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Transcription Factor Binding to a Putative Double E-Box Motif Represses *CYP3A4* Expression in Human Lung Cells

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Running Title: Repression of *CYP3A4* Transcription in Lung

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List of Abbreviations: P450, cytochrome P450; bp, base pair(s); EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; AP1, activator protein 1; Sp1, stimulatory protein 1; 2F1-LSF, CYP2F1 lung specific factor; LSF2, lung specific factor-2; C/EBP α , CCAAT/enhancer binding protein alpha; NHBE, normal human bronchial epithelial cells; and A549, human lung adenocarcinoma A549 cells.

ABSTRACT

Two vital enzymes of the CYP3A subfamily, CYP3A4 and CYP3A5, are differentially expressed in the human lung. However, the molecular mechanisms that regulate tissue-selective expression of the genes are poorly understood. The ability of the 5' upstream promoter region of these two genes to drive luciferase reporter activities in human lung A549 cells was dramatically different. The *CYP3A5* promoter region activated luciferase gene expression by 10-fold over the promoterless construct, while the *CYP3A4* promoter did not drive expression. Sequence comparisons of the promoters identified a 57 base pair insertion in the *CYP3A4* promoter region (-71 to -127), which was absent in the *CYP3A5* promoter. Deletion of the 57 bp motif from *CYP3A4* or insertion into the *CYP3A5* promoter, showed that this motif represses *CYP3A4* expression in lung. EMSA analysis using nuclear extracts from either A549 cells or human lung tissues showed two specific protein/DNA complexes formed with the ³²P-labeled *CYP3A4-57 bp* oligonucleotide. EMSA analyses identified two E-box motifs as the minimal specific *cis*-elements. Supershift assays with antibodies directed against known double or single E-box binding factors (TAL1, δ EF1, E2A, HEB, etc.) failed to identify this factor as a previously characterized *trans*-acting double E-box binding protein. These results demonstrated that the 5'-upstream region of *CYP3A4* contains an active putative double E-box repressor motif, not present in the 5'-upstream region of the *CYP3A5* gene, which attenuates *CYP3A4* expression in the human lung. We believe that this is the first documented case where a cytochrome P450 gene is actively repressed in a tissue-specific manner.

Members of the cytochrome P4503A (CYP3A) subfamily are major contributors to the metabolism of a variety of therapeutic, xenobiotic, and endogenous compounds (Guengerich, 1999; Sheweita, 2000). The organ expressing the highest levels of P450 is the liver (Watkins, 1994), but the importance of extrahepatic expression is becoming a subject of great interest (Ding and Kaminsky, 2003). The human CYP3A subfamily is comprised of four members, CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Nelson et al., 1996; Gellner et al., 2001). CYP3A4 plays the major metabolic role in adult livers (Watkins, 1994) and intestines (Kolars et al., 1994). CYP3A7 is the predominant fetal liver form (Stevens et al., 2003) and is polymorphically expressed in adults (Burk et al., 2002). CYP3A43 is the newest member of the CYP3A family (Gellner et al., 2001), but low levels of expression offer only minor contributions to total CYP3A metabolism. In comparison, CYP3A5 is expressed in the highest number of tissues (Kolars et al., 1994; Koch et al., 2002; Ding and Kaminsky, 2003), and its expression is limited by a single nucleotide polymorphism designated *CYP3A5*3* (an A>G conversion within intron 3 of the *CYP3A5* gene). However, the functional consequences of this polymorphism are controversial. Generally, individuals expressing the wild-type *CYP3A5*1* allele have the highest levels of CYP3A protein in the liver, where 3A5 protein is roughly equal to that of 3A4; but individuals expressing the homozygous *CYP3A5*3/*3* allele have very low to undetectable levels of CYP3A5 protein (Kuehl et al., 2001). Therefore, one would expect that people who are homozygous for *CYP3A5*3/*3* should turnover CYP3A substrates very poorly (Kuehl et al., 2001). However, this expectation has sometimes not been observed (Floyd et al., 2003; Westlind-Johnsson et al., 2003).

The regulation of cytochrome P450 gene expression has received much attention in recent years, and the mechanisms responsible for *CYP3A* gene expression vary widely and are often complex (Guengerich, 1999; Quattrochi and Guzelian, 2001; Gibson et al., 2002; Goodwin et al., 2002b). To address CYP3A's obvious importance in drug-drug interactions, a humanized mouse model of hepatic CYP3A4 regulation has been created (Zhang et al., 2003) and much work has been done in recent years to elucidate its regulatory mechanisms (Ding and Kaminsky, 2003; Schuetz, 2004; Xie et al., 2004). The 5'-flanking regions of the *CYP3A* subfamily of genes contain sequence motifs (i.e., *cis*-elements), which can regulate these genes in three generalized ways: 1) those that are involved in enhancing the enzyme's production (inducers; XREM, PXRE, GR etc.) (Hukkanen et al., 2000; Goodwin et al., 2002b; Hukkanen et al., 2003); 2) those that are involved in maintaining basal levels expression (e.g., NFY, NFκB, CAR, HNF3 etc.) (Iwano et al., 2001; Saito et al., 2001; Goodwin et al., 2002a; Bombail et al., 2004); and 3) those involved in turning genes off in the presence or absence of specific signals (e.g., C/EBPβ-LIP, SMRT) (Chen and Evans, 1995; Chen and Li, 1998; Jover et al., 2002; Johnson et al., 2006). The combined actions of these and other *cis*-acting elements ensure that the proper amount of CYP3A protein is produced only at the precise time it is needed.

The CYP3A enzymes expressed in the human lung are important determinants of pulmonary carcinogenesis caused by metabolism of several inhaled xenobiotic compounds (Piipari et al., 2000; Mollerup et al., 2001; Yeh et al., 2003). Other lung diseases cause significant morbidity and mortality, and specific P450 enzymes are at least partially responsible for these diseases (Ding and Kaminsky, 2003; Yeh et al., 2003).

These P450 enzymes probably participate in the metabolism and bioactivation of polycyclic aromatic hydrocarbons (PAHs) and other procarcinogens present in combustion products, tobacco smoke, and ambient particulate matter (Nelson et al., 1996; Guengerich and Shimada, 1998). CYP3A4 and CYP3A5 are active in the metabolic detoxication of benzo[a]pyrene, but they are also partly responsible for the activation of benzo[a]pyrene-7,8-diol to carcinogenic diol epoxides that are capable of covalently binding to DNA. *CYP3A4* and *CYP3A5* exhibit differences in expression patterns, both within tissues and among individuals (Ding and Kaminsky, 2003). Transcripts of *CYP3A4* have not been found in respiratory epithelial cell lines, such as the human A549 lung cell line and the immortalized bronchial epithelial BEAS-2B cell line. Nor have transcripts been detected in human bronchoalveolar macrophages or peripheral blood lymphocytes (Willey et al., 1996; Anttila et al., 1997; Hukkanen et al., 1997; Hukkanen et al., 2000; Piipari et al., 2000). In contrast, CYP3A5 is consistently expressed in all of these cell types. Moreover, tissue-specific expression of the CYP3A genes is thought to be a major factor influencing inter-individual variation in both drug response and lung disease susceptibility (Piipari et al., 2000; Lamba et al., 2002). Thus, the dominant mechanisms that regulate CYP3A expression in the human lung must be established in order to understand interindividual and interracial susceptibility to inhaled xenobiotics.

Materials and Methods

Reagents - Qiagen Plasmid/RNA isolation kits were purchased from Qiagen (Valencia, CA). Bacterial artificial chromosomes containing human sequences for the *CYP3A* loci [AC005020, AC011904, and AF280107 (Gellner et al., 2001)] were purchased from Incyte Genomics (Palo Alto, CA). Oligonucleotides used in PCR-

amplification of genomic sequences and EMSA analyses were purchased from Integrated DNA Technologies (Coralville, IA). The *pGL3-luciferase* reporter-plasmid and dual-luciferase reporter assay system were purchased from Promega (Madison, WI). SDS-polyacrylamide gel electrophoresis reagents (37.1:1 bis-acrylamide, ammonium persulfate, and N,N,N',N'-tetra-methyl-ethylene-diamine) were purchased from Bio-Rad Laboratories (Hercules, CA). Human lung tissue was acquired from consenting donors through the Rocky Mountain Donor Services (Salt Lake City, UT). Antibodies against known E-box binding proteins were generous gifts from Dr. Frans van Roy (SIP1) and Dr. Cornelis Murre (E2A and E47) or purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Platinum® *Pfx* DNA polymerase, Platinum® PCR Supermix High Fidelity, TOPO cloning kits, cell culture media, restriction enzymes, and all other molecular biology reagents were purchased from Invitrogen Technologies, BD Gentest or Cambrex. Real-Time PCR reagents were purchased from Bioline (Randolph, MA) and Invitrogen. All sequence manipulations were conducted *in silico* using the Clone Manager Suite 7.0 (Scientific & Educational Software, Durham, NC) software package.

Cell Culture - Human adenocarcinoma A549 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). A549 cells (p98) were seeded in 75 cm² flasks using cryo-preserved aliquots (~1 X 10⁶ cells; P87-90) and maintained for 48 hrs with Dulbelco's modified Eagle's medium/nutrient mixture with F12 (DMEM-F12) supplemented with 10% fetal bovine serum. For sub-culturing, cells were trypsin-disassociated and reseeded at 10% confluence in fresh media. For transfection assays, cells were trypsin-disassociated and reseeded in 96-well plates at a concentration of 1.0 X 10⁴ cells/well in 100 µl of the appropriate media. Normal Human Bronchial Epithelial

(NHBE) cells were obtained from Cambrex (Walkersville, MD) and were grown in bronchial epithelial growth medium and cultured as above in flasks and dishes coated with collagen, fibronectin and albumin. They were passaged no more than three times prior to transfection. Frozen primary hepatocytes were obtained from BD Genetec (Woburn, MA). They were thawed per the supplier's instructions in a 75 cm² flask and then reseeded as described above into 96 well plates in hepatocyte growth medium.

Construction of Promoter-Luciferase Reporter Plasmids - Luciferase reporter constructs containing promoter regions from *CYP3A5* (*pGL3A5-218*, *pGL3A5-443*, *pGL3A5-647*, *pGL3A5-872*, *pGL3A5-1150*, and *pGL3A5-1365*), and a fragment of the 5'-untranslated region of the gene (+31 base pairs), were adapted from previous work (Hukkanen et al., 2003). Chimeric *CYP3A4*-promoter- and *CYP3A5*-promoter-luciferase reporter plasmids were prepared by PCR amplification of the 5'-flanking regions of these genes from bacterial artificial chromosomes (i.e., AF_280107 for *3A4* clones; AC_005020 for *3A5* clones) using the primers listed in Tables 1 and 2. Both homology maps comparing the *CYP3A4* and *CYP3A5* promoters and stringent MATCH/TRANSFAC analyses were conducted to predetermine putative transcriptional binding motifs to guide primer design. Cloning primers introduced 5'-restriction sites for subsequent insertion into the multiple cloning site of the *pGL3-Basic* vector. For *CYP3A4*, a 5'-*NheI* site and a 3'-*HindIII* site were incorporated into the forward and reverse primers, respectively; for *CYP3A5* constructs, a 5'-*MluI* site and a 3'-*BglIII* site were utilized. *pGL3-3A4* constructs encompassed regions surrounding the transcriptional start site (i.e., from 3'-TSS +118 to 5'-TSS -35, -59, -72, -80, -150, -170, -222, -2795, and -13kb, respectively). A larger *pGL3-3A5-5.3kb* plasmid was also created by PCR-

amplification of a 2.3 kilobase pair region (bases 10195 to 13107) from a bacterial artificial chromosome containing *CYP3A5* (AC_005020). Using the oligonucleotides *Forward-3A5-5.3kb-KpnI* (5'-ggtaccATGCTCGTGTGCCTGATAAC-3'; sense, bases 10195 to 10216) and *A5_P1_Reverse* (5'-GCATTGCTTTGGGTAGTATGGAC-3'; antisense, bases 13107 to 13085), a product incorporating a *KpnI* restriction site at the 5'-end was created. Utilization of this new *KpnI* site, and a unique *AflIII* restriction site (bases -2837 to -2842) in the *CYP3A5* promoter, directed the proper insertion of this region into the original pGL3-3A5-3kb plasmid, which was prepared separately. All plasmids were screened with restriction digestions using enzymes, which cut at least once in both the vector and the insert, and then sequenced by the University of Utah core sequencing facility.

Site-directed mutagenesis - Site-directed mutagenesis of the *pGL3-CYP3A5-208* construct was performed with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutations were introduced using specific oligonucleotides that are listed in Table 2. Correct assembly of the mutations was confirmed by sequencing.

PCR-Based Mutations of CYP3A4 and CYP3A5 Constructs - The chimeric reporter plasmids created above were mutated to introduce or knockout the 57 bp region for the *CYP3A5* and *CYP3A4* constructs, respectively. Primers used in these methods are listed in Tables 1 and 2. The *CYP3A5-knock in* construct was made with forward and reverse primers with 3' ends that were complementary to the boundaries of homology within the *CYP3A5* promoter, but whose 5' ends were complementary to the *CYP3A4* 57 bp region. In order to reduce errors in oligonucleotide syntheses, the 57 bp insertion was

divided between the forward and reverse primers, and Platinum® *Pfx* DNA polymerase (a proof-reading polymerase that immediately terminates at the end of a template leaving blunt ends) protocols were applied as the manufacturer suggested. *CYP3A4* 57 bp deletion mutants were generated by long-template PCR amplification of the entire vector excluding the targeted 57 bp region. In order to accomplish these deletions, the *wild-type CYP3A4* constructs were subjected to a deletion protocol using Platinum® PCR Supermix High Fidelity containing 20 ng original template and 20 nM of each primer (*3A4-57 bp-mut F* and *3A4-57 bp-mut R*) in a total volume of 50 µL and subjected to an initial melting step of 94°C for 2 minutes, 20 cycles of amplification (94°C for 30 sec.; 55°C for 90 sec.; 68°C for 8.5 min.), and a final capping step of 68°C for 3.5 minutes. Ten PCR reactions were pooled and the template strands were removed with digestion with DpnI. The mutated products were purified on a 0.7% agarose gel with an expected product size of ~8 kb. Because the initial PCR-reaction mixture contained TAQ-DNA polymerase, the resulting products also contained 3'-poly-adenosine overhangs, which were subsequently removed with Mung Bean Nuclease (NEB, Beverly, MA), then gel-purified as before, and then self-ligated with T4-ligase in Rapid Ligation Buffer (Promega, Madison, WI). Due to the lack of flexibility in selection of the priming site, this protocol consistently yielded a *64 bp knockout CYP3A4* construct with 7bp more than the 57 bp region removed (the total region that was deleted was -71 to -132), but the *64 bp knockout* still closely resembled the homologous region of the *CYP3A5* gene. Multiple attempts to obtain a knockout construct with precisely 57 bp deleted were not successful. Similar attempts to restore this region to these constructs using the *knock-in* technique described above were also unsuccessful. However, a similar knockout was

created using the protocol described above with *3A4-SacII* (Table 1) primers to introduce two *SacII* restriction sites flanking the 57 bp region. Creation of the *CYP3A4-Spacer* construct was accomplished using the 64 bp *knockout* as a template, using primers that harbored the additional spacer sequence at the 5' end such that they would be included in the final PCR product. To insure that there were no other mutations introduced into the *pGL3-Basic* vector during these exceptionally long polymerization events, all completed promoter constructs were subcloned back into the original *pGL3-Basic* vector, screened with restriction enzymes, and sequenced.

Transient Transfection and Luciferase Assay - Parallel luciferase reporter assays were conducted in order to compare the genetic differences between *CYP3A4* and *CYP3A5* transcriptional activity in cultured human lung A549 cells. Approximately 50 mL cultures of each plasmid were purified using the EndoFree[®] Plasmid Maxi Kit (Qiagen, Valencia, CA.), yielding ~100-200 µg of DNA each. When confluence reached ~70%, the A549 cells were transfected with 0.25 µg of reporter plasmid and 0.005 µg of *Renilla* luciferase plasmid (pRL-SV40) using FuGene 6 (Roche, Indianapolis, IN), according to the manufacturer's suggestions. Primary cell cultures (NHBE and hepatocytes) were transfected using Effectene Reagent (Qiagen), following the manufacturer's suggestions. Cells were lysed for 36 hrs (A549) or 24 hours (primary cultures) after the transfections, and the respective luciferase activities were determined using the dual-luciferase assay system (Promega). Firefly luciferase activities for the experimental constructs were normalized for transfection efficiency and cell loading using *Renilla* luciferase activity and total protein concentration, respectively. Data from these experiments were expressed as fold luminescence over the activity of the

promoterless pGL3-*Basic* reporter plasmid. The data for A549 transfections were presented as mean fold luminescence (\pm S.D.) for three independent experiments performed in quadruplicate. NHBE and hepatocyte data were for a single experiment with nine replicates for each plasmid. Transfections of the mutated CYP3A5 -208 constructs to A549 cells were performed as described previously (Hukkanen et al., 2003).

Quantitative Real-Time PCR - Total RNA was purified from 10^6 NHBE or A549 cells using Trizol. One microgram of total RNA was used to synthesize first-strand cDNA using random hexomers and Superscript II (Invitrogen), diluted 1:5 and then evaluated by quantitative real-time PCR using a Chromo-4 cycler (Bio-Rad) and SYBR green (Invitrogen). The primers (a generous gift of Roger Gaedigk, University of Missouri, Kansas City) were: for *CYP3A4* detection 5'-CTTCATCCCAGACTTGGCCA-3' and 5'-ACAGGCTGTTGACCATCATAAAAG-3'; for *CYP3A5* detection 5'-GACCTCATCCCAAATTTGGCGG-3' and 5'-CAGGGAGTTGACCTTCATACGTT-3'; β -actin was utilized as a housekeeping control gene (primers: 5'-GACAACGGCTCCGGCATGTGCA-3' and 5'-TGAGGATGCCTCTCTTGCTCTG-3'). We calculated the relative expression by utilizing plasmid copy number standards.

Electrophoretic Mobility Shift Assay (EMSA) - Nuclear extracts from A549 or HepG2 cells and human lung tissues were prepared as previously described (Carr et al., 2003). EMSA was performed using the gel-shift assay system from Promega, essentially as described by the manufacturer. Binding reaction mixtures were preincubated at room temperature for 10 min. The mixtures contained 4 μ l of nuclear extract (4 μ g for lung tissue and 6 μ g for cell cultures), 0.005-0.01 pmol 32 P-labeled oligonucleotide probe, and

2 μ l of 5X binding buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 20% glycerol, 0.25 mg/ml poly(dI-dC)·poly(dI-dC)) in a total volume of 10 μ l. For competition experiments, a 100-fold molar excess of unlabeled double-stranded oligonucleotide was incubated for 15 min with nuclear extract prior to the addition of 1 μ l ³²P-labeled oligonucleotide probe (0.005-0.01 pmol). The mixtures were incubated for another 20 min at room temperature. Immediately before electrophoresis, gel loading dye (25 mM Tris-HCl, pH 7.5, 0.02% bromophenol blue, 4% glycerol) was added to all binding reaction mixtures. The protein/DNA complexes and unbound probes were separated by non-denaturing gel electrophoresis using 4% polyacrylamide gels (1-8 volts/length in cm) and detected by autoradiography.

Double-stranded oligonucleotides comprising the consensus binding sequences for AP1, Nf κ B, Sp1, OCT1, and the 2F1-LSF (Carr et al., 2003) were included (175-fold molar excess) as non-specific competitors in the gel shift assay system (Promega). Sequence-specific competitive oligonucleotides (1-11, Table 3) were generated by mutating five base pairs at a time, using the conversion of A>C and T>G; competitors M9-M55 (Table 3) contain single base pair mutations corresponding to the distance 5' - to 3' within the *57 bp insertion*; and E-box knockout competitors were created by converting the consensus sequence (CACCTG) to a sequence that is not known to bind any transcription factor (AAAAT). Competitive oligonucleotide probes were synthesized by either Integrated DNA Technologies (Coralville, IA) or the University of Utah Core Research Facilities. Sequences of the DNA probes used in competitive EMSA experiments are listed in Table 3.

Results

Transcriptional Activity of the 5' Promoter Regions of CYP3A4 and CYP3A5 in Human Lung A549 Cells - Transient transfection of the upstream promoter elements of the *CYP3A4* and *CYP3A5* luciferase constructs showed marked differences in the ability of these two promoters to drive luciferase expression in human lung A549 cells. On average, the *pGL3-CYP3A5* promoter increased luciferase activity by over 40-fold (Fig. 1A). In contrast, only the *CYP3A4-80* construct expressed luciferase at a higher level (2.8-fold) than the promotorless control vector (Fig. 1B), which was significantly lower than the *CYP3A5* construct. Comparison of the constructs with different lengths of the *CYP3A5* 5' flanking region showed that the majority of transcriptional activity was attributable to the proximal -208 base pairs. Sequence comparisons of the *CYP3A4* and *CYP3A5* 5'-upstream regions revealed ~85% homology between the first 500 base pairs of the two promoters. The most obvious difference between these two promoter regions is a 57 bp insertion within the promoter of *CYP3A4* (-71 to -127), which contains multiple near-consensus sequences of recognized transcription factors, including a functional C/EBP α element (-121 to -130) (Rodriguez-Antona et al., 2003), a putative Sp1 binding site (Bombail et al., 2004), and two putative E-box motifs (Fig. 2). A similar region is also found in the *CYP3A7* promoter, except it has a slightly smaller 56 bp insertion and the proximal E-box is not conserved.

We have previously shown that the *CYP3A5* pseudogene promoter, highly similar to *CYP3A5* promoter, is transcriptionally inactive (Hukkanen et al., 2003). In the current work, we utilized this information to identify regions in the *CYP3A5* promoter that are potentially required for the repression of transcription in A549 cells. Comparison of the promoter sequence present in the *pGL3-CYP3A5 -208* construct with the corresponding

CYP3A5 pseudogene sequence revealed 21 differing nucleotides preceding the TATA-box (Fig. 3a). All of these nucleotides in the *pGL3-CYP3A5 -208* construct were mutated into the corresponding nucleotide in the pseudogene individually or in combination of two or more closely located nucleotides. The mutated constructs were transfected into A549 cells and the luciferase activities were compared with the wild type construct. All mutations, except 3, and 20-21, significantly decreased luciferase activity ($p < 0.05$, One-Way ANOVA, LSD post hoc test). Mutations 2, 15-16, and 18-19 decreased transcriptional activity more than 50% and the mutation 17 decreased transcriptional activity 84%. These data suggested that the differences in the relative transcriptional activities of the *CYP3A4* and *CYP3A5* promoters (2-fold vs. 40-fold over the control vector respectively) are at least partially attributable to the low promoter activity of the *CYP3A4* cis-elements. Two of these elements are the previously published CCAAT-box and BTE that were crucial to the basal expression of the *CYP3A5* gene in HepG2 cell lines (Iwano et al., 2001). However, this data also confirmed that the complete 57 bp insertion is required for the full repression of the *CYP3A4* gene in A549 lung cells, because although a single nucleotide mutation (mutation 17, Fig. 3) in the CCAAT-box of the *CYP3A5* promoter did decrease transcription, the remaining level of expression was still higher than that of the *CYP3A4* promoter.

Function of the CYP3A4-57 bp Insertion in the Repression of CYP3A4 Gene Expression - We examined the repressive ability of the *CYP3A4-57 bp insertion* in the human A549 cell line by creating a series of mutated *CYP3A4* and *CYP3A5* promoter luciferase constructs. Introduction of *CYP3A4-57 bp insertion* into the analogous position of a *CYP3A5* promoter construct (*pGL3-3A5-3000*) significantly reduced

luciferase-driven expression of that construct in human A549 lung cells by roughly 50% (Fig. 4). Similar activity changes were observed for mutations in the smaller *pGL3-3A5-433*, and *pGL3-3A5-862* luciferase constructs (Fig. 4). These data demonstrated that the *CYP3A4-57 bp insertion* has direct functional consequences on the low expression of *CYP3A4* in human lung cells.

In comparison, deletion of various putative *cis*-elements within the 57 bp region of the *CYP3A4*-promoter constructs altered expression in a more complex manner (Fig. 5). For example, when the 57 bp region had been completely excised from the *CYP3A4* promoter (i.e., the 64 bp *knockout* construct), no increase in luciferase activity was observed when compared to wild type ($p > 0.1$; Unpaired t test w/ equal variance). However, if the C/EBP α binding motif (-121 to -130), shown (Rodriguez-Antona et al., 2003) to be essential for *trans*-activation in HepG2 liver cells, was maintained, but the rest of the motif deleted (i.e., the *SacII* construct; Fig. 5), luciferase expression increased substantially ($p < 0.05$; Unpaired t test w/ equal variance). Moreover, a similar *CYP3A4*-promoter construct harboring specific mutations that destroyed the two E-boxes, but maintained the C/EBP α motif, also increased expression (data not shown). The *CYP3A4-Spacer* construct was made to evaluate the hypothesis that changes in reporter gene activity by deletion of the 57 bp region were not caused by alterations of simple spatial interactions of *cis*-elements through the shortening of this region of the *CYP3A4* promoter. When this construct was tested, no difference in luciferase expression was observed ($p > 0.1$; Unpaired t test w/ equal variance; Fig. 5). Together, these findings suggest that this region is functionally active in A549 cells, and is therefore at least partially responsible for the repression of *CYP3A4* expression within the human lung.

The functionality of this region was validated by transfection experiments in primary human lung and liver cell cultures. (Fig. 6). Deletion of the region significantly ($p < 0.05$, One-Way ANOVA, LSD post hoc test) increased expression of a *CYP3A4* promoter construct in NHBE cells but not in primary hepatocytes. Likewise, NHBE expression of a *CYP3A5* promoter construct was significantly ($p < 0.05$, One-Way ANOVA, LSD post hoc test) decreased by insertion of this promoter element, while the insertion did not change hepatocyte expression. Quantitative real-time PCR was used to confirm the intrinsic expression of *CYP3A5* in A549 cells (~ 9000 copies/ μg total RNA) and NHBE cells (~ 850 copies/ μg total RNA), whereas *CYP3A4* was not detected in either the tumor cells or the primary lung cells (Fig. 7).

Analysis of Factor Binding to the CYP3A4 57 bp Region by EMSA - To demonstrate the specificity of nuclear factor(s) binding to the putative *cis*-element(s) identified by the luciferase experiments, EMSA experiments were conducted using ^{32}P -labeled double-stranded oligonucleotides corresponding to the wild-type sequence for the *CYP3A4-57 bp insertion* (-71 to -127), shown in Table 3, and nuclear extracts from A549 lung cells and whole lung nuclear extracts. When radiolabeled wild-type *CYP3A4-57 bp* probe was incubated with nuclear extract from either source, two sequence-specific DNA-protein complexes were observed (Fig. 8, data from whole lung extracts not shown). Binding was inhibited by the addition of 100-fold molar excess of unlabeled wild-type oligonucleotide. In contrast, complex formation was not inhibited by the addition of 175-fold molar excess of unlabeled competitors harboring consensus binding sequences for AP1, NF κ B, Sp1, OCT1, and the *CYP2F1-LSF1* motif (Table 3, Fig. 8). These results demonstrate the specific binding of nuclear factors within the human lung

and human lung adenocarcinoma A549 cells to the *CYP3A4-57 bp insertion*. Furthermore, because consensus oligonucleotides harbor the highest affinity binding sites for their respective nuclear factors, these data also strongly suggest that the *trans*-acting element(s) is not AP1, NFκB, Sp1, OCT1, nor CYP2F1-LSF1.

It seems feasible that selective expression of the *CYP3A5* gene in lung cells could be controlled by binding of specific transcription factors in lungs that are different from protein factors of liver cells. Therefore, we isolated nuclear proteins from the human liver cell line, HepG2, and compared binding of these proteins, and the nuclear factors from A549 cells, to the labeled 57 bp probe. The EMSA protein/DNA bands with liver nuclear factors (Fig. 9, lanes 4-6) elicited considerably less binding than A549 extracts (Fig. 9, lanes 1-3), and the two protein/DNA bands that were formed had considerably faster mobilities than the two bands using lung proteins. Thus, although liver transcription proteins appeared to bind specifically (the binding was abolished by 100-fold excess unlabeled probe) to the 57 bp motif, the proteins that bound appeared to have lower molecular weights than lung cell transcription factors.

In order to identify the essential bases for binding of A549 nuclear proteins, competitive EMSA experiments were conducted. A series of eleven mutated 57 bp oligonucleotides, designed to mutate 5 bp at a time, were incubated with the labeled wild-type 57 bp probe. These experiments (Table 3, Fig. 8) showed that only oligonucleotide probes 3,4 and 11 were unable to block binding of transcription factors to the native 57 bp sequence. A partial loss of competitive binding was observed for oligonucleotides 6, 9 and 10, but the DNA/protein bands were much lighter than the bands corresponding to the 5-bp mutated oligonucleotides from the E-boxes. These results suggested that the two

E-box motifs within this region are the putative binding regions for A549 nuclear transcriptional proteins. When oligonucleotide probes with only single mutations in either the first (Table 3, probe M15) or second (Table 3, M49 or M52) E-box elements were included in competitive EMSA assays, the single mutations in either E-box were sufficient to ameliorate competition of the transcription protein to the DNA of the 57 bp probe. These results demonstrated a requirement for the fidelity of both E-box motifs. This is a characteristic feature of double-E-box binding proteins such as the chicken δ EF1 (Remacle et al., 1999). These two E-box regions are in phase; approximately two complete turns of the double helix apart. The nuclear factor(s) prepared from human lung cells bound to the two E-box motifs in a nucleotide sequence-specific manner, and it is therefore possible that a single protein, like a member of the vertebrate polycomb proteins, could simultaneously bind to both E-boxes within this region.

In an attempt to identify the *trans*-acting factor involved in the active repression of the *CYP3A4* gene, supershift EMSA experiments were conducted using antibodies generated against the known double-E-box binding factors (SIP1, HEB and δ EF1), as well as single E-box binding proteins (e.g., E2A, Myc, MyoD, and E47). None of the antibodies retarded the mobility of the protein/DNA complex (data not shown), indicating that these are not the *trans*-acting factors in question. Multiple attempts to purify and identify the lung nuclear factor from lung cells or tissues by DNA affinity chromatography were not successful. When combined, these data suggest that an uncharacterized human lung specific transcription factor(s) binds specifically to a double E-box motif within the *CYP3A4-57 bp insertion* and actively represses *CYP3A4* expression in the human lung.

Discussion

Comparisons of the promoter regions of *CYP3A4* and *CYP3A5* show that they share over 90% homology within the first ~1 kb of upstream regulatory sequence and yet show striking differences in expression among individuals, races, developmental stages, tissues, and cell types. The regulation of the *CYP3A* cassette is also very complex with regard to its response to changes in physiological conditions.

Most studies on the *CYP3A* subfamily to date have concentrated on the expression of these isoforms in the liver, where polymorphisms have been shown to be important. An intriguing ramification of previous studies (Kuehl et al., 2001) is that 75% of a population (i.e., those homozygous for *CYP3A5*3*) would express an extremely limited amount of *CYP3A5* within their extrahepatic tissues, thus increasing the metabolic burden upon the *CYP3A4* enzyme. Because *CYP3A5* is probably the major *CYP3A* isoform expressed within human lung tissue (Anttila et al., 1997; Raunio et al., 1999), the overall metabolism of 3A substrates in lung cells would be drastically reduced or otherwise altered toward alternative metabolic pathways. Although procarcinogens metabolized by the *CYP3A* enzymes would not become bioactivated, drugs designed to target the lung might exhibit greater adverse side effects (e.g., long-term use of inhaled glucocorticoids might lead to superinfections) and carcinogens/toxins would exhibit extended half-lives. *CYP3A* individual genotypes have been correlated to lower incidence of lung and other types of cancer (Yeh et al., 2003; Keshava et al., 2004).

As expected in A549 cells, *CYP3A5-Luc* constructs exhibited over 20-fold higher luciferase activity than *CYP3A4-Luc* constructs. This dramatic difference in basal expression among these constructs becomes quite apparent within nested deletions

containing only the first 200 base pairs of the 5'-flanking region of these two genes (Fig. 1). Luciferase constructs which contained the 3A4 promoter gradually increased in activity as nested-deletions reached -80, but then dropped dramatically thereafter. In contrast, *CYP3A5* constructs rapidly reached maximal levels with the first -208 base pairs of the promoter; a pattern of expression that closely matches previous studies in HepG2 liver cells (Iwano et al., 2001). The experiments with mutated *CYP3A5* promoter constructs showed that the basic transcriptional element (BTE) and especially the CCAAT box are important for *CYP3A* transcriptional activation in A549 cells (Fig. 4); a finding that is surprisingly similar to the mechanisms of *CYP3A* transcriptional regulation in HepG2 cells.

We can conjecture about the genetic regulatory mechanisms in A549 lung cells by comparing the mechanisms governing expression of *CYP3A4* and *CYP3A5* in HepG2 cells. For instance, the basal regulation of *CYP3A5* is governed primarily by the cooperative effects of nuclear factor-Y (NF-Y) and specificity protein (Sp) family members binding to a CCAAT-box (-68 to -78) and the BTE (-46 to -67) in the proximal promoter of the *CYP3A5* gene (Iwano et al., 2001). Analogous motifs are identifiable within the proximal promoter of the *CYP3A4* gene. However, in comparison with the *CYP3A5* promoter, the *CYP3A4* CCAAT-box (-62 to -66) has a single mutation in the core binding motif and the BTE (-36 to -57) also shows a difference in two consecutive nucleotides. Both perturbations confer markedly less expression activity than their respective *CYP3A5* counterparts when tested in HepG2 cell lines (data not shown), and this is also the case in A549 cells (Fig. 1). In contrast, the transcriptional regulation of the *CYP3A4* gene in HepG2 cell lines is controlled by a complex circuit of transcription

factors that bind to motifs more distal to the related region of *CYP3A5*. Instead of being governed by the aforementioned regions, the basal expression of the *CYP3A4* gene in HepG2 cell lines is only partially controlled by the binding of C/EBP α and HNF-3 γ to a proximal ER-6 motif (-152 to -169) (Rodriguez-Antona et al., 2003); expression depends rather upon HNF-1 γ , HNF-4 γ , AP-1, and USF1 binding to a distal region (-10.9 to -11.4kb), which was referred to as the “Constitutive Liver Enhancer Module of *CYP3A4* (CLEM4)” (Matsumura et al., 2004). However, this is not the case in transient transfections of these same constructs in A549 cells, because the inclusion of the CLEM4 element did not increase expression (data not shown). What is important about these findings is that the underlying mechanisms responsible for the basal expression of these two genes are encoded within the sequences of the *CYP3A4* and *CYP3A5* promoters and that, although similar, the expression patterns within A549 lung cells are distinct from that of HepG2 liver cells.

When comparing the reporter activities of *CYP3A4-Luc* and *CYP3A5-Luc* in the human lung A549 cell line, dramatic differences in promoter-driven expression were observed within the first -150 and -208 base pairs for *CYP3A4* and *CYP3A5*, respectively. The most obvious difference between these two promoter regions is a *57-base pair insertion* in the *CYP3A4* promoter (-71 to -127; Fig. 2). More importantly, when introduced into the same region of the *CYP3A5* promoter, this insertion reduced the expression of *CYP3A5*-promoter constructs by roughly 50% (Fig. 4). It is important to note that the insertion of this 57 bp-region disrupts the regulatory CCAAT-box that is vital to drive basal expression in HepG2 cells (Iwano et al., 2001) and in A549s (this study). However, this insertion also includes a CCAAT-box at the 5'-end that

evolutionarily conserves this motif almost perfectly; suggesting either that another protein is interacting with this introduced region, or that distance from neighboring motifs is fundamental to maintaining this expression mechanism.

Two conclusions can be drawn from these observations: 1) differences between the proximal *cis*-elements of the *CYP3A4* and *CYP3A5* promoters reduce the expression of the *CYP3A4* gene in human lung cells; and 2) control of the lung-specific differences in expression observed in previous studies (Kivisto et al., 1996; Anttila et al., 1997; Hukkanen et al., 2000; Hukkanen et al., 2002) is a combination of these differences and the insertion of a repressor motif in the form of a 57 bp region containing two E-box motifs directly between the positive *cis*-elements and the transcriptional initiation site of the *CYP3A4* gene. A schematic diagram illustrating the mechanism by which the *CYP3A4* gene could be repressed through this region is depicted in Figure 10A.

The work described herein identifies a repressor motif (-71 to -127) within the 5'-flanking region of this gene that can be bound by at least one, currently unidentified nuclear protein expressed in human lung adenocarcinoma A549 cells and in normal human lung tissues. Nuclear proteins from both A549 lung cells and human lung tissues specifically bind the E-box motifs within this 57 bp region, and the fidelity of both motifs are required for the assembly of these specific regulatory protein complexes (Table 3, Fig. 8), suggesting that this region could associate with a double E-box binding protein resembling δ -EF1 (Remacle et al., 1999), a vertebrate polycomb protein involved in differentiation and long-term gene silencing. EMSA supershift experiments using antibodies generated against all known members of the δ -EF1 family of transcription factors failed to identify the protein associating with this region (data not shown);

suggesting the possibility of a novel member of the δ -EF1 family of transcription factors is binding to this region. Our repeated attempts to purify the protein(s) by DNA affinity chromatography were not successful, so we must refer to the protein(s) as a putative double E-box transcription factor.

The co-dependence on both E-box motifs could also be associated with cooperative binding of multiple transcription factors to this region. Although the detailed interactions between these motifs have not been rigorously established, these data strongly suggest that the active repression of *CYP3A4*, and not the lack of a transcriptional activator, is the operative mechanism in the human lung controlling the differential expression of these two very important genes. We believe that this is the first case where a cytochrome P4503A gene is actively 'silenced' in a tissue-specific manner. We have termed this *trans*-repressor protein as the Lung Specific Factor-2 (LSF2). Although the precise molecular mechanisms involved in LSF2 repression of *CYP3A4* in the human lung remain unclear, we hypothesize that LSF2 actively represses the expression of the *CYP3A4* gene in human lung cells.

The underlying mechanism could exhibit its effects through three different mechanisms (Johnson, 1995): 1) interfering directly with other *cis*-acting elements through competition for their cognitive *cis*-activation domain (Sekido et al., 1997), 2) physically blocking the guided assembly (or initiation) of the transcription machinery (Coumoul et al., 2002), or 3) having some chromatin remodeling capacity (Ringrose and Paro, 2004). A number of recent observations, both genetic and biochemical, suggest a different mechanism of CYP repression might be active in HepG2 liver cells. The PXR-SMRT mechanism of *CYP3A4* repression in the absence of ligand (Johnson et al., 2006),

involves both sequestration of *trans*-acting elements and direct recruitment of histone deacetylases involved in chromatin condensation. Supershift EMSA experiments using polyclonal goat antibodies raised against SMRT did not change the mobility of the A549 nuclear protein/DNA band. Therefore, it is highly unlikely that SMRT is the *trans*-element involved in the pulmonary repression mechanism.

Given that the 57 bp insertion is situated on the 3'-end of the promoter, the simplest mechanism would be that LSF2 would hinder the formation of the initiation complex either through steric hindrance or through the recruitment of chromatin remodeling complexes. Active repression by LSF2 is an attractive mechanism because it can override a number of different signal cascades with the same protein, regardless of which additional transcription factors might be actively transcribed within the cell.

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Footnotes

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Legends for figures

Fig. 1. Transcriptional activity of *pGL3-CYP3A4* and *pGL3-CYP3A5* constructs transiently transfected into human lung A549 cells as measured using the Dual Luciferase reporter system (Roche). Expression levels were normalized to activity of the co-transfected *Renilla* luciferase vector, and are expressed as fold luciferase activity over the promoterless *pGL3-Basic* plasmid. For all experiments, *pGL3-CYP3A5* constructs (B) expressed considerably higher luciferase activity than that of *pGL3-CYP3A4* constructs (A) (50-fold vs. 2-fold, respectively). Error bars represent standard deviations of four replicate experiments run in quadruplicate.

Fig. 2. Schematic representation of the first 150 base pairs of the *CYP3A4* and *CYP3A5* 5'-upstream regions. The *CYP3A4* sequence has a series of subtle (i.e., one or two base pairs) differences in the core binding sequences of the CCAAT-box, BTE-box, and the ER6 motif that have been previously shown (Iwano et al., 2001) to cooperate in driving basal expression of *CYP3A5* in human hepatoma HepG2 cells. The most obvious difference between these two regions is a 57 base pair insertion (-71 to -127) in the *CYP3A4* promoter, which includes another intact CCAAT-box (-119 to -124) [shown to constitute a portion of the functional C/EBP α element (-121 to -130) for *CYP3A4* regulation in HepG2 cells (Bombail et al., 2004)] and two E-box binding motifs, which are in phase, two full turns of the DNA double helix apart. Sizes and distances of salient features are not drawn to scale.

Fig. 3. Effect of site directed mutations on *CYP3A5* promoter transcriptional activity. A)

The proximal -208 bp of the *CYP3A5* promoter (H3A5) was compared with the corresponding region of the *CYP3A5* pseudogene (H3A5P1). The differing nucleotides are numbered and the transcription factor binding sites known to be important for *CYP3A5* transcriptional activation in liver (Iwano et al., 2001) are indicated. B) Mutated *pGL3-CYP3A5 -208* constructs were transfected into A549 lung cells and the luciferase activities were compared with the luciferase activity produced by the wild type (*CYP3A5-208*) construct. Luciferase activities were normalized to the activity of the co-transfected *Renilla* luciferase vector, and are expressed as fold luciferase activity over the wild type. The data represent means \pm S.D. of four separate replicates. The experiment was repeated three times with similar results.

Fig. 4. Insertion of the *CYP3A4-57 bp insertion* into the concomitant region of the *CYP3A5* promoter reduces expression in the human lung adenocarcinoma A549 cell line. Salient features of the *CYP3A5/luciferase* reporter constructs are depicted to the left of the bar graph with individual numbers representing relative distance from the published transcriptional start site for *CYP3A5*. Bar graphs represent the relative fold activity over the promoterless *pGL3-Basic* vector. Data represent means \pm S.D. from quadruplicate experiments that have been normalized for transfection efficiency and protein content.

Fig. 5. Transcriptional activity of *pGL3-3A4* constructs, with and without the *CYP3A4-57* bp region, transiently transfected into human lung A549 cells as measured using the Dual Luciferase reporter system (Roche). When the 57 bp region had been completely excised from the *CYP3A4* promoter, no increase in luciferase activity was observed (64

bp Knockout and *Spacer*; $p > 0.1$). However, if the C/EBP α binding motif (-121 to -130), shown to be essential for *trans*-activation in HepG2 liver cells (Rodriguez-Antona et al., 2003) was maintained (*SacII* construct), expression levels were increased by about 4-fold in comparison to the complete *CYP3A4* construct ($p < 0.05$ significantly different when compared to wild-type *pGL3-3A4* expression levels). Expression levels were normalized to activity of the co-transfected *Renilla* luciferase vector, and are expressed as fold luciferase activity divided by the promoterless *pGL3-Basic* plasmid. Error bars represent the standard deviations of four replicates that were normalized for transfection efficiency and protein content. The experiment was repeated twice with similar results.

Fig 6. Transcriptional activity of wild-type *pGL3-3A4* and *pGL3-3A5* constructs, with and without the *CYP3A4-57 bp* region, transiently transfected into primary cell cultures. The largest (13 kbp) *CYP3A4* promoter (a kind gift from Dr. Tetsuya Kamataki, Sapporo, Japan), which presumably contained all viable transcriptional elements, did not enhance luciferase expression in NHBE cells. The *SacII* deletion construct (57 bp KO) significantly ($p < 0.05$) increased luciferase activity over the intact *CYP3A4* construct in NHBE cells but this deletion did not alter promotion in primary hepatocytes, both of which showed activity above that of the control vector, *pGL-3 Basic*. Insertion of the *CYP3A4-57 bp* region (57 bp inserted) into the wild type 3 kbp *pGL3-3A5* construct significantly decreased ($p < 0.05$) luciferase activity in transfected NHBE cells but not in primary hepatocytes. Data represent means \pm S.D. from nine replicates that were normalized for transfection efficiency.

Fig. 7. Quantitative real-time PCR analysis of CYP3A4 and CYP3A5 transcripts in primary and immortalized human lung cells. Total RNA was extracted from 10^6 NHBE or A549 cells and one microgram of total RNA was used to synthesize first-strand cDNA and then analyzed by quantitative real-time PCR. Copy numbers were determined by extrapolation from a standard curve that was constructed with known amounts of CYP3A4 or CYP3A5 plasmid DNA, and standardized to actin levels. N.D. = not detected (the mRNA amounts for CYP3A4 were below detectable levels in both lung cell types).

Fig. 8. Competitive EMSA assays of serial mutations of the *CYP3A4* 57 bp promoter element. Putative E-boxes within the *CYP3A4* 57 bp insertion are capable of binding in a sequence-specific manner with human lung adenocarcinoma A549 cell nuclear proteins. The ^{32}P -labeled 3A4-57 bp probe was incubated with nuclear extracts (4 μg total protein) prepared from A549 lung cells with various competitors. When competitors were included in the incubations, they were added 10 minutes prior to the addition of the ^{32}P -labeled 3A4-57 bp probe. Therefore, the ability to compete for, rather than displace, the ^{32}P -labeled 3A4-57bp probe for A549 nuclear proteins was assayed. The two major protein/DNA complexes are marked with arrows. Competitive oligonucleotides were created by mutating five consecutive base pairs at a time, and are labeled with numbers above the lanes that correspond to the appropriate mutated sequences shown in Table 3. The CCAAT-box and the two putative E-box motifs are labeled above the 57 bp sequence and the consensus sequences are underlined. The five base pairs that were mutated for each competitive incubation are identified above each lane and demarcated

with vertical lines. Notations are as follows: Probe, ^{32}P -labeled 3A4-57 bp probe only; A549, ^{32}P -labeled 3A4-57 bp probe incubated with A549 nuclear extracts; Cold, 100-fold molar excess of unlabeled 3A4-57 bp; and NF κ B, 175-fold molar excess of NF κ B consensus oligonucleotide.

Fig. 9. EMSA analysis of lung and liver cellular nuclear extract binding to the 57 bp motif. Nuclear extracts (6 μg) from A549 (lanes 1-3) or HepG2 (lanes 4-6) cells were mixed with 0.005-0.01 pmol ^{32}P -labeled 57 bp oligonucleotide probe, and the protein/DNA complexes and unbound probes were separated by non-denaturing gel electrophoresis using 4% polyacrylamide gels and detected by autoradiography as described in Materials and Methods. Lanes 2 and 5 are samples from incubations that contained a 100-fold excess of unlabeled specific 57 bp oligonucleotide, incubated for 15 min with nuclear extract prior to the addition of labeled probe. Lanes 3 and 6 are samples from incubations that contained a 100-fold excess of non-specific oligonucleotide, incubated for 15 min with nuclear extract prior to the addition of labeled probe. Right-facing arrows point to the two major protein/DNA complexes of lung cell proteins with the 57 bp probe. Left-facing arrows point to the two major protein/DNA complexes of liver cell proteins with the 57 bp probe. Binding of liver nuclear extracts (lanes 4-6) to the probe was considerably weaker than lung extracts, because most of the radioactive probe stayed with the dye front at the bottom of the gel.

Fig. 10. A) Schematic representation of the LSF2 mechanism of repressing *CYP3A4* in the human lung. Actions of both the proximal (Rodriguez-Antona et al., 2003) and the

distal (Matsumura et al., 2004) elements converge upon an ER-6 motif and a CCAAT-box in order to recruit the RNA Pol II transcriptional machinery to the *CYP3A4* transcriptional start site. By binding to the 57 bp insertion located immediately downstream of this region (-71 to -128), LSF2 disrupts these events through one or a combination of actions: 1) altering the recognition of the CCAAT-box by these factors, 2) physically inhibiting the assembly of the complex, or 3) aiding the condensation of chromatin to mask the *cis*-elements. B) the sequence highlighting the protein/DNA interactions of the *CYP3A4* promoter region. Highlighted are the 57 bp insertion (red); the HNF-3 γ , ER-6, C/EBP α , and TATA transcription factor binding sites (grey); the E-box sites (underlined); and the core nucleotides for binding of A549 nuclear extracts to the 57 bp probe from EMSA experiments (Fig 7) or the core nucleotides for binding of HepG2 nuclear proteins (yellow). The transcription start site (+1) and various cloning sites are identified as well.

Table 1. Primer Sequences* Used to Generate the *CYP3A4* Nested Deletion and Mutation Reporter Plasmids.

Nested Deletions	
-2795	5'gctagcAGGGGAGAGGCATG 3'
-301	5'gctagcGAGACAAGGGCAAG 3'
-222	5'gctagcCCCTTGGACTCCCCA 3'
-170	5'gctagcTATGAACTCAAAGG 3'
-150	5'gctagcGTGAGTGGTGTGTG 3'
-80	5'gctagcCAGGTGGCCCTGC 3'
-35	5'gctagcCTAGCATATAACA 3'
-24	5'gctagcACAATCCAACAGC 3'
reverse	5'aagcttTGGGATGAGAGCCAT 3'
Mutations	
3A4-57 bp-KO F	5'/5Phos/-TGCTACTGGCTGCAGCTCCAGCCCTGCCTCCTTCTCTAGC 3'
3A4-57 bp-KO R	5'/5Phos/-GTTGGCAAAGAATCACACACACACCACTCACTGACCTCC 3'
3A4-SacII-F	5'CAGGCAGAGCACAGGTGcCgCgGCTACTGGCTGCAGCTCCAGC 3'
3A4-SacII-R	5'GCTTCTCCACCgcGGAAGTTGGCAAAGAATCACAC 3'
3A4CAATSpacer F	5'acgaacgaacgaacgaacgaacgaTGCTACTGGCTGCAGCTCCAGCCCTGCCTCCTTCTCTAGC3'
3A4CAATSpacer R	5'cgttcgttcggttcggttcgGGAAGTTGGCAAAGAATCACACACACCACTCACTGACCTCC3'

*Residues which match wild-type sequences are shown in upper case. Mutagenized residues are shown in lower case.

KO = knockout

F = forward primer

R = reverse primer

Table 2. Primer Sequences* Used to Generate the *CYP3A5* Nested Deletion and Mutation Reporter Plasmids.

Nested Deletions	
-5.3kb R (XhoI)	5'ctcgagTCTTCAGCCAAGCTGCTGAAA 3'
-3kb F (Mlu I)	5'GTGAGAacgcgtGAAAATGATTT 3'
reverse (Bgl II)	5'CTATGCagatctAGCGTCCAACACTTC 3'
Mutations	
3A5+57 bp-mut F	5'tgctacttccaactgcaggcagagcacaggtggcccTTGGCTGCAGCTATAGCCCTGCC 3'
3A5+57 bp-mut R	5'aggcttctccaccttgaagttgCAAAGAATCGCACACACCCTTTGCTGACCTCTTTTGA 3'
Mut 1	5'GCCAATGGCTCCACTTcAGTTCCTGATAAGAACC3'
Mut 2	5'ATGGCTCCACTTGAGTTtCTGATAAGAACCAGAAC3'
Mut 3	5'AGTTCCTGATAAGAACtCAGAACCCTTGGACT3'
Mut 4,5,6,7	5'CTTGAGTTCCTGATAAGAACCAGgttCCgTGGACTCCCCGATAAACTG3'
Mut 8,9	5'CAGAACCCTTGGACTCCctGATAAaACTGATTAAGCTTTTCATGAT3'
Mut 10,11,12	5'ACTCCCCGATAAACTGATTAAGtTgTTtATGATTCCTCATAGAACATGAACTC3'
Mut 13	5'CTGATTAAGCTTTTCATGATTCCcCATAGAACATGAACTCAAAGA3'
Mut 14	5'CTTTTCATGATTCCTCATAGAAaATGAACTCAAAGAGGTCAGC3'
Mut 15,16	5'TAGAACATGAACTCAAAGGAGGTaAGCAAAGGGGTGTGTG3'
Mut 17	5'GTGCGATTCTTTGCTAcTGGCTGCAGCTATAGCC3'
Mut 18,19	5'CTATTGGCTGCAGCTgcAGCCCTGCCTCCTTC3'
Mut 20,21	5'CTGCAGCTATAGCCCcaCCTCCTTCTCCAGCA3'

*The mutated nucleotides are indicated in lowercase. The reverse primers used were the reverse complements, but were otherwise identical.

F = forward primer

R = reverse primer

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Table 3. Sequences of the Competitive Oligonucleotides Used in EMSA

Label	Sequence (sense strand)*	Compete
Wild	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGTGTACC	+++
1	GTAAC ACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	++
2	TGCC CAGGA CAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	++
3	TGCCAACTT ACCTT TGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	-
4	TGCCAACTTCCAAG GTTC TAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	-
5	TGCCAACTTCCAAGGTGGAG CCTAA TCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	+++
6	TGCCAACTTCCAAGGTGGAGAAG CCGAGGA CAACTGCAGGCAGAGCACAGGTGGCCC	+
7	TGCCAACTTCCAAGGTGGAGAAGCCTCT TACC AGGCAGGCAGAGCACAGGTGGCCC	+++
8	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACT TACTT CAGAGCACAGGTGGCCC	+++
9	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAG GA CTCTCACAGGTGGCCC	++
10	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAG CACT TGTGGCCC	++
11	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAG TGTTACC	-
M9	TGCCAACT G CCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	+++
M12	TGCCAACTT CCG AGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	+++
M15	TGCCAACTTCCAAG T TGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	-
M18	TGCCAACTTCCAAGGT G TAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGTGTACC	+++
M25	TGCCAACTTCCAAGGTGGAGA A CACTCTTCCAACTGCAGGCAGAGCACAGGTGGCCC	+
M31	TGCCAACTTCCAAGGTGGAGAAGCCTCTT CA AACTGCAGGCAGAGCACAGTGTACC	+++
M49	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAG CACT TGGTGGCCC	-
M52	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAG G AGGCC	-
M55	TGCCAACTTCCAAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGT GGA CC	+++
AP1	CGCTTGATGAGTCAGCCGGAA	-
NFκB	AGTTGAGGGGACTTTCCAGGC	-
Sp1	ATTTCGATCGGGCGGGCGAG	-
OCT1	TGTCGAATGCAAATCACTAGAA	-
2F1- LSF1	CTCCACGGCACCTTTCCAGCTGGCTGTGAG	-

*The two putative E-boxes are shaded. Mutagenized residues are shown in bold type. -, no, +, weak, and +++, strong competition for lung nuclear proteins against ³²P-labeled wild-type probe.

Fig 1

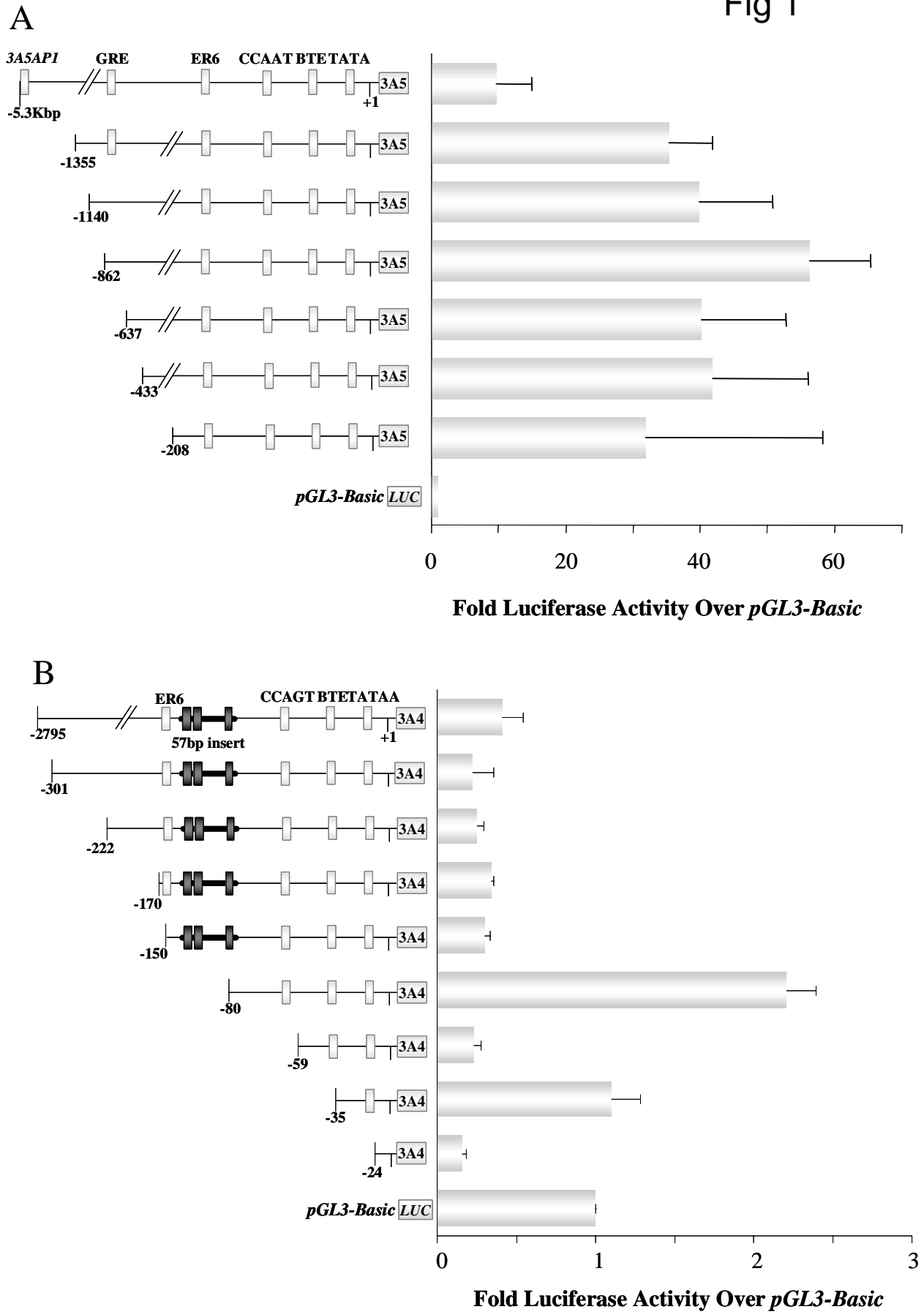


Fig 2

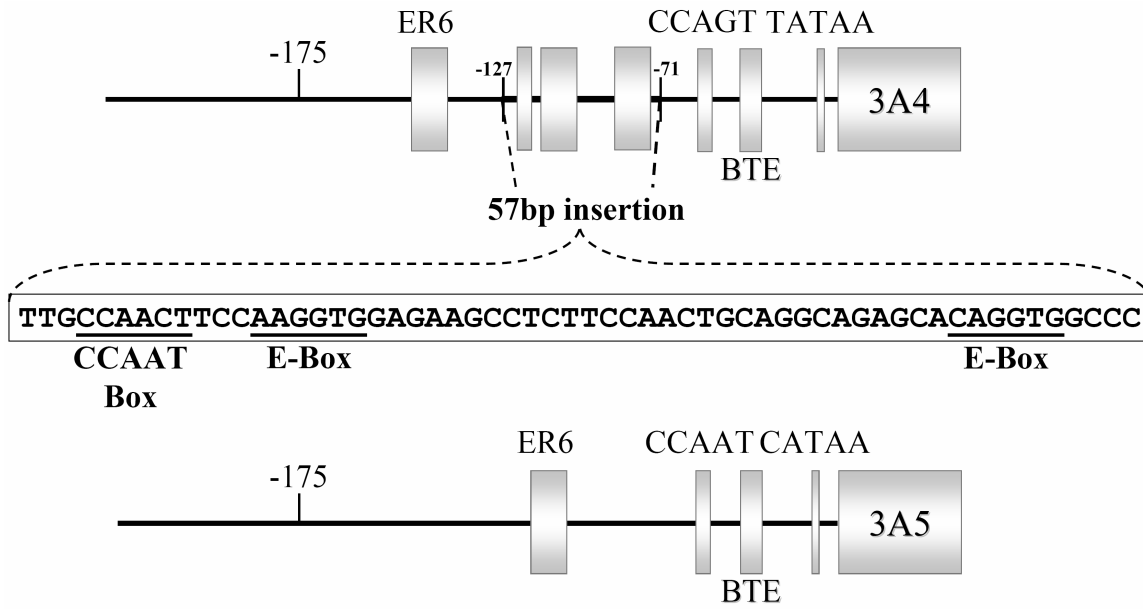


Fig 3

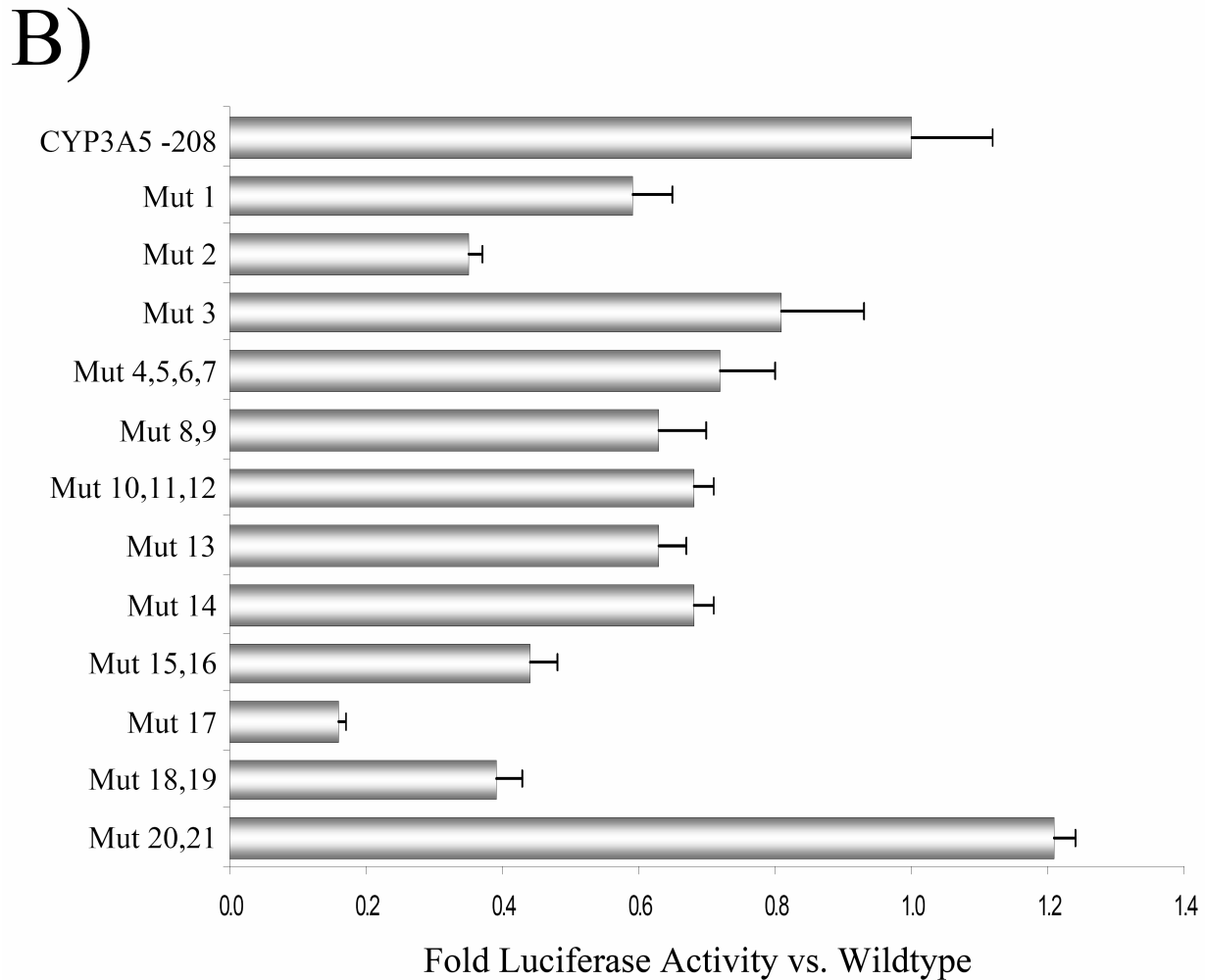
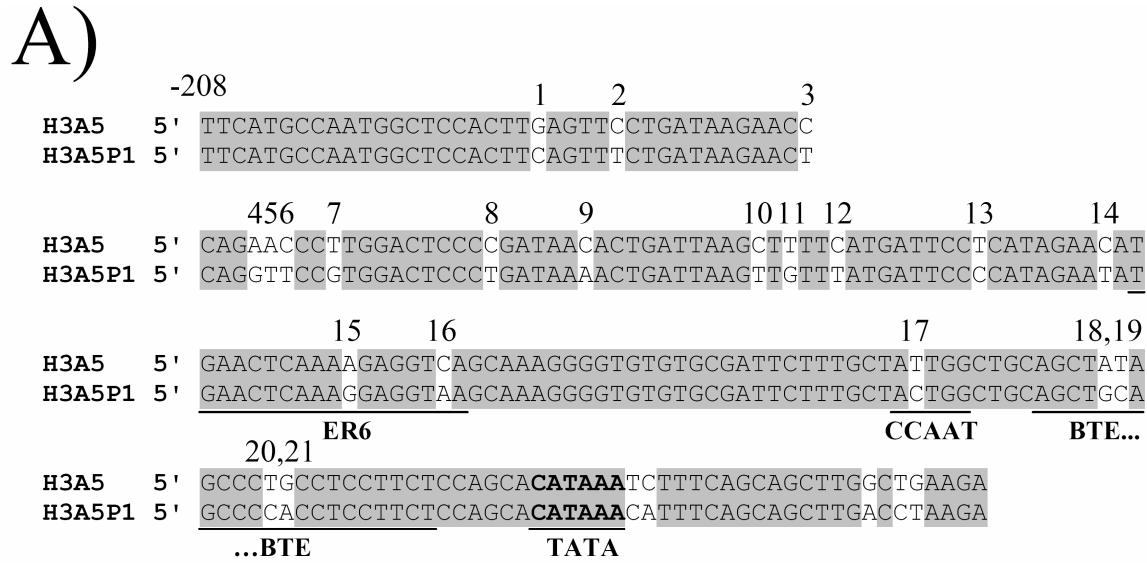


Fig 4

Luciferase Activity of Mutant *pGL3-3A5* Constructs in A549 Lung Cells

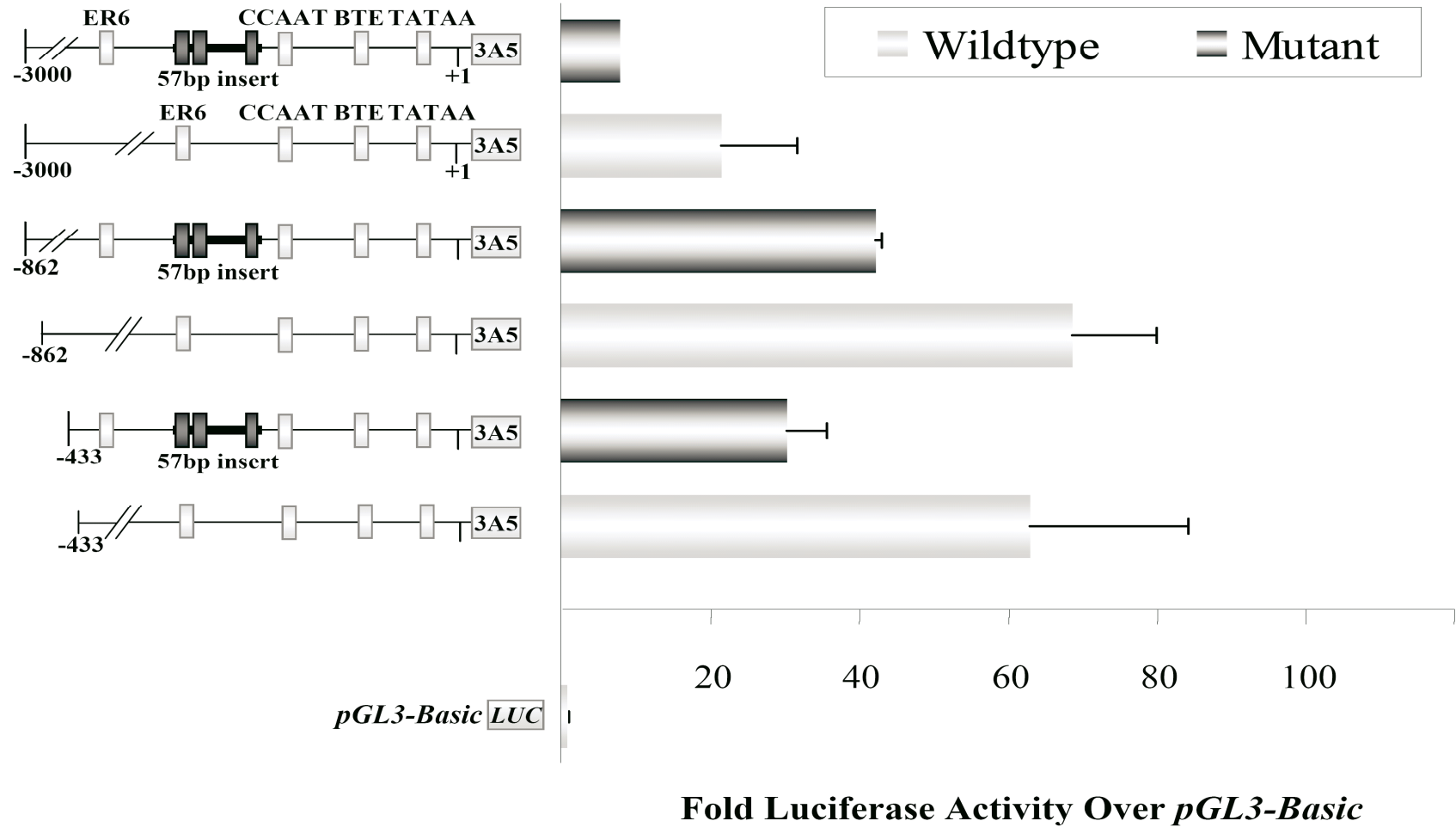


Fig 5

Luciferase Activity of Mutant *pGL3-3A4* Constructs in A549 Lung Cells

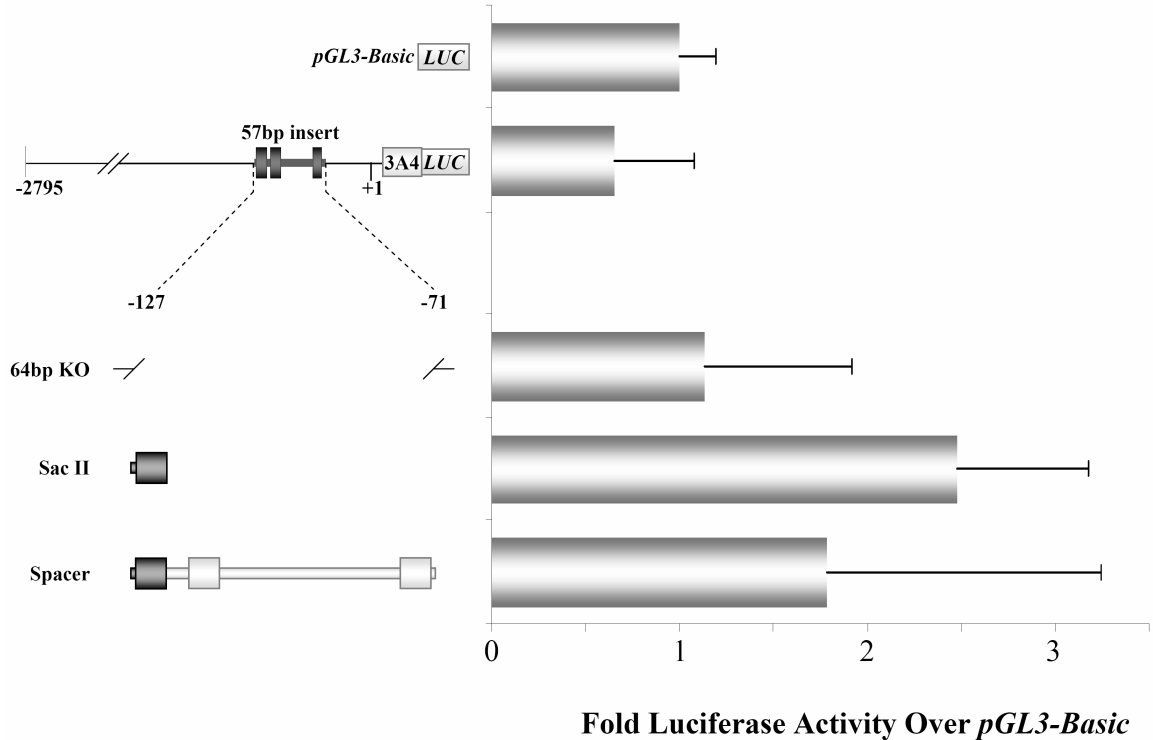


Fig 6

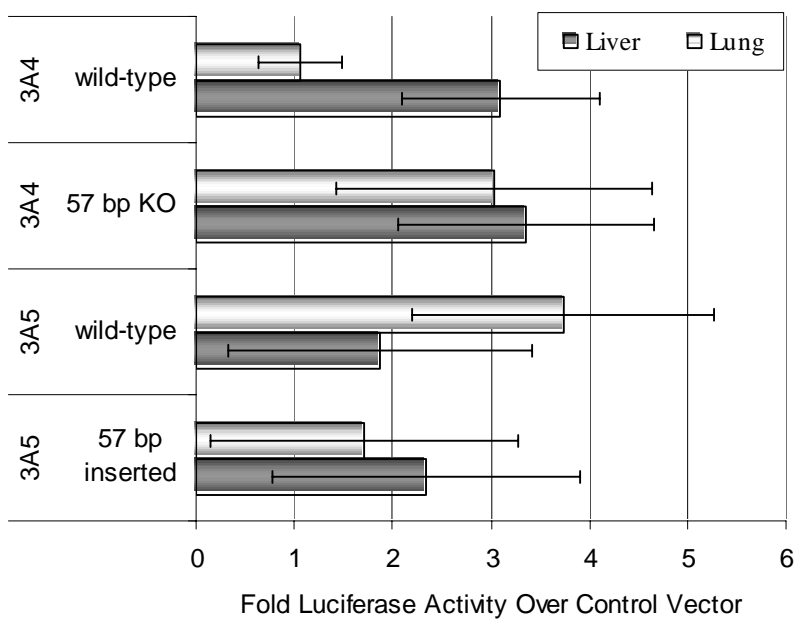


Fig 7

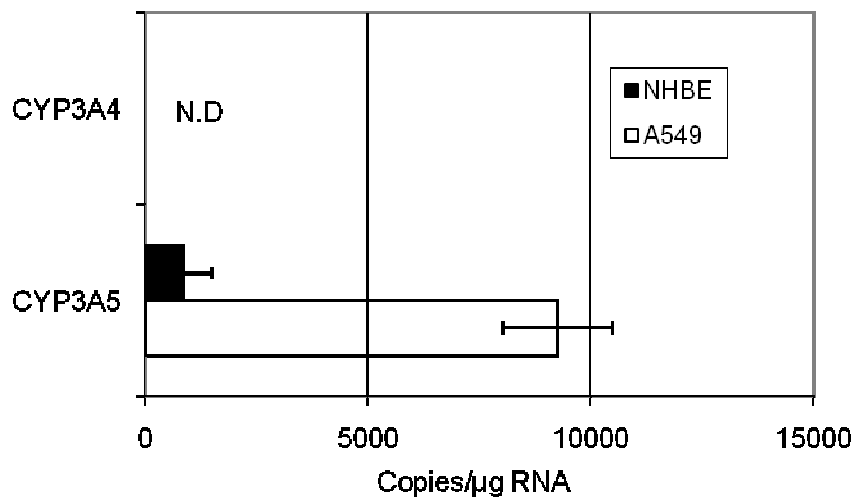


Fig 8

Probe A549 Cold

1	2	3	4	5	6	7	8	9	10	11										
CCAAT		E-box									E-box									
TTG	<u>CCA</u>	<u>ACT</u>	<u>TCC</u>	<u>AAG</u>	<u>TGG</u>	<u>GAG</u>	<u>AAG</u>	<u>CCT</u>	<u>CTT</u>	<u>CCA</u>	<u>ACT</u>	<u>GC</u>	<u>AGG</u>	<u>CAG</u>	<u>AGC</u>	<u>AC</u>	<u>AGG</u>	<u>TGG</u>	<u>CC</u>	
																				NFκB

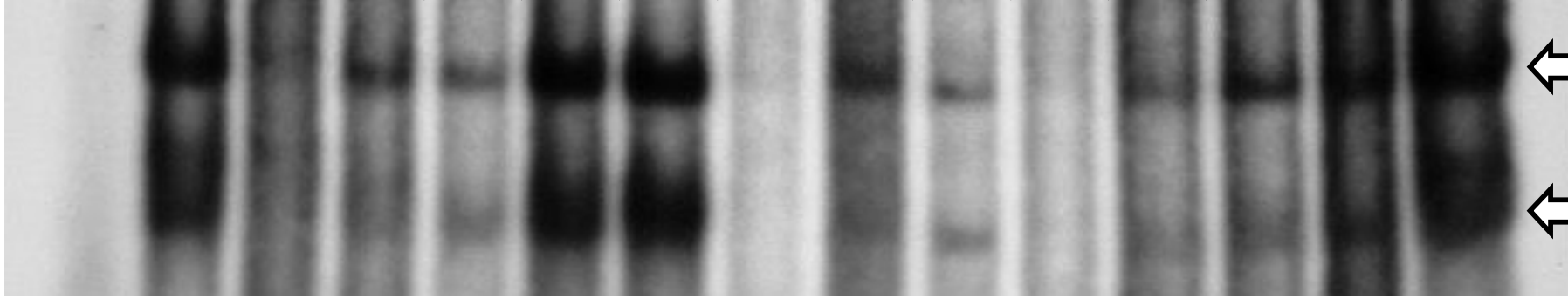


Fig 9

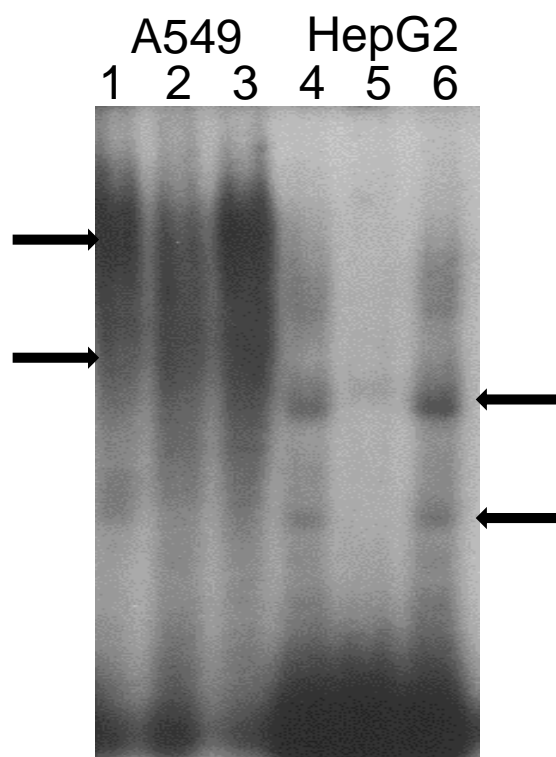


Fig 10

