung-selective positive element located between CYP4B1 position -1052 and -1008 that involves as yet unidentified transcription factor(s). Although the in vitro DNA/protein binding assays and the transient expression data are consistent with both Sp1 and Sp3 regulating CYP4B1 in a tissue-nonselective fashion, the in vivo ChIP data implicates the selective binding of Sp1 to the CYP4B1 proximal promoter element in lung tissue and as such, is consistent with the conclusion that the proximal regulatory element also contributes to CYP4B1 lung-selective expression.

A Match analysis of the proximal CYP4B1 element using the TRANSFAC Professional v 8.3 database identified six potential Sp/XKLF binding sites. However, only two of the sites appear to be functional (CYP4B1 position -118 to -114 and -77 to -73), both capable of binding the Sp/XKLF family members Sp1 and Sp3 and responding to both factors in transient expression assays. Sp1 and Sp3 are ubiquitously expressed, consistent with the observation that the CYP4B1 proximal element functioned equally well in both lung- and liver-derived cells. These latter data also are consistent with the inability of the lung-selective member of the Sp/XKLF family, the lung Krüppel-like factor (LKLF) (Anderson et al., 1995), to modulate the activity of the proximal element. Major differences between Sp1 and Sp3 lie with the ability of Sp1, but not Sp3, to form multimeric complexes and in doing so, synergistically up-regulate promoter activity (Su et al., 1991; Yu et al., 2003). In addition, the Sp1 inhibitory domain is located at the N terminus of the protein, whereas in Sp3 it is located next to the three conserved Cys2His2 zinc fingers involved in DNA binding (Suske, 1999). These differences in functional motifs permits Sp3 to commonly function as a repressor, although instances also exist where it serves as a strong, positively acting factor (Sowa et al., 1999; Rao et al., 2002). In the case of the CYP4B1 proximal element, Sp1 and Sp3 can compete for binding in vitro and both work to positively regulate promoter activity in the transient expression assays. However, Sp1 results in a more robust response.

Within the context of the proximal regulatory element alone, the mutagenesis studies suggest the two CYP4B1 proximal Sp1/Sp3 sites act in a largely additive and independent fashion. Thus, Sp/XKLF site 5 enhanced promoter activity 1.4-fold (Fig. 9, pRNH844 versus 698), whereas Sp/XKLF site 2 enhanced activity 2.1-fold (Fig. 9, pRNH845 versus 698). Together, a 3.7-fold enhancement was observed (Fig. 9, pRNH846 versus 698), essentially equal to the predicted additive response of 3.5-fold. In contrast, when examined in the context of the
full-length promoter, an enhancement somewhat greater than the predicted additive response was observed, i.e., a 5.2-fold enhancement (Fig. 9, pRNH843 versus 686) versus a predicted 4.3-fold additive response (Fig. 9, pRNH842 versus 686 and pRNH838 versus 686). Although these data are not convincing alone, synergy also is apparent in experiments where Sp1 or Sp3 expression vectors were cotransfected with CYP4B1/luciferase reporter constructs. These data showed that both Sp1 and Sp3 were able to further enhance promoter activity in the context of the proximal promoter alone. In contrast, Sp3 had no impact when overexpressed in the presence of the full-length construct, whereas cotransfection with the Sp1 expression vector resulted in a further 5-fold increase in promoter activity. Thus, the ability of Sp1 to form multimeric complexes involving multiple sites and synergistically activate transcription through protein-protein interactions has a minimal impact on the proximal promoter element alone but does function in the context of both the proximal and lung-selective distal element.

Cooperativity between Sp1 and lung-selective, as well as other tissue-selective, transcription factors has been documented (reviewed in Li et al., 2004). In this context, tissue-specific regulation involves the specific cellular environment, relative Sp1 and Sp3 expression levels, and protein modification. Thus, a significantly higher level of Sp1 was found in thymus, lung, and spleen tissue than was found in other tissues, including the liver (Saffer et al., 1991). Variation in Sp1/Sp3 ratios impacted cell-specific regulation of the kinase domain receptor in endothelial cells (Hata et al., 1998), the HPV-16 promoter in epithelial cells (Apt et al., 1996), and the COL2A1 promoter in chondrocytes (Chadjichristos et al., 2002). Protein modification of Sp1 and Sp3, including phosphorylation, glycosylation, sumoylation, and acetylation may also contribute to the cell-specific expression of a variety of proteins. Finally, a role for Sp1 and Sp3 in regulating the lung-selective expression of another cytochrome P450, i.e., CYP2F1, has recently been documented (Wan et al., 2005). The importance of Sp1 in regulating CYP4B1 lung-selective expression is apparent from the combined in vitro and in vivo studies presented in the current study and likely involves one or more of the above control mechanisms.

Tissue-selective CYP4B1 expression also is controlled by the combined effect of the lung-selective distal regulatory element localized between position −1052 to −1008 and the absence of regulation through the liver-selective negative regulatory element localized between position −457 and −216. Although the importance of the negative regulatory element is apparent by the marked decrease in reporter activity observed in transient expression assays with HepG2 cells, results with the 293 kidney-derived cell line would suggest a greater importance of the combinatorial regulation observed between the proximal and distal positively-acting elements. A more complete assessment of the negative element’s impact on CYP4B1 lung-specific expression will require a more exhaustive analysis of cell lines in which CYP4B1 is not expressed. Regulation through the CYP4B1 distal domain appears to involve transcription factor(s) binding to both CYP4B1 positions −1052 to −1042 and −1026 to −1008 but appears to require binding at the upstream site for subsequent binding to the downstream site. Although a search of the CYP4B1 distal regulatory element with Match using the TRANSFAC database suggested potential roles for several transcription factors known to be involved in lung-selective regulation, competitive DNA binding, antibody supershift experiments, and transient reporter assays with transcription factor expression vectors eliminated all of these potential
candidates. Identification of this unknown lung-specific regulatory factor(s) will require further study.

In summary, Sp1 acting through a proximal enhancer element and in combination with a distal, lung-selective enhancer, is critical for CYP4B1 lung-selective regulation. Also contributing to CYP4B1’s tissue-selective expression pattern is a liver-selective repressor motif. Although the activity of the human CYP4B1 protein is controversial (Imaoka et al., 2001; Zheng et al., 2003), studies have demonstrated CYP4B1 expression at the level of mRNA in lung tissue. Such lung-selective expression is distinctive and worthy of the mechanistic investigation reported herein. Thus, the current data contribute substantially to our understanding of the molecular mechanisms controlling cytochrome P450 lung-selective expression.

References

Apt D, Watts RM, Suske G, and Bernard HU (1996) High Sp1/Sp3 ratios in epithelial cells in order of increasing electrophoretic mobility) are indicated by solid arrows to the left of the autoradiogram. Competition was performed with either a 10- or 100-fold molar excess of unlabeled probe (lanes 3 and 4, respectively), with a 100-fold molar excess of double-stranded oligonucleotides representing sequences from position -1052 to -1008 (lane 5) and CYP4B1 position -1042 to -1008 (lane 6), and a 150-fold molar excess of consensus sequences for AP-4 (lane 7), C/EBP (lane 8), Elk-1 (lane 9), or HNF3 (lane 10).

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References


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