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Transcriptional Regulation of Human *CYP2A13* Expression in the Respiratory Tract by CCAAT/Enhancer Binding Protein and Epigenetic Modulation

Guoyu Ling, Yuan Wei, and Xinxin Ding

The Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York at Albany, Albany, New York

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ABSTRACT

CYP2A13, which is highly active in the metabolic activation of tobacco-specific nitrosamines, is selectively expressed in the respiratory tract, in which it is believed to play an important role in chemical carcinogenesis. The aim of this study was to determine the basis for tissue-specific regulation of *CYP2A13* gene expression. We have shown that expression of *CYP2A3*, the rat homolog of *CYP2A13*, is regulated by nuclear factor I (NFI) in a tissue-specific manner. In the present study, we found that the transcriptional regulation of human *CYP2A13* gene involves CCAAT/enhancer binding protein (C/EBP) transcription factors instead of NFI. DNase I footprinting and gel-shift assays with human lung nuclear extract identified two DNA elements bound by C/EBP. Reporter gene assays using a 216-base pair *CYP2A13* promoter fragment confirmed the activation of *CYP2A13* by transfected C/EBP factors, and results from chromatin immunoprecipitation assays indicated that C/EBP is associated with *CYP2A13* promoter in vivo in the olfactory mucosa of *CYP2A13*-transgenic mice. In NCI-H441 human lung cancer cells, we discovered that *CYP2A13* expression can be induced by a combined treatment with 5-aza-2′-deoxycytosine, a DNA demethylation agent, and trichostatin, a histone deacetylation inhibitor. In 5-aza-2′-deoxycytosine/trichostatin-treated NCI-H441 cells, overexpression of C/EBPδ, a lung-enriched C/EBP, led to additional increases in *CYP2A13* expression, whereas C/EBPδ knockdown by small interference RNA suppressed *CYP2A13* expression, findings that confirm a role for C/EBP in *CYP2A13* regulation. Our findings pave the way for further studies of the regulation of the *CYP2A13* gene, particularly the gene's potential suppression by airway inflammation, and the role of epigenetic modulation in the gene's tissue-selective expression.

The microsomal cytochrome P450 (P450) enzymes play important roles in the biotransformation of endogenous and exogenous compounds (Guengerich, 2004). P450-mediated metabolic activation often leads to the initiation of chemical toxicity, including carcinogenesis. Many P450 genes are expressed preferentially in organs that are in direct contact with the outside environment, such as the respiratory tract (Ding and Kaminsky, 2003). Of particular interest is the fact that several members of the *CYP2A* subfamily, including mouse *Cyp2a5*, rat *CYP2A3*, and human *CYP2A13*, are selectively expressed in the nasal mucosa and the lung (Su et al., 1996, 2000). Previous studies from our laboratory and others have demonstrated that these enzymes are highly effective in the metabolic activation of numerous xenobiotics compounds, including many procarcinogens (Ding and Kaminsky, 2003; Su and Ding, 2004).

Human CYP2A13 is the most efficient P450 enzyme in the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific, carcinogenic nitrosamine (Su et al., 2000; Wong et al., 2005). It is believed that the tissue-selective expression of CYP2A13 in the respiratory tract is an important contributing factor to smoking-related lung cancers, a notion also supported by recent epidemiological studies that linked *CYP2A13* genetic polymorphisms to incidence of smoking-related lung cancer (Wang et al., 2003). CYP2A13 also metabolizes many other toxic chemicals, such as 2,6-dichlorobenzonitrile (Su et al., 2000) and aflatoxin B1 (He et al., 2006).

Little is known about how the respiratory tract-selective

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ABBREVIATIONS: P450, cytochrome P450; ATCC, American Type Culture Collection; AzaC, 5-aza-2′-deoxycytosine; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; NFI, nuclear factor I; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; bZIP, basic region-leucine zipper; siRNA, small interference RNA; TBP, TATA box binding protein; TSA, trichostatin A; TSS, transcription start site; ANOVA, analysis of variance; HEK, human embryonic kidney; kb, kilobase(s); bp, base pair(s).

expression of *CYP2A13* and its rodent orthologs is achieved (Ling et al., 2004a). A better understanding of the transcriptional regulation of these P450 genes will help us to identify genetic, environmental, and pathophysiological factors that are responsible for the known, large interindividual variations in the levels of CYP2A13 expression in human lung (Zhang et al., 2004). Our recent studies on the regulation of rat *CYP2A3* expression indicated that nuclear factor I (NFI) transcription factors are bound in a tissue-selective fashion to the *CYP2A3* promoter in permissive tissues, to activate transcription, and that tissue-specific DNA methylation might be involved in the silencing of *CYP2A3* in nonpermissive tissues (Zhang and Ding, 1998; Ling et al., 2004b). However, it was not clear whether the same mechanisms occur in the regulation of human *CYP2A13*.

In the present study, we mapped the 5′- and 3′-ends of the CYP2A13 transcript, and we characterized the *CYP2A13* promoter by means of 1) reporter gene assays with promoter deletion constructs; 2) DNase I footprinting and gel-shift assays, using human lung nuclear extracts; and 3) in vivo chromatin immunoprecipitation assays, using tissues from CYP2A13-transgenic mice. We found that CCAAT-enhancer binding proteins (C/EBP), but not NFI, were bound to the *CYP2A13* promoter and that they activated *CYP2A13* expression in reporter gene assays. Furthermore, we established a human lung cancer cell-based model in which the endogenous *CYP2A13* gene can be reactivated by a combined treatment with 5-aza-2′-deoxycytosine (AzaC) and trichostatin A (TSA). We demonstrated that, in this model, *CYP2A13* expression is further stimulated by overexpression of C/EBPδ, whereas it is down-regulated by suppression of C/EBPδ expression using siRNA.

Materials and Methods

Identification of CYP2A13 cDNA 5′- and 3′-Ends by Rapid Amplification of cDNA Ends. 5′- and 3′-ends of CYP2A13 cDNA were identified using human lung Rapid Amplification of cDNA Ends (RACE)-ready cDNA (Ambion, Austin, TX) according to the manufacturer's instructions. HotStar *Taq*DNA polymerase (QIAGEN, Valencia, CA) was used to carry out nested-PCR reactions. CYP2A13 primers, designed to avoid amplification of CYP2A6 cDNA, included 5′-outer primer (5′-cacgccaaaacccttag-3′) and 5′-inner primer (5′-ccagactgacatcaagaccatc-3′) for 5′-RACE, and 3′-outer primer (5′-gctcagttcaccttgatgat-3′) and 3′-inner primer (5′-atgatgtccttcagagctgt-3′) for 3′-RACE. The nested-PCR products were gel-purified (Gel Purification Kit; QIAGEN) and cloned into pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA). Sequencing of isolated individual clones was performed in the Molecular Genetics Core Facilities of the Wadsworth Center (Albany, NY).

Plasmid Constructs. Flag-tagged C/EBPα, β, and δ expression vectors and the empty pMEX vector (Kim et al., 2002) were gifts of Dr. Simon Williams (Texas Tech University Health Science Center, Lubbock, TX). pGL3_Basic (containing a promoterless firefly luciferase reporter gene) and pGL3_PMO (containing a firefly luciferase reporter gene controlled by the SV40 promoter) were obtained from Promega. A 3.3-kb *CYP2A13* promoter fragment (−3344 to +17 bp) was amplified from a *CYP2A13* genomic clone (Hoffman et al., 1995) using the following primers: forward primer (5′-ggcggcgctagcactggacaaaatggcacaaaat-3′), containing an NheI site (underlined), and reverse primer (5′-ggcggcctcgagcagtgggatgatagatggtgat-3′), containing an XhoI site (underlined). The PCR product was first cloned into the pCR-XL-TOPO vector and then subcloned into the NheI/XhoI sites of the pGL3_Basic vector, resulting in p2A13. Reporter constructs p2A13_2153, p2A13_1008, p2A13_484, and p2A13_216 were made by inserting, respectively, SacI/XhoI (−2153 to +17), BglII/XhoI (−1008 to +17), NdeI/XhoI (−484 to +17), and PstI/XhoI (−216 to +17) fragments of p2A13 into the pGL3_Basic vector. p2A13_134 was made by PCR cloning using the same reverse primer as described above and a forward primer starting at −134 of the CYP2A13 promoter (5′-ggcggcgctagctggctgtgtcctaagctgtg-3′ containing an NheI site (underlined). The sequence of the subcloned *P450* promoter fragments in reporter constructs was confirmed to be identical with the *CYP2A13* genomic sequence (GenBank accession number NG_000008).

Cell Culture and Reporter Gene Assays. BEAS-2B human bronchial epithelial cells (ATCC CRL-9606; ATCC, Manassas, VA) were cultured in serum-free LHC9 medium (Biosource International, Camarillo, CA). Human hepatocellular carcinoma HepG2 cells (ATCC HB-8065) were cultured in modified Eagle's minimum essential medium (ATCC 30-2003) supplemented with 10% fetal bovine serum. NCI-H441 cells (ATCC HTB-174) were cultured in RPMI 1640 medium with 10% fetal bovine serum. Cells were maintained in humidified 5% CO_2 atmosphere at 37°C. Firefly luciferase reporter gene constructs (0.3 pmol each) and a *Renilla reniformis* luciferase construct pRL-SV40 (3 fmol, as an internal control; Promega, Madison, WI) were cotransfected into cells in six-well plates with the use of Lipofectamine 2000 (Invitrogen) or Fugene6 (Roche Applied Science, Indianapolis, IN). A549 human lung cancer cells (ATCC CCL-185), used for determining the effects of C/EBP overexpression on *CYP2A13* promoter activities, were cultured in Ham's F-12K medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum. Fifty nanograms of each of the expression vectors, or 25 to 100 ng of the C/EBPδ expression vector, was cotransfected with p2A13_216 and pRL_SV40 into A549 cells as described above. Cells were harvested 24 to 48 h after transfection. The Dual Luciferase Reporter Assay System (Promega) was used for determinations of the relative luciferase activities. Luminescence was measured using a luminometer (LB9501; Berthold Technologies, Bad Wildbad, Germany).

Nuclear Extract Preparation from Human Lung Tissue. Fresh lung tissues from an 18-year-old African-American male donor were provided by Tissue Transformation Technologies (Edison, NJ). The lung was shipped on wet ice and arrived at the Wadsworth Center ~16 h postmortem. Upon arrival of the specimens, the lung was cut into small pieces, snap-frozen in liquid nitrogen, and then stored at −80°C until use. The nuclear extract was prepared based on a published method with some modifications (Hattori et al., 1990). In brief, frozen tissues were crushed on dry ice and then homogenized in buffer H (Hattori et al., 1990) in the presence of a protease inhibitor cocktail (Roche 1836153, one tablet per 10 ml of homogenate), with use of a PT35 Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at a setting of 4 for 20 s followed by 5 strokes with a motor-driven Teflon pestle. The homogenates were filtered through four layers of cheesecloth and then spun at 1000*g* for 10 min. The pellets were resuspended in 10 volumes of buffer H supplemented with 0.1% Triton X-100, with use of a Dounce homogenizer (5 strokes, pestle B), and the suspension was kept on ice for 10 min. The samples were spun again, and the pellets were resuspended as before in buffer H, but without the detergent. The suspension was then mixed with 2 volumes of a cushion buffer (buffer H containing 2.2 M sucrose). The mixture was layered over 10 ml of the cushion buffer in ultracentrifuge tubes and then subjected to ultracentrifugation at 80,000*g* for 60 min. The nuclei were recovered from the bottom of the tubes after centrifugation. The nuclear extracts were prepared as described previously (Hattori et al., 1990) and dialyzed against 20 mM HEPES buffer, pH 7.6, containing 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 2 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mg/l each of aprotinin, leupeptin, and bestatin. After dialysis, the nuclear extracts were aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C.

DNase I Footprinting Assay. Probes for footprinting assays were generated by PCR with ^{32}P-end-labeled pGL vector sequencing primer *pGLprimer2* and unlabeled *Rvprimer3* (Promega). The tem-

plate for PCR was p2A13_216. The probe was gel-purified after PCR reaction. Approximately 90 μg of nuclear extract was incubated in the footprint buffer [20 mM HEPES, pH 7.6, containing 20% glycerol, 0.1 M KCl, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM $MgCl_2$, and 1.5 μg of poly(dI-dC)] for 10 min on ice, after which the labeled probe was added. The DNase I footprint assay was then performed as described in the Core Footprint System Manual (Promega).

Gel-Shift Assay. Gel-shift assay was performed as described previously (Ling et al., 2004b). Oligonucleotides containing a consensus C/EBP binding site were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and recombinant C/EBP proteins were purified from HEK293 cells transfected with a C/EBP expression vector using an anti-Flag affinity gel; column-bound recombinant protein was eluted with the FLAG peptide (Sigma). The binding reaction mixture, in a total volume of 20 μl, contained 10 mM HEPES buffer, pH 7.6, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 12% glycerol, 2 μg of poly(dI-dC), 0.04 pmol of a ^{32}P-end-labeled double-stranded DNA probe, and 4 μg of nuclear proteins or ~30 ng of a recombinant C/EBP. The mixture was first incubated on ice for 15 min in the absence of the probe; after addition of the probe, it was further incubated at room temperature for 30 min. For competition assays, a 50-fold excess of an unlabeled oligonucleotide probe, including the C/EBP element of the *CYP2B1* gene (5′-tctgaagttgcataactgagt-3′; Luc et al., 1996), was added before the addition of the labeled probe. In supershift assays, 2 μl of an anti-C/EBP antibody or a normal IgG (Santa Cruz Biotechnology; sc-9314, sc-150, sc-636, and sc-2027, for C/EBPα, β, and δ, and normal IgG, respectively) was added to the reaction mixture; the latter was incubated on ice for 45 min before the addition of the probe. The DNA-protein complexes and unbound probes were separated by electrophoresis through 5% nondenaturing polyacrylamide gels using Tris-glycine buffer and were visualized by use of a PhosphorImager instrument (Fuji BAS2000; FujiFilm, Tokyo, Japan) or by autoradiography.

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation (ChIP) assays for mouse olfactory mucosa were performed as described previously (Ling et al., 2004b) using pooled nasal tissues from *CYP2A13*-transgenic mice. Real-time PCR was performed to determine the abundance of *CYP2A13* and mouse *Cyp1a2* promoters in the immunoprecipitated samples. The differing extents of enrichment of *CYP2A13* and *Cyp1a2* promoters in various immunoprecipitated samples were calculated based on a method described by Friedman and coworkers (2004). The PCR primers used for detecting *CYP2A13* were 2A13−130F (5′-gtgtcctaagctgtgtgggatt-3′) and 2A13+16R (5′-gcagtgggatgatagatggtgat-3′), and those for detecting *Cyp1a2* were m1a2−187F (5′-ttatctccatggaccccaag-3′) and m1a2−78R (5′-tagctggatgctgcacaaag-3′).

AzaC/TSA Treatment, siRNA Transfection, and Real-Time RNA-PCR. For AzaC treatment, NCI-H441 cells at 40 to 50% confluence were exposed to AzaC (Sigma) at a final concentration of 2 μM continuously for 72 h. AzaC was added in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide was 0.02%. For TSA treatment, NCI-H441 cells at 90% confluence were treated with TSA (Sigma) at a final concentration of 0.5 μM for 24 h. TSA was added in ethanol; the final concentration of ethanol was 0.05%. For AzaC/TSA combined treatment, TSA was added 48 h after the addition of AzaC, and cells were harvested 24 h later. CYP2A13 mRNA was detected by RNA-PCR (Su et al., 2000). Quantitative analysis of CYP2A13 mRNA levels was carried out using real-time RNA-PCR as described previously (Zhang et al., 2004).

For determination of the effects of C/EBPδ on CYP2A13 expression in cells treated with AzaC/TSA, NCI-H441 cells were treated with AzaC at 0 h; then, at 24 h, they were transfected with 1.1 μg of the C/EBPδ expression vector or the pMEX empty vector, with use of Fugene6, in OptiMEM medium (Invitrogen) without AzaC. At 48 h, AzaC was added back to the cultures, followed by the addition of TSA at 72 h. Cells were harvested at 88 h for RNA preparation.

For down-regulation of C/EBPδ, siRNA duplex targeting human C/EBPδ was obtained from Ambion (sense, 5′-gacucagcaacgaccauatt-3′; antisense, 5′-uaugggucguugcugaguctc-3′). A scrambled siRNA duplex with no sequence homology to the human genome (Ambion) was used as a negative control. Transfection of siRNA duplexes was performed when cells reached 90% confluence using Lipofectamine 2000 (Invitrogen). Greater than 95% transfection efficiencies were routinely achieved, as indicated by the number of fluorescent cells after transfection with a fluorescein-labeled RNA duplex (Invitrogen) in parallel experiments. Cells were subcultured at a 1:2 ratio 48 h after transfection. At 64 h after transfection, AzaC was added to give a final concentration of 2 μM, and at 112 h after transfection, TSA was added to a final concentration of 0.5 μM. Cells were harvested at 136 h after transfection for RNA preparation.

RNA was prepared using an RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed using SuperScript III reverse transcriptases (Invitrogen). Real-time PCR quantitation of CYP2A13 cDNA was performed, in duplicate or triplicate, using a LightCycler instrument (Roche). PCR primers for C/EBPβ and δ quantitation were purchased from Qiagen. The levels of TBP in each RNA sample were also determined for data normalization (Zhang et al., 2004). For statistical analysis, significance of differences in pairwise comparisons between two groups was determined using Student's t test, whereas significance of differences in pairwise multiple group comparisons was determined using one-way ANOVA (Dunnett's method). Animal-use protocols were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

Results

Identification of CYP2A13 cDNA 5′- and 3′-Ends by RACE. CYP2A13 cDNA was originally cloned using a PCR approach (Su et al., 2000); the 5′- and 3′-untranslated sequences of endogenously expressed CYP2A13 mRNA have not been determined previously. The transcription start site (TSS) of *CYP2A13* was determined using the 5′-RACE approach. The nested-PCR products amplified from human lung cDNA, using *CYP2A13*-specific primers and a 5′-adapter primer, were cloned into the pCR-XL-TOPO vector. Four clones were analyzed, and they were found to have the same sequence, which, through a comparison with the sequence of the *CYP2A13* gene (GenBank accession number NG_000008), maps the TSS to 21 bp upstream of the ATG codon and 30 bp downstream of a TATA box (Fig. 1A).

Through the use of a similar strategy, the 3′-end of the CYP2A13 transcript was mapped to 251 bp downstream of the stop codon and 16 bp downstream of a putative polyadenylation signal (Fig. 1B). Four positive clones were identified, all of which had the same sequence. Our data did not reveal any other TSS or alternative polyadenylation sites.

Functional Analysis of Proximal Promoter Region by Reporter Gene Assays. Five promoter-deletion constructs, p2A13_2153, p2A13_1008, p2A13_484, p2A13_216, and p2A13_134, were generated and analyzed in human bronchial epithelial BEAS-2B cells and human hepatoma HepG2 cells. The rank order of relative transcriptional activities of the five differing constructs, as shown in Fig. 2, was similar in the two cell types. This result contrasts with the finding of a study using a different gene, *CYP2F1*, a gene that is also lung-selective; there, promoter constructs were active in BEAS-2B cells but silent in HepG2 cells (Carr et al., 2003). In the present study, the 134-bp and 216-bp *CYP2A13* promoter constructs were the most active; for the other constructs, activities decreased as the length of the promoter

fragment increased. For the 2.1-kb construct, activities were no more than 3-fold higher than that of the "promoterless" pGL3-Basic vector. Similar results were also seen in human embryonic kidney HEK293 cells and human choriocarcinoma JEG-3 cells (data not shown). These data indicate that the proximal promoter region (134 bp) can sustain basal expression, whereas distal sequences beyond −216 may serve as repressors.

Identification of Transcription Factor Binding Sites by DNase I Footprint Assays. DNase I footprint assays were performed using nuclear extracts from human lung and radiolabeled double-stranded DNA probes that cover the proximal promoter region (−216 bp to +17 bp). The lung tissue was obtained from a transplant donor (African-American male, 18 years old). As shown in Fig. 3A, two footprints were detected, one spanning −56 to −83 (Major) and the other spanning −114 to −133 (Minor). The Major footprint was clearer than was the Minor footprint, a fact that probably reflects a difference in either abundance of the cognate protein factors or else stability of the respective protein-DNA complexes. It is interesting that, as shown in Fig. 3, B and C, the Minor footprint overlapped with an NFI-like element; NFI elements are known to be present in several other *CYP2A* genes, such as mouse *Cyp2a5*, rat *CYP2A3*, and human *CYP2A6* (Zhang and Ding, 1998; Ling et al., 2004b; Ulvila et al., 2004). However, the NFI-like element in *CYP2A13* has a C>A substitution in the highly conserved CCAA site, a change that is expected to greatly decrease NFI binding affinity.

C/EBP Transcription Factors Bind to the *CYP2A13* Promoter In Vitro. The two footprints were further analyzed for the presence of binding sites of known transcription factors. A database search using Matinspector (Cartharius et al., 2005) and TFSEARCH (Heinemeyer et al., 1998) predicted potential binding sites for several bZIP transcription factors, including HLF and E4BP4. However, we did not find HLF or E4BP4 binding to the footprint region in antibody supershift assays performed using human lung nuclear extracts (data not shown). A potential, C/EBP-like binding site was identified by TFSEARCH within each of the two footprints (Fig. 3B), with a score of 82.2 and 73.1 for the Major and Minor footprint, respectively. Gel-shift and supershift assays were conducted using DNA probes corresponding to the Major and Minor footprints and human lung nuclear extracts. As shown in Fig. 4A, the two probes generated similar banding patterns, which consisted of multiple bands, indicating the interaction of the probes with multiple transcription factors. The majority of the band shifts was competed by an excess amount of the respective unlabeled probe or by a C/EBP element of the *CYP2B1* promoter reported previously (Luc et al., 1996), indicating specificity of protein binding (Fig. 4B). Supershifted bands were detected with the addition of antibodies to C/EBPα, β, and δ but not with normal IgG, anti-C/EBPγ or C/EBPε (Fig. 4A), anti-NFI, or anti-E4BP4 antibodies (data not shown). These data indicated that C/EBPα, β, and δ transcription factors were present in the protein-DNA complexes observed in the gel-shift assays. Nevertheless, because neither probe contained sequences highly homologous to classic C/EBP binding sites, it remained to be determined whether the association of C/EBP with the *CYP2A13* promoter was direct.

To determine whether the Major and Minor footprints can bind C/EBP proteins directly, we performed gel-shift assays

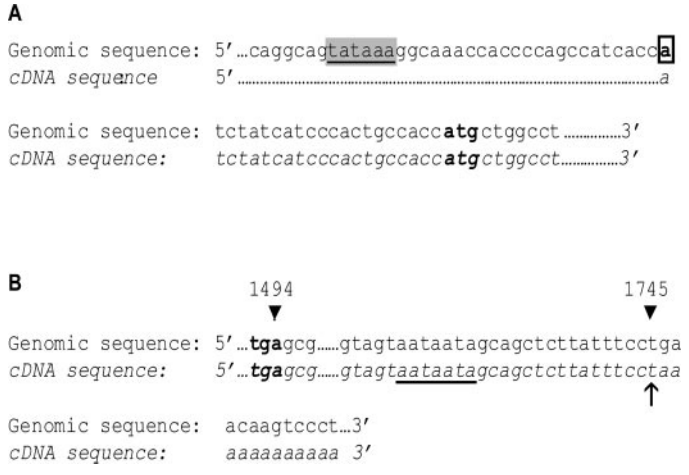

Fig. 1. The 5′- and 3′-ends of CYP2A13 transcript. A, sequence alignment of 5′-RACE cDNA with *CYP2A13* genomic DNA. The TSS is shown in an open box. The TATA box is shaded, and the ATG codon is shown in bold. B, sequence alignment of 3′-RACE cDNA with *CYP2A13* genomic DNA. The 3′-end is indicated by an arrow. The putative polyadenylation signal is underlined. The stop codon is shown in boldface type.

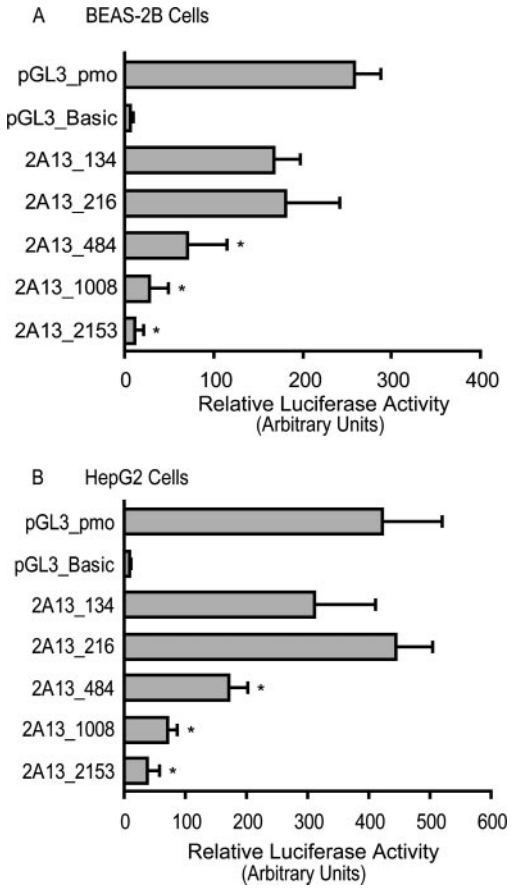

Fig. 2. *CYP2A13* promoter activity in BEAS-2B and HepG2 cells. BEAS-2B (A) and HepG2 (B) cells were harvested 24 h after transfection with one of the *CYP2A13* promoter constructs or a control vector (pGL3_Basic or pGL3_pmo). Activities of firefly luciferase in cell lysates were normalized to those of cotransfected *R. reniformis* luciferase, and the relative activities are expressed in arbitrary units. Data (means ± S.D.) represent three independent experiments. *, significantly lower than the activities in cells transfected with the 2A13_216 construct ($p < 0.05$; one-way ANOVA, Dunnett's method).

using purified Flag-tagged C/EBPδ recombinant proteins. A probe containing the consensus C/EBP element was included as a positive control. All probes were labeled with ^{32}P to similar specific activities. As shown in Fig. 4C, recombinant C/EBPδ formed a very strong complex with the consensus C/EBP probe. The C/EBPδ also formed a complex with the Major probe, but the density of the gel-shift band was ~10 times lower than the density observed for the complex formed with the consensus probe (based on estimates made at a lower exposure; data not shown). However, C/EBPδ did not form a complex with the Minor probe, contrary to findings from supershift assays using human lung nuclear extracts (Fig. 4A) and BEAS-2B lung cell lysate (data not shown). These data led us to conclude that C/EBP alone can bind directly to the Major footprint but not to the Minor footprint and that additional protein partners, which were present in human lung nuclear extracts, may be required for a higher-affinity association at either site.

C/EBP Is Associated with *CYP2A13* Promoter in Vivo in a *CYP2A13*-Transgenic Mouse Model. To detect in vivo interaction of C/EBP with *CYP2A13* promoter in a native chromosome environment, we performed ChIP assays. Because of difficulties associated with tissue availability and assay sensitivity, it was not possible to perform this assay with human lung or nasal mucosa. It was fortunate that a bacterial artificial chromosome *CYP2A13*-transgenic mouse model became available, in which the tissue-selectivity of human CYP2A13 was preserved, with predominant CYP2A13 expression in the nasal mucosa (Y. Wei and X. Ding, unpublished results). Therefore, we determined whether C/EBP and NFI are bound to the proximal promoter region of the *CYP2A13* transgene in mouse nasal mucosa. The ChIP assays were performed using anti-C/EBP and anti-NFI antibodies, and with olfactory mucosa obtained from male, adult, homozygous transgenic mice. The abundance of pulled-down promoter sequences for human *CYP2A13* and mouse *Cyp1a2* was measured by quantitative PCR for each sample. Normal IgG was used as a negative control for non-specific DNA binding during immunoprecipitation. *Cyp1a2*, which has a functional NFI binding site within its promoter (Zhang et al., 2000), was used as a positive control for NFI binding and, potentially, as a negative control for C/EBP binding to *CYP2A13*, because *Cyp1a2* is not known to be regulated by C/EBP.

As shown in Table 1, a significant, positive enrichment of *CYP2A13* (relative to control IgG-treated samples) was observed in samples immunoprecipitated with the C/EBPδ antibody but not in those precipitated with antibodies to C/EBPα, C/EBPβ, or NFI, indicating that C/EBPδ was bound to the *CYP2A13* promoter in vivo in the tissues analyzed. In contrast, a significant positive enrichment of *Cyp1a2* (relative to control IgG-treated samples) was observed only in samples immunoprecipitated with the NFI antibody, consistent with the known function of NFI in *Cyp1a2* regulation (Zhang et al., 2000). When the relative abundances of *CYP2A13* and *Cyp1a2* promoter fragments were compared in the same immunoprecipitated samples, significantly positive enrichment was found for C/EBPδ and significantly negative enrichment was found for NFI (Table 1). Thus, C/EBPδ, but not NFI, was associated with the *CYP2A13* promoter, whereas NFI, but not any of the C/EBPs studied, was associated with *Cyp1a2* promoter in vivo.

C/EBP Transactivates *CYP2A13* Promoter In Vitro. Effects of C/EBP transcription factors on *CYP2A13* gene

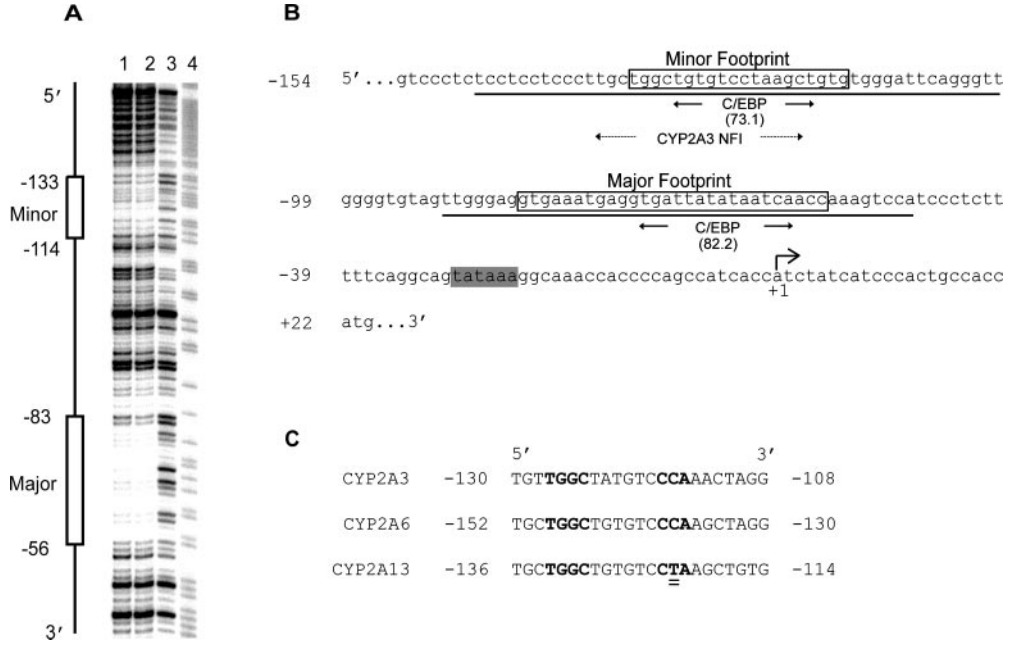

Fig. 3. DNase I footprint analysis of the *CYP2A13* proximal promoter region. A, DNase I footprinting assay was performed using a DNA probe (−216 to +17), labeled with ^{32}P at the 5′-end on the noncoding strand and human lung nuclear extracts. Two footprints were detected, one at −114 to −133 (Minor), and the other at −56 to −83 (Major). Lanes 1 and 2, complete reactions (duplicate); lane 3, reaction with nuclear extract omitted; lane 4, G/A sequencing ladder. B, sequences of *CYP2A13* proximal promoter region. The two footprints are boxed; the sequences of the probes used for gel-shift assays are underlined; and the positions of the putative C/EBP binding sites are indicated by double-headed arrows (with similarity scores shown in parentheses). The right-turn arrow indicates TSS (+1). The position corresponding to the NFI element in rat *CYP2A3* is delimited by the dotted arrow. C, comparison of the *CYP2A13* Minor footprint region with the conserved NFI binding site in the promoters of *CYP2A3* and *CYP2A6*. Important motifs for NFI binding are shown in bold. The cytosine-to-thymine change in the critical CCA motif is double-underlined.

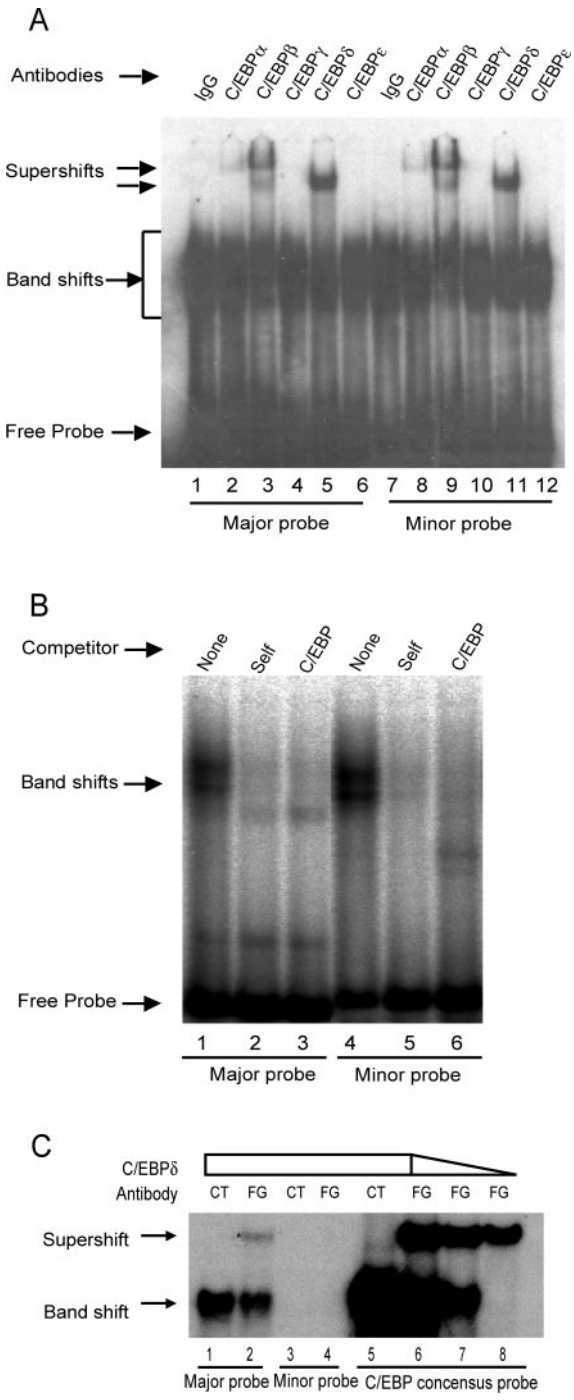

Fig. 4. Gel-shift assays for C/EBP binding to *CYP2A13* promoter. A, gel-shift assays with human lung nuclear extracts. The sequences of the Major probe and the Minor probe were shown in Fig. 3B. Human lung nuclear extracts (4 μg each) were incubated with the labeled probes in the presence of the indicated antibodies. The reaction mixtures were resolved by electrophoresis, and bands were visualized by autoradiography. Lanes 1 to 6, Major probe; lanes 7 to 12, Minor probe. The positions of the free probe and the shifted bands are indicated. B, the specificity of protein binding to the Major probe (lanes 1–3) and the Minor probe (lanes 4–6) was demonstrated in competition assays in which a 50-fold molar excess of either the unlabeled self-competitor (Self) or a C/EBP element of the *CYP2B1* promoter (C/EBP) was added. C, gel-shift assays with recombinant, FLAG-tagged C/EBP δ protein. The FLAG-tagged C/EBP δ protein was incubated with the Major probe (lanes 1 and 2), the Minor probe (lanes 3 and 4), or a consensus C/EBP binding site probe (5′-tgcagattgcgcaatctgca-3′; Santa Cruz Biotechnology) (lanes 5–8), in the presence of 2.0 μg of control IgG (CT; lanes 1, 3, and 5) or anti-FLAG IgG (Sigma; lanes 2, 4, and 6–8, labeled FG). The amount of FLAG-tagged C/EBP δ protein in the reaction mixtures was 30 ng for samples in lanes 1 to 6; however, it was 10 ng and 3.3 ng for the samples in lanes 7 and 8, respectively. C/EBP δ was bound to the Major probe and the consensus C/EBP binding site probe but not to the Minor probe; the band shift was supershifted by the anti-FLAG antibody.

expression were initially examined using the p2A13_216 reporter construct in lung-derived A549 cells, which have low endogenous C/EBP expression (Cassel et al., 2000). Cotransfection with C/EBPα, β, or δ each led to transactivation of the *CYP2A13* promoter (Fig. 5A). The transactivation occurred in a dose-responsive manner, as shown in Fig. 5B for C/EBPδ. These data indicate that C/EBP factors are capable of transactivating *CYP2A13* expression in reporter assays.

Activation of Endogenous *CYP2A13* Expression by C/EBPδ in a Chromosomal Context. A cell line that constitutively expresses the endogenous *CYP2A13* gene at readily detectable levels has not been identified, a fact that makes it difficult for us to examine *CYP2A13* expression in a chromosomal context. Here, we show that *CYP2A13* expression can be reactivated in NCI-H441 human lung adenocarcinoma cells. NCI-H441 cells are believed to be derived from Clara cells (Cassel et al., 2000), which have relatively high P450 expression level in vivo (Hukkanen et al., 2002). The NCI-H441 cell line has been used in studies of other lung-specific C/EBP target genes, such as the Clara cell secretory protein gene (Cassel et al., 2000). Basal expression of *CYP2A13* in the NCI-H441 cells was very low, at levels barely detectable by a highly sensitive reverse transcription-polymerase chain reaction protocol (Fig. 6A). Overexpression of C/EBPα, β, or δ did not significantly augment *CYP2A13* expression in these cells (data not shown), suggesting that other factors, such as the presence of coactivators and a permissive chromatin structure, are required for *CYP2A13* gene expression.

DNA demethylation and histone deacetylase (HDAC) inhibition are known to alter chromatin structure and to derepress gene expression in many cases (Cameron et al., 1999). We found that combined treatment with AzaC (a DNA demethylation agent) and TSA (an inhibitor of HDAC)—but not treatment with either AzaC or TSA alone—augmented *CYP2A13* expression by ~10-fold (Fig. 6, A and B). In the AzaC/TSA-treated cells, overexpression of C/EBPδ led to a further 2-fold increase in the expression of the endogenous *CYP2A13* (Fig. 6C). These data indicate that, under conditions permissive of constitutive expression of the *CYP2A13* gene, *CYP2A13* expression could be further increased by C/EBPδ overexpression. However, similar attempts to demonstrate the ability of transfected C/EBPα or C/EBPβ to further activate *CYP2A13* expression were unsuccessful (data not shown); the failure might have been due to, at least in part, interference by endogenous C/EBP factors.

C/EBPδ Knockdown by siRNA Decreases *CYP2A13* Expression. To examine more directly the roles of C/EBP in *CYP2A13* expression in vivo, we used an RNA interference approach to determine whether depletion of C/EBPδ affects *CYP2A13* expression. NCI-H441 cells were first transfected with C/EBPδ siRNA duplexes and then treated with AzaC and TSA at 64 and 112 h, respectively, after transfection. At 136 h after transfection, cells were harvested for RNA prep-

aration and subsequent quantitative RNA-PCR analysis. As shown in Fig. 7, C/EBPδ siRNA treatment led to ~70% decreases in C/EBPδ mRNA and corresponding deceases in CYP2A13 mRNA, compared with cells transfected with a scrambled (negative control) siRNA. As further evidence for targeting specificity, C/EBPβ mRNA levels were not decreased in cells treated with C/EBPδ siRNA. Thus, C/EBPδ knockdown by siRNA decreases *CYP2A13* expression.

TABLE 1

ChIP analysis of C/EBP and NFI binding to CYP2A13 and Cyp1a2 promoters in the olfactory mucosa of the CYP2A13-transgenic mice

Tissues were pooled from three male, 2-month-old homozygous mice. Equal amounts of chromatin were used for each immunoprecipitation experiment. Each immunoprecipitated sample was analyzed for the abundance of *CYP2A13* and *Cyp1a2* promoter fragments using real-time PCR relative to the amounts of each promoter detected in samples precipitated by the control IgG. The values presented (means ± S.D., $n = 3$) are in arbitrary units. The results shown are typical of three independent experiments performed.

Antibody	Relative Abundance of Promoter Fragments		*CYP2A13*/*Cyp1a2* Ratio	*p* Value[a]
	CYP2A13	*Cyp1a2*		
Normal IgG	100 ± 17	100 ± 16	1.00	
Anti-C/EBPα	131 ± 12	114 ± 11	1.15	0.17
Anti-C/EBPβ	93 ± 15	108 ± 7	0.86	0.17
Anti-C/EBPδ	174 ± 3[b]	121 ± 18	1.43	0.008
Anti-NFI	125 ± 21	259 ± 25[b]	0.48	0.002

[a] For differences between the relative amounts of *CYP2A13* and *Cyp1a2* in each sample (Student's *t* test).

[b] Significantly higher than in samples incubated with normal IgG ($P < 0.01$).

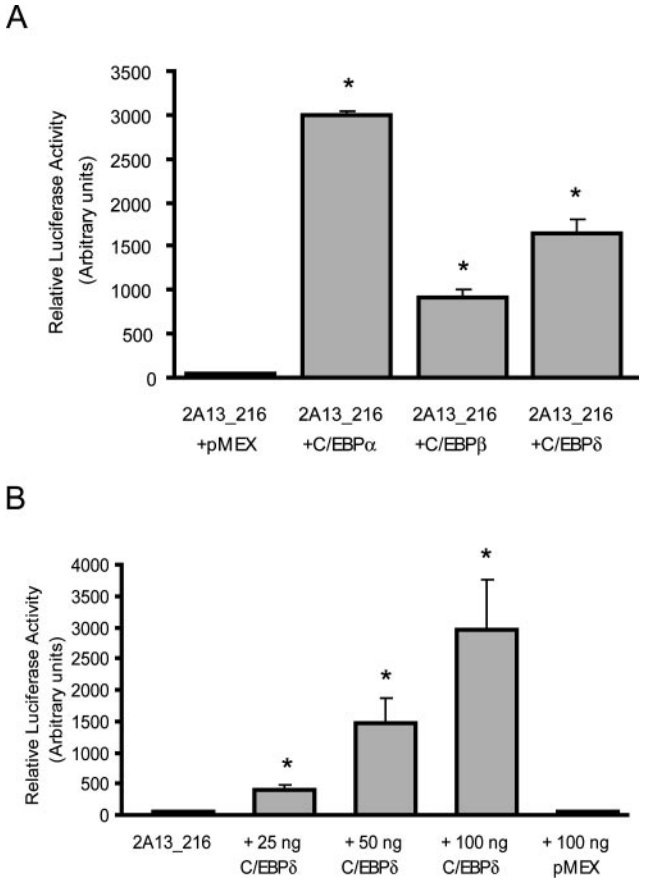

Fig. 5. Transactivation of *CYP2A13* promoter by C/EBP in A549 cells. Reporter construct p2A13_216 was cotransfected with the indicated vectors. Cells were harvested 24 h after transfection, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). A, activation of *CYP2A13* promoter by C/EBPα, β, and δ isoforms. B, activation of *CYP2A13* promoter by lung-enriched C/EBPδ in a dose-response manner. Values presented are means ± S.D. ($n = 3$). *, $p < 0.05$, compared with cells transfected with empty vector (one-way ANOVA, Dunnett's method).

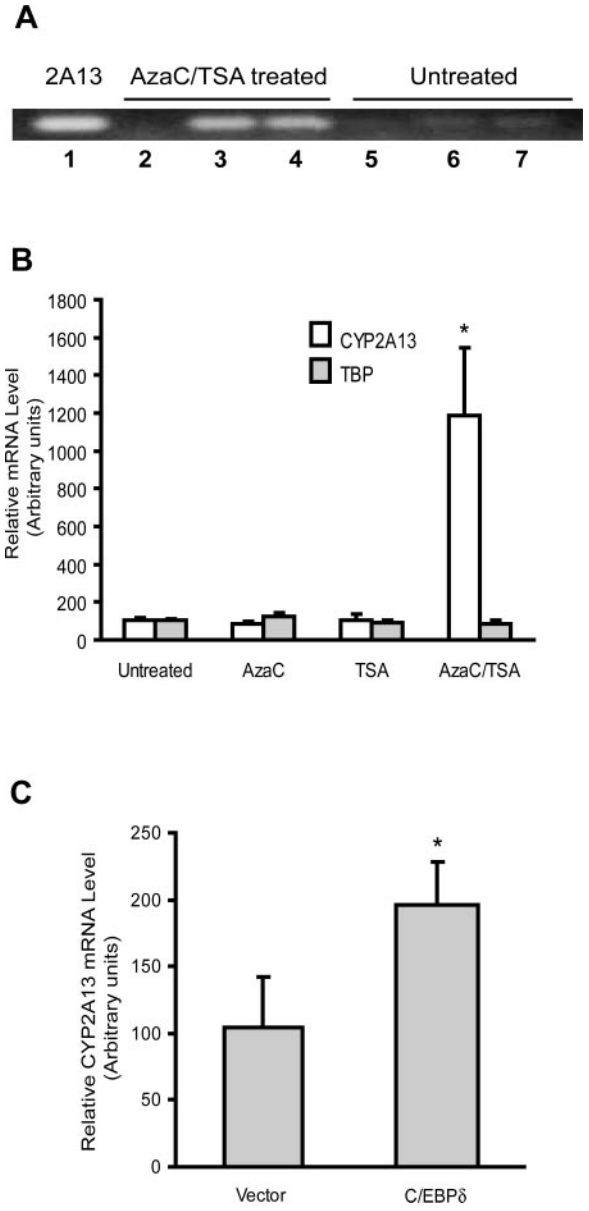

Fig. 6. Reactivation of endogenous *CYP2A13* expression in NCI-H441 cells by DNA demethylation and HDAC inhibition, and further activation by overexpression of C/EBPδ. NCI-H441 cells were untreated or treated with AzaC and TSA, as described under *Materials and Methods*. CYP2A13 mRNA was detected in cells using conventional PCR (A) and, for quantitative analysis, real-time PCR (B and C). In A, PCR products (10 μl each) were analyzed on an agarose gel and visualized by staining with ethidium bromide. PCR product from a CYP2A13 cDNA standard was used as a positive control (lane 1), whereas reverse transcriptase was omitted in the negative control (lanes 2 and 5). CYP2A13 was clearly detected in treated cells (lanes 3 and 4; duplicate reaction), but it was barely detected in untreated cells (lanes 6 and 7). In B, the levels of CYP2A13 and TBP in untreated cells were set to 100 (in arbitrary units). The data presented are means ± S.D. ($n = 3$). *, $p < 0.01$ compared with levels in untreated cells. In C, cells were transfected with C/EBPδ expression vector or the empty pMEX vector at 24 h after the initial AzaC treatment, and total RNA was prepared 64 h later, at 16 h after TSA treatment, as described under *Materials and Methods*. CYP2A13 mRNA levels (means ± S.D., $n = 3$) were significantly higher in cells transfected with C/EBPδ than in those transfected with the empty vector (*, $p < 0.01$; Student's *t* test).

Discussion

This is the first study to characterize the promoter of human CYP2A13, a P450 implicated in cigarette smoking-induced lung cancer in humans. Our findings will facilitate future efforts to study the developmental and pathophysiological regulation of the *CYP2A13* gene. Initial reporter gene assays indicated that the proximal promoter region (134 bp) can sustain basal expression, whereas distal sequences beyond −216 may serve as repressors. We are currently exploring whether, in the context of chromosomal structures in CYP2A13-expressing tissues such as the nasal mucosa and lung, the repressive function can be overcome, either through the activation of additional elements beyond the 2.1-kb region or else through transcriptional activators or tissue-selective epigenetic modifications that were not present in the cell lines and reporter gene system used here.

In the proximal promoter region, two transcription factor binding sites (Major and Minor) were identified by footprinting and gel-shift assays. Both sites interacted with C/EBP factors. The C/EBP family consists of six basic region-leucine zipper (bZIP) factors (α–ζ). They form homo- and heterodimers and bind to highly similar DNA sequences to regulate gene expression (Ramji and Foka, 2002; Cassel and Nord, 2003). These factors are involved in the regulation of important cellular processes such as differentiation, acute-phase responses, and energy metabolism (Ramji and Foka, 2002). C/EBPα, β, and δ are abundant in the lung epithelium, and they regulate expression of several lung-specific genes, including the surfactant proteins A and D, and the Clara cell secretory protein (Cassel and Nord, 2003). The C/EBPs also regulate the expression of *CYP2B1* in lung epithelial cells (Ramji and Foka, 2002).

It is interesting that the upstream (Minor) binding site contained an NFI-like sequence that differed by only one nucleotide from a previously characterized NFI element of rat *CYP2A3* (Ling et al., 2004b). An NFI element is also present at corresponding positions of the mouse *Cyp1a2*, *Cyp2a5*, and human *CYP2A6* genes (Zhang and Ding, 1998; Zhang et al., 2000; Ulvila et al., 2004). However, our gel-shift data indicate that the cytosine to thymine transition in the *CYP2A13* NFI-like sequence abolishes NFI binding. Thus, *CYP2A13* differs from rat *CYP2A3*, mouse *Cyp2a5*, and human *CYP2A6* in not having a functional NFI element in the proximal promoter region. Instead, the corresponding sequence (−133 to −114) contained a unique site that interacted with C/EBP factors in multiple protein complexes.

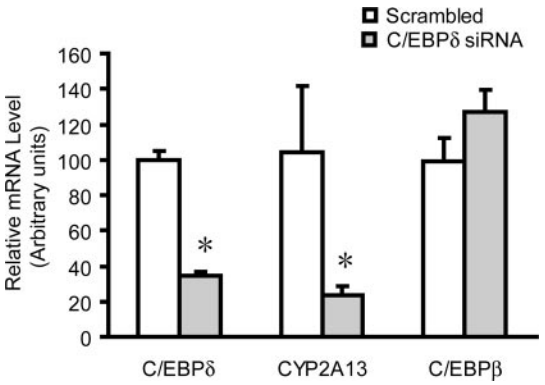

Fig. 7. CYP2A13 expression level was reduced by C/EBPδ siRNA treatment**.** NCI-H441 cells were transfected with C/EBPδ siRNA or a scrambled (negative control) siRNA. Cells were split into two aliquots after 48 h and were treated subsequently with AzaC and TSA as described under *Materials and Methods*. Total RNA was prepared at 24 h after the final treatment, and the amounts of C/EBPδ, CYP2A13, and C/EBPβ mRNAs were determined using real-time PCR. The data shown are means ± S.D. (n = 3) of the relative mRNA levels, normalized to the levels of TBP, with the levels in cells treated with the scrambled siRNA set to 100.

The sequence of the Major footprint (−56 to −83) that we have identified in the *CYP2A13* promoter is similar to the sequence of a functional C/EBP element that was recently identified in a corresponding position of the human *CYP2A6* promoter (Pitarque et al., 2005). This apparently conserved C/EBP element was found to be involved in the regulation of hepatic *CYP2A6* expression. Thus, C/EBP may play an important role in the regulation of both *CYP2A6* and *CYP2A13*. However, whereas C/EBPα is an activator of the hepatic *CYP2A6* expression and C/EBPβ a repressor (Pitarque et al., 2005), our data indicated that the α, β, and δ isoforms are all activators of a *CYP2A13* promoter construct in transfected A549 human lung cells, a finding that implicates promoter-specific interactions with differing C/EBP isoforms and associated coactivators or corepressors. It is noteworthy that we successfully demonstrated that the lung-enriched δ isoform is important in the expression of endogenous *CYP2A13* in NCI-H441 human lung cells under permissive conditions, and that C/EBPδ, but not C/EBPα or β, is bound to the *CYP2A13* promoter in vivo in the nasal mucosa of *CYP2A13* transgenic mice, where CYP2A13 is abundantly expressed. Further studies are warranted to determine whether, in vivo, the liver-predominant expression of *CYP2A6* and the respiratory tissue-predominant expression of *CYP2A13* are controlled by a preferential interaction of the conserved C/EBP element with differing C/EBP isoforms and associated factors and whether the unique presence of the upstream C/EBP interaction site in *CYP2A13* plays an important role.

Because of the close proximity of the Major and Minor footprints in *CYP2A13*, we could not determine the site(s) to which C/EBPδ was bound in the in vivo ChIP assay. Furthermore, the observed lack of in vivo association of C/EBPα and C/EBPβ with the transgenic *CYP2A13* promoter in mouse olfactory mucosa contradicted in vitro results from gel-shift and reporter assays performed using human lung nuclear extracts or lung cells. It remains to be determined whether this apparent inconsistency was due to differing effects of chromatin structure on the activities of the three C/EBP isoforms or whether it was a reflection of species or tissue differences in the relative abundance of the three C/EBP isoforms and their associated factors.

The isoform-specific recruitment of C/EBPδ to the *CYP2A13* promoter, as revealed by the in vivo ChIP assay, was probably assisted by C/EBPδ-interacting proteins, a notion supported by our gel-shift data showing that additional protein partners seem to be required for a higher-affinity association of C/EBPδ at either the Major or Minor footprint. It has been shown that C/EBPs interact with a number of bZIP and non-bZIP factors, including the p50 subunit of nuclear factor-κB, cAMP response element-binding protein/activating transcription factor, activator protein-1, glucocorticoid receptor, estrogen receptor-α, peroxisome proliferator-activated receptor-α, P53, and the retinoblastoma protein (Ramji and Foka, 2002). In some cases, heterodimers can be formed between C/EBPs and these factors, resulting in complexes with altered DNA binding specificity (Shuman et al.,

1997). Further studies to identify the C/EBP-interacting proteins and additional transcription factor binding sites on the *CYP2A13* promoter will help us better understand the regulation and function of this respiratory tract-selective P450.

The functions of C/EBP proteins can be regulated by both intracellular and extracellular signals, for example, inflammation. It has been shown that C/EBP is involved in the down-regulation of human *CYP3A4* in liver inflammation (Jover et al., 2002). Expression of many other *P450*s is also down-regulated by inflammation in the liver (Aitken et al., 2006), possibly through processes that involve C/EBP, given that C/EBPs have been shown to regulate the expression of a number of *P450*s in the liver or in liver-derived cells (Gonzalez and Lee, 1996); such P450s are *Cyp1a1* (Carrier et al., 1994), *CYP2A6* (Pitarque et al., 2005), *CYP2B1* (Park and Kemper, 1996), *CYP2B6*, *CYP2C9*, *CYP2D6* (Jover et al., 1998), and *CYP3A*s (Rodriguez-Antona et al., 2003; Martinez-Jimenez et al., 2005). On the other hand, only scant information is available on the effects of inflammation on extrahepatic P450 expression. One study showed that down-regulation of *CYP1A1* in the lung is associated with inflammation (Ghanem et al., 2004). In another study, lipopolysaccharide was found to induce *CYP2E1* in astrocytes via C/EBP β and δ binding sites in the *CYP2E1* promoter (Kelicen and Tindberg, 2004). Based on our data, we speculate that, as a target of C/EBP, nasal and pulmonary *CYP2A13* expression can be modulated by airway inflammation, a common pathological condition in humans; however, additional studies are needed for an assessment of whether *CYP2A13* is up- or down-regulated in such a context.

Another interesting finding of the present study is that *CYP2A13* expression can be reactivated in NCI-H441 human lung cancer cells through a combined treatment with AzaC and TSA. It has been long known that the expression levels of most P450s are drastically reduced in established cell lines and even in primary cell cultures after propagations; that fact has indeed hindered the application of in vitro cell models to studies of P450 gene regulation (Castell et al., 2005). Our finding that combined AzaC and TSA treatment can reactivate *CYP2A13* gene expression in human lung cancer cells confirms speculations that epigenetic factors, such as DNA methylation and histone modifications, are involved in the suppression of P450 expression in cancer cell lines and that the silencing can be at least partially reversed by demethylation and inhibition of histone deacetylases. Our findings further imply that epigenetic silencing of the *CYP2A13* promoter plays a role in the gene's tissue-selective expression. Three lines of evidence support this notion. First, the *CYP2A13* promoter was equally active in lung-derived cells and cells of hepatic origin in our reporter gene assays, in which the promoter sequence was not subject to epigenetic modification. This result contrasts with the known respiratory tissue-selective expression of *CYP2A13* in humans. Second, combined treatment with AzaC and TSA increased *CYP2A13* expression in NCI-H441 lung cancer cells and in human embryonic kidney HEK293 cells (data not shown), again suggesting that, in the absence of epigenetic modification, *CYP2A13* expression is not tissue-selective. Finally, our previous studies on rat *CYP2A3*, an orthologous gene with a tissue distribution similar to that of *CYP2A13*, had demonstrated a correlation between tissue-specific DNA methylation and gene repression (Ling et al., 2004b). Thus, we believe that epigenetic modifications are critical for repressing *CYP2A13* expression in nonpermissive tissues and that this mechanism could also contribute to the large interindividual and interallelic variations in *CYP2A13* expression in human lung tissues (Zhang et al., 2004).

In summary, we have presented compelling evidence that C/EBP transcription factors, notably the δ isoform, bind to the *CYP2A13* promoter and activate *CYP2A13* expression in human lung cells; such a finding implies that *CYP2A13* expression can be modulated by airway inflammation. We also obtained data that further support the notion that tissue-selective expression of this P450 is controlled at multiple levels (Ling et al., 2004a). Relative enrichment of tissue-specific transcription factors, such as C/EBPδ, could contribute to the respiratory tract-predominant *CYP2A13* expression, whereas epigenetic modifications could provide more critical on-off control of gene expression. Further studies on the details of these regulatory mechanisms are warranted so that we can better understand the mechanisms underlying the known interindividual and interallelic variations of *CYP2A13* expression in human lung tissues and the consequent variability in susceptibility to chemical toxicity in the respiratory tract.

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Address correspondence to: Dr. Xinxin Ding, Wadsworth Center, New York State Department of Health, Empire State Plaza, Box 509, Albany, NY 12201-0509. E-mail: xding@wadsworth.org