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Transcriptional Activation of *CYP2C9*, *CYP1A1*, and *CYP1A2* by Hepatocyte Nuclear Factor 4α Requires Coactivators Peroxisomal Proliferator Activated Receptor-γ Coactivator 1α and Steroid Receptor Coactivator 1[S]

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ABSTRACT

Hepatocyte nuclear factor 4α (HNF4α) is a key transcription factor for the constitutive expression of cytochromes P450 (P450s) in the liver. However, human hepatoma HepG2 cells show a high level of HNF4α but express only marginal P450 levels. We found that the HNF4α-mediated P450 transcription in HepG2 is impaired by the low level of coactivators peroxisomal proliferator activated receptor-γ coactivator 1α (PGC1α) and steroid receptor coactivator 1 (SRC1). Reporter assays with a chimeric CYP2C9-LUC construct demonstrated that the sole transfection of coactivators induced luciferase activity in HepG2 cells. In HeLa cells however, CYP2C9-LUC activity only significantly increased when coactivators were cotransfected with HNF4α. A deletion mutant lacking the two proximal HNF4α binding sites in the *CYP2C9* promoter did not respond to PGC1α or SRC1, demonstrating that coactivators were acting through HNF4α response elements. Adenovirus-mediated transfection of PGC1α in human hepatoma cells caused a significant dose-dependent increase in CYP2C9, CYP1A1, and CYP1A2 and in the positive control CYP7A1. PGC1α also showed a moderate activating effect on CYP3A4, CYP3A5, and CYP2D6. Adenoviral transfection of SRC1 had a lessened effect on P450 genes. Chromatin immunoprecipitation assay demonstrated in vivo binding of HNF4α and PGC1α to HNF4α response sequences in the *CYP2C9* promoter and to three new regulatory regions in the common 23.3 kilobase spacer sequence of the *CYP1A1/2* cluster. Insulin treatment of HepG2 and human hepatocytes caused repression of PGC1α and a concomitant down-regulation of P450s. Our results establish the importance of coactivators PGC1α and SRC1 for the hepatic expression of human P450s and uncover a new HNF4α-dependent regulatory mechanism to constitutively control the *CYP1A1/2* cluster.

Cytochromes P450 (P450s) are a superfamily of monooxygenases that play a key role in the detoxification of xenobiotics, the metabolic activation of chemical carcinogens, and the oxidative metabolism of endogenous compounds such as steroids, fatty acids, and prostaglandins. Most foreign compound-metabolizing P450s are highly expressed in the liver, although lower levels of particular P450 forms are also found in extrahepatic tissues such as intestine, lung, and kidney. The molecular mechanism sustaining the hepatic-specific P450 expression is not completely understood, although recent studies have shown that P450 expression in the liver is primarily governed at the transcriptional level and relies on the combinatorial action of a set of liver-enriched transcription factors such as C/EBPα, C/EBPβ, HNF1α, HNF3γ, and HNF4α (Akiyama and Gonzalez, 2003; Rodriguez-Antona et al., 2003; Bort et al., 2004; Martinez-Jimenez et al., 2005).

Current research supports the notion that among the multiple liver-enriched transcription factors, HNF4α is one of the

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ABBREVIATIONS: P450, cytochrome P450; HNF, hepatocyte nuclear factor; PGC1α, peroxisomal proliferator activated receptor-γ coactivator 1α; SRC, steroid receptor coactivator; m.o.i., multiplicity of infection; Ad, recombinant adenovirus; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); PBGD, porphobilinogen deaminase; PXR, pregnane X receptor; C/EBP, CCAAT/enhancer-binding protein; CAR, constitutive androstane receptor; AHR, aryl hydrocarbon receptor; XRE, xenobiotic response element; PCAF, p300/CBP-associated protein.

most important for the expression of hepatic-specific genes. HNF4α is a highly conserved member of the nuclear receptor superfamily that is preferentially expressed in the liver (Sladek et al., 1990). HNF4α homodimers bind to specific DNA sequence elements (e.g., DR1) (Fraser et al., 1998) and stimulate transcription in the absence of exogenously added ligands, which is in line with studies suggesting that HNF4α is constitutively bound to endogenous fatty acids (Wisely et al., 2002). Genome-scale location analysis revealed surprising results for HNF4α in hepatocytes. The number of genes enriched in HNF4α ChIPs (>1500 genes) was much larger than that observed for other typical hepatic-specific regulators such as HNF1 or HNF6 (Odom et al., 2004). Therefore, HNF4α emerges as a widely acting transcription factor in the liver, consistent with the fact that it is involved in the transcriptional regulation of multiple genes for glucose, urea, cholesterol, and fatty acid metabolism, and in the synthesis of blood coagulation factors (Li et al., 2000; Hayhurst et al., 2001). HNF4α also plays critical roles in the specification of the hepatic phenotype during liver development. Indeed, disruption of the *HNF4α* gene leads to an early embryonic lethal phenotype associated with a failure of visceral endoderm differentiation (Chen et al., 1994).

Several lines of evidence also point to HNF4α as an important factor for the constitutive expression of P450s in the liver. Analysis of promoter and enhancer sequences and multiple studies using mobility shift and recombinant promoter analysis have shown that this factor plays a positive role in the regulation of several rodent and human P450s (Akiyama and Gonzalez, 2003). Moreover, studies in HNF4α-deficient mice (Wiwi et al., 2004) and in human hepatocytes transfected with HNF4-antisense RNA (Jover et al., 2001) have also proved the wide relevance of this transcription factor in the expression of hepatic drug-metabolizing P450 genes.

One fact that goes against the key role of HNF4α on hepatic P450 expression is that well-characterized hepatoma cells, such as HepG2, have a high level of HNF4α (Rodriguez-Antona et al., 2002), yet they lack the functional expression of most relevant human liver P450s, and only very sensitive techniques (i.e., RT-PCR) allow for the detection and quantification of the transcribed genes. Indeed, most of the P450 mRNAs examined in HepG2 showed a 100 to 1000 times lower expression than in cultured human hepatocytes (Rodriguez-Antona et al., 2002).

The initial objective of the present study was to investigate the factors causing the dissociation between high HNF4α expression and low P450 transcription in human hepatoma cells. We found that HepG2 cells express low levels of HNF4α coactivators, PGC1α and SRC1, and that their re-expression by means of adenoviral vectors caused a significant activation of several human P450 genes, especially *CYP2C9*, *CYP1A1*, and *CYP1A2*. Based on our experimental evidence, we conclude that SRC1 and notably PGC1α play a prominent role in the transcriptional activation of several major human P450s involved in drug metabolism and that this effect occurs via HNF4α. Our data also uncover a new regulatory mechanism for the coordinated expression of the *CYP1A1/2* cluster and provide a mechanistic explanation for the P450 variability caused by the feeding-fasting cycle.

Materials and Methods

Cell Culture. Human hepatoma cells (HepG2 and Mz-Hep-1) were plated in Ham's F-12/Leibovitz L-15 media [1:1 (v/v)] supplemented with 6% fetal calf serum and cultured to 70 to 80% confluence. HeLa (human cervix carcinoma) and 293 cells (AdE1A-transformed human embryonic kidney) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and maintained as monolayer cultures. Culture medium for 293 cells was also supplemented with 3.5 g/l glucose. Human hepatocytes were isolated from liver biopsies (1–3 g) of patients undergoing liver surgery after informed consent. None of the patients regularly consumed alcohol or other drugs. Six liver biopsies (four from men and two from women, ages ranging from 46 to 65 years) were used. Hepatocytes were isolated using a two-step perfusion technique (Gomez-Lechon and Castell, 2000) and were seeded on plates coated with fibronectin (3.6 $\mu g/cm^2$) at a density of 8×10^4 cells/cm^2. The culture medium was Ham's F-12/Leibovitz L-15 [1:1 (v/v)] supplemented with 2% newborn calf serum, 5 mM glucose, 0.2% bovine serum albumin, and 10^{-8} M insulin. The medium was changed 1 h later to remove unattached hepatocytes. After 24 h, the culture medium was changed to serum-free medium containing 10^{-8} M dexamethasone. Cultures were routinely supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin.

Development of Adenoviral Vectors. A recombinant adenovirus for the expression of human HNF4α was prepared as follows: HNF4α2 cDNA was released from the expression vector pMT2-HNF4B (Dr. I. Talianidis, IMBB, Heraklion, Greece) by EcoRI digestion and subcloned into the EcoRI site of the adenoviral shuttle vector pAC/CMVpLpA. This plasmid was cotransfected with pJM17 containing the full-length adenovirus-5 genome (dl309) into 293 cells by calcium phosphate/DNA coprecipitation. Homologous recombination between adenovirus sequences in the shuttle vector pAC/CMV-pLpA and in the pJM17 plasmid generates a genome of a packageable size in which most of the adenovirus early region 1 is lacking, thus rendering the recombinant virus replication defective (Jover et al., 2001). The resulting virus (named Ad-HNF4α) was plaque-purified, expanded into a high-concentration stock, and titrated by plaque assay.

A recombinant adenovirus for the coactivator SRC1 was prepared by using the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA). SRC1α cDNA was released from pCR3.1-SRC1α (Dr. B. W. O'Malley, Baylor College of Medicine, Houston, TX) by ApaI digestion, subcloned into the pSPORT vector (Invitrogen, Carlsbad, CA), and ligated into the BglII and KpnI sites of the adenoviral pShuttle-CMV vector (Stratagene). The linearized plasmid (PmeI digestion) was transferred into BJ 5183 cells containing pretransferred Ad-Easy-1 vector to generate recombinant adenovirus. Colonies containing the correct recombinant adenovirus were identified using restriction enzymes and PCR with insert-specific primers. The recombinant adenovirus DNA was then linearized by PacI and transfected into human embryonic kidney 293 cells by the calcium phosphate precipitation method. After a culture period of several days, infected 293 cells were collected and subjected to three freezing/thawing cycles. The generation of a high-titer adenovirus stock was performed as described previously (Jover et al., 2001).

The adenoviral vector for the expression of PGC1α was kindly provided by Dr. P. Puigserver (Johns Hopkins University School of Medicine, Baltimore, MD) (Yoon et al., 2001). The Ad-PGC-1 vector contains, in tandem, the GFP cDNA and the PGC1α cDNA (containing Flag and hemagglutinin epitope tags) downstream of separate CMV promoters.

Cell lines and primary hepatocytes were infected with recombinant adenoviruses for 120 min at a multiplicity of infection (m.o.i.) ranging from 1 to 48 plaque-forming units/cell. Thereafter, cells were washed, and fresh medium was added. Forty-eight hours after transfection, cells were analyzed or directly frozen in liquid N_2.

Transfection and Reporter Gene Assays. *CYP2C9* promoter region (1836 bp) was PCR-cloned using the Human GenomeWalker Kit (Clontech, Mountain View, CA), as described previously (Bort et al., 2004) and ligated into the enhancerless, promoterless pGL3-Basic vector (Promega, Madison, WI). The resulting CYP2C9 chimeric luciferase reporter construct, CYP2C9-LUC, contains two HNF4α-response elements at −152 and −185 bp (Chen et al., 2005). For the generation of the subsequent deletion mutant AvrII-Del-CYP2C9-LUC, the original reporter construct (CYP2C9-LUC) was cleaved with AvrII to release a short fragment between −114 and −250 bp, which contained the two HNF4α binding sites. Expression vectors for transcription factors and coactivators were the following: pMT2-HNF4B (Dr. Talianidis), pcDNA3-hemagglutinin-hPGC1 (Dr. A. Kralli, The Scripps Research Institute, La Jolla, CA), pCR3.1-SRC1α (Dr. O'Malley), pCMX-Flag-P300/cAMP response element-binding protein-binding protein-associated factor (Dr. Talianidis), pSG5-GRIPI (Dr. M. R. Stallcup, University of Southern California, Los Angeles, CA), pSG5-TIF-II (Dr. H. Gronmeyer, Institut de Génétique et de Biologie Moleculaire et Cellulaire, Strasbourg, France), and pCMX-ACTR (Dr. R. M. Evans, Howard Hughes Medical Institute, La Jolla, CA).

Plasmid DNAs were purified with Qiagen Maxiprep kit columns

TABLE 1
Oligonucleotides used for quantitative RT-PCR

Oligonucleotide Name	Oligonucleotide Sequence	Fragment Size	T_m
		bp	*°C*
HNF4α		255	61
Forward	5′-GCC TAC CTC AAA GCC ATC AT-3′		
Reverse	5′-GAC CCT CCC AGC AGC ATC TC-3′		
SRC1		358	61
Forward	5′-CAG GAA AGA GGA CGA AAT GAG T-3′		
Reverse	5′-CAA TAA AAG CAG AAA AAA CAG GC-3′		
PGC1α		229	54
Forward	5′-AAT GTG TCT CCT TCT TGT TCT T-3′		
Reverse	5′-GGT GTC TGT AGT GGC TTG A-3′		
CYP 1A1		371	60
Forward	5′-TCC AGA GAC AAC AGG TAA AAC A-3′		
Reverse	5′-AGG AAG GGC AGA GGA ATG TGA T-3′		
CYP 1A2		453	62
Forward	5′-AAC AAG GGA CAC AAC GCT GAA T-3′		
Reverse	5′-GGA AGA GAA ACA AGG GCT GAG T-3′		
CYP 2C9		137	60
Forward	5′-CCT CTG GGG CAT TAT CCA TC-3′		
Reverse	5′-ATA TTT GCA CAG TGA AAC ATA GGA-3′		
CYP 2D6		289	62
Forward	5′-CTA AGG GAA CGA CAC TCA TCA C-3′		
Reverse	5′-CTC ACC AGG AAA GCA AAG ACA C-3′		
CYP 3A4		382	60
Forward	5′-CCT TAC ATA TAC ACA CCC TTT GGA AGT-3′		
Reverse	5′-AGC TCA ATG CAT GTA CAG AAT CCC CGG TTA-3′		
CYP 3A5		678	60
Forward	5′-GAA GAA AAG TCG CCT CAA C-3′		
Reverse	5′-AAG AAG TCC TTG CGT GTC TA-3′		
CYP7A1		343	58
Forward	5′-CTG CTA CCG AGT GAT GTT TGA A-3′		
Reverse	5′-GAA AGT CGC TGG AAT GGT GTT-3′		
PBGD		294	58
Forward	5′-CGG AAG AAA ACA GCC CAA AGA-3′		
Reverse	5′-TGA AGC CAG GAG GAA GCA CAG T-3′		

TABLE 2
Oligonucleotides used for ChIP assay

Oligonucleotide Name	Oligonucleotide Sequence	Fragment Size	T_m
		bp	*°C*
CYP2C9-PR		225	60
Forward	5′-AAC GAA GGA GAA CAA GAC CA-3′		
Reverse	5′-AGC ACA AGG ACC ACA AGA GA-3′		
CYP1A1/2 region 1		445	57
Forward	5′-GGA ATA GAA AGA GGG GAA AAG-3′		
Reverse	5′-ATC ATT CTT GGG TGT TTC TCG-3′		
CYP1A1/2 region 2		345	57
Forward	5′-CTC GCT CCA TTC GTG CTC TC-3′		
Reverse	5′-GGC CTG ACC ACT TTG CAA TCT A-3′		
CYP1A1/2 region 3		244	57
Forward	5′-GAA AGA AGC CCA GAT CAG TC-3′		
Reverse	5′-GAA AGC TGG ATC ACT GAA CAA-3′		
CYP2C9 (Exon 3)		150	60
Forward	5′-TTT CGT TTC TCT TCC TGT TAG-3′		
Reverse	5′-GAT AGT AGT CCA GTA AGG TCA GTG-3′		
CYP3A4 (Exon 2)		686	60
Forward	5′-ATC TCA TCA TCA CTG CGG AAG A-3′		
Reverse	5′-AGA GCC CTT GGG TAA ACA TTG C-3′		

(QIAGEN, Valencia, CA) and quantified by optical density at 260 nm and fluorescence using PicoGreen (Invitrogen, Leiden, The Netherlands). The day before transfection, cells were plated in 35-mm dishes with 1.5 ml of Dulbecco's modified Eagle's medium/Nut F12 (Invitrogen, Barcelona, Spain), and were supplemented with 6% newborn calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Firefly luciferase expression constructs (pGL3-Basic, CYP2C9-LUC, and AvII-del-CYP2C9-LUC) (0.75 μg) were transfected with varying amounts of expression plasmids (0.2–3.0 μg) by the calcium phosphate precipitation method, as indicated in the figures. The total amount of expression vector was kept constant by adding empty expression vector. In parallel, 0.05 μg of pRL-CMV (a plasmid expressing *Renilla reniformis* luciferase under the CMV immediate early enhancer/promoter) was cotransfected to correct variations in transfection efficiency. Calcium phosphate/DNA coprecipitates were directly added to cultures, and cells were incubated for a further 48 h. Luciferase activities were assayed using the Dual-Luciferase Reporter Kit (Promega).

Quantification of mRNA Levels. Total cellular RNA was extracted with the RNeasy Total RNA Kit (QIAGEN), and contaminating genomic DNA was removed by incubation with DNase I Amplification Grade (Invitrogen). RNA (1 μg) was reverse-transcribed as described previously (Perez et al., 2003). Diluted cDNA (3 μl) was amplified with a rapid thermal cycler (LightCycler Instrument; Roche Diagnostics, Indianapolis, IN) in 15 μl of LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN), 5 mM $MgCl_2$, and 0.3 μM concentration of each oligonucleotide. We designed specific primer sets for 10 different cDNAs including P450s, transcription factors, and coactivators (Table 1). In parallel, we always analyzed the mRNA concentration of the human housekeeping porphobilinogen deaminase (PBGD, hydroxymethylbilane synthase) as an internal normalization control (Table 1). PCR amplicons were confirmed to be specific by both size (agarose gel electrophoresis) and melting curve analyses. After denaturing for 30 s at 95°C, amplification was performed in 40 cycles of 1 s at 94°C, 5 s at 62°C, and 15 to 20 s at 72°C. The real-time monitoring of the PCR reaction and the precise quantification of the products in the exponential phase of the amplification were performed with the LightCycler quantification software according to the manufacturer's recommendations. To ensure that specificity was high enough to discriminate between CYP3A4 and CYP3A7, amplified DNA was digested with HindIII, which recognizes only CYP3A4 amplicons. Agarose gel analysis revealed that almost 100% of the PCR product was HindIII-digested.

Chromatin Immunoprecipitation Assay. Cells were infected with Ad-PGC1α and Ad-HNF4α for 48 h and then treated with 1% formaldehyde in phosphate-buffered saline buffer by gentle agitation for 10 min at room temperature to cross-link proteins to DNA. Thereafter, cells were collected by centrifugation, washed, resuspended in lysis buffer, and sonicated on ice for 6 $\times$ 10-s steps at 75% output in a Branson Sonicator (Branson, Danbury, CT). Cross-linking and sonication of chromatin from human liver tissue (750 mg) were carried out by following a partially different protocol (Sandoval et al., 2004). Sonicated samples were centrifuged to clear supernatants. DNA content was carefully measured by fluorescence with PicoGreen dye (Invitrogen) and properly diluted to maintain an equivalent amount of DNA in all samples (input DNA). For immunoprecipitation of HNF4α-DNA complexes, a 10 μg/ml concentration of a specific antibody against a C-terminal epitope of the human HNF4α (Santa Cruz Biotechnology, Santa Cruz, CA) was added. Samples were incubated overnight at 4°C on a 360° rotator (antibody-bound DNA fraction). For each cell preparation, an additional mock immunoprecipitation with rabbit preimmune IgG (Santa Cruz Biotechnology) was performed in parallel (background DNA fraction). The immunocomplexes were affinity-absorbed with 10 mg of protein A/G Sepharose (prewashed with lysis buffer for 4 h at 4°C by gentle rotation) and collected by centrifugation (6500*g*, 1 min). The antibody-bound and background DNA fractions were washed as described previously (Sandoval et al., 2004). ANTI-FLAG M2 Affinity Gel (Sigma, St. Louis, MO) directed against epitope-tagged PGC1α was used for immunoprecipitation of recombinant PGC1α-DNA complexes in Ad-transfected cells. In this case, the antibody-bound DNA fractions were washed according to the manufacturer's recommendations. A control cell sample, in which PGC1α had not been transfected, was processed in parallel to determine the background level

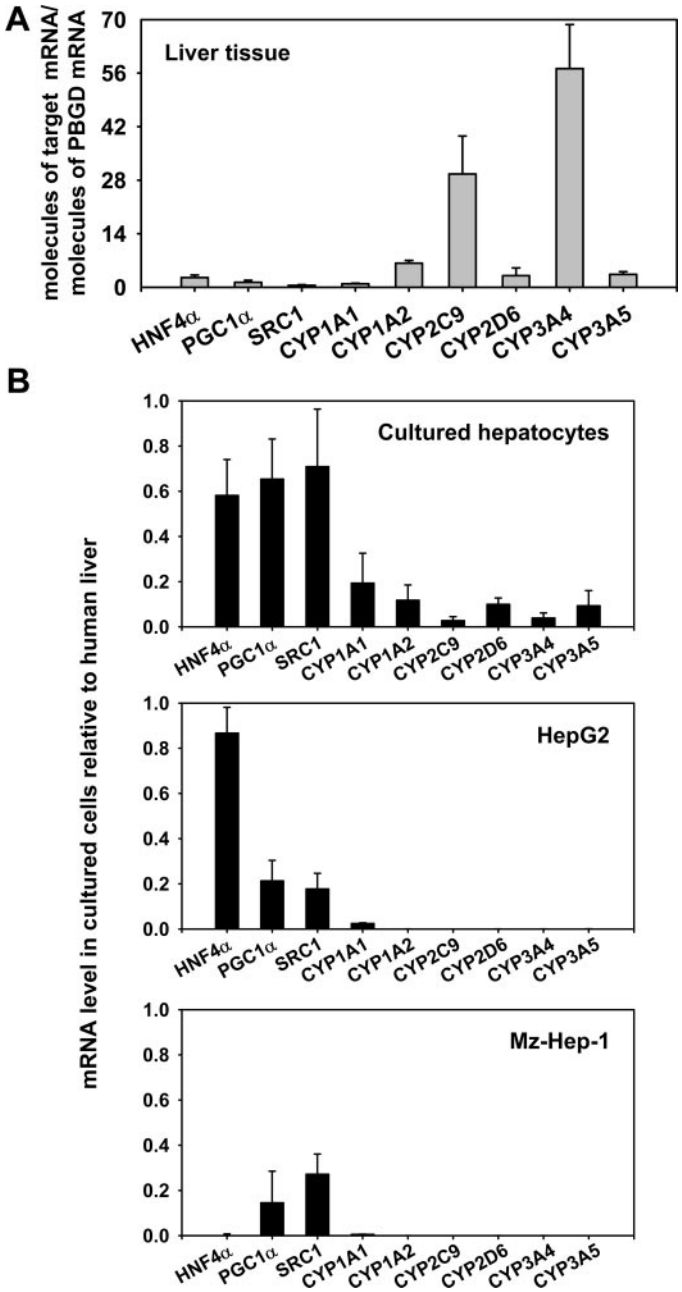

Fig. 1. Expression level of HNF4α, coactivators, and P450s in human liver and hepatic cell models. A, absolute mRNA concentrations were determined by real-time quantitative RT-PCR analysis in a human liver cDNA pool (n = 12). Concentration values were obtained by interpolation in standard curves and expressed as molecules of target mRNA per molecule of the human housekeeping PBGD mRNA. Data represent the mean ± S.D. from four independent RT-PCR analyses. B, total RNA was purified from human cultured hepatocytes (top), HepG2 (middle), and Mz-Hep-1 (bottom), and the mRNA levels of HNF4α, PGC1α, SRC1, and P450s were determined by quantitative RT-PCR analysis. Results were normalized with PBGD values. Data were expressed as relative to human liver tissue (liver = 1) and represent the mean ± S.D. from four to six independent cell cultures.

of unspecific binding in the immunoprecipitates. All of the cross-links were reversed by heating samples overnight at 65°C. The DNA from bound and input fractions was purified, diluted (1/10, bound fractions and 1/400 input fractions), and subjected to quantitative real-time PCR with a LightCycler instrument. Amplification was real-time monitored and allowed to proceed in the exponential phase until fluorescent signal from input samples reached a significant value (i.e., 10 arbitrary units). Amplified DNA was then analyzed by agarose gel electrophoresis. Amplification of P450 gene sequences (5′-flanking, intergenic, and exons) among the pull of DNA was performed with specific primers flanking these regions (Table 2).

Results

P450, HNF4α, and Coactivator Expression Levels in Different Hepatic Cell Models. First, we measured the absolute mRNA level of HNF4α, PGC1α, SRC1, and P450s in human liver by quantitative real-time RT-PCR analysis. The resulting expression profile in human liver was similar to that described previously by other authors, in which CYP3A4 and CYP2C9 were the most abundant P450s, and CYP1A1 was the isoform with the lowest expression level (Fig. 1A). HNF4α and coactivators showed a similar, relatively low expression level (Fig. 1A), which is in consonance with the limiting concentrations that regulatory proteins should have. Second, we quantified the same transcripts in three hepatic cellular models, human cultured hepatocytes and HepG2 and Mz-Hep-1 hepatoma cells, and compared the expression levels with those found in human liver (Fig. 1B). Human hepatocytes in culture (24 h) maintained a high level of both HNF4α and coactivators (PGC1α and SRC1), whereas drug-metabolizing P450 mRNAs were expressed at 10 to 20% of human liver content (Fig. 1B, top). These results suggest that, despite certain phenotypic instability, cultured human hepatocytes maintain a balanced expression of transcription factors and P450s. Analysis of human hepatoma HepG2 cells showed that HNF4α is expressed at levels as high as in cultured hepatocytes or liver tissue. However, the HNF4α coactivators PGC1α and SRC1 were down-regulated, and P450 mRNA levels were several orders of magnitude lower than those in the liver (Fig. 1B, middle). A very similar scenario is observed in Mz-Hep-1 hepatoma cells, with the only difference being that this cell line shows a very low HNF4α mRNA concentration (Fig. 1C, bottom). Therefore, a major divergence at the transcriptional level between cultured human hepatocytes and HepG2 cells is the lower expression of the HNF4α coactivators PGC1α and SRC1 in the human hepatoma cell line.

PGC1α and SRC1 Transactivate *CYP2C9* Promoter in HepG2 Cells. We investigated the relevance of HNF4α coactivators in the transactivation of the *CYP2C9* promoter, which contains two proximal HNF4α binding sites.

In HepG2 cells, the basal luciferase activity of a construct containing −1.8 kb of *CYP2C9* 5′-flanking region (CYP2C9-LUC) was 10 times higher than the background luciferase activity of the promoterless pGL3-Basic vector (Fig. 2A). Transfection of HNF4α did not increase CYP2C9-LUC activity (Fig. 2A), which suggests that other missing factors may limit the response. Transfection of coactivators SRC1 and, notably, PGC1α caused a significant increase in the CYP2C9-LUC reporter activity in HepG2 cells (3.6- and 12.6-fold, respectively, Fig. 2A). Other coactivators (SRC2, SRC3, and PCAF) did not substantially change CYP2C9 promoter activity (Fig. 2A). Cotransfection of HepG2 cells with SRC1α and PGC1α caused an additive effect on CYP2C9-LUC reporter activity (Supplemental Fig. S1). On the other hand, *CYP2C9* basal promoter activity was non significant in the nonhepatic cell line HeLa, and transfection of HNF4α or coactivators caused only low-range effects (Fig. 2B).

Experimental evidence suggests that endogenous HNF4α is functional in HepG2 cells, but its activity is limited by the low concentration of SRC1 and PGC1α. This was further demonstrated by cotransfection experiments. Figure 3 illustrates how the cotransfection of HNF4α and coactivators in

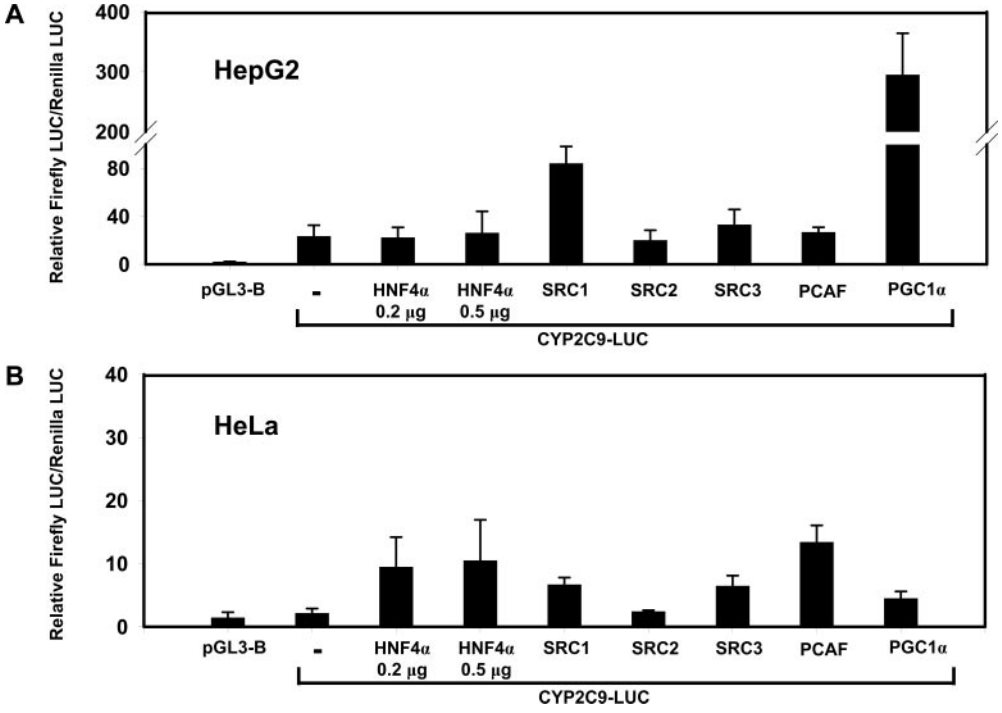

Fig. 2. Coactivators PGC1α and SRC1 activate CYP2C9 promoter in HepG2 cells. Chimeric CYP2C9-LUC reporter vector (0.75 μg) containing two HNF4α binding sites was either transfected alone or cotransfected with expression vectors for HNF4α (0.2 and 0.5 μg) or for coactivators (3.0 μg). The plasmid pRL-CMV (0.05 μg) was used to normalize transfection efficiency. The transactivating effect of HNF4α and several coactivators was evaluated individually in HepG2 (A) or HeLa cells (B). The insertless plasmid pAC/CMVpLpA was added to maintain a constant amount of expression vector. Cells were harvested 48 h after DNA transfection and luciferase activities were assayed. Values represent firefly luciferase/*R. reniformis* luciferase enzymatic activity ratios. Data represent the mean ± S.D. from at least four independent experiments performed in triplicate.

HepG2 cells caused a response very similar to that obtained with the sole transfection of coactivators SRC1 (Fig. 3A) or PGC1 (Fig. 3B). However, a clear synergistic effect of HNF4α and coactivators was observed in HeLa cells, where cotransfection of both factors was needed to largely induce *CYP2C9* promoter activity (Fig. 3).

Experimental data suggest that *CYP2C9* promoter activation by exogenous coactivators in HepG2 cells is likely to be mediated through HNF4α. To prove this idea, we performed reporter experiments with a CYP2C9-LUC construct after deleting HNF4α binding sites. AvrII was used to produce a short deletion of 136 bp containing the two HNF4α binding sites at −152 and −185 bp of the *CYP2C9* start site. The resulting AvrII-Del-CYP2C9-LUC construct showed no response to the transfection of coactivators SRC1 or PGC1α in HepG2 cells (Fig. 4A) or to the cotransfection of coactivators and HNF4α in HeLa cells (Fig. 4B). This blockade in response caused by deleting HNF4α binding sites reinforces

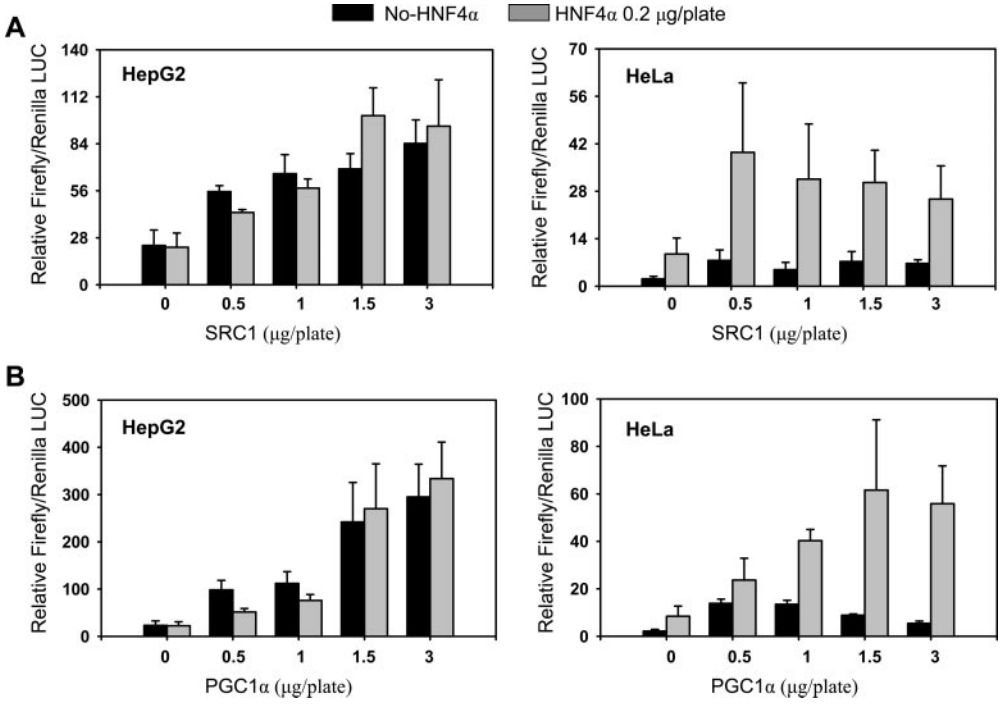

Fig. 3. Cotransfection of HNF4α and coactivators activates *CYP2C9* promoter in HeLa cells. Chimeric CYP2C9-LUC reporter construct (0.75 μg) was cotransfected with expression vectors in HepG2 (left-hand side) and HeLa cells (right-hand side). The transactivating effect of SRC1 (A) or PGC1α (B) was evaluated dose-dependently (0.5–3.0 μg) in cotransfection with HNF4α (0.2 μg, ▨) or empty vector (0.2 μg, ■). Normalized luciferase activities were obtained as described in the legend to Fig. 2. Data represent the mean ± S.D. from at least three independent experiments performed in triplicate.

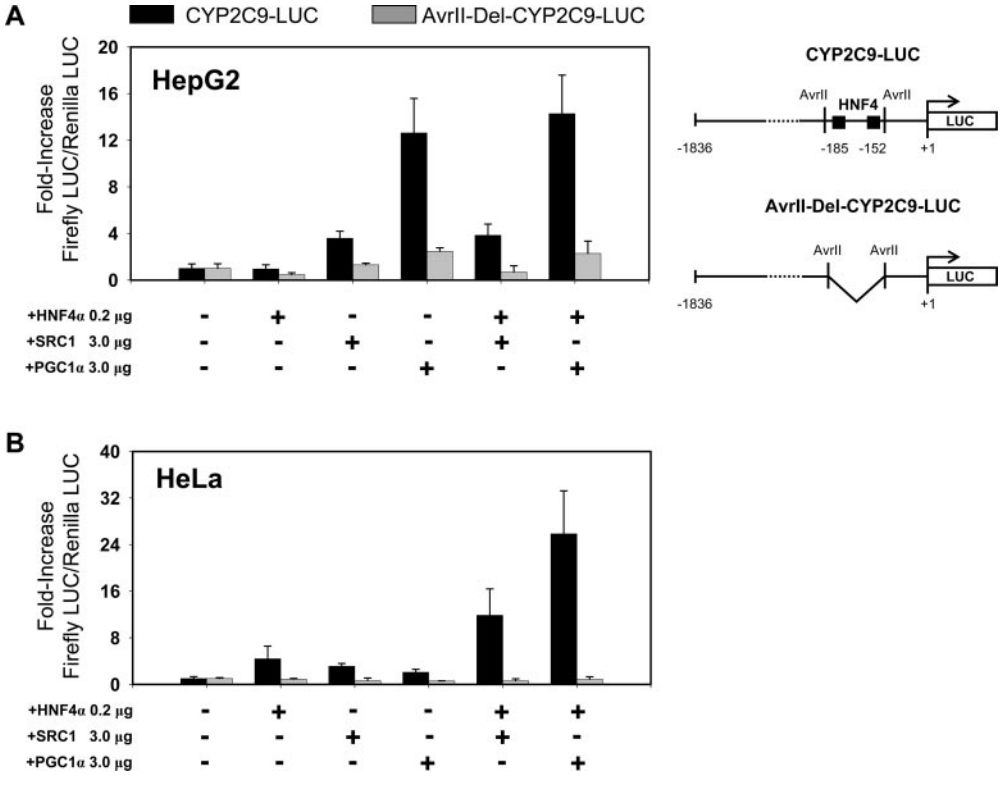

Fig. 4. *CYP2C9* promoter response to coactivators is abolished in a deletion mutant construct lacking HNF4α binding sites. Chimeric reporter gene constructs CYP2C9-LUC (■) and AvrII-Del-CYP2C9-LUC (▨) were transfected with expression vectors for HNF4α, SRC1, and PGC1α as indicated. The transactivating effect of HNF4α and coactivators was evaluated in either HepG2 (A) or HeLa cells (B). Normalized luciferase activities were obtained as described in the legend to Fig. 2. Data represent the mean ± S.D. from at least three independent experiments performed in triplicate. Top, right side, schematic luciferase reporter constructs with 1836 bp of *CYP2C9* 5′-flanking region. The two HNF4α binding sites in CYP2C9-LUC and the AvrII sequence deleted to abolish HNF4α response motifs in AvrII-Del-CYP2C9-LUC are depicted.

the idea that the activating effect of PGC1α and SRC1 on *CYP2C9* promoter is mediated by HNF4α at the −152-bp and −185-bp binding sites. Our experimental evidence suggests that the re-expression of limiting PGC1α and SRC1 coactivators would probably promote the critical interactions and cooperation needed to stimulate HNF4-mediated P450 transcription in hepatoma cells.

Adenovirus-Mediated Re-Expression of PGC1α and SRC1 Activates P450 Genes in Human Hepatoma Cells. We developed adenoviral vectors for the expression of SRC1

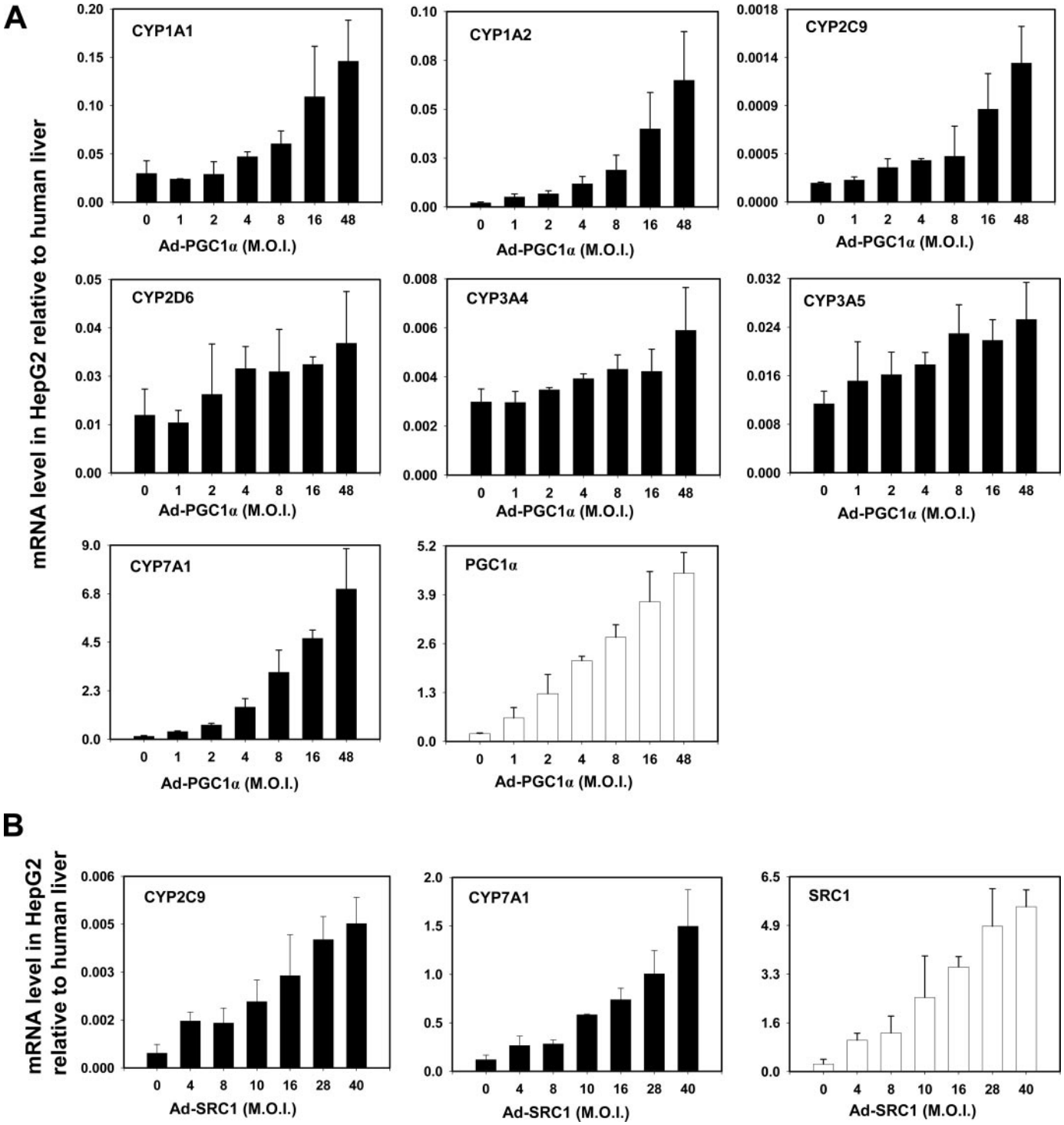

Fig. 5. Adenoviral-mediated transfection of PGC1α and SRC1 reactivates the expression of P450 genes in HepG2 cells. HepG2 cells were transduced with increasing doses of Ad-PGC1α (1–48 m.o.i.) (A) or Ad-SRC1 (4–40 m.o.i.) (B), and 48 h later, the mRNA concentrations of six relevant human drug-metabolism P450s were measured by quantitative RT-PCR. As a positive target gene, we evaluated the mRNA level of the cholesterol-7α-hydroxilase (CYP7A1). The expression of the transfected coactivators (□) was also analyzed by RT-PCR, after a previous treatment of purified RNA with DNaseI. Normalized data were expressed as relative to human liver tissue (liver = 1). Data represent the mean ± S.D. from three to four independent cultures.

and HNF4α. The adenoviral vector for PGC1α was kindly provided by Dr. Puigserver. HepG2 cells were infected with increasing doses of Ad-PGC1α and Ad-SRC1, and their effect on endogenous P450 genes was evaluated. The adenoviral-mediated transfection of PGC1α in HepG2 cells caused a significant dose-dependent increase in several drug-metabolizing P450 mRNAs: CYP2C9, CYP1A1, and CYP1A2. PGC1α also showed a moderate activating effect on CYP3A4, CYP3A5, and CYP2D6 (Fig. 5A). Otherwise, adenoviral transfection of SRC1 had a lessened effect on P450 genes, in which a significantly increased expression was only observed in CYP2C9 mRNA (Fig. 5B). *CYP7A1* is a known target gene activated by both PGC1α and SRC1. Its expression was measured as a positive control. Parallel transfection with a control adenoviral vector did not modify the basal level of P450 mRNA. Infection with Ad-GFP demonstrated a transfection efficiency of approximately 100%. The activating effect observed with coactivators revealed no significant improvement by the cotransfection of HepG2 with an adenoviral vector for HNF4α. Therefore, we postulate that re-expression of PGC1α and SRC1 can restore HNF4α activity in HepG2 hepatoma cells, which in turn leads to the transcription activation of several key drug-metabolizing P450 genes.

To further demonstrate that the transactivating effects caused by PGC1α on endogenous P450 genes were mediated by HNF4α, we carried out transfection experiments in MZ-Hep-1, a hepatoma cell line showing very low levels of HNF4α (Fig. 1B, bottom). Transfection of PGC1α or HNF4α caused slight increases in P450 gene expression, whereas cotransfection triggered a remarkable activation of *CYP2C9*, *CYP1A1*, and *CYP1A2* (Fig. 6). The combined effect of PGC1α and HNF4α on other P450s was not as significant. Our experimental evidence demonstrates that both HNF4α and PGC1α are required for an efficient transcriptional activation of *CYP2C9, CYP1A1*, and *CYP1A2* in hepatic cells.

HNF4α and PGC1α Are Bound in Vivo to HNF4 Response Sequences in P450 Regulatory Regions. Chromatin immunoprecipitation was used to investigate whether HNF4α and PGC1α were bound to the *CYP2C9* proximal promoter region and to several intergenic regions in the common spacer regulatory sequence of the *CYP1A1/2* cluster. The two HNF4α binding elements in the *CYP2C9* promoter have already been characterized in detail by electrophoretic mobility shift and supershift analyses (Chen et al., 2005; Kawashima et al., 2006). In relation to the *CYP1A1/2* cluster, we analyzed its 23.3-kb intergenic sequence with the NUBISCAN software by using the HNF4α matrix for comparison and a threshold raw score of 0.85 (Podvinec et al., 2002). Three putative HNF4 binding regions were identified. Region 1 between −4.0 and −4.2 kb (two hits), region 2 between −7.8 and −8.0 kb (three hits), and region 3 between −23.0 and −23.2 kb (two hits) from the starting transcription site of the *CYP1A1* gene (Fig. 7A). First, we performed a ChIP assay to demonstrate any binding of HNF4α to these sequences. The analysis was carried out on cultured human hepatocytes and on two different human liver samples. Results not only confirmed a strong binding of HNF4α to the *CYP2C9* promoter region but also demonstrated binding of HNF4α to the three predicted regions in the *CYP1A1/2* intergenic sequence (Fig. 7B). However, no significant binding was observed in exons of P450 genes, lacking consensus HNF4α binding sites (Fig. 7B). The amount of immunopre-

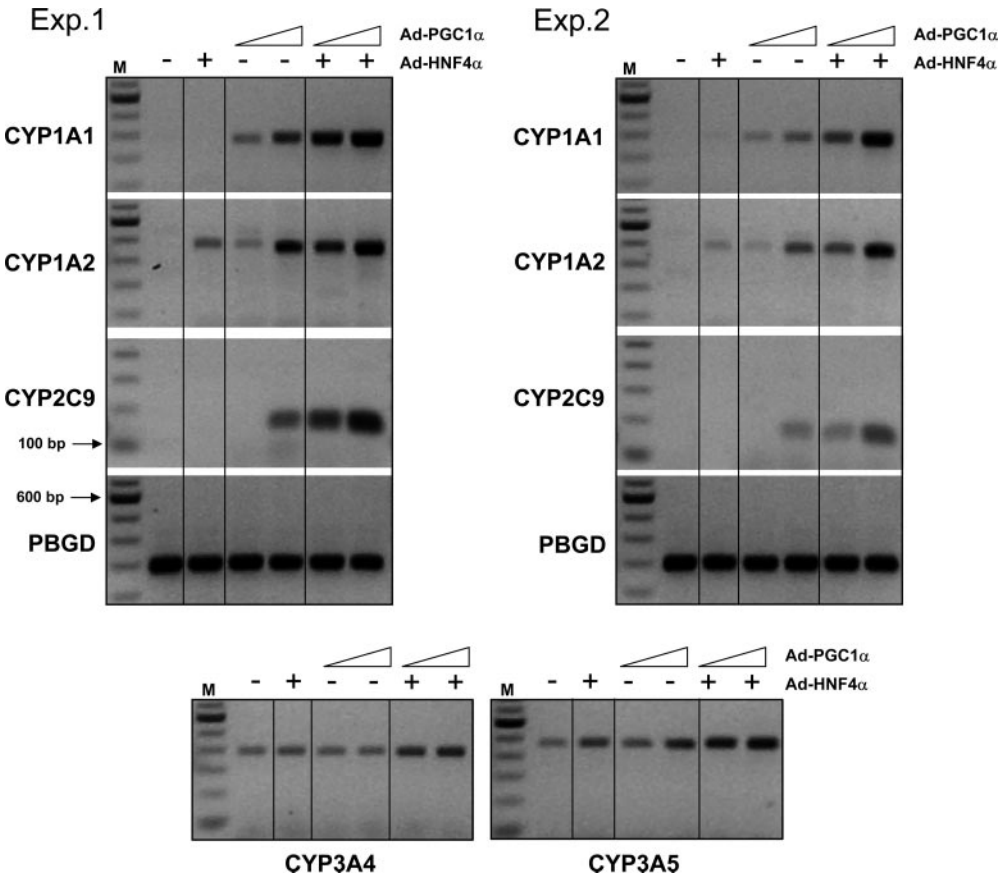

Fig. 6. Efficient reactivation of P450 genes by PGC1α requires HNF4α expression. Mz-Hep-1 cells were transduced with Ad-PGC1α (16 or 32 m.o.i.) and/or Ad-HNF4α (32 m.o.i.) as indicated, and 48 h later, the mRNA concentrations of CYP2C9, CYP1A1, CYP1A2, CYP3A4, and CYP3A5 and housekeeping PBGD were measured by RT-PCR. Appropriate cDNA dilutions and cycles were empirically determined for each gene to ensure that the PCR products did not reach the plateau of the amplification. Aliquots (20 μl) of the PCR reaction were subjected to electrophoresis on 1.5% agarose gel and stained with ethidium bromide. M, 100-bp DNA ladder.

cipitated DNA was also measured by quantitative PCR (Supplemental Fig. S2). These results demonstrated that in vivo binding of HNF4α to DNA response elements in cultured human hepatocytes is always lower than in human liver tissue, ranging from 30 to 80% depending on the specific regulatory region. Our data are in agreement with a lower HNF4α expression in cultured hepatocytes (60% of liver; Fig. 1B, top).

Second, we attempted to confirm whether PGC1α was bound to the same HNF4α binding regions. To this end, Mz-Hep-1 cells were transfected with Ad-PGC1α and Ad-HNF4α, and 48 h later, cells were processed for ChIP assay. Immunoprecipitation was performed with a specific antibody against the FLAG peptide, an epitope tag sequence fused to the expressed PGC1α protein. Results in Fig. 7C show that immunoprecipitates of PGC1α-DNA in transfected hepatoma cells (bound fractions, BN) were enriched in both *CYP2C9* promoter DNA and *CYP1A1/2* intergenic DNA regions. Comparative analysis of the ChIP results in the three *CYP1A1/2* regions demonstrated that region 2 had the strongest PGC1α binding signal. On the contrary, region 3 showed no significant signal over background levels. By combining information from the HNF4α and PGC1α ChIP assays, we conclude that region 2, at −8 kb from *CYP1A1* and at −15 kb from *CYP1A2*, is the most relevant for the coordinated activation by HNF4α and PGC1α.

Our results demonstrate binding of PGC1α to chromatin DNA of drug-metabolizing P450 genes for the first time and suggest that a direct interaction between PGC1α and HNF4α is required for the efficient transactivation of these P450 genes in hepatic cells.

Insulin Represses PGC1α and P450 Expression in HepG2 Hepatoma Cells and in Cultured Human Hepatocytes. PGC1α is modulated during the feeding-fasting cycle. Likewise, variations in human diet energy produce marked effects on drug metabolism and P450 expression. It can therefore be suggested that modulation of PGC1α could influence P450 levels during the feeding-fasting cycle. To investigate this possibility, we treated hepatoma HepG2 cells with insulin and measured the levels of both PGC1α and its target P450 genes. Cells were cultured in serum- and hormone-free medium and treated with insulin for 12 h. We found that insulin caused a 50% decrease in PGC1α mRNA and a concomitant 30 to 50% decrease in CYP1A1, 1A2, and 2C9 (Fig. 8A). Transfection of HepG2 cells with Ad-PGC1α (16 m.o.i.) increased basal P450 levels and prevented repres-

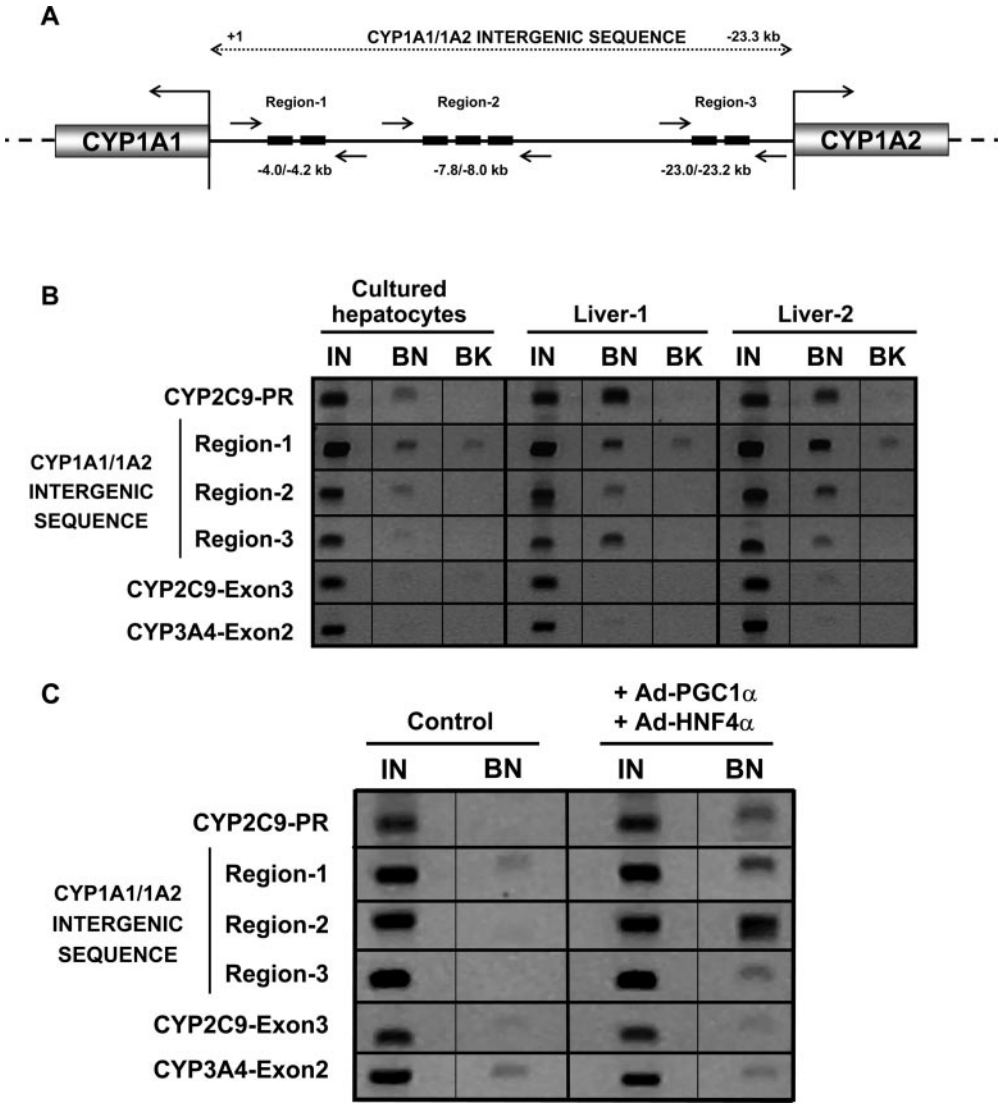

Fig. 7. HNF4α and PGC1α bind to P450 DNA regions containing HNF4α elements. A, schematic chart of the *CYP1A1/2* intergenic sequence showing the predicted HNF4α binding regions 1, 2, and 3 and the flanking primers designed for ChIP assays. B, formaldehyde cross-linked chromatin from human liver samples was incubated with anti-HNF4α antibody, and immunoprecipitated DNA (antibody-bound DNA fraction, BN) was analyzed by PCR with primers specific to regulatory regions containing HNF4α binding elements in the *CYP2C9* promoter (CYP2C9-PR) and *CYP1A1/2* intergenic sequence. As a negative control, PCR amplifications with primers flanking the *CYP2C9* exon 3 (CYP2C9-Ex-3) and *CYP3A4* exon 2 (CYP3A4-Ex-2) were performed. Parallel PCR reactions with input DNA (input DNA fraction, IN) and with mock immunoprecipitated DNA (background DNA fraction, BK) were also carried out. C, Mz-Hep-1 hepatoma cells were transfected with Ad-PGC1α (32 m.o.i.) in combination with Ad-HNF4α (32 m.o.i.) for 48 h. Formaldehyde cross-linked chromatin was incubated with anti-FLAG antibody, and ChIP assay was performed. Control Mz-Hep-1 cells were processed in parallel to determine the level of unspecific bound DNA (background signal).

sion by insulin (data not shown). Similar experiments in cultured human hepatocytes confirmed that insulin causes a parallel down-regulation of PGC1α and P450s (Fig. 8B). Altogether, our results suggest that PGC1α could be involved, at least partially, in the modulation of P450 expression in response to diet energy changes or diabetes.

Discussion

The high expression level of many P450 genes in the liver is sustained by the concerted action of both tissue-specific and ubiquitous transcription factors. However, gene control is also directed by coactivators, acting as the primary targets of differentiation or physiological signals. Coactivators allow the functional integration of multiple transcription factors and facilitate specialized biological programs (Spiegelman and Heinrich, 2004). Coactivators interact with transcription factors to recruit histone acetyltransferases and methyltransferases to specific enhancer/promoter regions facilitating chromatin remodelling, assembly of general transcription factors on promoter regions, and transcription of target genes.

For the first time, the present work demonstrates a key role of coactivators in the constitutive expression of several human drug-metabolizing P450 genes. Previous studies have demonstrated a relevant role of coactivators in the induction of P450 genes by ligand-activated nuclear receptors. Induction of CYP3A4 by rifampicin requires the interaction of PXR with SRC1 and PGC1α (Li and Chiang, 2006). Rifampicin also causes an exchange of the corepressor silencing mediator for retinoid and thyroid hormone receptor with the p160 coactivator SRC3 in PXR (Johnson et al., 2006). Likewise, the induction of CYP3A4 by 1,25-dihydroxyvitamin D_3 requires vitamin D receptor-retinoid X receptor heterodimers and recruitment of SRC1 (Jurutka et al., 2005). On the other hand, induction of CYP2B1 by CAR involves SRC1 (Muangmoonchai et al., 2001), whereas the p160 coactivator SRC2 mediates CAR nuclear accumulation in untreated animals (Xia and Kemper, 2005). Finally, the three p160 coactivators (particularly SRC1) are bona fide coactivators for the AHR/aryl hydrocarbon receptor nuclear translocator transcription factors and enhance XRE-driven *CYP1A1* transcription (Beischlag et al., 2002). Our results have demonstrated that SRC1 and particularly PGC1α are also required for the basal, non-induced transcription of several P450 genes in hepatic cells, in which this effect is mediated by the liver-enriched transcription factor HNF4α.

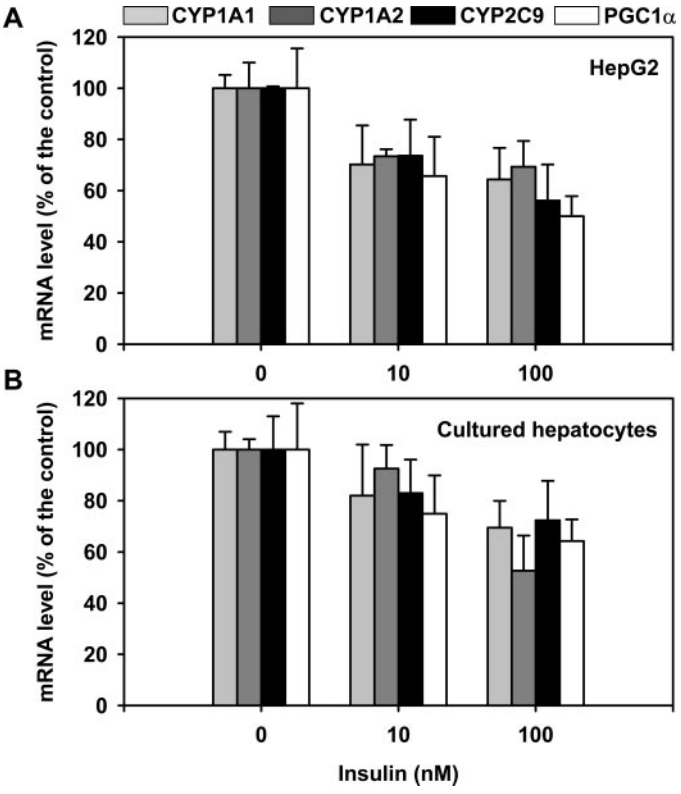

Fig. 8. Insulin represses PGC1α and causes the down-regulation of CYP1A1, 1A2, and 2C9 in human hepatic cells. HepG2 cells (A) and cultured human hepatocytes (B) were cultured in serum- and hormone-free medium and treated with insulin for 12 h, as indicated. Total RNA was purified, and the mRNA levels of PGC1α and P450s were determined by real-time quantitative RT-PCR analysis. In parallel, we also analyzed the mRNA concentration of the housekeeping PBGD for normalization. Data were expressed as a percentage of the control and represent the mean ± S.D. from two to three independent experiments.

CYP2C9 was the only P450 to show a significant response to both PGC1α and SRC1. CYP2C9 is preferentially expressed in the liver and seems to be regulated by various hepatic transcription factors such as HNF4α, HNF3γ, and C/EBPα (Jover et al., 1998, 2001; Bort et al., 2004; Chen et al., 2005). Two HNF4 binding sites have been identified and functionally characterized in the *CYP2C9* basal promoter region (Chen et al., 2005; Kawashima et al., 2006). These proximal HNF4 binding sites are required for the optimal induction of CYP2C9 by both CAR and PXR, through cross-talk between both distal CAR/PXR sites and proximal HNF4α elements in the *CYP2C9* promoter. We have demonstrated that these HNF4α response elements are also critical for the transcriptional activation of *CYP2C9* by coactivators and for sustaining a high expression level of CYP2C9 in hepatic cells.

CYP1A1 and CYP1A2 also responded significantly to PGC1α in hepatoma cells, in which this response was mediated by HNF4α. *CYP1A1* gene regulation has been extensively studied. The proximal 5′-flanking region (1300 bp) in the human *CYP1A1* gene contains at least seven XREs, which are needed for transcriptional induction by the ligand-activated AHR receptor. In contrast, the regulation of the *CYP1A2* gene is not as clearly understood. Whereas the induced expression is also mediated by the AHR, only one XRE has been identified in the *CYP1A2* proximal 5′-flanking region (at −2903 bp) (Corchero et al., 2001). Even less is known about what factors regulate the constitutive expression of CYP1A2 in hepatocytes. The expression of CYP1A2 is mainly hepatic-specific. Several transcription factors have been implicated as regulators of the constitutive and tissue-specific expression of CYP1A2, including members of the nuclear factor 1 family of transcription factors (Narvaez et al., 2005) and the liver-enriched transcription factor HNF1 (Chung and Bresnick, 1997). Moreover, DNA binding sequences of several putative regulatory factors have also been characterized, including activator protein-1 element (Quattrochi et al., 1998) and multiple E-box motifs (Narvaez et al., 2005). However, the organization of the *CYP1A* cluster on chromosome 15, in which a common 23.3-kb sequence separates *CYP1A1* and *CYP1A2*, suggests the possibility that distal regulatory elements may be shared (Corchero et al., 2001). Indeed, it has

been shown that this is the case for the induction of human CYP1A1 and CYP1A2, which are simultaneously controlled through bidirectional and common regulatory XREs (Ueda et al., 2006). Thus, it can be suggested that controlling the constitutive expression of *CYP1A* genes may also require interactions of promoter sequences with distant shared enhancer elements (Corchero et al., 2001). This study has demonstrated the existence of such shared distal sequences for the coordinated response to PGC1α via HNF4α. The re-expression of PGC1α in HepG2 cells activated *CYP1A1/2* transcription to levels closer to those found in human hepatocytes. Moreover, cotransfection experiments with adenoviral vectors demonstrated that PGC1α and HNF4α are involved in the activation of the *CYP1A* cluster in hepatic cells.

Transfection of coactivators caused an important enhancement of P450 expression in human hepatoma cells, although the P450 mRNA levels attained were still lower than those found in human liver. The low expression level of P450s in hepatoma cells is a consequence of multiple phenomena, including the loss of multiple tissue-specific transcription factors, which act coordinately to enhance gene transcription. Thus, the re-expression of only a regulatory protein may not be enough to restore P450 expression to liver levels. In the transfection experiments with Ad-PGC1α, the mRNA level of coactivator attained ranged from 0.2- to 4-fold the liver level. The dose-response profile of transfected PGC1α was very similar to that of CYP7A1, a well-characterized PGC1α target gene. However, the responses of CYP1A1 and CYP2C9 were not significant at low doses of Ad-PGC1α (1–2 m.o.i.). This lack of correlation between PGC1α mRNA level and target gene mRNA level may have several explanations: 1) some target P450 genes (e.g., 2C9) are expressed marginally in HepG2, and a low increase in limiting coactivators may not be sufficient for a significant activation of P450 transcription in hepatoma cell lines; other factors may be missing; 2) each P450 gene may need a different time for maximal response after transfection because they have different transcription rates and mRNA half-lives; and 3) the PGC1α mRNA expressed from the adenoviral vector is different from the endogenous natural PGC1α mRNA. They have distinct 5′ and 3′ untranslated sequences that play critical roles in translation efficiency as a result secondary structural features, glycine-cysteine content, ribosomal recruitment and positioning, etc. This could lead to a discrepancy between PGC1α mRNA and protein levels in transfected cells.

PGC1α is found in tissues with a high energy demand. In heart, brown adipose tissue, and skeletal muscle, PGC1α expression is induced in response to stimuli such as cold or physical exercise (Knutti and Kralli, 2001). In the human liver, the constitutive expression of PGC1α is relatively low (Esterbauer et al., 1999; Larrouy et al., 1999; Knutti et al., 2000). Under fasting conditions however, glucagon, cAMP, and glucocorticoids induce PGC1α expression, leading to the activation of all key enzymes of gluconeogenesis and the increase of hepatic glucose production (Yoon et al., 2001). The opposite effects are observed with insulin, which suppresses basal PGC1α levels in hepatoma cells (Herzig et al., 2001; Daitoku et al., 2003). We have also shown that PGC1α repression by insulin correlates with the down-regulation of CYP1A1, 1A2, and 2C9 mRNAs; it might therefore be speculated that PGC1α could play a role in the regulation of P450s during the feeding/fasting cycle. Our data are in agreement with previous studies showing the negative effect of insulin on both basal and induced P450 levels (Sidhu and Omiecinski, 1999; Tamaki et al., 2005) and provide a mechanistic explanation for this phenomenon for the first time.

CYP2C9 is the major member of the CYP2C subfamily in the human liver and metabolizes more than 16% of clinically used drugs. CYP1A1 and CYP1A2 mediate the rate-limiting step in the metabolism of many drugs and in the oxygenation of polycyclic aromatic hydrocarbons and the bioactivation of procarcinogens. Individual variability in the metabolism of CYP2C9, 1A1, and 1A2 substrates in humans may result in tolerance, therapeutic failure, or increased susceptibility to procarcinogens. Interindividual variability can be caused by structural polymorphisms in the P450 genes, although regulatory polymorphisms or exogenous factors could also be involved. In this sense, any signal or genetic variation leading to changes in expression of coactivators PGC1α and SRC1 could influence the expression of CYP2C9, 1A1, and 1A2 and cause variability in biotransformation activity.

In short, our results establish the importance of the coactivators PGC1α and SRC1 for the HNF4α-mediated expression of human CYP2C9, CYP1A1, and CYP1A2 in hepatic cells. Moreover, our data provide new insights into the regulatory mechanisms of the *CYP1A1/2* cluster and the negative effect of insulin on the expression of human P450s.

Acknowledgments

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