SP1 AND SP3 REGULATE BASAL TRANSCRIPTION OF THE HUMAN CYP2F1 GENE

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Abbreviations used are: P450, cytochrome P450 protein; LSF, lung-specific factor; C/EBP, CCAAT enhancer binding protein; TSS, transcription start site; EMSA, electrophoretic mobility shift assay; BTE, basic transcription element; BTEB, basic transcription element binding protein; XKLF, Krüppel-like transcription factors; LKLF, lung-enriched Krüppel-like transcription factor; RT, room temperature.
ABSTRACT

Selective transcription of the human \textit{CYP2F1} gene in lung tissues may control the susceptibilities of this organ to diverse pneumotoxicants and lung carcinogens. However, the mechanisms responsible for \textit{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 \textit{CYP2F1}. Four putative Sp1-like sites were identified in the \textit{CYP2F1} promoter. Competitive EMSA 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 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. Co-transfection of a Sp1 expression vector with the reporter construct dramatically increased luciferase activity in either A549 cells or Sp1-deficient \textit{Drosophila} SL-2 cells. However similar cotransfections with an Sp3 expression vector failed to increase activity. Co-transfection 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 \textit{CYP2F1} gene transcription, and that Sp1 and Sp3 factors interact to modulate constitutive \textit{CYP2F1} transcription in lung cells.
INTRODUCTION

Lung cancer and other chronic lung diseases may be caused by the bioactivation of procarcinogens or protoxicants by cytochrome P450 enzymes that are selectively expressed in respiratory tissues (Ding and Kaminsky, 2003; Yost, 2001). 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, which 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 (TGFβ-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 (Kaczynski et al., 2002; Zhang et al., 1998). These studies showed the role of BTEB and 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 C/EBP proteins in rat liver control selective transcription of the \( \text{CYP2B1} \) gene, and NF1-like factors regulate \( \text{CYP1A2} \) (Zhang et al., 2000) and \( \text{CYP2A3} \) (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 \( \text{CYP4B1}, \text{CYP2S1}, \text{CYP3A5}, \) or \( \text{CYP2F1} \).

The human lung CYP2F1 enzyme catalyzes the bioactivation of many potent environmental toxicants such as 3-methylindole (Lanza and Yost, 2001), naphthalene (Lanza et al., 1999), styrene (Nakajima et al., 1994), 1,1-dichloroethylene (Simmonds et al., 2004), and benzene (Powley and Carlson, 2001; Sheets et al., 2004). For many of these toxicants, the selective damage that occurs in specific lung cells is caused by the specific expression of the \( \text{CYP2F1} \) gene [and activity of the gene product (Nichols et al., 2003)], without concomitant expression in other organs such as liver and kidney. Thus, an important objective in our research has been to characterize the mechanisms responsible for selective transcriptional regulation of P450 genes in human lung cells.

Previous studies from our laboratory (Carr et al., 2003) have demonstrated that a novel lung-specific binding motif exists between \( \text{CYP2F1} \) position -182 to -152. Nuclear proteins from human lung tissues and cells bind specifically to this putative regulatory region, whereas hepatic nuclear proteins did not form protein-DNA complexes with this motif. Binding to this element alone was not solely responsible for lung-specific promoter activation, because a minimal basic promoter region (from position -129 to -1, which did not contain the LSF) drove reporter gene
activities in A549 human lung alveolar cells or BEAS-2B lung epithelial cells, but did not support luciferase activity in HepG2 human liver-derived cells. However, larger constructs that incorporated the LSF-binding motif did not enhance promoter activity in BEAS-2B or A549 lung cells. It is possible, however, that these studies did not incorporate sufficient upstream elements which might contribute in a combinatorial manner to enhance LSF function. Therefore, because a functional property for the LSF-binding domain could not be identified in these specific lung cells, additional studies were needed to determine which cis-acting elements control lung-selective transcriptional activation in the CYP2F1 minimal promoter.

EXPERIMENTAL PROCEDURES:

**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 (Hagen et al., 1994) (Sapetschnig et al., 2002), Philipps-University Marburg, Germany. 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 (Santa Cruz, CA). The pGL3 luciferase reporter vectors and Dual-Luciferase Reporter Assay system were purchased from Promega (Madison, WI). The *Drosophila* SL-2 cells were kindly provided by Dr. Terry Elton, Brigham Young University, Utah. 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 maxi 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, Canada). Rabbit serum against Lung Krüppel-like Factors (LKLF) was
kindly provided by Dr. Landy Kangaloo, Harvard School of Public Health. Human lung tissue
was obtained from Rocky Mountain Donor Services. Human Liver tissue was the generous gift
of Dr. Michael Franklin.

**Cell Culture**—A549 and HepG2 cells were obtained from American Type Culture
Collection (ATCC, Manassas, VA) and cultured as described by the supplier. Briefly, A549
cells were maintained with Dulbecco’s modified Eagle's medium/nutrient mixture F12 containing
10% fetal bovine serum. HepG2 cells were cultured in Dulbecco's modified Eagle's medium
supplemented with 10% fetal bovine serum and 10 mM sodium pyruvate. Drosophila SL-2 cells
were cultured in Schneider’s medium supplemented with heat-inactivated 10% fetal bovine
serum at 25°C as described in Zhao, et al. (Zhao et al., 2001).

**Preparation of Nuclear Extracts**—Nuclear extracts from cultured cells were prepared as
described previously (Carr et al., 2003). Protein concentrations of all nuclear extracts were
determined using the Bio-Rad Protein Assay Kit I.

**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 DTT, 250 mM NaCl, 0.25
mg/ml poly(dI-dC), 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% non-denaturing 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 assays, representing the consensus sequences for nonspecific AP1 and Sp1/3 were supplied in the Promega Gel Shift Assay System. EMSA supershift assays were performed with antibodies to Sp1 and Sp3 as previously described (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 (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 previously described (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 Molecular Biochemical) in 96-well
plates. In co-transfection 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 assayed 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 ± SE. Results were assessed using a single factor ANOVA. 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 co-transfected with 0.3 µg of the listed CYP2F1 reporter constructs and 0.3 µg of the pPac/Sp1 expression vector, 0.3 µg of the pPac/USp3 expression vector, or 0.3 µg of the pPac empty vector as described previously (Jiang et al., 2002). The empty control vector pPac (0.3-0.6 µg) was added to keep the total amount of plasmid DNA constant at 0.9 µg for each transfection. In addition, each transfection included 0.006 µg of pRL-TK, the internal control plasmid. The DNA and FuGENE 6 were added, at a 3:1 ratio (µL FuGENE 6: µg DNA), to Hank’s 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 ± SE relative to the promoter-less pGL3 basic vector.

**Western Blotting**—Western blotting was performed as described (Wang et al., 1998) with some modifications. Briefly, samples (cell and tissue 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 either 7% or 10% SDS-polyacrylamide gel electrophoresis for tissues or cells, respectively, at 100V for 1-2 h. For cells, proteins were transferred to PVDF membrane and the membrane was blocked with 1XPBST (1X phosphate buffered-saline and 0.1% Tween) containing 2% 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 1XPBST containing 0.5% nonfat milk at 4°C. Following the overnight incubation, the membrane was rinsed four times with 1XPBST and was then incubated with HRP-conjugated second antibody, diluted 1:1000 in 1XPBST containing 1% nonfat milk for 1 h at room temperature. The secondary antibody was removed and the membrane was washed four times with 1XPBST. For tissues, proteins were transferred to PVDF membrane and the membrane was blocked with 1XPBST 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 1XPBST containing 0.25% nonfat milk at 4°C. Following the overnight incubation, the membrane was rinsed four times with 1XPBST and was then incubated with affinity purified peroxidase-conjugated second antibody purchased from ICN (Catalog #55689), diluted 1:1200 (Sp1) or 1:2000 (Sp3) in 1XPBST containing 0.25% nonfat milk (Sp1) or 0.5% milk plus 1.25% FBS (Sp3) for 1 h at room temperature. The secondary antibody was removed and the membrane was washed four times with 1XPBST. The PVDF membrane was developed with SuperSignal West Pico Trial Kit (Pierce, Rockford, IL) at room temperature and exposed to Kodak film. The densities of the bands were analyzed by NIH image version 1.61.

Chromatin Immunoprecipitation—Chromatin Immunoprecipitation was performed as described (Shang et al., 2000) with some modifications. Approximately 200 mg of human lung
or liver tissue were pulverized in liquid nitrogen, then transferred immediately to a 1% formaldehyde solution at room temperature and fixed for 15 minutes. Fixed tissue was washed twice in cold phosphate buffered-saline (PBS), then incubated 15 minutes at 30°C in 100 mM Tris HCl pH 9.4 and 10 mM dithiothreitol. Tissue was again washed in ice cold PBS, then washed in ChIP buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), then ChIP buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), then resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitor cocktail. Tissue was sonicated 10x 10 seconds with a W-220 Ultrasonic Processor (Heat-System Ultrasonics, Inc. Plainview, NY). Sonicated samples were spun for 10 minutes 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 in 3 equal parts and brought to 1 mL with cold dilution buffer. 50 µL of salmon sperm DNA/protein A Agarose (Upstate Biotechnology, Lake Placid, NY) was added to each sample and rocked at 4°C for 2 hours to immunoclear the solutions. Samples were spun at maximum speed and supernatants transferred to new tubes. Samples were immunoprecipitated overnight rocking at 4°C with antibodies against either Sp1, Sp3 or no antibody. 50 µL of salmon sperm DNA/protein A Agarose was then added to each sample and rocked for 1 hour at 4°C. Samples were spun at maximum speed and supernatants removed. Beads were washed for ten minutes subsequently with TSE I (150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100), TSE II (500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100), and Chip buffer III (0.25 M LiCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1, 1% NP-40, 1% deoxycholate,). They were then washed 3 times with Tris HCl EDTA pH 8.0 buffer and eluted 3 times with 50 µL of elution buffer.
Eluants and genomic aliquots were incubated over night at 65°C to reverse crosslinking then DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). PCR analysis was carried out using oligonucleotides 5’TACCCCAAAACCAGGCCCCAGGC and 5’GCTGGGATGCGCTAGAGCCTGC and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA).

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 TSS (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 TSS are similar to the reported Sp1/Sp3 binding sites (Boisclair et al., 1993; Jiang et al., 2002; Suske, 1999), 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 end-labeled with [γ-32P]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 (Fig. 2 and Fig. 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 are blurred (due to overexposure) in Fig. 2 because the film had to be exposed for a longer time period to observe the supershifted bands. When these same gels were exposed for shorter times
to the films, the slower mobility bands were quite distinct (data not shown). Preincubation of anti-Sp1 antibody 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 Fig 2B and 2D and Fig 3C. Thus, Sp1 immunoreactive nuclear protein appeared to bind to all four Sp1-like sites to produce one predominant protein/DNA complex, and Sp3 nuclear proteins appeared 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 appear to contain sufficient nuclear Sp1 protein capable of complexing with the CYP2F1 Sp1-like sites, particularly when compared to the presence of these transcription factors in A549 cells. When anti-Sp3 antibody was preincubated with nuclear proteins from HepG2 cells, prior to 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 were able to effectively compete for binding to the radioactive DNA probe (data 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 appears 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 oligonucleotide failed to compete for DNA binding.

A model has been proposed (Kriwacki et al., 1992) in which the G’s at positions 2, 3, 4 and 6 in GC boxes are critical for Sp1 binding. In order to study the specificity of Sp1/Sp3 binding to these GT boxes and to make mutants that would abolish GT-box binding for functional assays, we mutated 3 bases corresponding to the critical 2, 3, and 4 positions (Kriwacki et al., 1992) in the GT-rich boxes in all four Sp1/3 double-stranded oligonucleotide probes (Table 1). As shown in Fig. 4, the consensus Sp1/3 probe appeared to bind the A549 nuclear proteins with higher affinity than the Sp A element and with less affinity than the Sp C element. The relative binding affinities of the consensus Sp1/3 probe with the Sp B and Sp D elements appeared 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 these mutations effectively abolished protein/DNA binding and eliminating 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 did not detect any supershifted bands, or a
decrease in protein/DNA complex density, when either A549 or HepG2 nuclear extracts were
utilized (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 (positive control data
not shown). This result is consistent with our earlier report (Carr et al., 2003).

In order 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 pGL3/−292/+115 construct. The
mutations were the same as those utilized for the double-stranded oligonucleotides that were
used in the EMSA assays (Table 1), namely mutA, mutB, mutC, mutD, mutAB, mutBC,
mutABC. All of the mutants were sequenced to confirm their identity. We conducted cotransfection experiments in A549 and HepG2 cells using the wild-type pGL3/-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 pGL3/-292/+115 vector increased luciferase activity four-fold compared to pGL3/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 saturated 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 while mutation of the A site has a more
dramatic effect. With co-mutation of the A, B, and C sites, the D site appears 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. Co-mutation of sites A, B and C did result in a decrease of expression relative to wild type (p-value=0.014) but, as with A549 cells, was not sufficient to completely eliminate response to Sp1 over-expression.

*Cotransfection of the CYP2F1 reporter construct with an Sp1 expression construct increases luciferase activity, while 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 co-expression with the Sp3 factor produced only about 2-fold increases, which were similar to co-expression 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 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 weights 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 weights 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 level 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 was much lower in liver nuclear extracts. Although Sp1 protein was not observed in the blot, when longer film exposure times were employed, 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 posttranslational 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 weight of 60 kDa, was detected in liver. Conversely to Sp1, Sp3 immunoreactive protein was not detected in lung tissues in the blot shown in Figure 9B. However, when longer film exposure times were used this 60 kDa 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 (Figure 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 while 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 reporter 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 (Li et al., 2004; Philipsen and Suske, 1999). Although Sp1 is expressed in most cell types, its expression levels are altered during development and can vary in different cell types (Lania et al., 1997; Saffer et al., 1991; 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 co-expressed in a variety of tissues that also express Sp1, and it has an affinity comparable to 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 (Conn et al., 1996; Ghayor et al., 2001; Hagen et al., 1994; Ihn and Trojanowska, 1997; Majello et al., 1997). 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; Ghayor et al., 2001; Marin et al., 1997). This is interesting, since all four Sp1-like sites in CYP2F1, albeit GC-rich, are still quite different from each other and from the perfect Sp1 consensus sequence (GGGCGGGG). 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 is highly inducible by compounds found in cigarette smoke. It was demonstrated 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 gut-lung enriched Krüppel-like factor (GKLF), 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. GKLF 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 GKLF 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 co-transfection studies with Sp1 and/or Sp3 expression vectors. These studies showed that over-expression 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 appear to repress normal transcription levels, since over-expression did not reduce reporter activities below basal levels. In fact, a slight enhancement of reporter activity was observed in A549 cells when Sp3 was over-expressed. This is intriguing as one would expect that Sp3 over-expression 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 (Dennig et al., 1995; Hagen et al., 1994; Majello et al., 1997). To precisely evaluate transcriptional activation in an Sp-deficient background, studies were performed with Drosophila melanogaster Schneider line 2 (SL-2) cells. These results
confirmed that Sp1 is capable of trans-activating CYP2F1 reporter constructs, and in these cells, Sp3 completely abolished Sp1-mediated activation. Also, it appeared that basal promoter-driven luciferase activity was decreased by over-expression 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 initiation complex (Blake et al., 1990; Boisclair et al., 1993; Chen et al., 1992). Similar to the results presented here, Chen, et al. (Chen et al., 1992) found that the introduction of mutations into any of several putative Sp1 elements in the rat transforming growth factor alpha promoter region inhibited transcription from the -58 site, with the most proximal element having the largest effect.

Tjian and co-workers (Pugh and Tjian, 1991; Ryu et al., 1999) proposed that Sp1 bound to a GC box would recruit the TFIID complex or the transcriptional cofactor complex, CRSP, through protein-protein interactions. In this model, the TATA binding protein does not bind directly to the promoter but is anchored to the DNA by a tethering protein bound to Sp1. Alternatively, the TATA binding protein has been shown to bind with low affinity to the -30 region of many TATA-less promoters and may be stabilized by Sp1 (Wiley et al., 1992).

Our combined results with several different cell lines indicate that lung-selective trans-activation of several Sp1-like sites, but predominantly the most proximal site in the CYP2F1
promoter, occurs through binding with an Sp1 factor and that repression of \textit{CYP2F1} transcription in liver cells is mediated by Sp3 binding to the same Sp1-like sites. Some investigators believe that transcription rates depend on the ratio of Sp1/Sp3 proteins, and this concept appears to be important in \textit{CYP2F1} tissue-selective expression (Li et al 2004). We demonstrated that Sp1 levels were considerably higher in A549 cells than in HepG2 cells, and the converse was true for the expression of Sp3. More importantly, it was shown that the Sp1 protein was expressed to a much higher level in human lung than in liver tissues, and Sp3 protein was expressed much higher in liver nuclear extracts than in lung. Moreover, chromatin immunoprecipitation studies provided compelling evidence that the \textit{CYP2F1} promoter is specifically bound to Sp1 in lung or to Sp3 in liver. Based on this observation and the cumulative results of our \textit{CYP2F1} promoter analysis, we conclude that the Sp1/Sp3 ratio is a critical factor for lung-specific \textit{CYP2F1} expression and perhaps for many other lung-specific genes as well.

In summary, these studies provide significant evidence for the contribution of the ubiquitous factors, Sp1 and Sp3, to the mechanisms that govern \textit{CYP2F1} basal promoter activity in lung epithelial cell lines and establish a molecular basis for the regulation of pulmonary specific \textit{CYP2F1} gene expression. Four proximal promoter elements, that contain the Sp1-like motif, appear to regulate organ-selective \textit{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


FOOTNOTES

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FIGURE LEGENDS:

Fig. 1. **Nucleotide sequence of the proximal promoter of the CYP2F1 gene.** The nucleotide sequence shown is from -293 to +115 bp, relative to the previously reported CYP2F1 transcription start site (Carr et al., 2003). The four putative Sp1-like sites: Sp A, Sp B, Sp C, and Sp D, are shaded and the sequences of the oligonucleotide probes used for EMSA are underlined and bolded. Exon 1 of the CYP2F1 gene is italicized and bolded. The major transcription start site is numbered +1.

Fig. 2. **One Sp1 and two Sp3 protein/DNA complexes are formed with A549 nuclear extracts to each of the four putative Sp1-like sites.** Double-stranded oligonucleotides derived from the CYP2F1 promoter, each containing a putative Sp1-like site, were synthesized and end-labeled with $^{32}$P-ATP. EMSA was performed by preincubating nuclear extract prepared from A549 cells with 100-fold molar excess of unlabeled/cold Sp1/3 probe (Cold P), consensus Sp1/3 probe (Cold Sp1/3), or nonspecific AP1 probe (Cold NP) at RT for 15 min. For supershift analysis, 2 µL of antibody to Sp1 (Anti-Sp1) or Sp3 (Anti-Sp3) were preincubated with nuclear extract at RT for 15 min. After the preincubation, the $^{32}$P-labeled probe Sp A, Sp B, Sp C, or Sp D (0.005-0.01 pmol) was added to the mixtures and incubated at RT for another 20 min. The protein/DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel. The gel was dried then exposed to film overnight. The positions of relevant DNA-protein complexes are indicated with arrows. The sequences of the Sp probes are listed in Table 1 and the nonspecific AP1 probe was provided with the Gel-Shift Assay System (Promega).

Fig. 3. **Only two Sp3 protein/DNA complexes are formed with liver HepG2 cell nuclear extracts to each of the four putative Sp1 sites.** Double-stranded oligonucleotides derived from the CYP2F1 promoter, each containing a putative Sp1/3 site, were synthesized and end-labeled with $^{32}$P-ATP. EMSA was performed by preincubating nuclear extract prepared from HepG2 cells with 100-fold molar excess (for Sp A, Sp B and Sp C probes) or 500-fold molar excess (for Sp D) of unlabeled/cold Sp1/3 probe (Cold P), consensus Sp1/Sp3 probe (Cold Sp1/3), or nonspecific AP1 probe (Cold NP) at RT for 15 min. For supershift analysis, 2 µL of antibody to Sp1 (Anti-Sp1) or Sp3 (Anti-Sp3) were preincubated at RT for 15 min. After the preincubation, the $^{32}$P-labeled probe Sp A, Sp B, Sp C, or Sp D (0.005-0.01 pmol) was added to the mixtures and incubated at RT for another 20 min. The protein/DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel. The gel was dried then exposed to film overnight. The positions of the relevant complexes are indicated with arrows. The sequences of the Sp probes are listed in Table 1 and the nonspecific AP1 probe was provided with the Gel-Shift Assay System (Promega).

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 $^{32}$P-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 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.

**Fig. 5. Mutations in the four Sp1-like sites abolish all protein/DNA complexes.** Nuclear extracts prepared from A549 cells were incubated for 20 min at RT with $^{32}$P-labeled wild-type (wt) oligonucleotide probes Sp A (lane 1), Sp B (lane 3), Sp C (lane 5), or Sp D (lane 7), and compared to incubations with $^{32}$P-labeled mutated (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% nondenaturing polyacrylamide gel. The gel was dried 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*.

**Fig. 6.** All four Sp1-like sites are required for maximal activation of the proximal CYP2F1 promoter in lung cells, but do not drive luciferase activities in liver cells. Five luciferase reporter-constructs containing various truncated CYP2F1 promoter sequences were compared to 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 bars) or HepG2 liver cells (shaded bars), to investigate the function of the four putative Sp sites. Reporter constructs (0.1 µg) and internal control pRL-TK (0.002 µg) were transfected using 3:1 (µl/µg) ratio of Fugene 6 (Roche) as described under “Experimental Procedures.” Cells were lysed 36 hr 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 pGLB activity. The data are presented as fold luminescence ± SE, n=3. * indicates significantly different from basic. # indicates statistically different from the corresponding HepG2 readings (p<0.05).

**Fig. 7.** The Sp A site of the CYP2F1 promoter dominates functional promoter activity in A549 cells, but neither wild-type nor Sp site mutants of the CYP2F1 promoter act as functional promoters in HepG2 cells. The pGL3/basic vector, containing the wild-type (-292/+115) or mutated (at individual or multiple Sp sites as indicated) CYP2F1 promoter constructs (100 ng), were transfected (hatched bars) into A549 cells (A) or HepG2 cells (B) with 0.05 µg of the empty pEVR2 expression vector (open bars) or the Sp1 expression construct (black bars). 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. The mutated sequences are shown in Table 1. * indicates significantly different from basic. # indicates significantly different from the corresponding white bar (p<0.05).

**Fig. 8.** Sp3 inhibits Sp1-mediated activation of CYP2F1 reporter constructs in A549 cells, and ablates Sp1-mediated activation of CYP2F1 reporter constructs in Sp1-deficient Drosophila SL-2 cells. Transient transfection studies were performed in A549 cells (A) or in Drosophila SL-2 cells (B), the cells with an Sp1-deficient background, to investigate the extent of Sp1 and Sp3 protein interactions with the CYP2F1 promoter constructs -19 to +115 (shaded bars) or -292 to+115 (solid bars). Control values (open bars) were cells transfected with
promoter-less vectors. For A549 cells, expression vectors (0.05 or 0.1 µg) containing Sp1, Sp3, or empty control vectors (pEVR2 for Sp1 and pRC/CMV for Sp3) were co-transfected with the reporter construct (0.1 µg) and internal control pRL-TK (0.002 µg), providing a constant total amount of 0.202 µg DNA/experiment, using 3:1 (µl/µg) ratio of Fugene 6 (Roche) as described under “Experimental Procedures.” For SL-2 cells, expression vectors (0.3 µg) of Sp1, Sp3, or empty control vector (pPac, 0.6 µg), were co-transfected with the reporter construct (0.3 µg) and internal control pRL-TK (0.006 µg), providing a constant total amount of 0.906 µg DNA/experiment. Cells were lysed 48 hr 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 activation over the promoter-less pGL3 basic vector. The data are presented as fold activation ± SE, n=3. * indicates significantly different from empty vector transfected. # indicates significantly different from Sp1 transfected. ¥ indicates statistically different from Sp3 transfected (p<0.05).

Fig. 9. Western blots of cellular or tissue nuclear extracts demonstrate abundant Sp1 protein in lung and abundant Sp3 protein in liver. (A) A549 and HepG2 nuclear cell extracts (20 µg/lane), or (B) human lung or liver nuclear tissue extracts (25 µg/lane for Sp1 and 10 µg/lane for Sp3) were separated on 10% or 7% Tris-HCl polyacrylamide gels and blotted to PVDF membranes. Immunoblotting was performed by incubating membranes with Sp1 or Sp3 purified polyclonal anti-peptide antibodies from Santa Cruz Biotechnology. Immunoreactive proteins were detected using peroxidase-conjugated secondary antibodies with chemiluminesence detection. Molecular weight marker mobilities are shown on the right edge of each frame. The arrows show calculated molecular weights of putative Sp1 or Sp3 proteins based on the original autoradiogram. The antibodies should (according to the manufacturer) identify proteins with the following molecular weights: Sp1 – 95 and 105 kDa; Sp3 – 60 and 100 kDa. (C) Chromatin from formaldehyde-fixed lung and liver tissues were immunoprecipitated with antibodies against Sp1 or Sp3. DNA fragments from these precipitations were then un-cross-linked, purified, and used as templates for detection of the CYP2F1 promoter region in PCR reactions. PCR product was run on a 1.8% agarose gel containing ethidium bromide and a 310 base pair product was detected via ultraviolet illumination. For positive controls (+ lane), purified DNA from the chromatin that was sampled prior to immunoprecipitation was used as the template. For negative controls (- lane), the immunoprecipitation was performed without antibodies.
Table 1.

<table>
<thead>
<tr>
<th>Oligonucleotide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Position&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>Sp D</td>
<td>5'-GGGCAGCCCCCTCCCACGGGACCTTTCCA-3'</td>
<td>-193/-164</td>
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<tr>
<td>Sp Dmut</td>
<td>5'-GGGCAGCCCCCTaaaACGGCACCTTTCCA-3'</td>
<td>-193/-164</td>
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<tr>
<td>Sp C</td>
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<td>-125/-103</td>
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<tr>
<td>Sp Cmut</td>
<td>5'-AAGATGATTttAGGATTTTAAAG-3'</td>
<td>-125/-103</td>
</tr>
<tr>
<td>Sp B</td>
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<td>-72/-49</td>
</tr>
<tr>
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<tr>
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<td>Sp1/3</td>
<td>5'-ATTCGATCGGGCGCGCGCGGCAGC-3'</td>
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<sup>a</sup> Single-stranded oligonucleotides were synthesized by IDT DNA (Coralville, IA). Oligonucleotides were resuspended (100 µM). Equal volumes of each oligonucleotide and its anti-sense oligonucleotide were annealed to form 50 µM of double-stranded oligonucleotide, by heating to 95°C then slowly cooling to RT. One pmol of double-stranded oligonucleotide probe was end-labeled with [γ<sup>32</sup>P]ATP using T4 kinase. Sp1/3 is the sequence of a perfect consensus oligonucleotide supplied with the Promega Gel Shift System.

<sup>b</sup> Putative Sp sites are bolded and underlined. Mutated nucleotides are lower-case.

<sup>c</sup> Position of the oligonucleotides relative to the transcriptional start site.
Figure 1

-291 ATGACTACCC CAAAACCAGG CCCAGGCTCC AACTGGCAAA GCAAAAAAAA
-241 AAAACAAAGA AAGAAAGAAA GCTTTGCCCC ATCCCCAGGAG GGCCCCCAAGG

Sp D
-191 CGCAGCCCC CCCACGGCA CTTTCCA GC TGGCTGTGAG CCCTAGGGCC

Sp C
-141 GCAGGAAAGG TATGCA AAGA TGATGGGAGG GATTTAAAG GC TGGCTGTGAG CCCTAGGGCC

Sp B
-91 AACAAGAAAA GTGGGGTTAA A GAGGAGGT GTGGCAGAA ACA ACTTAAG

Exon 1
+10 AGCGCATCCC AGCCAGTGTCA CTTTCAGCAGT CAGCAGTAA AGGGCCCGC
+60 TGAGGGAGGC AGAGAAAGGAG GGGGTGTCTC CCAAGAGAGG GTGGGTCGCC

+110 AGCTCCAA
Figure 2

Sp A

Lane 1 2 3 4 5 6

Sp1
Sp3
Sp3

Anti-Sp1 - + - - - -
Anti-Sp3 - - + - - -
Cold P - - - + - -
Cold Sp1/3 - - - + -
Cold NP - - - - +

Sp B

Lane 1 2 3 4 5 6

Sp1
Sp3
Sp3

Anti-Sp1 - + - - - -
Anti-Sp3 - - + - - -
Cold P - - - + - -
Cold Sp1/3 - - - + -
Cold NP - - - - +

Sp C

Lane 1 2 3 4 5 6

Sp1
Sp3
Sp3

Anti-Sp1 - + - - - -
Anti-Sp3 - - + - - -
Cold P - - - + - -
Cold Sp1/3 - - - + -
Cold NP - - - - +

Sp D

Lane 1 2 3 4 5 6

Sp1
Sp3
Sp3

Anti-Sp1 - + - - - -
Anti-Sp3 - - + - - -
Cold P - - - + - -
Cold Sp1/3 - - - + -
Cold NP - - - - +
Figure 3

Sp A

Lane 1 2 3 4 5 6

Sp B

Lane 1 2 3 4 5 6

Sp C

Lane 1 2 3 4 5 6

Sp D

Lane 1 2 3 4 5 6

Anti-Sp1  - + - - - -
Anti-Sp3  - - + - - -
Cold P    - - - + - -
Cold Sp1/3- - - - + -
Cold NP   - - - - + -

Anti-Sp1  - + - - - -
Anti-Sp3  - - + - - -
Cold P    - - - + - -
Cold Sp1/3- - - - + -
Cold NP   - - - - + -

Anti-Sp1  - + - - - -
Anti-Sp3  - - + - - -
Cold P    - - - + - -
Cold Sp1/3- - - - + -
Cold NP   - - - - + -

Anti-Sp1  - + - - - -
Anti-Sp3  - - + - - -
Cold P    - - - + - -
Cold Sp1/3- - - - + -
Cold NP   - - - - + -
Figure 4

Sp A  Sp B

Sp C  Sp D

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

Lane 1 2 3 4 5 6 7 8

Sp A Sp B Sp C Sp D
wt mut wt mut wt mut wt mut

Sp1
Sp3
Figure 6

-292

-141

-70

-50

-19

+115

Basic

Fold Luminescence

HepG2

A549

0 2 4 6 8

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Figure 7

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 8

A

Fold Activation

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<th>0.1 µg pEVR2</th>
<th>0.05 µg Sp1</th>
<th>0.05 µg Sp3</th>
<th>0.05 µg Sp1+ 0.05 µg Sp3</th>
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<tr>
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<td>-19 to +115 CYP2F1</td>
<td>-292 to +115 CYP2F1</td>
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B

Fold Activation

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<th>0.3 µg Sp3</th>
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<td>-19 to +115 CYP2F1</td>
<td>-292 to +115 CYP2F1</td>
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</tr>
</tbody>
</table>

* Significant difference
# Significant difference
¥ Significant difference
Figure 9

A

Sp1

106
93

98
67

Liver Lung

Sp3

106

77

50.8

A549 HepG2

B

Sp1

95

108

90

50.7

Liver Lung

Sp3

61

108

90

50.7

A549 HepG2

C

+ Sp1 Sp3 -

+ Sp1 Sp3 -

Lung Liver

300 bp