0026-895X/08/7302-451–460$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2008 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 73:451–460, 2008

Vol. 73, No. 2
39354/3294922
Printed in U.S.A.

Dexamethasone-Mediated Up-Regulation of Human *CYP2A6* Involves the Glucocorticoid Receptor and Increased Binding of Hepatic Nuclear Factor 4α to the Proximal Promoter

Tania Onica, Kathleen Nichols, Meghan Larin, Lorraine Ng, Ann Maslen, Zdenek Dvorak, Jean-Marc Pascussi, Marie-Josée Vilarem, Patrick Maurel, and Gordon M. Kirby

Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada (T.O., K.N., M.L., L.N., A.M., G.M.K.); Institut National de la Santé et de la Recherche Médicale U632, Montpellier, France, and Univ Montpellier1, Montpellier, France (J.-M.P., M.-J.V., P.M.); and Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacky University, Olomouc, Czech Republic (Z.D.)

Received July 12, 2007; accepted October 31, 2007

ABSTRACT

Human cytochrome P450 2A6 (CYP2A6) metabolizes various clinically relevant compounds, including nicotine- and tobacco-specific procarcinogens; however, transcriptional regulation of this gene is poorly understood. We investigated the role of the glucocorticoid receptor (GR) in transcriptional regulation of *CYP2A6*. Dexamethasone (DEX) increased CYP2A6 mRNA and protein levels in human hepatocytes in primary culture. This effect was attenuated by the GR receptor antagonist mifepristone (RU486; 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl]estra-4,9-dien-3-one), suggesting that induction of CYP2A6 by DEX was mediated by the GR. In gene reporter assays, DEX caused dose-dependent increases in luciferase activity that was also prevented by RU486 and progressive truncations of the *CYP2A6* promoter delineated DEX-responsiveness to a −95 to +12 region containing an hepatic nuclear factor 4 (HNF4) α response element (HNF4-RE). Mutation of the HNF4-RE abrogated HNF4α- and DEX-mediated transactivation of *CYP2A6*. In addition, overexpression of HNF4α increased CYP2A6 transcriptional activity by 3-fold. DEX increased HNF4α mRNA levels by 4-fold; however, the amount of HNF4α nuclear protein was unaltered. Electrophoretic mobility shift, chromatin immunoprecipitation (ChIP), and streptavidin DNA binding assays revealed that DEX increased binding of HNF4α to the HNF4-RE and that an interaction of GR and HNF4α occurred at this site. Moreover, ChIP assays indicated that histone H4 acetylation of the CYP2A6 proximal promoter chromatin was increased by DEX that may allow for increased binding of HNF4α to the HNF4-RE in human hepatocytes. These findings indicate that increased expression of *CYP2A6* by DEX is mediated by the GR via a nonconventional transcriptional mechanism involving interaction of HNF4α with an HNF4-RE rather than a glucocorticoid response element.

CYP2A6 is expressed predominantly in the liver, representing between 1 and 10% of total hepatic P450s. CYP2A6 activity shows a wide degree of interindividual variability, largely as a result of polymorphic gene variants (Raunio et al., 1999). Twenty-six allelic variants of *CYP2A6* have been identified to date, resulting in altered enzyme activity that affects the metabolism of substrates ranging from pharmaceuticals to toxins including procarcinogens (Raunio et al., 2001). Although interindividual variation in CYP2A6 levels has been attributed to physiological factors (Ujjin et al., 2002), inflammatory disease (Kirby et al., 1996), pharmaceutical exposure (Sotaniemi et al., 1995; Pelkonen et al., 2000), and genetic polymorphisms within the *CYP2A6* coding region (Raunio et al., 2001), little is known about *CYP2A6* transcriptional regulation and its involvement in the observed variation in expression.

In smokers, CYP2A6 is responsible for approximately 80% of the hepatic metabolism of nicotine to cotinine via C-oxidation (Tutka et al., 2005). Nicotine is the major

This research was funded by the Natural Sciences and Engineering Research Council of Canada.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.039354.

ABBREVIATIONS: P450, cytochrome P450; GRE, glucocorticoid response element; GR, glucocorticoid receptor; DEX, dexamethasone; HNF4, hepatic nuclear factor 4; HNF4-RE, HNF4α response element; RU486, mifepristone, 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl]estra-4,9-dien-3-one; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; UTR, untranslated region; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay(s); ChIP, chromatin immunoprecipitation; DTBP, dimethyl-3,3-dithiobispropionimidate-2HCl; TAT, tyrosine aminotransferase; GRIP, glucocorticoid receptor interacting protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

determinant for establishing and maintaining smoking dependence, because smoking behavior is adjusted to maintain plasma and brain nicotine levels in a constant range (Sellers et al., 2003). In addition, it has been shown that CYP2A6 has the capacity to bioactivate several procarcinogens, such as tobacco-specific nitrosamines. Indeed, interindividual variation in CYP2A6 expression results in altered nicotine inactivation rates and smoking patterns (Pianezza et al., 1998), increasing the propensity for addiction to tobacco products, which ultimately affects the incidence of tobacco-related cancers (Kamataki et al., 2005). Thus, CYP2A6 is pivotal in influencing smoking initiation and tobacco dependence, the number of cigarettes smoked, and the risk of tobacco-related cancers (Raunio et al., 2001; Sellers et al., 2003). For example, it is hypothesized that CYP2A6-poor metabolizers differ in their smoking habits from extensive metabolizers and that they may be protected from developing cancer at the target sites of these carcinogens, mainly the lung and the liver (Raunio et al., 2001; Nakajima et al., 2002; Malaiyandi et al., 2005).

Although the wide variation in CYP2A6 activity and levels has been largely attributed to polymorphisms within the coding region of the gene, recent studies have identified single-nucleotide polymorphisms within the 5′- and 3′-flanking regions, affecting gene expression and activity (Pitarque et al., 2001; Wang et al., 2006). Moreover, response elements for HNF-1α, CCAAT/enhancer-binding protein α, CCAAT/enhancer-binding protein β, and Oct-1 transcription factors in the proximal promoter are involved in CYP2A6 transcriptional regulation (Pitarque et al., 2005). Characterization of the *CYP2A6* promoter conducted in this study examines the mechanisms of glucocorticoid-mediated induction. Glucocorticoids have been previously shown to regulate several *P450* genes, including *CYP2C8* (Ferguson et al., 2005), *CYP2C9* (Gerbal-Chaloin et al., 2002), and *CYP2C19* (Chen et al., 2003). Computer analysis of a 2.5-kilobase section of the *CYP2A6* promoter region revealed numerous putative glucocorticoid response elements (GREs), which suggests that *CYP2A6* may be regulated in part by the GR. The frequency of clinical use of glucocorticoids underscores the importance of determining how treatment affects *CYP2A6* transcriptional activity because any potential changes in gene regulation will alter metabolism of CYP2A6 substrates, particularly nicotine- and tobacco-related carcinogens. The results of this study demonstrate that DEX induces CYP2A6 expression via GR-mediated transcriptional activation by increasing HNF4α binding to an HNF4-RE in the proximal promoter.

Materials and Methods

Materials. Cell culture media and supplements were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Dexamethasone, mifepristone (RU486), and dimethyl-3,3-dithiobispropionimidate-2HCl (DTBP) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Antibodies against human HNF4α were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). [γ-^{32}P]ATP (3000 mCi/mmol) was purchased from GE Healthcare (Piscataway, NJ). All other compounds were readily available commercially.

Cell Culture. Hepatocytes were prepared from lobectomy segments resected from adult patients for medical reasons unrelated to our research program. Tissue encompassing the liver tumors was removed surgically and assessed by histopathology, whereas the remaining tissue was used for hepatocyte isolation. Apart from age, sex, and the reason for surgery, no further patient information was available for this study. It is noteworthy that pathological examination of the surgical specimen was in no way hindered by the procedure used to obtain primary hepatocytes; otherwise, the tissues used for this study would have been immediately discarded. In all patients, serologic analysis for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus was negative. Primary human hepatocytes were prepared and cultured as described previously (Pichard-Garcia et al., 2002). Cells were plated onto collagen-coated dishes at 1.7×10^5 cells/cm^2 in a hormonally and chemically defined medium consisting of a mixture of William's E and Ham's F-12 media (1:1 in volume). Forty-eight hours after plating, cells were cultured for 16 h in glucocorticoid-free media before exposure to DEX for 24 h.

HeLa cells were cultured in a humidified incubator infused with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, and 1% penicillin and streptomycin.

RNA Isolation and Comparative Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was extracted from cells following the TRIzol reagent method (Invitrogen Canada Inc.). Messenger RNA analysis was conducted by comparative real-time reverse transcription-polymerase chain reaction (RT-PCR) using a Roche Diagnostics (Indianapolis, IN) LightCycler instrument and the DNA Master SYBR Green I kit (Roche Diagnostics, Mississauga, ON, Canada). RNA (1 μg) was first treated with 1 unit of DNase (RQ1 RNase-free DNase; Promega, Madison, WI). Complementary DNA was synthesized from RNA using 0.1 μg of random primers, 20 units of RNase inhibitor (RNasin; Promega), and 200 units of murine Moloney leukemia virus reverse transcriptase (Invitrogen Canada Inc.). PCR was performed in a 10-μl volume containing 1 μl of SYBR Green I and 5 μM concentrations of each primer. The final Mg^+ concentration was 2 mM and the PCR parameters were as follows: an initial step at 95°C for 10 min followed by 45 cycles of 95°C, 15 s; 70°C, 5 s; 70°C, 15 s). The gene-specific primers are listed in Table 1.

Plasmid Construction. The pTAT-TK-GRE-LUC (containing one copy of the consensus GRE upstream of a minimal herpes simplex thymidine kinase promoter and a luciferase reporter gene) and HNF4α expression plasmid (pHNF4α) were kindly provided by Dr. L. Poellinger (Karolinska Institute, Stockholm, Sweden). A *LUC* reporter gene plasmid construct containing 2469 base pairs of the 5′-flanking region of the *CYP2A6* promoter and deletion plasmids consisting of sequential truncations of this region were prepared by PCR with primers listed in Table 2. The antisense primer was the same for all the constructs.

PCR was performed using genomic DNA extracted from human whole blood as a template. The amplified fragment was cloned into the pCR-TOPO cloning vector (Invitrogen Canada Inc.) following manufacturer's instructions, and then it was restriction digested with KpnI and XhoI to facilitate subcloning into the pGL3 luciferase

TABLE 1
HNF4, CYP2A6, and GAPDH oligonucleotide primers used in RT-PCR

Target Gene	Sequence
HNF4	
Sense	5′-CAGGTGTTGACGATGGGCAATG-3′
Antisense	5′-AGCGGCACTGGTTCCTCTTG-3′
CYP2A6	
Sense	5′-TGCAAGGGCTGGAGGACTTC-3′
Antisense	5′-AGGGTGGTGCTGACGGTCTC-3′
TAT	
Sense	5′-AGGCCAGGTGGTCTGTGAGG-3′
Antisense	5′-AGGGGTGCCTCAGGACAGTG-3′
GAPDH	
Sense	5′-ACAGTCCATGCCATCACTGCC-3′
Antisense	5′-GCCTGCTTCACCACCTTCTTG-3′

vector (Promega) for reporter gene assays. DNA was precipitated and transformed into competent XL1 blue cells, and plasmid DNA was then harvested and purified using a Plasmid Maxi kit (QIAGEN Inc., Mississauga, ON, Canada). The CYP2A6 promoter sequences were confirmed by direct sequencing and the resultant plasmids were designated as p2A6–2469-LUC, p2A6–2065-LUC, p2A6–1551-LUC, p2A6–1038-LUC, p2A6–472-LUC, p2A6–239-LUC, and p2A6–95-LUC according to the length of the 5′-UTR insert.

Site-Directed Mutagenesis. The HNF4-RE(GCCAAAGTCCA, −69 to −79) within the proximal promoter region was mutated within p2A6–2469-LUC using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide primers were designed to anneal to the same sequence on opposite strands of the plasmid flanking the HNF4α site and to carry a two-base pair CC→AA mutation. The primer sequences are as follows: sense: GGTAATTATGTAATCA**GCCAAAGTAAA**TCCCTCTTTTTCAGGCAG; and antisense: CCA TTAATACATTAGT**CGGTTTCATTT**AGGGAGAAAAAGTCCGTC.

The response element is indicated by bold letters, and the mutated bases are underlined. The presence of the mutation was confirmed by direct sequencing and the resultant plasmid was designated as p2A6–2469-ΔHNF4-LUC.

Transfection and Dual-Luciferase Reporter Assays. HeLa cells were transfected in suspension with a density of 1.5×10^5 cells/well in a 24-well plate. A DNA mixture containing 800 ng/μl of p2A6–2469-LUC reporter plasmid, 10 ng/μl pRL-TK plasmid (*Renilla reniformis*, luciferase plasmid), and increasing concentrations of the pHNF4α were transfected using LipofectAMINE (Invitrogen Canada Inc.) according to the manufacturer's recommendations. As a positive control, cells were transfected with the pGL3-control plasmid (Promega), which constitutively expresses the luciferase gene through a simian virus-40 promoter. As a negative control, cells were transfected with the promoter-less pGL3-basic. After 24 h, medium containing 10 nM DEX was added. Twenty-four hours later, cells were harvested and washed twice with phosphate-buffered saline, cell extracts were prepared, and aliquots of the lysates were assayed for luciferase enzyme activities as described by the dual-luciferase reporter assay system (Promega). Luciferase activities were normalized to *Renilla* luciferase activity to correct for differences in transfection efficiency and cell numbers. All transfection studies were repeated at least three times.

Preparation of Nuclear Extracts. Nuclear extracts were prepared from HeLa cells (5.0×10^6 cells) as described by Dignam et al. (1983). In brief, cells were harvested, washed, and resuspended in 200 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2 μg/ml pepstatin, 10 μg/ml aprotinin, 0.5 M DTT, 0.575 mM PMSF, and 2 μg/ml leupeptin). Cell lysis was facilitated by passing the cells repeatedly through a 1-ml syringe fitted with a 25-gauge needle. Nuclei were recovered by centrifugation at 14,000*g* for 3 min at 4°C, resuspended in 60 μl of buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 2 μg/ml pepstatin, 10 μg/ml aprotinin, 0.5 M DTT, 0.575 mM PMSF, and 2 μg/ml leupeptin), and incubated on ice for 15 min. The samples were then centrifuged at 14,000*g* for 3 min at 4°C to obtain supernatants containing the nuclear fractions. Nuclear extracts were resuspended in 60 μl of buffer D (20 mM HEPES, pH 7.9, 25% glycerol, 0.2 mM EDTA, 2 μg/ml pepstatin, 10 μg/ml aprotinin, 0.5 M DTT, 0.575 mM PMSF, and 2 μg/ml leupeptin). Extracts were stored at −70°C until further analysis. Protein concentrations of nuclear extracts were determined by the Bradford assay using the Bio-Rad protein assay kit (Bio-Rad, Mississauga, ON, Canada).

TABLE 2
Oligonucleotide primers used for PCR amplification of the *CYP2A6* promoter region

Primer	Sequence
CYP2A6-2469 sense	5′-CCCAACCTGTCTCTCCCCATC-3′
CYP2A6-2065 sense	5′-AAAGGTGCAACCCTAAATGGT-3′
CYP2A6-1551 sense	5′-TCCCAGCTCCCTCACTTACTCC-3′
CYP2A6-1038 sense	5′-CCACTGTCTGTTTTCTGTCCTCTG-3′
CYP2A6-472 sense	5′-CCCTGCATCCTCCACAACAG-3′
CYP2A6-239 sense	5′-GGCACCCCTCCTGAAGTACC-3′
CYP2A6-95 sense	5′-GGTAATTATGTAATCAGCCAAAGTCCA-3′
CYP2A6 antisense	5′-TGAGGCCAGCATGGTGGTAG-3′

Western Blot Analysis and Immunoprecipitation. CYP2A6 was identified in cell extracts of primary human hepatocytes and GR and HNF4α proteins from nuclear extracts of HeLa cells by Western blot analysis as described previously (Romero et al., 2002). In brief, 15 μg of protein from cell or nuclear extracts was separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose, and incubated for 1 h with monoclonal antibodies to human CYP2A6 (BD Biosciences, Mississauga, ON, Canada) at a dilution of 1:2000, and with polyclonal antibodies against HNF4α (H-171) or GR (H-300) (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200. After incubation with either goat anti-mouse (CYP2A6) or goat anti-rabbit (GR and HNF4α) peroxidase secondary antibody (Vector Laboratories, Burlington, ON, Canada), bands were detected by chemiluminescence (ECL Plus; GE Healthcare) and visualized using a Typhoon 9410 scanner (GE Healthcare). The nitrocellulose membrane was later stained with Amido Black (Bio-Rad) staining solution to ensure equal loading of protein. The relative amounts of HNF4α protein levels were subsequently determined by densitometry using National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij/). Three independent experiments were performed, and Western blots are presented from one representative experiment.

GR protein was immunoprecipitated from nuclear extracts prepared from 100 nM DEX-treated HeLa cells using a Nuclear Complex Co-IP kit (Active Motif Inc., Carlsbad CA). Nuclear protein levels were quantified using the Bradford assay (Bradford 1976), and 170 μg of nuclear extract was combined with 1 μg of anti-GR antibody (Santa Cruz Biotechnology, Inc.) diluted to 500 μl in immunoprecipitation incubation buffer in low-stringency conditions and incubated overnight at 4°C with gentle rotation. The antibody-protein mix was then incubated with 10 μl of protein A/G antibody binding beads (Santa Cruz Biotechnology, Inc.) at 4°C. The bead pellet was washed several times followed by centrifugation, and pellets were resuspended in SDS-PAGE loading dye and boiled for 5 min in preparation for SDS-PAGE and Western blotting.

Electrophoretic Mobility Shift Assay. Protein binding reactions were carried out in a 20-μl volume using 10 μg of nuclear extract and the Gel Shift Assay kit (Promega) according to the manufacturer's instructions. For competition studies, samples were incubated with a 50-fold molar excess of unlabeled oligonucleotide. The sequence of the HNF4-RE double-stranded oligonucleotide probe used for the gel shift assay was 5′-GTTGGGAGGTGAAATGAGGTAAT-3′. Competitor probe sequences were as follows: Sp1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′; ApoCIII, 5′-CGAGCGCTG-GGCAAAGGTCACCTGC-3′; and CYP2A6ΔHNF4-RE, 5′-GTAATCAGCCAAAGTCCATCCCTCT-3′.

In competition experiments, a 50-fold excess of the unlabeled oligonucleotides reaction mixes was preincubated with unlabeled oligonucleotides before adding the radiolabeled probe. In the supershift experiment, 2 μg of anti-HNF4α polyclonal antibody was added to the binding reaction and incubated at 4°C for 1 h. The DNA-protein complexes were resolved on a 6% acrylamide gel in 0.5× TBE buffer at 200 V for 19 min. The gel was dried and then imaged by phosphorimaging using a Typhoon 9410 scanner (GE Healthcare). The relative amounts of protein-bound ^{32}P-labeled oligonucleotide were subsequently determined by densitometry using ImageJ software. Samples were analyzed in triplicate, and gel shift assays results presented from one representative experiment.

Streptavidin-Agarose DNA Binding Assay. DNA binding reactions (100 μl) were carried out using 50 μg of nuclear extract with 5 μg of a biotinylated double-stranded CYP2A6 HNF4 RE probe (Invitrogen Canada Inc.) consisting of the same CYP2A6 HNF4 RE sequence indicated in EMSA above and incubated with 200 μl of 50%

streptavidin-agarose beads (Novagen, Madison, WI). The samples were incubated at room temperature with mixing for 1 h. Samples were then centrifuged at 5000*g* for 30 s and washed three times with ice-cold phosphate-buffered saline before being resuspended in 50 μl of Western loading dye. Samples were then analyzed for the presence of bound HNF4 and GR protein by Western blotting as described above.

Chromatin Immunoprecipitation Assay. Human hepatocytes were cultured in the absence of glucocorticoids for 16 h and then treated with 100 nM DEX for 6 h. For experiments with HeLa cells, ChIP signals for proteins that bind indirectly to DNA were improved by incubating cells with the homobifunctional, cleavable cross-linking reagent DTBP on ice for 30 min. Formaldehyde (1%) was then added to the tissue culture media, and the plates were incubated for 10 min at room temperature on a rocker. The cross-linking reaction was stopped with 125 mM glycine for 5 min. Soluble chromatin was further isolated as described previously (Pascussi et al., 2003). The chromatin solution was sonicated and precleared with salmon sperm DNA/protein A-Sepharose (Upstate Biotechnology, Charlottesville, VA). The precleared chromatin solution was incubated for 16 h with 5 μg of antibodies to either anti-acetylated histone-4 (Upstate Biotechnology), anti-glucocorticoid receptor (Santa Cruz Biotechnology, Inc.), or anti HNF4α (Santa Cruz Biotechnology, Inc.). Immune complexes were collected with salmon sperm DNA/protein A-Sepharose, washed, and eluted in 1% SDS and 0.1 M $NaHCO_3$. Cross-links were reversed and chromatin-associated proteins were digested with proteinase K. The DNA was recovered via phenol/chloroform extraction and ethanol precipitation. Pellets were resuspended in 50 μl of water, and 20-μl aliquots were used as a template for PCR reaction (40 cycles) with the Expand Long Template PCR System (Roche Diagnostics). The primers for PCR of CYP2A6 promoter proximal fragment (−274 to +28) were 5′-CTTATCTGTTGCCCCCTCCT and 5′-CACCAGAAGCATCCCTGAG and for the distal fragment (−2978 to −2659) were 5′-CCTCCTTCCCAAAGGAAAAG and 5′-TTTCTGAATGGATGGTGCAA.

Statistical Analysis. Statistically significant differences among groups were identified by one-way analysis of variance. Data were further evaluated by Fisher's least significant difference test if groups were found to be significant. Significance was established at $p < 0.05$.

Results

DEX-Mediated Induction of CYP2A6 mRNA in Human Primary Hepatocytes Occurred via the GR. To determine the influence of glucocorticoids on *CYP2A6* expression, human hepatocytes in primary culture were treated for 24 h with DEX (100 nM) and CYP2A6 mRNA levels were assessed by real-time RT-PCR (Fig. 1A). Increases in CYP2A6 protein were also observed in human hepatocytes treated with increasing concentrations of DEX (Fig. 1A, inset). Messenger RNA levels for tyrosine aminotransferase (TAT) gene, a conventional biomarker of glucocorticoid genomic effects was also determined (Fig. 1B). DEX treatment resulted in a 3.3-fold increase in CYP2A6 mRNA and a 5.5-fold increase in TAT mRNA relative to control hepatocytes ($p < 0.05$). Cotreatment with the GR antagonist RU486 significantly attenuated the DEX-mediated induction in CYP2A6 and TAT mRNA levels ($p < 0.05$). CYP2A6 and TAT mRNA levels were not significantly altered by RU486 treatment alone.

To determine whether induction of CYP2A6 mRNA by DEX was mediated directly or indirectly by the GR, we assessed whether de novo protein synthesis was necessary. Hepatocytes were treated with 100 mM DEX in the presence or absence of cycloheximide, a typical inhibitor of protein synthesis and CYP2A6 mRNA was assessed by real-time RT-PCR. In Fig. 1C, we show that cycloheximide had no effect on the 9-fold induction of CYP2A6 by DEX. These findings suggest that CYP2A6 induction by DEX is not dependent on de novo protein synthesis and occurs either in direct response to hGR or the interaction of hGR with transcriptional coactivators.

Responsiveness to DEX-Mediated CYP2A6 Transactivation Was Localized to the Proximal Promoter. To determine whether DEX elevates CYP2A6 mRNA levels by increasing the transcriptional activity of the CYP2A6 promoter, reporter gene assays were performed in HeLa cells, which constitutively express the endogenous GR, transfected with p2A6–2469 + 12-LUC. Treatment of transfectants with DEX resulted in a dose-dependent increase ($p < 0.05$) in

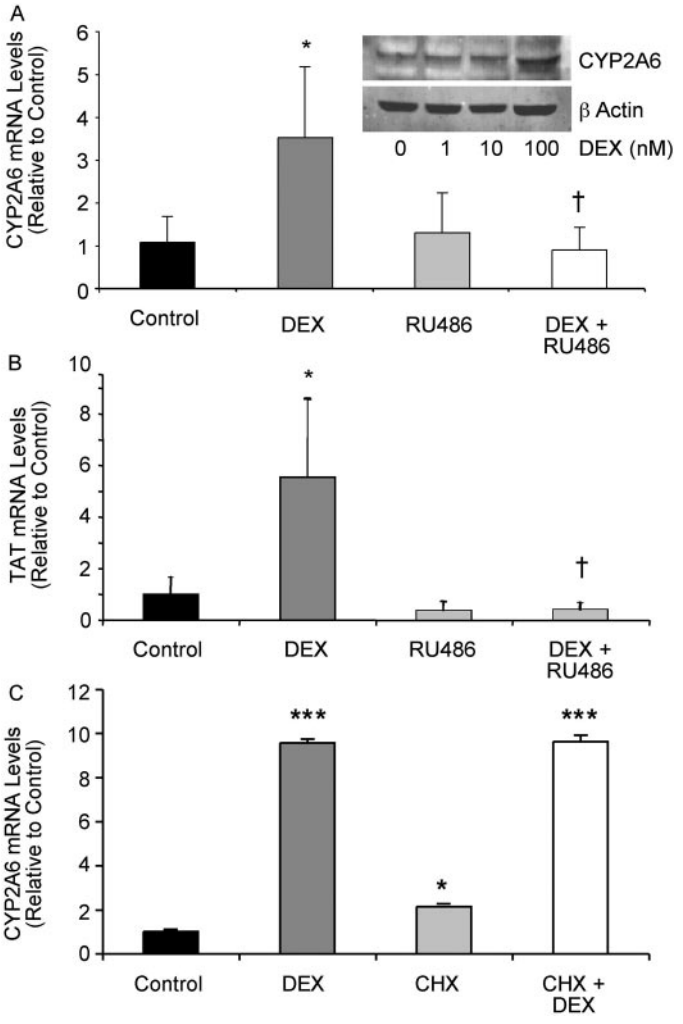

Fig. 1. Dex-mediated induction of CYP2A6 mRNA in human primary hepatocytes occurs via the GR. A and B, primary human hepatocytes were treated for 24 h with 100 nM DEX and/or 100 nM RU486. C, primary human hepatocytes were treated for 24 h with 100 nM DEX in the presence of absence of 10 μg/ml cycloheximide. Differences in CYP2A6 (A and C) and TAT mRNA (B) levels were determined by real-time RT-PCR and normalized to GAPDH mRNA levels as described under *Materials and Methods*. A, inset, primary human hepatocytes were treated with DEX (1, 10, and 100 nM), and cell extracts were assessed for CYP2A6 protein expression by Western blot analysis as described under *Materials and Methods*. Values represent the mean + S.E. of results from four different primary human hepatocyte cultures (A and B) or three independent experiments of a single culture of human hepatocytes (C) expressed relative to control hepatocytes. *, $p < 0.05$, significantly from controls. †, $p < 0.05$significantly from cells treated with 100 nM DEX.

luciferase activity to a maximum of 3.2-fold at a concentration of 1000 nM (Fig. 2A). Treatment with RU486 attenuated the DEX-mediated transactivation differing significantly from cells treated with 1 μM DEX only ($p < 0.05$). The response of cells transfected with p2A6–2469 + 12-LUC was similar to findings in HeLa cells transfected with pTAT-GRE-luc in which a 4-fold induction of luciferase activity ($p < 0.05$) was observed after treatment with 1 μM DEX, a response that was abolished by RU486.

To delineate the location responsible for DEM-mediated transactivation of CYP2A6, HeLa cells were transfected with reporter plasmids containing progressive deletions of the CYP2A6 5′-flanking region and treated with 100 nM DEX. All deletion constructs, including the smallest consisting of −95 to +12 base pairs of the CYP2A6 promoter, responded to DEX with significant increases in luciferase activity (Fig. 2B). Accordingly, treatment of transfectants with RU486 inhibited the DEX-mediated transactivation of these regions (data not shown).

DEX-Mediated Increases in CYP2A6 Transcriptional Activity Occurred via an HNF4-RE. Although DEX responsiveness was localized to the proximal CYP2A6 promoter, computer analysis of this region failed to identify a putative GRE. However, an HNF4-RE present in this region has previously been shown to be pivotal in the transcriptional regulation of CYP2A6 (Pitarque et al., 2005). To determine whether the HNF4-RE plays a role in DEX-responsiveness, the HNF4-RE within p2A6–2469-LUC was mutated by site-directed mutagenesis and used in luciferase reporter assays as described above. Treatment of cells transfected with p2A6–2469ΔHNF4 with increasing concentrations of DEX did not have any significant effect on luciferase activity (Fig. 3A). To confirm the role of HNF4-RE in CYP2A6 transcriptional activation, HeLa cells were cotransfected with p2A6–2469-LUC and increasing amounts of the expression plasmid pHNF4α. (Fig. 3B). Without HNF4 overexpression (control in Fig. 3B), basic luciferase activity was reduced by approximately 50% in cells transfected with p2A6–2469-ΔHNF4-LUC compared with transfectants with p2A6–2469-LUC. HNF4 overexpression resulted in dose-dependent increases ($p < 0.01$) in luciferase activity up to 3-fold of control levels; however, mutation of the HNF4-RE abolished this increase.

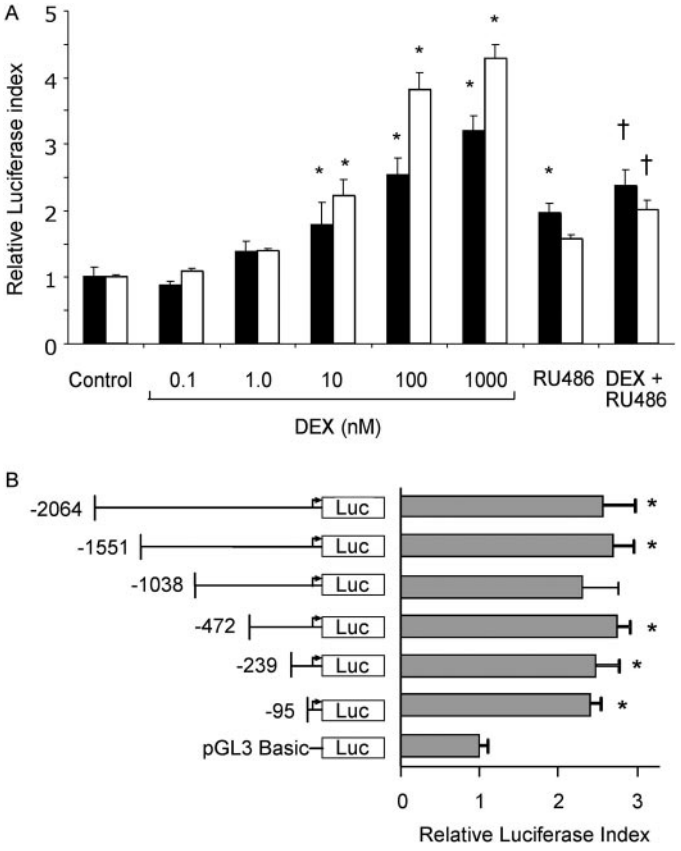

Fig. 2 Responsiveness to DEX-mediated CYP2A6 transactivation is localized to the proximal promoter. A, HeLa cells were transiently transfected with p2A6–2469-LUC (solid bars) and treated for 24 h with DEX ± RU486. B, luciferase reporter constructs incorporated various lengths of the 5′-UTR of CYP2A6 were transiently transfected into HeLa cells and treated with 100 nM DEX for 24 h. Cell extracts were assayed for luciferase activity, which was normalized to *Renilla* luciferase activity. Cell extracts were assayed for luciferase activity that was normalized to *Renilla* luciferase activity. As a positive control, HeLa cells were transiently transfected with pTAT-TK-GRE-LUC (open bars) and treated with DEX and/or RU486. ∗, $p < 0.05$, significantly different from controls. †, $p < 0.05$, significantly different from cells treated with 1000 nM DEX.

Effect of DEX on HNFα mRNA and Nuclear Protein Levels in Human Hepatocytes. To determine whether DEX induces CYP2A6 by a mechanism involving HNF4 overexpression, HNF4α mRNA and protein levels were assessed in DEX-treated human hepatocytes in primary culture. DEX resulted in a 4-fold increase of HNF4 mRNA levels compared with control hepatocytes ($p < 0.05$; Fig. 4A). However, levels of HNF 4α nuclear protein were unaltered by DEX treatment (Fig. 4B).

DEX Increased Nuclear GR Protein and Complex Formation with HNFα in HeLa Cells. We hypothesized that DEX-mediated transactivation of CYP2A6 in HeLa cells was due, in part, to the formation of complexes between GR and HNF4α. We first examined the effect of DEX on GR and HNF4α nuclear protein levels. Treatment of HeLa cells with increasing concentrations of DEX (1, 10, and 100 nM) resulted in dose-dependent increases in GR protein in nuclear extracts; however, HNF4α protein remained unchanged (Fig. 5A). To determine whether interactions between GR and HNF4α were occurring in response to DEX, GR protein was immunoprecipitated from nuclear extracts of HeLa cells. Western blot analysis of immunoprecipitates revealed the presence of HNF4α protein, the levels of which were elevated with DEX treatment (Fig. 5B). Similar immunoprecipitation experiments conducted on cell extracts of human hepatocytes demonstrated GR-HNF4α protein interactions; however, DEX-mediated effects were not evident.

DEX Increased Binding of HNFα to the HNF4-RE in the CYP2A6 Promoter. To determine whether DEX increases CYP2A6 transcription by increasing binding of HNF4α to the HNF4-RE in the proximal CYP2A6 promoter, EMSA was performed by incubating nuclear extracts from control and DEX-treated human hepatocytes with a radiolabeled HNF4-RE probe (Fig. 6A). The shift of HNF4-RE probe-protein complexes observed with nuclear extracts from control cells was abolished in the presence of 50-fold excess of nonradioactive HNF4-RE probe (CYP2A6 HNF4-RE) and a probe consisting of the well characterized HNF-4-RE in the apoCIII gene promoter. However, the mutated probe (CYP2A6 ΔHNF4-RE) and the nonspecific competitor probe (Sp1) had no effect. The presence of a

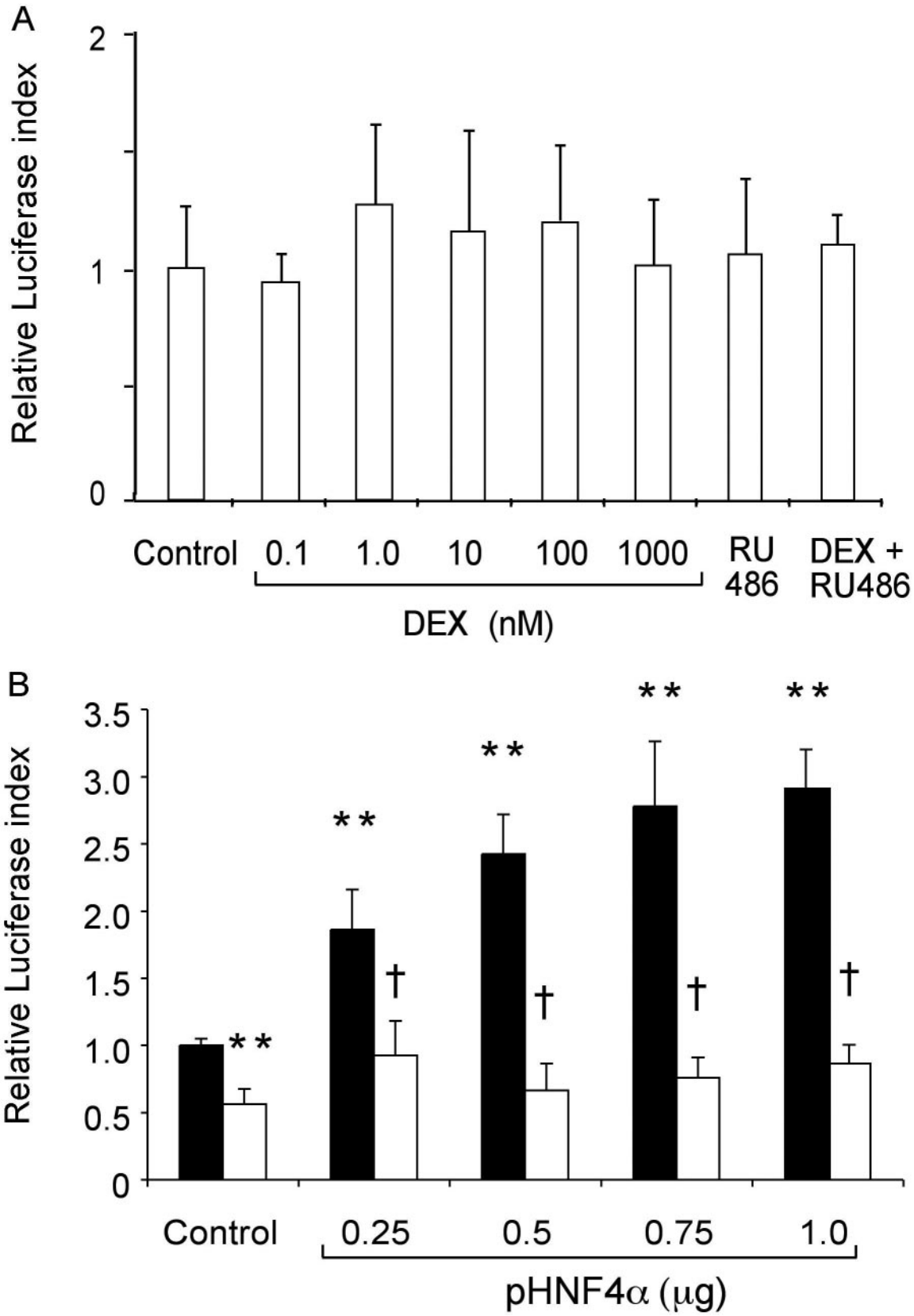

Fig. 3. The HNF4-RE is necessary for DEX-mediated *CYP2A6* transactivation. A, site-directed mutagenesis of the *CYP2A6* HNF4α site abrogates the DEX-mediated luciferase response. HeLa cells were transiently cotransfected with p2A6–2469-ΔHNF4-LUC and treated for 24 h with DEX and/or 100 nM RU486. B, HNF4 overexpression induces *CYP2A6* transactivation. HeLa cells were transiently cotransfected with either p2A6–2469-LUC (solid bars) or p2A6–2469-ΔHNF4-LUC (open bars) and increasing amounts of pHNF4α. Cell extracts were assayed for luciferase activity, which was normalized to *Renilla* luciferase activity. **, $p < 0.01$, significantly different from control cells with no HNF4 overexpression. †, $p < 0.05$, significantly different from cells transfected with p2A6–2469-LUC and corresponding amounts of pHNF4α.

supershift with the use of polyclonal antibodies identified HNF4α as the protein forming the HNF4-RE probe-protein complex.

To determine whether both HNF4α and GR were associated with the HNF4-RE and to determine the capacity of increasing concentrations of DEX to alter HNF4α-HNF4-RE complex formation, a DNA binding assay using a biotinylated HNF4-RE probe nuclear extracts from DEX-treated HeLa cells was performed (Fig. 6B). Proteins bound to the HNF4-RE were identified by purifying the biotinylated probes on streptavidin-agarose beads followed by immunoblot analysis using polyclonal antibodies to either human HNF4α or GR. DEX treatment resulted in a dose-related increase in HNF4α and GR binding to HNF4-RE to levels up to a maximum of 2.5-fold and 1.9-fold higher than controls.

DEX Increased Histone H4 Acetylation and HNF4α Binding to the Proximal CYP2A6 Promoter in Human Hepatocytes. Histone acetylation results in chromatin structure relaxation, allowing for greater accessibility of transcription factors and gene transactivation. Using the ChIP assay, we investigated the degree of histone acetylation in the proximal CYP2A6 promoter in human hepatocytes (Fig. 7A). We also determined the extent to which HNF4 binds to the HNF4-RE in the CYP2A6 promoter in the presence and absence of DEX. Hepatocytes were first maintained 16 h in a glucocorticoid-free medium, and then they were treated with 100 nM DEX for 6 h. DEX increased histone H4 acetylation in the proximal CYP2A6 promoter region and binding of HNF4α to this region was also augmented with DEX. The specificity of the ChIP assay was verified using a pair of oligonucleotide primers spanning a region in the distal promoter, far removed from the identified HNF4-RE, for which a DEX-mediated effect was not observed. These data suggest that DEX causes changes in chromatin structure in the vicinity of CYP2A6 promoter, allowing for an increased binding of HNF4α to the HNF4-RE and resulting in increased transcription of the CYP2A6 gene.

To determine whether interactions occur between GR and HNF4 at the HNF4-RE in the CYP2A6 promoter, we performed a ChIP assay in HeLa cells using DTBP. DTBP is a homobifunctional, cleavable cross-linking reagent that forms amidine bonds with primary amines that are cleavable by mild reduction. Thus, DTBP improves signals in ChIP assays for proteins that bind indirectly through protein-protein interactions. The results of this modified ChIP assay indicate an increased presence of GR at the HNF4-RE in HeLa cells treated with 100 nM (Fig. 7B).

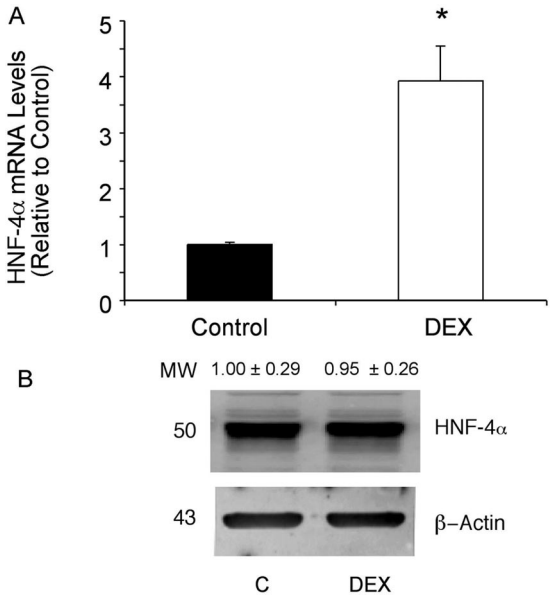

Fig. 4. Effect of DEX on HNF4α expression levels in primary human hepatocytes. Hepatocytes were treated for 24 h with 100 nM DEX and/or 100 nM RU486. HNF4α mRNA and protein levels were assessed by real-time RT-PCR (A) and Western blot analysis (B), respectively, as described under *Materials and Methods*. Messenger RNA levels represent the mean + S.E. of four different hepatocyte cultures relative to untreated hepatocytes. For Western blots, the mean band intensities are shown from three independent experiments from different hepatocyte cultures assessed by densitometry and expressed relative to untreated controls. A representative western blot is shown. *, $p < 0.05$, significantly different from controls.

Discussion

The purpose of this study was to investigate the mechanism by which the GR modulates transcriptional regulation of *CYP2A6*. Several lines of evidence suggest that the GR is involved in the induction of *P450* genes. Functional GREs have been identified within the promoter regions of human *CYP2C8* (Ferguson et al., 2005), *CYP2C9* (Gerbal-Chaloin et al., 2002), and *CYP2C19* genes (Chen et al., 2003). However,

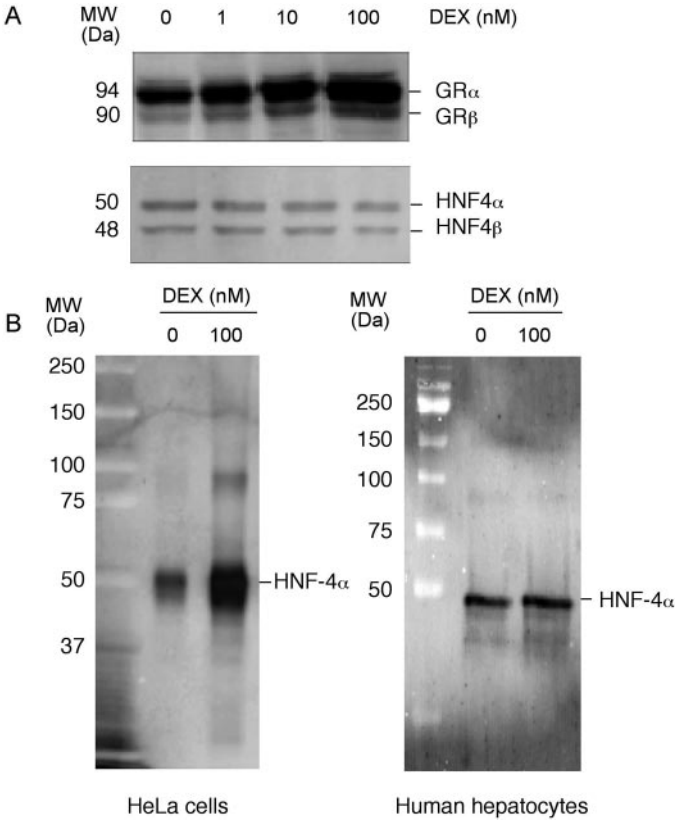

Fig. 5. DEX increases GR protein and interaction with HNFα in nuclei of HeLa cells. A, HeLa cells were treated for 24 h with increasing concentrations of DEX (1, 10, and 100 nM), and Western blot analyses were performed on nuclear extracts using antibodies to GR and or HNF4α and HNF4β. B, GR protein was immunoprecipitated from nuclear extracts of HeLa cells and cell extracts of human hepatocytes cultured in the presence or absence of 100 nM DEX. Western blotting was then performed on immunoprecipitates using antibodies to HNFα as described under *Materials and Methods*. Blots represent the results of two experiments with similar results.

other investigations have raised concerns regarding glucocorticoid-mediated induction of *P450* genes because many genes lack GREs. The results of this study demonstrate that DEX-mediated induction of *CYP2A6* occurs via the GR and that it is mediated through interaction of HNF4α with HNF4-RE rather than a GRE.

We present several findings indicating GR-mediated regulation of *CYP2A6* gene expression. We observed that induction of CYP2A6 mRNA in primary human hepatocytes treated with DEX paralleled DEX-mediated increases in CYP2A6 promoter transactivation in luciferase reporter assays. Dose-dependent induction of *CYP2A6* at submicromolar concentrations of DEX supports GR involvement as the dissociation constant (K_d) for DEX is in the nanomolar range (Hirschelmann et al., 1990; Bernasconi et al., 1997). In addition, cotreatment with RU486, a prototypical human GR antagonist, abrogated increases in CYP2A6 mRNA levels, and it significantly inhibited DEX-mediated *CYP2A6* transactivation, further implicating the involvement of the GR. Finally, transactivation of the prototypical glucocorticoid-inducible gene *TAT* was also observed at the same concentrations of DEX. We have reported previously correlations between *TAT* and *CYP2C9* promoter responsiveness to DEX in luciferase reporter assays (Gerbal-Chaloin et al., 2002).

Through deletion analysis of the 5′ flanking region of *CYP2A6*, we delineated the region of DEX responsiveness to the proximal promoter region (−95 to +12). However, computer analysis of this region failed to identify GRE equivalents, suggesting that the observed DEX responsiveness was due to a nonconventional glucocorticoid response. Site-directed mutagenesis of an HNF4-RE within the proximal promoter abrogated the DEX-mediated transactivation of

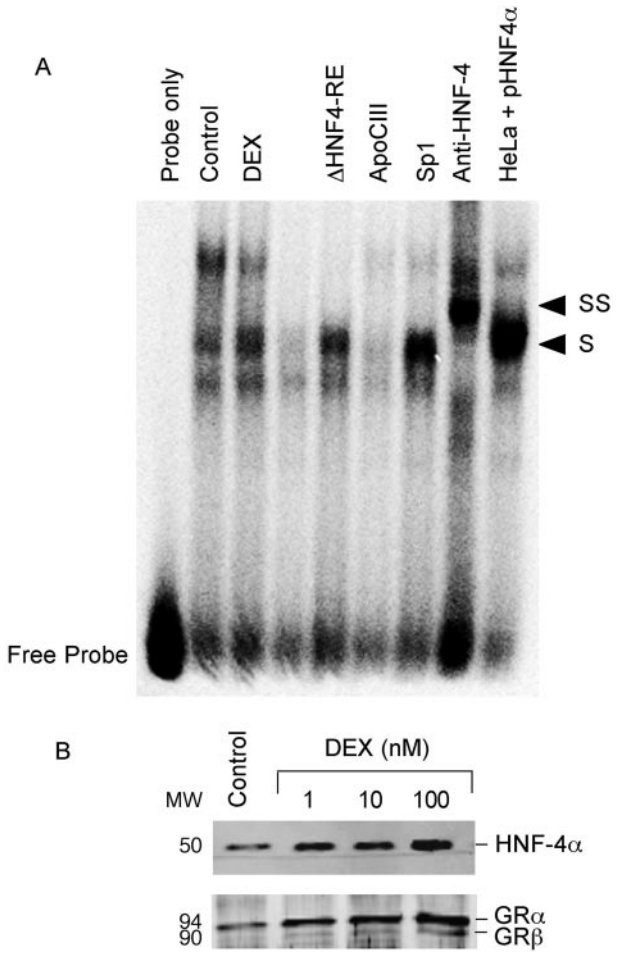

Fig. 6. DEX increases binding of HNF4α-GR complexes to the HNF4-RE in human hepatocytes. A, EMSA analysis of nuclear extracts from DEX-treated primary human hepatocytes. In EMSA experiments, nuclear extracts from human hepatocytes that were either untreated (control), treated with 100 nM DEX or from HeLa cells transfected with an HNF4α expression vector (HeLa + pHNF4α) were incubated with a ^{32}P-labeled HNF4-RE oligonucleotide probe and electrophoresed on a 6% polyacrylamide gel as described under *Materials and Methods*. Competition experiments comprised a radiolabeled probe incubated with nuclear extract from DEX-treated human hepatocytes and 50-fold excess of various unlabeled oligonucleotides in the binding reaction. Shifted (S) and supershifted (SS) probe-protein complexes are indicated by arrowheads. B, DNA binding reactions for the identification of interaction HNF4α-GR complexes with HNF4-RE probes. Nuclear extracts from HeLa cells treated with increasing concentrations of DEX were incubated with a biotinylated HNF4-RE probe. Binding reactions were then incubated with streptavidin-agarose, and protein bound to the biotinylated probe was analyzed by Western blot analysis using polyclonal antibodies to HNF4α or GR.

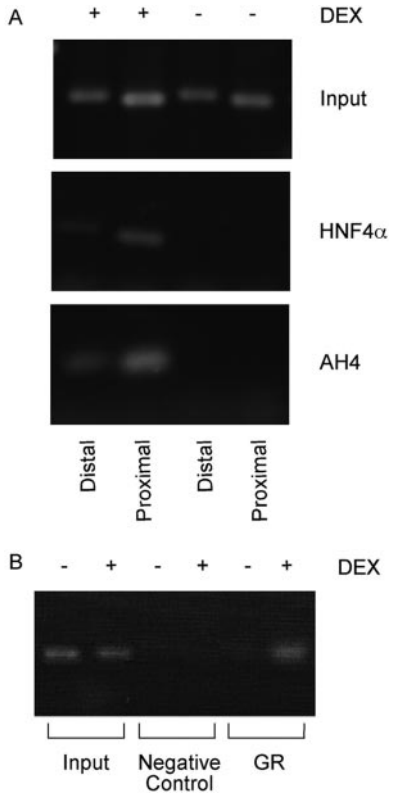

Fig. 7. DEX increases histone acetylation of the proximal CYP2A6 promoter and binding of HNF4α to this region in human hepatocytes. A, human hepatocytes were cultured in a glucocorticoid-free medium for 24 h. Thereafter, cells were cultured for 6 h with 100 nM DEX. Using a ChIP assay, the association of acetylated histone H4 (AH4) and HNF4α with the CYP2A6 promoter was detected by immunoprecipitation followed by PCR amplification of the proximal (−274 to +28) and distal (−2978 to −2659) regions of the CYP2A6 promoter region. Five microliters of each of the analyzed samples were used for PCR amplification as the input control (Input) before immunoprecipitation. B, HeLa cells were incubated with DEX for 24 h before performing a modified ChIP assay that included dimethyl-3,3-dithiobispropionimidate-2HCl to improve detection of protein-protein interactions by cross-linking primary amines. PCR amplification of samples immunoprecipitated with anti-GR antibodies revealed the presence of GR at the proximal (−274 to +28) CYP2A6 promoter. Input sample DNA was PCR-amplified before immunoprecipitation, and the negative control was immunoprecipitated in the absence of antibodies.

CYP2A6, thereby confirming this suspicion. It has been previously demonstrated, by adenovirus-mediated antisense targeting in cultured human primary hepatocytes, that HNF4α is a general activator of *CYP2A6* (Pitarque et al., 2005). HNF4-REs have also been identified in the 5′ flanking region of the *hCYP2C8* (Ferguson et al., 2005) and *hCYP2C9* (Chen et al., 2005) genes. In addition, *CYP2A5* the mouse ortholog of *CYP2A6* is regulated by HNF4α through an HNF4-RE identified at position −63 to −47 within the proximal promoter (Ulvila et al., 2004) that displays considerable sequence homology with the CYP2A6 HNF4-RE. Thus, the current study confirms the role of HNF4α in transcriptional regulation of *CYP2A6*, and it suggests that GR may contribute to CYP2A6 induction by interacting with HNF4α.

There are several possible mechanisms by which DEX influences HNF4α-mediated *CYP2A6* transcriptional activation, including GR-mediated up-regulation of HNF4α expression, increased access to and/or binding of HNF4α to the HNF4-RE or various combinations thereof. Several of our findings suggest that increased expression of HNF4α is not required for GR-mediated induction of CYP2A6. Although DEX increased HNF4α mRNA levels in primary human hepatocytes, HNF4α nuclear protein levels were clearly not altered. Moreover, cycloheximide treatment did not reduce DEX-mediated increases in CYP2A6 transcript levels, indicating that synthesis of de novo protein is not required for CYP2A6 transcription. Results of ChIP assays demonstrated that DEX treatment facilitates binding of HNF4α to the HNF4-RE perhaps by increasing histone H4 acetylation, thereby altering chromatin structure and enhancing accessibility to the CYP2A6 proximal promoter. We have previously shown a similar DEX-mediated increase in histone H4 acetylation in the proximal CAR promoter (Assenat et al., 2004). However, changes in histone H4 acetylation cannot explain the effect of DEX on HNF4α interactions with HNF4-RE observed in EMSA and luciferase reporters assays in which the true chromatin structure is not present.

Although HNF4α is clearly required for DEX-mediated induction of CYP2A6, the precise relationship between GR and HNF4α in facilitating binding to the HNF4-RE is currently unclear. Previous studies have shown that high-affinity binding of GR to the phosphoenolpyruvate carboxykinase gene promoter is strictly dependent on associated accessory factor elements, including HNF4 (Stafford et al., 2001). Moreover, we have recently shown that CYP3A4 and CYP2C9 promoters are activated by GR in placental cells only after cotransfection with HNF4α (Pavek et al., 2007). However, the novelty of the findings of the current study is that the mechanism by which GR and HNF4α cooperatively transactivate CYP2A6 is independent of a GRE. Nevertheless, direct GR-DNA interactions may not be necessary for CYP2A6 transactivation because GR may act through the formation of complexes with HNF4α and possibly other coactivators of transcription. Results of the ChIP and streptavidin DNA binding assays indicate that both HNF4α and GR are present at the HNF4-RE suggesting that GR may perform a tethering role through direct protein-protein interaction that stimulates transcriptional activity of HNF4α. Such a physical interaction has been observed with GR and the chicken ovalbumin upstream promoter-transcription factor II that plays a critical role in the metabolism of glucose, cholesterol, and xenobiotics (De Martino et al., 2004). Moreover, the Glucocorticoid receptor interacting protein 1 (GRIP1) in known to associate with the HNF4α receptor (Wang et al., 1998) and the GR (Li et al., 2003), raising the possibility that activated GR may recruit GRIP1 to act as a bridging factor between the GR and HNF4α, resulting in transactivation via the HNF4-RE. However, a necessary assumption would be that interaction between the GR and GRIP1 occurs independently of a GRE. In preliminary streptavidin DNA binding experiments using biotinylated HNF4-RE probes, we have shown that complexes among GR, HNF4α, and GRIP1 do occur at the HNF4-RE.

In conclusion, the results of this study demonstrate that DEX-mediated induction of *CYP2A6* occurs by transcriptional activation via a mechanism involving the GR and enhanced interactions between HNF4α and an HNF4-RE present in the proximal CYP2A6 promoter. Glucocorticoid-mediated induction of CYP2A6 expression has profound implications on the rate of nicotine metabolism and clearance and tobacco dependence.

References

Assenat E, Gerbal-Chaloin S, Larrey D, Saric J, Fabre JM, Maurel P, Vilarem MJ, and Pascussi JM (2004) Interleukin 1beta inhibits CAR-induced expression of hepatic genes involved in drug and bilirubin clearance. *Hepatology* **40:**951–960.

Bernasconi AG, Rebuffat AG, Lovati E, Frey BM, Frey FJ, and Galli I (1997) Cortisol increases transfection efficiency of cells. *FEBS Lett* **419:**103–106.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Chen Y, Ferguson SS, Negishi M, and Goldstein JA (2003) Identification of constitutive androstane receptor and glucocorticoid receptor binding sites in the CYP2C19 promoter. *Mol Pharmacol* **64:**316–324.

Chen Y, Kissling G, Negishi M, and Goldstein JA (2005) The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4alpha to synergistically activate the human CYP2C9 promoter. *J Pharmacol Exp Ther* **314:**1125–1133.

De Martino MU, Alesci S, Chrousos GP, and Kino T (2004) Interaction of the glucocorticoid receptor and the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII): implications for the actions of glucocorticoids on glucose, lipoprotein, and xenobiotic metabolism. *Ann N Y Acad Sci* **1024:**72–85.

Dignam JD, Lebovitz RM, and Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:**1475–1489.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M, and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4α. *Mol Pharmacol* **68:**747–757.

Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ, and Maurel P (2002) Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J Biol Chem* **277:**209–217.

Hirschelmann R, Schade R, Burger W, and Bekemeier H (1990) Nonspecific action of glucocorticosteroids after high-dose pulse therapy? *Agents Actions* **31:**148–151.

Kamataki T, Fujieda M, Kiyotani K, Iwano S, and Kunitoh H (2005) Genetic polymorphism of CYP2A6 as one of the potential determinants of tobacco-related cancer risk. *Biochem Biophys Res Commun* **338:**306–310.

Kirby GM, Batist G, Alpert L, Lamoureux E, Cameron RG, and Alaoui JMA (1996) Overexpression of cytochrome P-450 isoforms involved in aflatoxin B1 bioactivation in human liver with cirrhosis and hepatitis. *Toxicol Pathol* **24:**458–467.

Li X, Wong J, Tsai SY, Tsai MJ, and O'Malley BW (2003) Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. *Mol Cell Biol* **23:**3763–3773.

Malaiyandi V, Sellers EM, Tyndale RF, Nakajima M, Kuroiwa Y, and Yokoi T (2005) Implications of CYP2A6 genetic variation for smoking behaviors and nicotine dependence. *Clin Pharmacol Ther* **77:**145–158.

Nakajima M, Kuroiwa Y, and Yokoi T (2002) Interindividual differences in nicotine metabolism and genetic polymorphisms of human CYP2A6. *Drug Metab Rev* **34:**865–877.

Pascussi JM, Busson-Le Coniat M, Maurel P, and Vilarem MJ (2003) Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. *Mol Endocrinol* **17:**42–55.

Pavek P, Cerveny L, Svecova L, Brysch M, Libra A, Vrzal R, Nachtigal P, Staud F, Ulrichova J, Fendrich Z, et al. (2007) Examination of glucocorticoid receptor alpha-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines. *Placenta* **28:**1004–1011.

Pelkonen O, Rautio A, Raunio H, and Pasanen M (2000) CYP2A6: a human coumarin 7-hydroxylase. *Toxicology* **144:**139–147.

Pianezza ML, Sellers EM, and Tyndale RF (1998) Nicotine metabolism defect reduces smoking. *Nature* **393:**750.

Pichard-Garcia L, Gerbal-Chaloin S, Ferrini JB, Fabre JM, and Maurel P (2002) Use of long-term cultures of human hepatocytes to study cytochrome P450 gene expression. *Methods Enzymol* **357:**311–321.

Pitarque M, Rodriguez-Antona C, Oscarson M, and Ingelman-Sundberg M (2005)

Transcriptional regulation of the human CYP2A6 gene. *J Pharmacol Exp Ther* **313:**814–822.
Pitarque M, von Richter O, Oke B, Berkkan H, Oscarson M, and Ingelman-Sundberg M (2001) Identification of a single nucleotide polymorphism in the TATA box of the CYP2A6 gene: impairment of its promoter activity. *Biochem Biophys Res Commun* **284:**455–460.
Raunio H, Rautio A, Gullsten H, and Pelkonen O (2001) Polymorphisms of CYP2A6 and its practical consequences. *Br J Clin Pharmacol* **52:**357–363.
Raunio H, Rautio A, and Pelkonen O (1999) The CYP2A subfamily: function, expression and genetic polymorphism. *IARC Sci Publ* **1999:**197–207.
Romero L, Higgins MA, Gilmore J, Boudreau K, Maslen A, Barker HJ, and Kirby GM (2002) Down-regulation of alpha class glutathione S-transferase by interleukin-1beta in human intestinal epithelial cells (Caco-2) in culture. *Drug Metab Dispos* **30:**1186–1193.
Sellers EM, Tyndale RF, and Fernandes LC (2003) Decreasing smoking behaviour and risk through CYP2A6 inhibition. *Drug Discov Today* **8:**487–493.
Sotaniemi EA, Rautio A, Backstrom M, Arvela P, and Pelkonen O (1995) CYP3A4 and CYP2A6 activities marked by the metabolism of lignocaine and coumarin in patients with liver and kidney diseases and epileptic patients. *Br J Clin Pharmacol* **39:**71–76.
Stafford JM, Wilkinson JC, Beechem JM, and Granner DK (2001) Accessory factors facilitate the binding of glucocorticoid receptor to the phosphoenolpyruvate carboxykinase gene promoter. *J Biol Chem* **276:**39885–39891.
Tutka P, Mosiewicz J, and Wielosz M (2005) Pharmacokinetics and metabolism of nicotine. *Pharmacol Rep* **57:**143–153.
Ujjin P, Satarug S, Vanavanitkun Y, Daigo S, Ariyoshi N, Yamazaki H, Reilly PE, Moore MR, and Kamataki T (2002) Variation in coumarin 7-hydroxylase activity associated with genetic polymorphism of cytochrome P450 2A6 and the body status of iron stores in adult Thai males and females. *Pharmacogenetics* **12:**241–249.
Ulvila J, Arpiainen S, Pelkonen O, Aida K, Sueyoshi T, Negishi M, and Hakkola J (2004) Regulation of Cyp2a5 transcription in mouse primary hepatocytes: roles of hepatocyte nuclear factor 4 and nuclear factor I. *Biochem J* **381:**887–894.
Wang J, Pitarque M, Ingelman-Sundberg M, Rodriguez-Antona C, and Oscarson M (2006) 3′-UTR polymorphism in the human CYP2A6 gene affects mRNA stability and enzyme expression. *Biochem Biophys Res Commun* **340:**491–497.
Wang JC, Stafford JM, and Granner DK (1998) SRC-1 and GRIP1 coactivate transcription with hepatocyte nuclear factor 4. *J Biol Chem* **273:**30847–30850.

Address correspondence to: Dr. Gordon M. Kirby, Department of Biomedical Sciences, University of Guelph, Guelph, ON, Canada, N1G 2W1. E-mail: gkirby@uoguelph.ca