SP1 AND SP3 REGULATE BASAL TRANSCRIPTION OF THE HUMAN CYP2F1 GENE

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ABSTRACT:

Selective transcription of the human CYP2F1 gene in lung tissues may control the susceptibilities of this organ to diverse pneumotoxicants and lung carcinogens. However, the mechanisms responsible for CYP2F1 organ-selective transcription have not been elucidated. The objectives of the current studies were to identify and characterize basal transcription elements within the TATA-less promoter region of CYP2F1. Four putative Sp1-like sites were identified in the CYP2F1 promoter. Competitive electrophoretic mobility shift assay analysis with mutated oligonucleotide probes and lung A549 cell nuclear extract, along with supershift studies using antibodies to either Sp1 or Sp3 proteins, demonstrated that all four sites formed three specific protein-DNA complexes. Mutations in any of the four core Sp1-like motifs abolished protein-DNA binding. Western blot analysis of both human tissues and cells showed that Sp1 was considerably higher in lung than liver and that Sp3 was much higher in liver than lung. Promoter activation of a luciferase reporter construct was sequentially increased by addition of each of the four Sp1-like motifs in lung A549 cells but not in liver HepG2 cells. Cotransfection of a Sp1 expression vector with the reporter construct dramatically increased luciferase activity in either A549 cells or Sp1-deficient Drosophila Schneider line 2 (SL-2) cells. However, similar cotransfections with an Sp3 expression vector failed to increase activity. Cotransfection of both the Sp1 and Sp3 expression vectors considerably decreased Sp1-mediated activity in A549 cells and abolished activity in SL-2 cells. Thus, these studies demonstrated that four Sp1-dependent proximal promoter elements drive organ-selective CYP2F1 gene transcription, and that Sp1 and Sp3 factors interact to modulate constitutive CYP2F1 transcription in lung cells.

Lung cancer and other chronic lung diseases may be caused by the bioactivation of procarcinogens or proteotoxicants by cytochrome P450 enzymes that are selectively expressed in respiratory tissues (Yost, 2001; Ding and Kaminsky, 2003). Bioactivation refers to the oxidation of essentially inert xenobiotics to highly reactive electrophiles or reactive oxygen species that initiate mutagenic lesions or dysregulate cell growth (or death) processes. A considerable number of cytochrome P450 genes are selectively expressed in the respiratory tract (Ding and Kaminsky, 2003), and most of these gene products are responsible for the bioactivation of toxicants and/or carcinogens in lung tissues.

The ubiquitous transcription factor Sp1 is one of the most commonly studied transcription factors and has emerged as an integral part of the cellular machinery that mediates gene expression (Li et al., 2004). Sp1 belongs to a recently defined family of proteins known as Sp/XKLF that contain three highly homologous C-terminal zinc-finger motifs that bind sequences known as GC-boxes and the closely related GT/CACC boxes found in the promoters of a diverse number of genes (Philipsen and Suske, 1999). The Sp/XKLF family contains 17 members divided into three subgroups: 1) the four Sp transcription factors; 2) the closely related BTEB and TIEG1/2 (transforming growth factor β-induced early gene) proteins; and 3) the Krüppel-like factors (XKLF), e.g., the lung-enriched Krüppel-like factor (LKLF). An illustration of the mechanisms whereby these factors regulate P450 tissue-specific transcription has been described for CYP1A1 (Zhang et al., 1998; Kaczynski et al., 2002). These studies showed the role of BTEB and gut-lung enriched Krüppel-like factor (GKLF) in the basal transcription of CYP1A1, including a physical interaction of these factors with Sp1 and Sp3 proteins. Therefore, any combination of Sp/XKLF proteins may interact with a given promoter element to either activate or repress transcription, often in a tissue-specific manner. Some XKLFs are expressed in both lung and liver; however, LKLF is expressed primarily in lung (Philipsen and Suske, 1999).

For other P450 genes, little is known about the trans-acting factors that control tissue-selective transcription. Studies have shown (Luc et al., 1996) that CCAAT enhancer binding proteins in rat liver control selective transcription of the CYP2B1 gene, and nuclear factor 1-like factors regulate CYP1A2 (Zhang et al., 2000) and CYP2A3 (Zhang and Ding, 1998) olfactory mucosa-selective expression. However, no extensive studies have described the mechanisms controlling transcriptional regulation of other important genes such as CYP4B1, CYP2S1, CYP3A5, or CYP2F1.

The human lung CYP2F1 enzyme catalyzes the bioactivation of many potent environmental toxicants such as 3-methylindole (Lanza

ABBREVIATIONS: XKLF, Krüppel-like transcription factors; BTEB, basic transcription element binding protein; LKLF, lung-enriched Krüppel-like transcription factor; P450, cytochrome P450; SL-2, Schneider line 2; GKLF, gut-lung enriched Krüppel-like factor; LSF, lung-specific factor; EMSA, electrophoretic mobility shift assay; RT, room temperature; PCR, polymerase chain reaction; PBST, 1× phosphate buffered-saline and 0.1% Tween; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; BTE, basic transcription element.
were prepared as described previously (Carr et al., 2003). Protein concentra-

.. table:: Nucleotide sequences of the Sp1-like and mutant oligonucleotides used for EMSA

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Position</th>
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<tbody>
<tr>
<td>Sp D</td>
<td>5'-GGGCGCACGACGACGTCGAGA-3'</td>
<td>-193/-164</td>
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<tr>
<td>Sp Dmut</td>
<td>5'-GGGCGCGACGACGACGTCGAGA-3'</td>
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<tr>
<td>Sp C</td>
<td>5'-AGAGAGGGAGATTATTTAAGG-3'</td>
<td>-125/-103</td>
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<tr>
<td>Sp Cmut</td>
<td>5'-AGAGAGGGAGATTATTTAAGG-3'</td>
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<tr>
<td>Sp B</td>
<td>5'-AGAGGGAGGTTGAGAAAGAAACA-3'</td>
<td>-72/-49</td>
</tr>
<tr>
<td>Sp Bmut</td>
<td>5'-AGAGGGAGGTTGAGAAAGAAACA-3'</td>
<td></td>
</tr>
<tr>
<td>Sp A</td>
<td>5'-CCTTCTCCCCAAACCCCCCAGA-3'</td>
<td>-36/-14</td>
</tr>
<tr>
<td>Sp Amt</td>
<td>5'-CCTTCTCCCCAAACCCCCCAGA-3'</td>
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</tr>
<tr>
<td>Sp1/3</td>
<td>5'-AATCTATGTTGGGCGGACGACG-3'</td>
<td>Consensus</td>
</tr>
</tbody>
</table>

* Single-stranded oligonucleotides were synthesized by IDT DNA. Oligonucleotides were resuspended (100 μM). Equal volumes of each oligonucleotide and its antisense oligonucleotide were annealed to form 50 μM double-stranded oligonucleotide, by heating to 95°C and then slowly cooling to RT. One picomole of double-stranded oligonucleotide probe was end-labeled with [γ-32P]ATP using T4 kinase. Sp1/3 is the sequence of a perfect consensus oligonucleotide supplied with the Promega gel shift system.

* Putative Sp sites are in bold and underlined. Mutated nucleotides are lowercase.

* Position of the oligonucleotides relative to the transcriptional start site.

Materials and Methods

Materials. Expression plasmids pEVR2/Sp1, pRC/CMV/Sp3, pPac/USp3, and corresponding empty vectors pEVR2, pRC/CMV, and pPac were kindly provided by Dr. Guntram Suske (Philipps University, Marburg, Germany) (Hagen et al., 1994; Sapetschnig et al., 2002). Expression plasmid pPac/Sp1 was provided by Dr. Robert Tjian (University of California, Berkeley, CA). Anti-Sp1 (sc-59X or sc-14027X) and anti-Sp3 antibodies (sc-644X) for EMSA supershift analysis, Western blotting, and chromatin immunoprecipitation were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The pGL3 luciferase reporter vectors and dual-luciferase reporter assay system were purchased from Promega (Madison, WI). The Drosophila Schneider line 2 (SL-2) cells were kindly provided by Dr. Terry Elton (Brigham Young University, Provo, UT). The TOPO cloning kits, cell culture media, restriction enzymes, and all other molecular biology reagents were purchased from Invitrogen (Carlsbad, CA). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). The Endo-free plasmid max kit for plasmid purification was purchased from QIAGEN (Valencia, CA). Human lung (lot 10-41402319) and liver (lot 10-71215053) nuclear extracts were purchased from Geneka (Montreal, QC, Canada). Rabbit serum against LKLF was kindly provided by Dr. Landy Kangaloo (Harvard School of Public Health, Boston, MA). Human lung tissue was obtained from Rocky Mountain Health, Boston, MA). Human lung cells.

Preparation of Nuclear Extracts. Nuclear extracts from cultured cells were prepared as described previously (Carr et al., 2003). Protein concentra-

tions of all nuclear extracts were determined using the Bio-Rad protein assay kit 1 (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assays. EMSA was performed using the Promega gel shift assay system essentially as described by the manufacturer. Binding reaction mixtures, preincubated at RT for 10 min, contained 4 μl of nuclear extract (4 μg) and 2 μl of binding buffer [50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 mg/ml poly(d-I-c), and 20% glycerol] in a total volume of 10 μl. After the 10-min incubation, 1 μl of 32P-labeled probe (0.005–0.01 pmol) was added, and the mixtures were incubated for another 20 min at RT. For competition experiments, unlabeled competitor double-stranded oligonucleotide (25-, 50-, or 100-fold molar excess) was incubated for 15 min before addition of the 1 μl of 32P-labeled probe. The mixtures were then incubated for another 20 min at RT. The DNA-protein complexes and unbound probes were separated by electrophoresis using 4% nondenaturing polyacrylamide gels and detected by autoradiography. Sequences of the synthesized DNA probes are listed in Table 1. Single-stranded oligonucleotide probes were synthesized by IDT DNA (Coralville, IA). Other oligonucleotides used for competition experiments, representing the consensus sequences for nonspecific Sp1 and Sp3, were supplied in the Promega gel shift assay system. EMSA supershift assays were performed with antibodies to Sp1 and Sp3 as described previously (Carr et al., 2003). Antibodies (2 μl) were incubated with the nuclear protein for 20 min at RT before the labeled probe was added. After electrophoresis, dried gels were exposed for various times to Kodak film (Eastman Kodak, Rochester, NY) with an enhancer sheet and developed for analysis.

Transient Transfection Studies in Mammalian Cell Lines. Luciferase reporter assays were performed to identify functional promoter regions as described previously (Carr et al., 2003). CYP2F1 reporter constructs were produced using PCR amplification with multiple primers that introduced a SacI site at positions –292, –141, –70, –50, and –19 paired with a single 3′-antisense primer that generated a BglII site at position +115. After digestion with SacI/BglII, the fragments were cloned into the pGL3 basic vector. The mutations at the Sp A, Sp B, Sp C, and Sp D sites were constructed from the wild-type –292/+115 pGL3 vector using the QuikChange site-directed mutagenesis kit. All mutations were verified by sequence analysis. Reporter constructs, the pGL3 basic negative control vectors, and Sp1 or Sp3 expression vectors were used for transient transfection studies. The human lung cell line A549 and the human liver cell line HepG2 were transfected with 0.1 μg of the reporter constructs and 0.001 μg of pRL-TK plasmid using a 3:1 ratio of FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN) in 96-well plates. In cotransfection assays, cells were transfected with 0.1 μg of the listed reporter constructs and 0.002 μg of pRL-TK and 0.05 or 0.1 μg of Sp1, Sp3, or empty expression vectors, maintaining the total transfected DNA at 0.2 μg. Cells were lysed 36 h post-transfection, and luciferase activities were measured using the dual luciferase assay. Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity (pRL-TK) and expressed as fold luminescence over the activity observed with the promoter-less pGL3 basic vector for each individual transfection assay. The data are presented as
mean fold luminescence ± S.E. Results were assessed using a single-factor analysis of variance. A value of \( p < 0.05 \) was accepted as a significant difference.

Transient Transfection Studies in the SL-2 Cell Line. SL-2 cells seeded in 24-well plates were cotransfected with 0.3 \( \mu \)g of the listed CYP2F1 reporter constructs and 0.3 \( \mu \)g of the pPac/Sp1 expression vector, 0.3 \( \mu \)g of the pPac/USp3 expression vector, or 0.3 \( \mu \)g of the pPac empty vector as described previously (Jiang et al., 2002). The empty control vector pPac (0.3–0.6 \( \mu \)g) was added to keep the total amount of plasmid DNA constant at 0.9 \( \mu \)g for each transfection. In addition, each transfection included 0.006 \( \mu \)g of PRL-TK, the internal control plasmid. The DNA and FuGENE 6 were added, at a 3:1 ratio (microliters of FuGENE 6:micromgrams of DNA), to Hanks’ balanced salt solution and incubated at room temperature for 30 min before addition to the cells. Cells were lysed 48 h post-transfection, and luciferase activities were assayed using the dual luciferase assay. Luciferase activities were normalized to cellular protein, measured using the Bio-Rad protein assay kit I, and reported as fold activation ± S.E. relative to the promoter-less pGL3 basic vector.

Western Blotting. Western blotting was performed as described previously (Wang et al., 1998) with some modifications. Briefly, samples (cell and nuclear extracts) were prepared by addition of an equal volume of loading dye followed by heating for 5 min at 100°C. Samples were immediately fractionated by electrophoresis for 2 h at 120 V. For cells, proteins were transferred to PVDF membrane, and the membrane was blocked with 1 \( \times \) PBST (1 \% Tween-20, pH 8.1) containing 5 \% nonfat milk for 1 h at room temperature. The PVDF membrane was incubated overnight with anti-Sp1 or anti-Sp3 antibodies diluted 1:3000 in 1 \( \times \) PBST containing 0.5 \% nonfat milk at 4°C. After the overnight incubation, the membrane was rinsed four times with 1 \( \times \) PBST, then washed with 1 \( \times \) PBST containing 1 \% nonfat milk for 1 h at room temperature. The secondary antibody was removed, and the membrane was washed four times with 1 \( \times \) PBST. For tissues, proteins were transferred to PVDF membrane, and the membrane was blocked with 1 \( \times \) PBST containing 1 \% nonfat milk for 1 h at room temperature. The PVDF membrane was incubated overnight with anti-Sp1 or anti-Sp3 antibodies diluted 1:1500 (Sp1) or 1:2500 (Sp3) in 1 \( \times \) PBST containing 0.25 \% nonfat milk at 4°C. After the overnight incubation, the membrane was rinsed four times with 1 \( \times \) PBST, and then incubated with horseradish peroxidase-conjugated secondary antibody, diluted 1:5000 in 1 \( \times \) PBST containing 1 \% nonfat milk for 1 h at room temperature. The secondary antibody was removed, and the membrane was washed four times with 1 \( \times \) PBST. For tissues, proteins were transferred to PVDF membrane, and the membrane was blocked with 1 \( \times \) PBST containing 1 \% nonfat milk for 1 h at room temperature. The PVDF membrane was incubated overnight with anti-Sp1 or anti-Sp3 antibodies diluted 1:1500 (Sp1) or 1:2500 (Sp3) in 1 \( \times \) PBST containing 0.25 \% nonfat milk (Sp1) or 0.5 \% milk plus 1.25 \% fetal bovine serum (Sp3) for 1 h at room temperature. The secondary antibody was removed, and the membrane was washed four times with 1 \( \times \) PBST. The PVDF membrane was developed with SuperSignal West Pico trial kit (Pierce Chemical Company) or enhanced chemiluminescence solution and incubated at room temperature for 30 min before addition to the membrane. The PVDF membrane was washed in ice-cold PBS and then incubated 15 min at 30°C in ice-cold phosphate buffered saline (PBS) and then incubated 15 min at 30°C in ice-cold phosphate buffered-saline (PBS) and then incubated 15 min at 30°C in ice-cold phosphate buffered-saline (PBS) and then incubated 15 min at 30°C in ice-cold phosphate buffered-saline (PBS) and then incubated 15 min at 30°C in ice-cold phosphate buffered-saline (PBS) and then incubated 15 min at 30°C in 100 mM Tris-HCl, pH 9.4, and 10 mM dithiothreitol. Tissue was again washed in ice-cold PBS and then washed in ChIP buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES, pH 6.5) and ChIP buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES, pH 6.5) and then resuspended in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1) with protease inhibitor cocktail. The sonicated samples were spun for 10 min at maximum speed in a microcentrifuge to remove debris. An aliquot was taken at this point for evaluation of DNA fragmentation and for use as a positive control for PCR analysis. Remaining samples were divided into three equal parts and brought to 1 ml with cold (4°C) dilution buffer. Fifty microliters of salmon sperm DNA/protein A agarose (Upstate Biotechnology, Charlottesville, VA) was added to each sample and rocked at 4°C for 2 h to immunoprecipitate the solutions. Samples were spun at maximum speed, and supernatants were transferred to new tubes.

Results

Identification of the Sp1-Like Sites in the Promoter of the Human CYP2F1 Gene. Although no TATA box element was found within the vicinity of the transcription start site (Fig. 1), sequence analysis revealed a cluster of GT-rich boxes. Since Sp1 and Sp3 have been shown to bind to these boxes, and the GT-rich domains located close to the CYP2F1 transcription start site are similar to the reported Sp1/Sp3 binding sites (Boisclair et al., 1993; Suske, 1999; Jiang et al., 2002), we performed supershift EMSA using anti-Sp1 or anti-Sp3 antibodies to map the regions that might interact with the Sp1/Sp3 proteins. Four double-stranded oligonucleotides, derived from the CYP2F1 promoter, each containing a putative Sp1-like site (Table 1), were synthesized and preincubated with [\( ^{32} \)P]ATP. A consensus Sp1 probe was provided in the gel shift assay system (Promega). Supershift EMSA was conducted by incubating nuclear extracts from A549 cells and HepG2 cells (Figs. 2 and 3, respectively) with the radiolabeled Sp1-like probes (Sp A, Sp B, Sp C, and Sp D) in the presence of anti-Sp1 or anti-Sp3 antibodies or a 100-fold molar excess of unlabeled double-stranded oligonucleotide. Three major protein-DNA complexes were observed with all four Sp1 probes in the EMSA assays with A549 cell extracts. The slower mobility bands were blurred (due to overexposure) in Fig. 2 because the film had to be exposed for a longer time to observe the supershifted bands. When these same gels were exposed for shorter times to the films, the slower mobility bands were distinct (data not shown). Preincubation of anti-Sp1 antibody

Chromatin Immunoprecipitation. Chromatin immunoprecipitation was performed as described previously (Shang et al., 2000) with some modifications. Approximately 200 mg of human lung or liver tissue was pulverized in liquid nitrogen and then transferred immediately to a 1% formaldehyde solution at room temperature and fixed for 15 min. Fixed tissue was washed twice in ice-cold phosphate buffered-saline (PBS) and then incubated 15 min at 30°C in 100 mM Tris-HCl, pH 9.4, and 10 mM dithiothreitol. Tissue was again washed in ice-cold PBS and then washed in ChIP buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES, pH 6.5) and ChIP buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES, pH 6.5) and then resuspended in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1) with protease inhibitor cocktail. The sonicated samples were spun for 10 min at maximum speed in a microcentrifuge to remove debris. An aliquot was taken at this point for evaluation of DNA fragmentation and for use as a positive control for PCR analysis. Remaining samples were divided into three equal parts and brought to 1 ml with cold (4°C) dilution buffer. Fifty microliters of salmon sperm DNA/protein A agarose (Upstate Biotechnology, Charlottesville, VA) was added to each sample and rocked at 4°C for 2 h to immunoprecipitate the solutions. Samples were spun at maximum speed, and supernatants were transferred to new tubes.
with nuclear factors abrogated the formation of the slowest mobility complex when all four probes (Sp A, Sp B, Sp C, and Sp D) were used. Anti-Sp1 antibody did not affect the migration of the other two major protein-DNA complexes with any of the four Sp1-like elements and the A549 nuclear extracts. Anti-Sp3 antibodies decreased the densities (lane 3 with all four probes) of the highest mobility complex for all four Sp1-like elements and the complex with intermediate mobility for several of the probes. However, the band shifts of the intermediate mobility complex with the anti-Sp3 antibody were ambiguous in Figs. 2, B and D, and 3C. Thus, Sp1 immunoreactive nuclear protein seemed to bind to all four Sp1-like sites to produce one predominant protein-DNA complex, and Sp3 nuclear proteins seemed to form complexes with most of the Sp1 sites to produce two distinct protein-DNA complexes.

In contrast to what was observed with lung cell nuclear extracts, when HepG2 cell nuclear extracts were used, only two major complexes were observed with any of the four probes, and addition of anti-Sp1 antibody to the binding reaction failed to either inhibit the formation or further retard the mobilities of the two bands (Fig. 3). Therefore, HepG2 cells did not seem to contain sufficient nuclear Sp1 protein capable of complexing with the CYP2F1 Sp1-like sites, particularly compared with the presence of these transcription factors in A549 cells. When anti-Sp3 antibody was preincubated with nuclear proteins from HepG2 cells, before the binding reactions, the two specific protein-DNA complexes disappeared, and supershifted bands were observed with all four probes. However, binding of putative Sp3-like factors to the Sp D site was considerably lower than the other sites when using HepG2 cell nuclear extract. Neither a 100-fold molar excess of the unlabeled probe nor the consensus Sp1/3 probe was able to effectively compete for binding to the radioactive DNA probe.
the Sp A element and with less affinity than the Sp C element. The seemed to bind the A549 nuclear proteins with higher affinity than probes (Table 1). As shown in Fig. 4, the consensus Sp1/3 probe GT-rich boxes in all four Sp1/3 double-stranded oligonucleotide critical 2-, 3-, and 4-positions (Kriwacki et al., 1992) in the functional assays, we mutated three bases corresponding to the boxes and to make mutants that would abolish GT-box binding for binding. To study the specificity of Sp1/Sp3 binding to these GT Gs at positions 2, 3, 4, and 6 in GC boxes are critical for Sp1 oligonucleotide failed to compete for DNA binding. [Image 44x396 to 296x737]

FIG. 4. Competitive EMSA analysis show all four Sp1-like sites formed specific complexes with A549 cell protein factors. Nuclear extracts prepared from A549 cells were preincubated with 25-, 50-, and 100-fold molar excess of the unlabeled probes (listed in Table 1) at RT for 15 min. After the preincubation, the 32P-labeled oligonucleotides probe Sp A, Sp B, Sp C, or Sp D (0.005–0.01 pmol) was added and incubated at RT for another 20 min. The protein-DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel. The gel was dried and then exposed to film overnight. The positions of the three relevant protein-DNA complexes are indicated with arrows, but multiple additional Sp1/3-specific complexes with higher mobilities can be noted.

not shown). However, a 500-fold molar excess of the unlabeled specific probe, or the consensus Sp1/3 probe, disrupted the protein-DNA complexes, whereas the nonspecific probe did not abolish the DNA-protein complex (Fig. 3). Thus, it seems that a partially selective Sp3-like protein that differs from the protein in A549 cells that bound to the Sp D site exists in HepG2 cells. This difference in binding efficiency among the four probes was confirmed in Fig. 4. Using A549 cell nuclear extracts, competition was observed with the addition of 25-, 50-, or 100-fold molar excess of unlabeled probe or the consensus Sp1/3 oligonucleotide. Addition of the nonspecific AP1 mut oligonucleotides probes Sp A mut (lane 2), Sp B mut (lane 4), Sp C mut (lane 6), or Sp D mut (lane 8). The protein-DNA complexes were resolved on a 4% nonadenaturing polyacrylamide gel. The gel was dried and then exposed to film overnight. Probe sequences are listed in Table 1. The positions of the three relevant protein-DNA complexes are indicated with arrows.

relative binding affinities of the consensus Sp1/3 probe with the Sp B and Sp D elements seemed to be similar. Mutation of the Sp1-like sites in all four probes abolished competition for protein binding with the corresponding Sp1 probes. In additional experiments both wild-type and mutated Sp1-like probes were radiolabeled for EMSA analysis using A549 nuclear extracts. As shown in Fig. 5, no strong protein-DNA complexes were detected using the mutated Sp1-like probes, indicating that these mutations effectively abolished protein/DNA binding and eliminated the possibility that new, unforeseen binding sites were introduced with these mutations that might lead to artifacts in the data. We also used antibodies against LKLF for supershift experiments with the Sp1 probes, and we did not detect any supershifted bands, or a decrease in protein-DNA complex density, when either A549 or HepG2 nuclear extracts were used (data not shown). This result suggested that LKLF probably does not participate in tissue-specific transcription of CYP2F1 in this promoter region. However, we have not addressed the possibility that LKLF might regulate CYP2F1 through interactions with more distal elements of the 5’-upstream promoter region.

The Sp1-Like Sites (Sp A, Sp B, Sp C, and Sp D) Drive Luciferase Reporter Gene Expression in A549 Cells but Not in HepG2 Cells To examine the role of these GC- and GT/CACC-rich boxes in regulating CYP2F1 promoter activity, we conducted transfection experiments in both A549 cells and HepG2 cells using CYP2F1 promoter-driven luciferase reporter constructs in which the Sp1-like sites were consecutively deleted, i.e., construct −141/−115 removed Sp1 D; −70/+115 removed Sp1 D and Sp1 C; −50/+115 removed Sp1 D, Sp1 C, and Sp1 B; and −19/+115 removed all Sp1 sites. As shown in Fig. 6, when each Sp1-like site was removed, the activity in A549 cells was decreased by about the same amount, consistent with an additive effect of each element. However, CYP2F1 promoter-driven reporter activity was effectively nonexistent in HepG2 cells with any of the constructs. The reporter activity of the construct that eliminated all four Sp1 sites was identical to that of pGL3/basic vector in HepG2 cells, despite the fact that transfection efficiency was much higher in HepG2 cells than in A549 cells.
from the corresponding HepG2 readings (pHepG2 cells using the wild-type pGL3/
mutBC, and mutABC. All of the mutants were sequenced to confirm
EMSA assays (Table 1), namely, mutA, mutB, mutC, mutD, mutAB,
used for the double-stranded oligonucleotides that were used in the
pGL3/H11001
115 construct. The mutations were the same as those
292/
pGL3/H11002
pEVR2/Sp1 expression vector that was used for the construct that
promoter sequences were compared with a promoter-less basic construct. The positions
of the deletion constructs relative to the transcriptional start site are marked.
Putative Sp1-like sites are indicated by boxes. Transient transfection studies were
performed in A549 lung cells (open columns) or HepG2 liver cells (shaded columns), to
investigate the function of the four putative Sp sites. Reporter constructs (0.1 µg) and internal control pRL-TK (0.001 µg) were transfected using 3:1 (microliters per microgram) ratio of FuGENE 6 (Roche Diagnostics) as described
under Materials and Methods. Cells were lysed 36 h post-transfection, and luciferase activities were assayed using the Promega dual luciferase assay. Firefly luciferase activity was normalized for transfection efficiency using the internal control Renilla luciferase activity and calculated as fold luminescence over the
promoter-less pGL3 activity. The data are presented as fold luminescence ± S.E., n = 3, * indicates significantly different from basic; # indicates statistically different from the corresponding HepG2 readings (p < 0.05).

(positive control data not shown). This result is consistent with our previous report (Carr et al., 2003).

To investigate the function of the individual Sp1-like sites in detail, we
mutated each Sp1-like site alone or in several combinations in the
gpGL3/−292/+115 construct. The mutations were the same as those
used for the double-stranded oligonucleotides that were used in the
EMSA assays (Table 1), namely, mutA, mutB, mutC, mutD, mutAB, mutBC, and mutABC. All of the mutants were sequenced to confirm
their identity. We conducted cotransfection experiments in A549 and
HepG2 cells using the wild-type gpGL3/−292/+115 construct and a
series of mutant constructs alone or with the empty pEVR2 vector or the pEVR2/Sp1 expression vector that was used for the construct that
contained the Sp1 cDNA. As shown in Fig. 7A, the wild-type gpGL3/−292/+115 vector increased luciferase activity 4-fold compared with
gpGL3/basic in A549 cells, whereas mutA (Sp A mutant), mutAB (Sp A and Sp B were mutated simultaneously), and mutABC (Sp A, Sp B, and Sp C were mutated simultaneously) effectively ablated luciferase activity. However, single mutations at the Sp B, C, or D sites were
much less effective at decreasing activity. We cannot explain why the
mutAC did not ablate activity, since all other constructs with mutations
in the Sp A motif produced dramatic reductions in the abilities
to drive reporter activity. It seems feasible, however, that this mutation might have inhibited the effects of a negatively acting interaction. However, the combined results were still highly consistent with a
conclusion that the Sp A site is the most important of the four motifs.

When these wild-type and mutated reporter constructs were
cotransfected with an Sp1 expression vector, the activities for all constructs were significantly enhanced. The activity of the wild-type
construct with overexpression of Sp1 protein was increased by almost
10-fold, with the lowest induction seen in all constructs containing
any combination of the Sp A mutation, alone or with other mutated
sites. These increases may have been caused by a lack of saturation of
the Sp1-like sites with Sp1 protein in normal cells, which was satu-
rated with excess Sp1 protein by transfection of the Sp1 construct. In
the presence of excess Sp1, mutation of the B, C, and D sites have a
similar small decrease in expression, whereas mutation of the A site
has a more dramatic effect. With conulation of the A, B, and C sites,
the D site seems to be sufficient to stimulate expression but only in
the presence of overexpressed Sp1.

In HepG2 cells (Fig. 7B), similar to previous experiments, the
wild-type construct showed no activity when transfected alone nor
did any of the mutations produce appreciable increases in reporter
activity. However, cotransfection of the normal reporter construct
with the Sp1 expression vector induced luciferase activity by approximately 5-fold. Mutations of the Sp A, B, C, and D sites did not decrease activity. Cotransfection of sites A, B, and C did result in approximately 5-fold. Mutations of the Sp A, B, C, and D sites did with the Sp1 expression vector induced luciferase activity by 1250 WAN ET AL.

Cotransfection of the CYP2F1 Reporter Construct with an Sp1 Expression Construct Increases Luciferase Activity, whereas Cotransfection with an Sp3 Expression Construct Has No Effect by Itself, or Abrogates Sp1-Mediated Increases. To identify the role of Sp1 and Sp3 trans-acting factors in regulating CYP2F1 promoter activity, we conducted cotransfection experiments in A549 cells using the CYP2F1 promoter-driven luciferase reporter construct, plus the empty expression vectors or constructs encoding Sp1 or Sp3 cDNAs. As shown in Fig. 8A, cotransfection of A549 cells with the −292/ +115 reporter construct and an Sp1 expression construct induced luciferase gene expression by 10-fold, but coexpression with the Sp3 factor produced only about 2-fold increases, which were similar to coexpression with the empty vector. When Sp1 and Sp3 proteins were cotransfected together, the induction by Sp1 was significantly decreased by the Sp3 factor.

The Sp-deficient Drosophila SL-2 cell line was also used to study the function of Sp1 and Sp3. As shown in Fig. 8B, cotransfection of SL-2 cells with the −292/+115 construct and Sp1 expression vector induced luciferase gene expression by 13-fold. Cotransfection with the Sp3 expression vector did not induce luciferase gene expression. When Sp1 and Sp3 were cotransfected together, the induction effect on luciferase gene expression by Sp1 was completely abrogated by Sp3. These results imply that, by itself, Sp3 can induce transcription of CYP2F1 but to a much lesser extent than that of Sp1. In a cellular context where multiple Sp proteins are expressed, Sp3 can compete for binding with the more potent Sp1 and thus has a net effect of repressing expression.

Sp1/Sp3 Protein Ratio Was Higher in A549 Cells Than in HepG2 Cells. Because in our previous report (Carr et al., 2003) and in this study, CYP2F1 promoter reporter constructs showed no activity in HepG2 cells, but cotransfection with Sp1 could induce the luciferase gene expression, we hypothesized that the Sp1/Sp3 ratio might be low in HepG2 cells. Therefore, Western blot analyses were performed to detect the expression levels of Sp1 and Sp3 proteins in A549 and HepG2 cell nuclear extracts. Two distinguishable bands with molecular masses at about 106 and 93 kDa were detected using the anti-Sp1 antibody in both A549 and HepG2 nuclear extracts (Fig. 9A). According to the company data sheet, this antibody should detect 105- and 95-kDa Sp1 isoforms. The Sp1 protein level in A549 cells was 2.5 times higher (by densitometry analysis) than the expression in HepG2 cells. When the anti-Sp3 antibody was used, two bands with molecular masses of 98 and 67 kDa, which were close to the putative 100- and 60-kDa proteins from the product literature, were detected in both A549 and HepG2 cells. The Sp3 protein level in HepG2 cells was approximately 1.5 times higher (by densitometry analysis) than the expression in A549 cells. Therefore, the relative Sp1/Sp3 ratio in A549 cells was at least 3.5 times higher than that in HepG2 cells. Additional Western blot analyses were performed to detect the expression levels of Sp1 and Sp3 proteins in human lung and liver nuclear extracts (Fig. 9B). The 95-kDa Sp1 level was present at high levels in human lung tissue extracts, but it was much lower in liver nuclear extracts. Although Sp1 protein was not observed in the blot, when longer film exposure times were used, the 95-kDa band for human liver extracts were observed, although at much lower levels than lung extracts. It was interesting to note that the 105-kDa post-translational modification product (phosphorylation and glycosylation) of Sp1 was not detected in either lung or liver nuclear proteins. When using the Sp3 antibody, only one band with a molecular mass of 60 kDa was detected in liver. Conversely to Sp1, Sp3 immunoreactive protein was not detected in lung tissues in the blot shown in Fig. 9B. However, when longer film exposure times were used this 60-kDa
was used as the template. For negative controls (purified DNA from the chromatin that was sampled before immunoprecipitation) a pair product was detected via ultraviolet illumination. For positive controls (product was run on a 1.8% agarose gel containing ethidium bromide and a 310-base templates for detection of the

liver tissues were immunoprecipitated with antibodies against Sp1 or Sp3. DNA proteins based on the original autoradiogram. The antibodies should (according to each frame. The arrows show calculated molecular weights of putative Sp1 or Sp3

immunoblotting was performed by incubating membranes with Sp1 or Sp3 purified polyclonal anti-peptide antibodies from Santa Cruz Biotechnology, Inc. Immunoreactive proteins were formed by incubating membranes with Sp1 or Sp3 purified polyclonal anti-peptide antibodies from Santa Cruz Biotechnology, Inc. Immunoreactive proteins were detected using peroxidase-conjugated secondary antibodies with chemiluminescence detection. Molecular weight marker mobilities are shown on the right edge of

performed using antibodies for Sp1 and Sp3 (Fig. 9C). The

expression in lung cells (Rundlof et al., 2001), was also precipitated from lung chromatin with the Sp1 antibody but not the Sp3 antibody.

band was faintly present. The 100-kDa alternatively spliced form of Sp3 (G. Suske, personal communication) was not detected in either tissue.

To confirm that different transcription factors occupied these sites in human liver and lung tissues, chromatin immunoprecipitation was performed using antibodies for Sp1 and Sp3 (Fig. 9C). The CYP2F1 promoter region was precipitated from lung chromatin with the Sp1 antibody but not the Sp3 antibody. Conversely, an Sp3 antibody precipitated this same promoter region from liver chromatin, whereas an Sp1 antibody failed to do so. The thioredoxin reductase 1 gene proximal promoter, which has also been shown to depend on Sp1 for expression in lung cells (Rundlof et al., 2001), was also precipitated from lung chromatin with the Sp1 antibody but not the Sp3 antibody (data not shown), suggesting that Sp1 or Sp3 relative levels play a role in pulmonary expression of additional genes.

Discussion

This work described studies on the mechanisms regulating constitutive expression of CYP2F1 in lung cells. Specifically, the proximal promoter region was analyzed and shown to have basal promoter activity in A549 lung cells. Sequence analysis of this region identified four putative GC- and GT/CACC-boxes that are putative Sp1 binding sites. In TATA-less promoters, Sp1 often regulates constitutive promoter activity; therefore, we examined whether the Sp1 protein, or the highly homologous Sp3 protein, could bind the CYP2F1 promoter elements and regulate constitutive expression. These studies show that all four sites are able to bind both Sp1 and Sp3, that they are important in regulating expression of CYP2F1, and that the relative levels of Sp1 and Sp3 protein play a vital role in the tissue-specific expression of this gene.

The ubiquitous transcription factor Sp1 is well characterized and is involved in the expression of many genes, including structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors, and signaling receptors (Philipsen and Suske, 1999; Li et al., 2004). Although Sp1 is expressed in most cell types, its expression levels are altered during development and can vary in different cell types (Saffer et al., 1991; Lania et al., 1997; Suske, 1999). It is also the founding member of a redefined family of transcription factors, known as Sp/XKLF proteins, that contain three highly homologous C-terminal zinc-finger motifs and are capable of binding similar DNA sequences (Philipsen and Suske, 1999). Any combination of Sp/XKLF proteins may compete or interact for a given promoter element to either activate or repress transcription, often in a tissue-specific manner. Therefore, it is difficult to define the function of Sp1, because of the potentially redundant or antagonistic actions of its related family members. However, Sp1 is essential for embryogenesis, because Sp1−/− mouse embryos display growth retardation and die during gestation (Marin et al., 1997).

Sp3, also a member of the Sp/XKLF transcription factor family, is closely related to Sp1. It is coexpressed in a variety of tissues that also express Sp1, and it has an affinity comparable with that of Sp1 for responsive elements. However, Sp3 can function as either a transcription activator or repressor by binding to and competing with Sp1 for the transcription regulatory sites (Hagen et al., 1994; Conn et al., 1996; Ihn and Trojanowska, 1997; Majello et al., 1997; Ghayor et al., 2001). The experimental conditions under which Sp3 functions as a repressor are not fully understood. However, it has been shown that the ratio of Sp1/Sp3 can sometimes determine whether Sp3 acts as an activator or a repressor of gene expression (Suske, 1999).

Specific binding of the Sp1 and Sp3 proteins was determined for each of the Sp1-like sites investigated. An EMSA binding pattern consisting of one Sp1/DNA band and two Sp3/DNA bands are often seen with cis-elements that bind Sp1/Sp3 proteins (Dennig et al., 1995; Marin et al., 1997; Ghayor et al., 2001). This is interesting, since all four Sp1-like sites in CYP2F1, albeit GC-rich, are still very different from each other and from the perfect Sp1 consensus sequence (GGGGCCGGG). Conversely, each Sp1-like site possesses considerable similarity to GT/CACC-boxes that bind other Sp1 family factors. Therefore, future studies should investigate whether other members of the Sp1 family of factors bind to the Sp1-like sites of CYP2F1.

The BTEB factors, which are members of the Sp/XKLF family of factors, have been shown to regulate the expression of the CYP1A1 gene. Usually, CYP1A1 expression in the lung is low, but it is highly inducible by compounds found in cigarette smoke. It was demon-
strated that BTEB3 and BTEB4 bind and repress the CYP1A1 promoter, even in the presence of inducer (Kaczynski et al., 2002). Another Sp/XKLF family factor, the GKL, binds to the CYP1A1 promoter BTE and inhibits CYP1A1 promoter activity in transient transfection assays (Zhang et al., 1998), demonstrating the cooperativity of Sp/XKLF factors. GKL was also demonstrated to inhibit Sp1-mediated CYP1A1 promoter activation and enhance Sp3-dependent CYP1A1 promoter suppression by physical interaction with the Sp1 and Sp3 proteins, respectively. The CYP2F1 proximal promoter displays lung cell-specific activation and binds Sp1 and Sp3. Therefore, interaction of LKLF or GKL with Sp1 or Sp3 may be related to CYP2F1 pulmonary-specific expression. However, our studies did not support this hypothesis, because the LKLF antibody did not produce a supershifted band in our EMSA studies with lung cell extract.

Activation of the CYP2F1 proximal promoter was demonstrated using cotransfection studies with Sp1 and/or Sp3 expression vectors. These studies showed that overexpression of Sp1 resulted in a significant enhancement of luciferase reporter activity with all the deletion constructs, whereas Sp3 was ineffective at trans-activation of the CYP2F1 proximal reporter. Sp3 was an efficient inhibitor of Sp1-mediated activation, however, demonstrating that Sp3 functions by competing with Sp1 for the Sp/XKLF-like CYP2F1 consensus sites. Surprisingly, Sp3 did not seem to repress normal transcription levels, since overexpression did not reduce reporter activities below basal levels. In fact, a slight enhancement of reporter activity was observed in A549 cells when Sp3 was overexpressed. This is intriguing because one would expect that Sp3 overexpression would inhibit binding of endogenous Sp1. An activation of transcription by Sp3 protein in the absence of copious amounts of Sp1 protein has been observed previously, despite the fact that Sp3 inhibits Sp1-mediated activation and is a common transcription regulatory mechanism (Hagen et al., 1994; Dennig et al., 1995; Majello et al., 1997). To precisely evaluate transcriptional activation in an Sp-deficient background, studies were performed with Drosophila SL-2 cells. These results confirmed that Sp1 is capable of trans-activating CYP2F1 reporter constructs, and in these cells, Sp3 abolished Sp1-mediated activation. Also, it seemed that basal promoter-driven luciferase activity was decreased by overexpression of Sp3, implying that repression of normal cellular transcription was caused by the Sp3 protein in these Sp-deficient cells. However, these results are not consistent with the activation of luciferase by overexpression of only Sp3 in A549 cells, which may be caused by the complex effects of several Sp proteins acting on the same Sp1-like motifs in the promoter region of CYP2F1.

In TATA-less promoters, mutation of Sp1 sites located near transcription initiation sites decreases the extent of transcription and alters the relative usage of alternative initiation sites, suggesting that Sp1 is involved both in the activation of transcription and in positioning the relative usage of alternative initiation sites, suggesting that Sp1 is a critical factor for lung-specific CYP2F1 expression and perhaps for many other lung-specific genes as well.

In summary, this study provides significant evidence for the contribution of the ubiquitous factors Sp1 and Sp3 to the mechanisms that govern CYP2F1 basal promoter activity in lung epithelial cell lines and establish a molecular basis for the regulation of pulmonary-specific CYP2F1 gene expression. Four proximal promoter elements that contain the Sp1-like motif seem to regulate organ-selective CYP2F1 gene transcription. In lung cells, increased transcription occurs because of the high levels of Sp1 protein factors that enhance transcription and the low levels of Sp3 protein factors that repress transcription.

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References


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