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Transcriptional Regulation of Human CYP3A4 Basal Expression by CCAAT Enhancer-Binding Protein α and Hepatocyte Nuclear Factor-3γ

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ABSTRACT

Cytochrome P450 3A4 (CYP3A4) is involved in the metabolism of more than 50% of currently used therapeutic drugs, yet the mechanisms that control *CYP3A4* basal expression in liver are poorly understood. Several putative binding sites for CCAAT/enhancer-binding protein (C/EBP) and hepatic nuclear factor 3 (HNF-3) were found by computer analysis in *CYP3A4* promoter. The use of reporter gene assays, electrophoretic mobility shift assays, and site-directed mutagenesis revealed that one proximal and two distal C/EBPα binding sites are essential sites for the *trans*-activation of *CYP3A4* promoter. No *trans*-activation was found in similar reporter gene experiments with a HNF-3γ expression vector. The relevance of these findings was further explored in the more complex DNA/chromatin structure within endogenous *CYP3A4* gene. Using appropriate adenoviral expression vectors, we found that both hepatic and nonhepatic cells overexpressing C/EBPα had increased *CYP3A4* mRNA levels, but no effect was observed when HNF-3γ was overexpressed. In contrast, overexpression of HNF-3γ simultaneously with C/EBPα resulted in a greater activation of the *CYP3A4* gene. This cooperative effect was hepatic-specific and also occurred in *CYP3A5* and *CYP3A7* genes. To investigate the mechanism for HNF-3γ action, we studied its binding to *CYP3A4* promoter and the effect of the deacetylase inhibitor trichostatin A. HNF-3γ was able to bind *CYP3A4* promoter at a distal position, near the most distal C/EBPα binding site. Trichostatin A increased C/EBPα effect but abolished HNF-3γ cooperative action. These findings revealed that C/EBPα and HNF-3γ cooperatively regulate *CYP3A4* expression in hepatic cells by a mechanism that probably involves chromatin remodeling.

The cytochromes P450 (P450) are a superfamily of heme-containing enzymes that catalyze the metabolism of a wide range of endogenous substrates as well as the detoxification/metabolic activation of exogenous compounds (Guengerich, 1993). Human CYP3A4 is the primary catalyst of testosterone 6β-hydroxylation (Waxman et al., 1991) and is involved in the metabolism of more than 50% of currently used therapeutic drugs (Li, 1995). The major role of CYP3A4 in xenobiotic metabolization and the large intra- and interindividual variability to which it is subjected (Forrester et al., 1992) strongly contribute to the important differences in the therapeutic and toxic effects of many drugs.

As with most xenobiotic-metabolizing P450s, CYP3A4 is highly expressed in liver, where its is one of the most abundant enzymes (Yamashita et al., 2000), but low levels are also found in extrahepatic tissues. Detailed studies of typical hepatic genes have shown that liver-specific gene expression is accomplished by the concerted action of a small number of liver-enriched transcription factors (LETFs) (Cereghini, 1996). Although the mechanisms that control *CYP3A4* high and variable basal expression in human hepatocytes are still unknown, it has been shown that the LETFs hepatocyte nuclear factor-1 (HNF-1), HNF-3, HNF-4, and CCAAT/enhancer-binding protein (C/EBP) play important roles in regulating the expression of P450 genes (Gonzalez and Lee, 1996) and that in most cases, two or more LETFs are responsible for the expression of a hepatic gene.

C/EBPα is a member of the basic region leucine zipper family of transcription factors (Antonson and Xanthopoulos, 1995) and its expression controls, among others, the terminal

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ABBREVIATIONS: P450, cytochrome P450; LETF, liver-enriched transcription factor; HNF, hepatocyte nuclear factor; C/EBP, CCAAT enhancer-binding protein; MOI, multiplicity of infection; EMSA, electrophoretic mobility shift assay; Ad-C/EBPα, recombinant adenovirus encoding C/EBPα; Ad-HNF-3γ, recombinant adenovirus encoding HNF-3γ; Ad-pAC, recombinant adenovirus encoding pAC/CMVpLpA; TSA, trichostatin A; PCR, polymerase chain reaction; bp, base pair(s); CMV, cytomegalovirus; RT, reverse transcription.

differentiation of adipocytes and hepatocytes (Shugart and Umek, 1997). In the liver, C/EBPα plays a major role in the maintenance of energy homeostasis by regulation of glycogen synthase, phosphoenolpyruvate carboxykinase, and glucose-6-phosphatase (Wang et al., 1995), as well as in the inflammatory response (Burgess-Beusse and Darlington, 1998). A direct demonstration of C/EBPα implication in P450 expression was first obtained in Hep G2 cells, which showed augmented levels of CYP2B6, -2C9, and -2D6 mRNAs, when they were stably transfected with a C/EBPα expression vector (Jover et al., 1998). Although the expression of *CYP3A4* in these cells was not investigated in detail, previous preliminary evidence indicating that C/EBPα *trans*-activates *CYP3A4* promoter was gained in gene reporter assays (Ourlin et al., 1997).

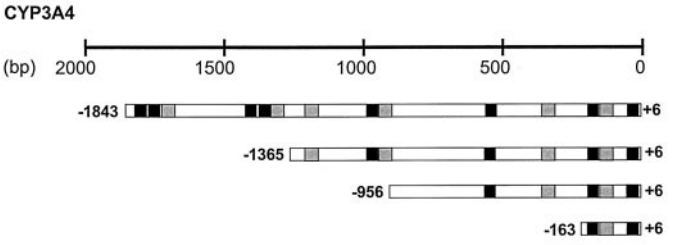
HNF-3 belongs to a large family of transcription factors that is characterized by the presence of a winged helix/forkhead domain. This domain is similar to the globular domain of linker histone (Clark et al., 1993) and enables HNF-3 to directly control nucleosome position (Shim et al., 1998). The HNF-3 proteins are involved in the regulation of numerous liver-specific genes (Kaestner et al., 1998; Wang et al., 2000). They regulate the expression of human *CYP2Cs* (R. Bort, R. Jover, C. Rodríguez-Antona, M. J. Gómez-Lechón, and J. V. Castell, manuscript in preparation), and recombinant promoter analysis has demonstrated that HNF-3 *trans*-activates rat *CYP2C6* and *CYP2C12* (Shaw et al., 1994; Delesque-Touchard et al., 2000). In addition, footprint analysis revealed HNF-3 binding sites in the rat *CYP2C13* promoter (Legraverend et al., 1994). From the three HNF-3 isoforms expressed in liver, α, β, and γ, we focused our studies on HNF-3γ based on its temporal expression during embryogenesis (Kaestner et al., 1994) and on knock-out mice data: inactivation of HNF-3γ resulted on an altered expression of liver specific genes in contrast to the HNF-3α and HNF-3β knock-out mice (Kaestner et al., 1998, 1999; Sund et al., 2001).

In the present study, we establish the role of C/EBPα and HNF-3γ in the basal expression of human *CYP3A4* by assaying the *trans*-activating ability of C/EBPα and HNF-3γ on *CYP3A4* promoter deletions and identifying the precise location of the binding sites by EMSA analysis. By using adenoviral expression vectors encoding both LETFs, we found that C/EBPα up-regulated *CYP3A4*, whereas HNF-3γ had a synergistic effect. This cooperative effect, which was also detected in the *CYP3A5* and *CYP3A7* genes, was hepatic specific and probably occurs via chromatin remodeling.

Materials and Methods

Construction of Plasmids. Putative binding sites for the transcription factors C/EBPα and HNF-3γ were identified within the -1843, $+6$ region of the human *CYP3A4* promoter using computer programs (positions are relative to the transcription start site, $+1$). The MatInspector software (Wingender et al., 2000) was used to identify HNF-3 putative binding sites using search conditions of 100% similarity in core and 82.5% in matrix. Because C/EBPα can bind as an α-α homodimer or an α-β heterodimer, C/EBPα putative binding sites were selected using TFSearch software (Heinemeyer et al., 1998) with search conditions of 80% similarity for C/EBPα sites and 82.5% similarity for C/EBPβ sites. Six C/EBP and eight HNF-3 putative binding sites were identified in this search (Fig. 1). Based on this data and using human genomic DNA isolated from human liver, we generated by PCR different deletion fragments of the *CYP3A4* promoter containing different putative binding sites. The amplified fragments were: -1843, -1365, -956, -163, and -104 to $+6$ (the PCR primers used had restriction enzymes sites for *Kpn*I or *Xho*I at the 5′ end and are described in Table 1). After the PCR reaction, the fragments were double-digested with *Kpn*I and *Xho*I and ligated to the pGL3-Basic vector (Promega) that had previously been digested with the same enzymes. Plasmids isolated from transformed bacterial colonies were sequenced to confirm the inserted sequence. The complete cDNA of rat C/EBPα (a kind gift of Dr. J. Patrick Condreay) was cloned by sticky-blunt ligation of a *Xba*I-*Kpn*I fragment into the pAC/CMVpLpA vector (Gómez-Foix et al., 1992) predigested with *Xba*I-*Hin*dIII, generating an expression vector for C/EBPα (pAC-C/EBPα). The expression plasmid for HNF-3γ (pAC-HNF-3γ) was constructed by PCR amplification of the complete human HNF-3γ cDNA and ligation into the pAC/CMVpLpA (R. Bort, R. Jover, C. Rodríguez-Antona, M. J. Gómez-Lechón, and J. V. Castell, manuscript in preparation).

PCR Mutagenesis of the C/EBP DNA-Binding Site at -121/-130 in CYP3A4 Promoter. The CTTTGCCAAC wild-type C/EBP DNA binding site at -121/-130 in the *CYP3A4* promoter was mutated to CTAGAGAGAC. Two separate PCR reactions were set up to amplify 56- and 152-bp fragments with mutations within the C/EBP binding site using -163/$+6$ pGL3-Basic plasmid as a template. The C/EBP binding site in the 56- and 152-bp fragments is within 25 overlapping nucleotides that can subsequently be annealed together to serve as templates for further amplification of a full-length 183-bp fragment containing selective point mutations in the C/EBP binding site. The 56- and 152-bp fragments were amplified in independent reactions containing 1 ng of -163/$+6$ pGL3-Basic, 0.2 μM of sense and antisense oligonucleotide primers, 200 μM of each nucleotide, Expand High Fidelity buffer with 1.5 mM $MgCl_2$ (Roche Applied Science, Indianapolis, IN), and 2 units of Expand high-fidelity *Taq* polymerase (Roche Applied Science) in a total volume of 50 μl. DNA was amplified for 30 cycles (denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s). The following specific primers were used for the 56-bp PCR fragment: -163-FP and C/EBPmut-RP and the 152-bp PCR fragment: C/EBPmut-FP and $+6$-RP (primer sequences are shown in Table 1). The DNA fragments of expected mobility were excised from 2% agarose gels and purified with the UltraClean DNA purification kit (Mo Bio Laboratories, Inc.,



CONSENSUS SITE	POSITION	CONSENSUS SITE	POSITION
C/EBP	-121/ -130	HNF-3	-22/ -30
C/EBP	-315/ -324	HNF-3	-184/ -192
C/EBP	-964/ -973	HNF-3	-567/ -575
C/EBP	-1202/ -1211	HNF-3	-989/ -997
C/EBP	-1393/ -1402	HNF-3	-1432/ -1440
C/EBP	-1659/ -1668	HNF-3	-1446/ -1454
		HNF-3	-1718/ -1726
		HNF-3	-1722/ -1730

Fig. 1. *CYP3A4* promoter constructs and putative binding sites for C/EBPα and HNF-3γ. Schematic nucleotide sequences of the *CYP3A4* promoter constructs cloned in pGL3-Basic, showing putative binding sites for C/EBP (▨) and HNF-3 (■). The positions are relative to the transcriptional start site + 1 and the location of the putative binding sites for C/EBP and HNF-3 in the *CYP3A4* promoter are shown in the table.

Carlsbad, CA). To generate a full-length −163/+6 promoter fragment with mutations within the C/EBP binding site, 3 ng of each of the purified 56- and 152-bp DNA fragments were annealed in a reaction mixture containing 200 μM of each nucleotide and Expand high-fidelity buffer with 1.5 mM $MgCl_2$ at 94°C for 2 min and 55°C for 5 min. Two units of Expand high-fidelity *Taq* polymerase (Roche Applied Science) were added to the reaction mixture in a total volume of 50 μl. DNA was amplified for 10 cycles (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min). Sense −163-FP and antisense +6-RP oligonucleotide primers (0.2 μM) were subsequently added to the reaction mixture, and DNA was amplified for an additional 30 cycles (1 cycle = 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) with a final extension at 72°C for 6 min. The PCR products were precipitated, washed with 70% ethanol, and digested with *Kpn*I and *Xho*I. The digestion product was electrophoretically fractioned in a 1.5% agarose gel, purified as described above, and cloned into the pGL3-Basic vector. The mutation was confirmed by DNA sequencing.

Cell Culture and Transfection Assays. Hep G2 cells were plated in Ham's F-12/Leibovitz L-15 media [1:1 (v/v)], supplemented with 7% newborn calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, and cultured to 70% confluence. HeLa and human embryonic kidney 293 cells were maintained as monolayer cultures and grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin; 293 cell medium was supplemented with 3.5 g/liter of glucose.

Plasmid DNAs were purified with QIAGEN Maxiprep kit columns (QIAGEN, Valencia, CA) and quantified by absorbance at 260 nm and fluorescence using PicoGreen (Molecular Probes, Eugene, OR). The day before transfection, cells were plated in 35-mm diameter dishes with 1.5 ml of medium. Two hours before transfection, medium was changed to Dulbecco's modified Eagle's medium/Nut F12 (Invitrogen, Carlsbad, CA) supplemented with 10% newborn calf serum, 50 U/ml penicillin, and 50 mg/ml streptomycin. Firefly luciferase pGL3-Basic constructs (0.5 to 1 μg) were transfected with or without pAC-C/EBPα and pAC-HNF-3γ (0.5 to 1 μg) by calcium phosphate precipitation. 0.1 μg of pRL-CMV (a plasmid expressing *Renilla reniformis* luciferase under the CMV immediate early enhancer/promoter) was cotransfected to correct for variation in transfection efficiency. Calcium phosphate/DNA coprecipitates were added directly to each culture and incubated for 6 (Hep G2) or 20 h (HeLa). Then, the medium was replaced; 48 h after transfection, firefly and *R. reniformis* luciferase activities were determined using the dual-luciferase reporter assay system (Promega, Madison, WI). In all experiments, luciferase activity was normalized to transfection efficiency (*R. reniformis* luciferase activity by pRL-CMV) and protein content.

When infecting cells with adenoviral vectors, cells were incubated for 90 min with the recombinant adenovirus at 0.75 to 15 multiplicity of infection (MOI). Thereafter, cells were washed with phosphate-buffered saline, medium was replaced, and 48 h after infection, cells were harvested and frozen in liquid N_2.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay. Nuclear extracts from Hep G2 cells infected with C/EBPα or HNF-3γ adenovirus were prepared as described previously (Schreiber et al., 1989). Briefly, cells were scraped, washed with ice-cold phosphate-buffered saline, homogenized, and centrifuged to pellet nuclei. The nuclei were incubated with a high-salt buffer at 4°C for 15 min. After centrifugation, the supernatant was stored at −70°C. For EMSA, 12 μg of nuclear extract were preincubated at 37°C for 20 min with 1.5 μg of poly(dI/dC), 100 mM of NaCl, 15 mM HEPES, pH 7.9, 0.25 mM EDTA, 0.25 mM EGTA, 0.25 mM dithiothreitol, and 5% glycerol. The double-stranded oligonucleotide was radiolabeled (50,000 to 100,000 cpm) using [^{32}P]dATP and T4 polynucleotide kinase (Roche Applied Science), added to the reaction mixture, and incubated for 40 min at 37°C. The binding of proteins to the oligonucleotides was determined by fractionating the reaction mixture by electrophoresis through a nondenaturing 4% polyacrylamide gel at 150 V for 3 to 4 h at 4°C, using a Tris-glycine-EDTA buffer (50 mM Tris, 375 mM glycine, 2 mM EDTA, pH 8.5). Where appropriate, a 50-fold excess (unless another amount is indicated) of competitor DNA was included in the preincubation, before the addition of the ^{32}P labeled DNA. For antibody supershifts, 1 μg of C/EBPα and HNF-3γ or 4 μg of C/EBPβ antiserums (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added after the incubation with the labeled probe and incubated for 45 min on ice. Gels were dried and exposed at −70°C to an X-ray film with intensifying screens.

Development of Adenoviral Vectors Encoding C/EBPα and HNF-3γ. Recombinant adenovirus encoding C/EBPα and HNF-3γ were prepared as described elsewhere (Gómez-Foix et al., 1992). Briefly, pAC-C/EBPα was cotransfected with pJM17 into 293 cells (AdE1A-transformed human embryonic kidney cells) by calcium phosphate/DNA precipitation. The CMV-driven cassette of pAC/CMVpLpA is located between the sequences representing 0 to 1.3 map units and 9.2 to 16 map units of the adenovirus type 5, whereas pJM17 encodes a full-length adenovirus-5 genome (dl309) interrupted by the insertion of the bacterial plasmid pBRX at position 3.7 map units, thereby exceeding the packaging limit. Homologous recombination between adenovirus sequences in the transfer plasmid (recombinant pAC/CMVpLpA) and in the pJM17 plasmid results in the substitution of the pBRX sequences in pJM17 by the chimeric gene. This generates a genome of packageable size in which most of the adenovirus early region 1 is lacking, thus rendering the replication defective recombinant virus. The resulting virus named Ad-C/EBPα was plaque-purified, expanded into a high-concentration stock, and titrated by plaque assay as described previously (Castell et al., 1997). The preparation of Ad-HNF-3γ was performed in a similar way (R. Bort, R. Jover, C. Rodríguez-Antona, M. J. Gómez-Lechón, and J. V. Castell, manuscript in preparation). To confirm that the C/EBPα protein expressed with the adenoviral vector was functional, we measured albumin synthesis and mRNA contents of C-reactive protein in Hep G2 cells infected with the C/EBPα virus, finding that both were increased. A recombinant adenovirus encoding the insertless vector pAC/CMVpLpA (Ad-pAC) was used as control.

RNA Purification and Semiquantitative RT-PCR Analysis. Cellular RNA was extracted with RNeasy Total RNA Kit (QIAGEN),

TABLE 1

Oligonucleotide PCR primers for cloning *CYP3A4* promoter fragments and for PCR mutagenesis of the C/EBP DNA-binding site at −121/−130 in *CYP3A4* promoter

CYP3A4	Primer Sequence	Restriction Enzyme
+6-RP	5′-cct ctc gag cag tga ttc agt gag gct gtt gga ttg ttt-3′	*Xho*I
−104-FP	5′-gga ggt acc tct tcc aac tgc agg cag agc aca-3′	*Kpn*I
−163-FP	5′-atg gta cca aag gag gtc agt gag tgg tgt gtg tgt ga-3′	*Kpn*I
−956-FP	5′-agg gta cct cta act tgc tat cta tgg cag gac ct-3′	*Kpn*I
−1365-FP	5′-gag gta cca ctt ggg tgt gag tga cga taa tga gc-3′	*Kpn*I
−1843-FP	5′-ccg gta cct gtc ctt ttt ggt ttg atg ctt gct gt-3′	*Kpn*I
C/EBPmut-FP	5′-tgt gtg att cta gag aga ctt cca agg tgg aga agc-3′	
C/EBPmut-RP	5′-ttg gaa gtc tct cta gaa tca cac a-3′	

FR, forward primer; RP, reverse primer.

contaminating genomic DNA was removed by incubation with DNase I Amplification Grade (Invitrogen), and 1 μg of total RNA was reverse transcribed. cDNA fragments of C/EBPα and HNF-3γ were amplified by PCR using 3 μl of diluted cDNA in 40 μl of 20 mM Tris-HCl, pH 8.4, containing 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM concentrations of each deoxynucleotide triphosphate, 1 unit of *Taq* DNA polymerase (Invitrogen), 0.2 μM concentrations of each specific primer (Table 2), and, in the case of amplifying C/EBPα cDNA, 2.8 μl of glycerol. After denaturing for 4 min at 94°C, amplification was performed by 30 to 35 cycles (94°C for 35 s; 60°C or 57°C for C/EBPα or HNF-3γ, respectively, for 30 s; and 72°C for 45 s) and a final extension of 72°C for 7 min. mRNA levels of CYP3A4, CYP3A5, CYP3A7, and β-actin were quantified by RT-PCR with the LightCycler Instrument (Roche Applied Science) using the LightCycler-DNA Master SYBR Green I (Roche Applied Science). Aliquots (15 μl) of the PCR reactions were subjected to electrophoresis on 1.5% agarose gel, for size and purity confirmation. Sample-to-sample variations were normalized by analysis of β-actin content in the same cDNA series. Primers used for PCR amplification are shown in Table 2.

Immunoblot Analysis. Protein extracts were electrophoresed in a SDS-polyacrylamide gel (20 μg protein/lane). Proteins were transferred to Immobilon-P membranes (Millipore) and incubated with appropriate polyclonal antibodies (Santa Cruz Biotechnology). After washing, blots were developed with horseradish peroxidase-labeled IgG using an enhanced chemiluminescence kit (Amersham Biosciences).

Statistical Analysis. Statistical analysis was done by Student's *t* test. A *P* value less than 0.05 was considered significant.

Results

C/EBPα but Not HNF-3γ *trans*-Activates *CYP3A4* Promoter Constructs. Computer analysis of *CYP3A4* promoter revealed the existence of several putative binding sites for C/EBP and HNF-3 (Fig. 1). Their biological relevance was examined by reporter gene assays using progressive 5′ deletions of the *CYP3A4* promoter fused upstream of the firefly luciferase gene in the pGL3-Basic plasmid. The transfection experiments were carried out in a human cervix carcinoma cell line (HeLa) and in a human hepatic cell line (Hep G2), to determine possible differences in *trans*-activation depending on cell/tissue specific factors.

The reporter expression of the deletion constructs was similar in both cell lines tested. The basal luciferase activity of promoter constructs increased with the deletion of upstream sequences from −1843 to −956, as shown in Fig. 2A, suggesting the existence of negative regulatory elements in this region. With a further deletion to −163, the activity decreased, but it was still higher than that of the promoterless pGL3-Basic, indicating that within −956 to +6, where two C/EBP and three HNF-3 putative binding sites were located (Fig. 1), there might be positive regulatory elements. The similar behavior of the two cell lines examined indicates that the transcription factors interacting with these positive elements are present at an operating level in both cell lines. However, the average response was higher in the hepatic cell line, as expected for a hepatic-specific gene like *CYP3A4*.

The effect of the liver-specific transcription factors C/EBPα and HNF-3γ on the human *CYP3A4* promoter was investigated by cotransfection of expression plasmids for C/EBPα (pAC-C/EBPα) or HNF-3γ (pAC-HNF-3γ) with the *CYP3A4* promoter constructs (Fig. 2B). C/EBPα was able to *trans*-activate the different *CYP3A4* constructs, the maximal *trans*-activatory effect corresponded to the −163 fragment (7.4- and 5.3-fold induction for HeLa and Hep G2 cell lines, respectively) giving relevance to a C/EBP responsive element located in the −163 to +6 fragment (Fig. 1). In Hep G2 but not in HeLa cells, the luciferase activity of the −1843 construct was higher than that of the −1365 construct, suggesting that within −1843 and −1365, there are C/EBP binding sites that are active in hepatic cells. The effect of HNF-3γ on *CYP3A4* promoter was studied in the same cell lines using transfection conditions identical to those used for C/EBPα. Despite the presence of multiple HNF-3 putative binding sites in the *CYP3A4* promoter, no increase in luciferase activity was found (Fig. 2B). To investigate whether HNF-3γ could enhance the *trans*-activation exerted by C/EBPα, we cotransfected both transcription factors. Again, no HNF-3γ effect was found, and C/EBPα *trans*-activatory effect was not modified.

Functional C/EBP Binding Sites Are Present in the Proximal *CYP3A4* Promoter at −121/−130 and in the Distal CYP3A4 Promoter at −1393/−1402 and −1659/−1668. In the −163/+6 region, where the maximal C/EBPα *trans*-activation was detected, sequence analysis located at positions −121/−130 the motif CTTTGCCAAC, which shows the features of a consensus C/EBPα binding site (Osada et al., 1996). To investigate whether C/EBPα could bind to this site, we performed EMSA analysis with nuclear extracts from Hep G2 cells overexpressing C/EBPα.

Using a labeled probe matching the −163/+6 region of the *CYP3A4* promoter (P1), different complexes were detected (Fig. 3A). Complexes 1 and 2 were specific, because their formation was prevented by addition of unlabeled probe but not by a 25-mer with an unrelated sequence (U). C/EBPα was identified as the protein contained in complexes 1 and 2 because competition with the *CYP3A4* promoter sequence between −115 and −139 (P2), which contains the −121/−130 C/EBPα putative binding site, prevented the formation of these complexes. Competition with a probe identical to P2 but with the putative C/EBP binding site mutated (P2m) did not prevent the formation of these complexes. Finally, preincubation with an antibody directed against the C/EBPα iso-

TABLE 2
Oligonucleotide PCR primers for cDNA amplification

Gene	Forward Primer Sequence (5′ to 3′)	Reverse Primer Sequence (5′ to 3′)	Annealing Temperature	Fragment Size
			°C	bp
CYP3A4	cct tac aca tac aca ccc ttt gga agt	agc tca atg cat gta cag aat ccc cgg tta	55.3	382
CYP3A5	gaa gaa aag tcg cct caa c	aag aag tcc ttg cgt gtc ta	52.8	679
CYP3A7	cac cta tga tac tgt gct aca gt	tca ggc tcc tac ggt ct	52.0	478
β-Actin	cgt acc act ggc atc gtg at	gtg ttg gcg tac agg tct ttg	58.7	452
C/EBPα	ccc gtg ccc agc cct cat	cac ctt ctg ctg cgt ctc cac	62.4	264
HNF-3γ	atg ctg ggc tca gtg aag at	ttg aga atg gct gct acc tg	59.3	615

form retarded the migration of both complexes 1 and 2 (Fig. 3A, lane 7).

These results were confirmed by EMSAs using labeled −115/−139 probe (P2). In this case, nonspecific complexes were absent, probably because of the shortage of the probe; again, however, it was shown that C/EBPα binds the −121/−130 site (Fig. 3*A*, right). C/EBP isoforms α and β are both abundant in liver and are known to form heterodimers and recognize the same DNA sequence (Shugart and Umek, 1997). Preincubation with specific C/EBP antibodies revealed that the formed complex largely corresponded to C/EBPα and to a lesser extent to endogenous C/EBPβ, in agreement with the high expression of C/EBPβ in Hep G2 cells (Rodriguez-Antona et al., 2002).

To ascertain whether the observed C/EBPα *trans*-activation of *CYP3A4* proximal promoter constructs occurred through its effective binding to the identified site at −121/−130, we compared the effect elicited by C/EBPα on different *CYP3A4* promoter constructs: −163 to +6, −104 to +6 (lacking the −121/−130 C/EBP binding site), and −163 to +6 with the −121/−130 C/EBP binding site mutated as in P2m, all of them cloned in pGL3-Basic. The abolishment of C/EBPα dependent *trans*-activation when the C/EBP binding site at −121/−130 was either absent or mutated showed that this was a functional site (Fig. 3B).

The −1843/−1365 region of the *CYP3A4* promoter increased C/EBPα *trans*-activation in Hep G2 cells, indicating that it contained functional C/EBPα sites (Fig. 2B). In this region, two putative C/EBP binding sites were identified at positions −1393/−1402 and −1659/−1668 by sequence analysis (Fig. 1). To investigate whether C/EBPα could bind these sites, we performed EMSAs using, as labeled probes, oligonucleotides containing the putative C/EBP binding sites and matching the sequence of CYP3A4 within positions −1384/−1408 and −1652/−1676. In both cases, we could identify complexes that were competed by an excess of unlabeled probe, but not by an excess of an oligonucleotide with an unrelated sequence (Fig. 3C, lanes 3 and 4, respectively). The supershift of these complexes after incubation with a specific C/EBPα antibody identified C/EBPα as the protein forming the complexes (Fig. 3C, lane 5).

Expression of P450s in Cells Transfected with C/EBPα and HNF-3γ Adenoviral Vectors. The results obtained with the reporter assays need further confirmation in a more complex system because in plasmid constructs, the DNA lacks the native chromatin structure, which is an important feature for gene expression (van Holde, 1997). To investigate the regulation of the *CYP3A4* gene with its native structure, we constructed adenoviral vectors encoding C/EBPα (Ad-C/EBPα) and HNF-3γ (Ad-HNF-3γ) as tools to overexpress these transcription factors in cells. In these experiments, we used hepatic Hep G2 cells, which have lost the expression of *CYP3A4* and other hepatic-specific genes (Fig. 4A), and HeLa cells, which are derived from cervix carcinoma cells and have no *CYP3A4* expression (Fig. 4C). In both cases, the cells were infected with Ad-C/EBPα, Ad-HNF-3γ, or Ad-pAC, and 48 h after infection, *CYP3A4* mRNA content was analyzed by RT-PCR. The expression of C/EBPα and HNF-3γ was also measured by RT-PCR (data not shown) and Western blot to examine the efficiency of the infection; in all cases, a dose-proportional expression of the corresponding transcription factor was obtained (Fig. 4*A*).

In the adenoviral infected cells, the individual effects of C/EBPα or HNF-3γ on the native *CYP3A4* gene promoter were in agreement with those found in reporter assays (e.g., 7.5 MOI of Ad-C/EBPα increased by 4-fold the *CYP3A4* mRNA content of Hep G2 cells, whereas Ad-HNF-3γ had no effect) (Fig. 4A). Remarkably, infection of Hep G2 cells with increasing amounts of Ad-HNF-3γ (0.75–4.5 MOI) simultaneously with 7.5 MOI of Ad-C/EBPα revealed a dose-depen-

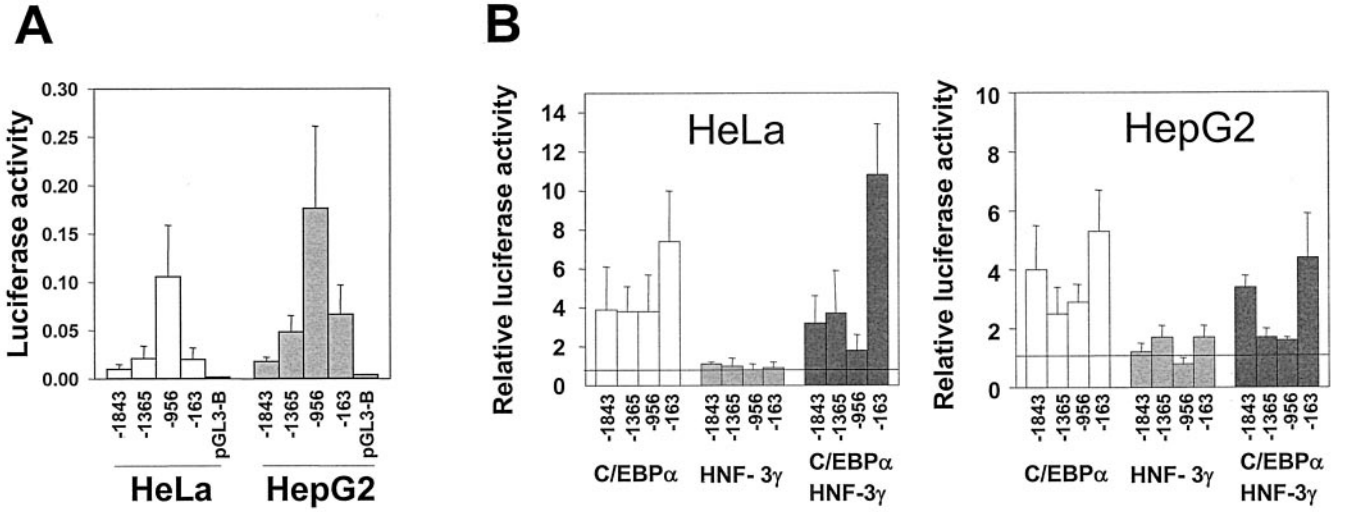


Fig. 2. Basal activity and *trans*-activation by C/EBPa and HNF-3γ of *CYP3A4* promoter constructs in HeLa and Hep G2 cells. Deletions of the 5′ flanking region of *CYP3A4* promoter were cloned in the firefly luciferase reporter plasmid pGL3-Basic. The numbers given indicate the 5′ end of the promoter fragment. Forty-eight hours after transfection, cells were scraped, lysed, and both firefly and *R. reniformis* luciferase activities were measured. A, basal activity of *CYP3A4* promoter deletion constructs. These constructs (1 μg) and 0.1 μg of the plasmid pRL-CMV, as a transfection efficiency control, were transfected with calcium phosphate in HeLa and Hep G2 cells. B, *trans*-activation by C/EBPα and HNF-3γ of *CYP3A4* promoter constructs. The *CYP3A4* promoter constructs (1 μg) were cotransfected with pAC-C/EBPα (1 μg) or/and pAC-HNF3γ (1 μg) expression vectors into HeLa and Hep G2 cells. The insertless plasmid pAC/CMVpLpA was added, to have a constant amount of total expression vector, and 0.1 μg of the plasmid pRL-CMV was used to control transfection efficiency. Values represent firefly/*R. reniformis* luciferase activity ratios divided by protein content. Bars represent the mean ± S.D. of four independent experiments.

dent, cooperative effect that was not found in reporter assays. This cooperative effect was most clearly observed in cells infected with a submaximal concentration of Ad-C/EBPα (7.5 MOI) and 4.5 MOI of Ad-HNF-3γ, where the *CYP3A4* mRNA levels were 10-fold higher than in cells infected with only Ad-C/EBPα.

The expression test applied to *CYP3A4* could be applied to any gene. Therefore, considering that the 5′ flanking region of *CYP3A4* is highly similar to that of *CYP3A5* and *CYP3A7* (60 and 90% identical in the 1 kilobase upstream of the transcriptional start site, respectively) (Hashimoto et al., 1993; Jounaidi et al., 1994), we investigated whether C/EBPα and HNF-3γ also enhanced the expressions of *CYP3A5* and *CYP3A7* mRNAs in the Hep G2-infected cells. Figure 4B shows that C/EBPα and HNF-3γ up-regulated the expression of *CYP3A5* and *CYP3A7* in a similar manner, although to a lower extent, than that of *CYP3A4*.

When C/EBPα was overexpressed in the nonhepatic HeLa cells, the CYP3A4, -3A5, and -3A7 mRNAs increased from undetectable levels to PCR-detectable levels, whereas the expression of the CYP1A1, -1A2, -2B6, -2D6, and -2E1 did not change (Fig. 4C). This demonstrated that the C/EBPα effect was specific for the CYP3A family and that it also occurred in nonhepatic cells. However, when C/EBPα and HNF-3γ were coexpressed in HeLa cells, no difference in CYP3A expressions could be observed compared with cells infected with C/EBPα alone (data not shown), indicating that the cooperativity between C/EBPα and HNF-3γ was hepatic-specific.

HNF-3γ Binds *CYP3A4* Distal Promoter. To determine whether a direct effect of HNF-3γ in *CYP3A4* promoter was responsible for the cooperativity with C/EBPα, EMSAs were performed with seven labeled oligonucleotides containing the eight different HNF-3 putative binding sites predicted by sequence analysis (Fig. 1) (the two more distal HNF-3 sites overlap, and both were contained in one single probe) and nuclear extracts from Hep G2 cells infected with HNF-3γ adenovirus. When a oligonucleotide containing the consensus binding sequence for HNF-3 [T(A/G)TTTNNTT] was used for competition, only the −1710/−1738 probe, which contains the two overlapping HNF-3 sites (TGTTTATTTGTCT), showed competed complexes (data not shown and Fig. 5). In agreement with this, when a HNF-3γ–specific antibody was

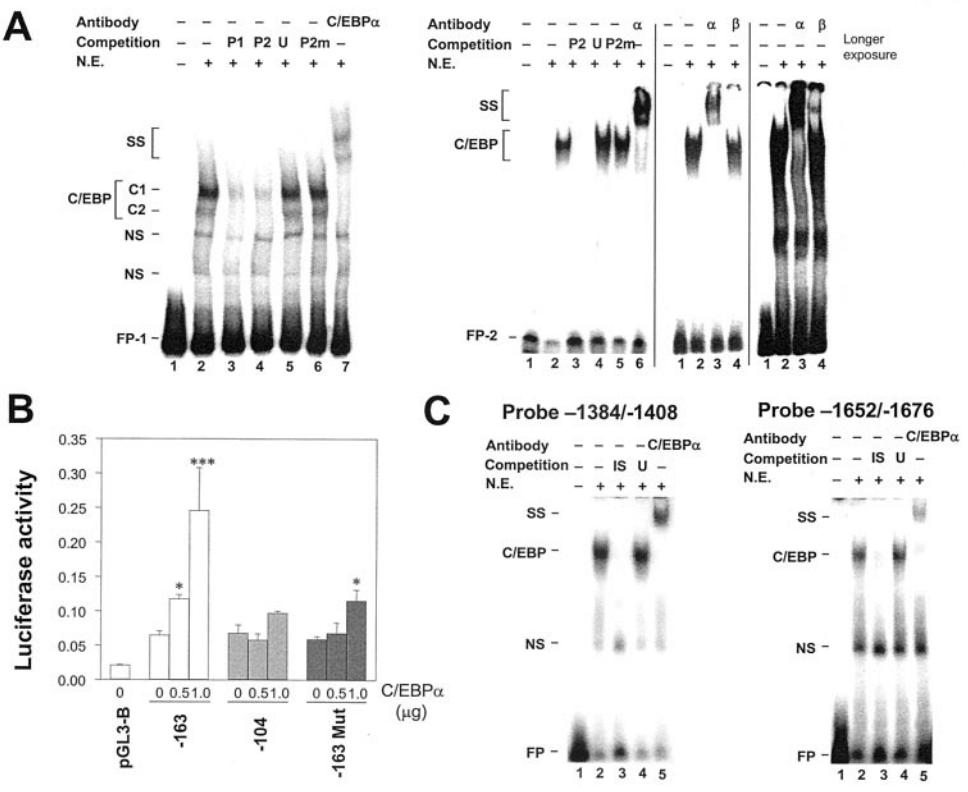


Fig. 3. Binding of C/EBPα to *CYP3A4* promoter and functionality of the sites. For EMSAs, double-stranded DNAs matching *CYP3A4* promoter sequence were labeled and incubated with nuclear extracts of Hep G2 cells overexpressing C/EBPα. Unlabeled oligonucleotides were incubated at 50-fold molar excess for competition. A, binding of C/EBPα to the proximal *CYP3A4* promoter. EMSAs were performed using labeled P1 (−163 to +6) in the left or P2 (−115 to −139) in the right. Specific C/EBPα (α) and C/EBPβ (β) antibodies were used and gels were exposed to X-ray films for different times to see C/EBPβ supershift. B, functionality of the C/EBPα binding site located at −121/−130. Different *CYP3A4* promoter fragments: −163 to +6 (−163); −104 to +6 (−104); and −163 to +6 with −121/−130 C/EBPα binding site mutated (−163 Mut) were cloned in pGL3-Basic. These constructs (0.5 μg) were cotransfected with increasing amounts of pAC-C/EBPα expression plasmid. The insertless plasmid pAC/CMVpLpA was added to have a constant amount of total expression vector and 0.1 μg of the plasmid pRL-CMV was used to control transfection efficiency. Forty-eight hours after transfection, cells were scraped, lysed, and both firefly and *R. reniformis* luciferase activities were measured. Values represent firefly/*R. reniformis* luciferase activity ratios divided by protein content. Bars represent the mean ± S.D. of four independent experiments. Significantly different (*, $p < 0.05$; ***, $p < 0.005$) from cells transfected with the same promoter construct and no pAC-C/EBPα expression plasmid. C, binding of C/EBPα to the distal *CYP3A4* Promoter. Double-stranded oligonucleotides matching the *CYP3A4* promoter sequence between positions −1384/−1408 and −1652/−1676 that contained the putative C/EBP binding sites at −1393/−1402 and −1659/−1668, were used as labeled probes. N.E., nuclear extract; IS, itself; U, oligonucleotide with an unrelated sequence; P2m, −115 to −139 oligonucleotide with the central nucleotides of the putative C/EBPα binding site at −121/−130 mutated; FP, free probe; NS, nonspecific complex; C1 and C2, specific complexes; C/EBP, DNA/C/EBPα complex; SS, supershifted complex.

added to the EMSA binding reaction, supershifted complexes could only be detected with the −1710/−1738 labeled probe (Fig. 5A, lane 14). As shown in Fig. 5B, the specific complex of the −1710/−1738 probe had a relatively high affinity (100-fold excess of unlabeled probe was required for complete competition) (Fig. 5B, lanes 3 and 4). HNF-3γ was identified as the protein present in this complex by competition with a consensus HNF-3 binding sequence and by supershift with a specific HNF-3γ antibody. These data support a direct effect of HNF-3γ in *CYP3A4* promoter.

To further investigate whether the HNF-3γ cooperative effect with C/EBPα was direct or mediated by other transcription factors, we measured the expression of the nuclear receptors HNF-4α, pregnane X receptor, constitutive androstane receptor, and retinoid X receptor-α, which are important for *CYP3A4* expression. No changes in the expression of these factors could be detected in Hep G2 cells overexpressing HNF-3γ (data not shown).

HNF-3γ Cooperative Effect Is Prevented by a Deacetylase Inhibitor. HNF-3 proteins can modify nucleosome positioning, disrupt the local chromatin structure, and in this way facilitate the accession of other transcription factors to their binding sites (Crowe et al., 1999; Cirillo et al., 2002). To investigate whether this mechanism could be responsible for the cooperative effect observed between C/EBPα and HNF-3γ, we treated Hep G2 cells overexpressing C/EBPα and/or HNF-3γ with trichostatin A (TSA), a compound that remodels the chromatin to a transcriptional competent state by inhibiting histone deacetylases (Yoshida et al., 1995). TSA alone had no effect on *CYP3A4* expression (Fig. 6), but it increased by 13-fold the C/EBPα activatory effect (Fig. 6, compare bars 2 and 6) and clearly abolished HNF-3γ cooperative effect (Fig. 6, bars 6 and 8 are not significantly different, whereas bars 2 and 4 are statistically different). These results suggest an important role of chromatin structure in the cooperativity between C/EBPα and HNF-3γ on the expression of *CYP3A4*.

Discussion

The LETFs are *trans*-activating factors that control the expression of hepatic genes acting within a network of cooperative and synergistic effects. C/EBPα and HNF-3γ have been identified as key signals in the regulation of many liver-specific genes, including several P450s (Ourlin et al., 1997; Jover et al., 1998; Delesque-Touchard et al., 2000). However, their role in the regulation of the constitutive expression of *CYP3A4* in hepatocytes, which is much higher than in nonhepatic cells, has not been investigated. Among the different C/EBP consensus binding sequences found by computer analysis in *CYP3A4* promoter (Fig. 1), C/EBPα *trans*-activated a luciferase reporter gene specifically binding the −121/−130 site (Fig. 3, A and B). The similar results obtained in hepatic and nonhepatic cell lines transfected with

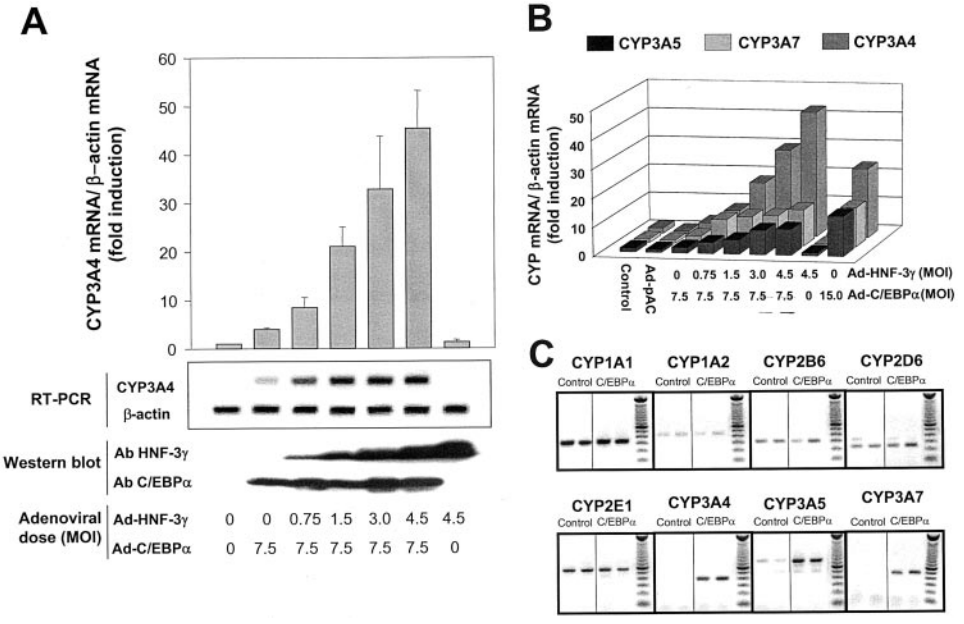


Fig. 4. Effect of C/EBPα and HNF-3γ adenoviral vectors on P450 expression. Cells were infected with Ad-C/EBPα and/or Ad-HNF-3γl; 48 h after infection, cells were harvested. Total RNA was isolated and P450 and β-actin mRNA contents were measured by RT-PCR as described under *Materials and Methods*. For quantitative measurements, the P450 mRNA contents were normalized dividing by their respective β-actin mRNA contents and were expressed as fold induction relative to the levels of control cells infected with Ad-pAC. Bars are the mean of four independent experiments ± S.D. A, effect of C/EBPα and HNF-3γ adenoviral vectors on Hep G2 *CYP3A4* expression. Representative PCR reactions for *CYP3A4* and β-actin are depicted after ethidium bromide staining. C/EBPα and HNF-3γ protein levels were analyzed by immunoblotting using 20 μg of total protein extracts. A representative Western blot is depicted after detection with the specific antibodies. The doses of Ad-C/EBPα and Ad-HNF-3γ used in the experiments are indicated. B, effect of C/EBPα and HNF-3γ adenoviral vectors on Hep G2 *CYP3As* expression. Hep G2 cells were infected with different amounts of adenoviral vectors (Ad-C/EBPα, Ad-HNF-3γ, and Ad-pAC), total RNA was isolated and *CYP3A4*, *CYP3A5*, *CYP3A7*, and β-actin mRNAs were measured by RT-PCR. Control, adenoviral-untreated cells; Ad-pAC, Ad-pAC treated cells. C, P450 expression in HeLa cells infected with C/EBPα adenoviral vector. Total RNA was isolated from HeLa cells infected with Ad-C/EBPα or Ad-pAC adenoviral vectors (7.5 MOI). After reverse transcription, cDNA fragments of CYP1A1, -1A2, -2B6, -2D6, -2E1, -3A4, -3A5, and -3A7 were amplified by 35 PCR cycles using specific primers. Two identical RT-PCR reactions were carried out using the RNA of Ad-pAC infected cells (control) or Ad-C/EBPα-infected cells (C/EBPα). After gel electrophoresis of the PCR products and staining with ethidium bromide, the fluorescent bands were recorded with video camera. Marker, 100-bp DNA ladder.

the proximal promoter constructs, suggested that the mechanisms mediating C/EBPα action at the −121/−130 site did not depend on specific hepatic factors (Fig. 2B). In addition to the proximal site, two other C/EBPα binding sites were located at distal positions in the promoter (−1393/−1402 and −1659/−1668, Fig. 3*C*). In contrast to the proximal site, the luciferase reporter gene constructs revealed that the distal sites were functional in hepatic cells but not in nonhepatic cells (Fig. 2B), which may lack hepatic-specific activators or express inhibitors that avoid C/EBPα action. On the other hand, HNF-3γ neither had any *trans*-activatory effect by itself nor modified the C/EBPα-dependent *trans*-activation.

Because the reporter plasmids are not organized into the nucleosome array characteristic of cellular chromatin (Smith and Hager, 1997), we tested whether the results found with the gene reporter assays could be extrapolated to the endogenous *CYP3A4* gene. For this purpose, we developed replicant-defective recombinant adenoviral vectors encoding C/EBPα or HNF-3γ. These expression vectors allow transfection of foreign genes into cells with almost 100% efficiency in a rather nondisturbing manner for the cells (Castell et al., 1997) and were an excellent tool for the expression of different levels of the transcription factors (Fig. 4). As predicted by the reporter assays, C/EBPα increased the *CYP3A4* mRNA content of Hep G2 cells (4-fold for 7.5 MOI), whereas HNF-3γ did not modify *CYP3A4* expression. In contrast, unpredicted by the reported assays, when both factors were expressed simultaneously, the *CYP3A4* mRNA levels were increased 45-fold, evidencing a cooperative effect between C/EBPα and HNF-3γ. The lack of effect of HNF-3γ when C/EBPα was not coexpressed provides evidence that the intrinsic levels of C/EBPα in Hep G2 cells were insufficient to bring about the HNF-3γ cooperative effect (Fig. 4A). Low levels of C/EBPα in Hep G2 cells have been described previously (Jover et al., 1998).

The observed HNF-3γ action could occur through a direct binding of HNF-3γ to *CYP3A4* promoter or by a HNF-3γ-mediated increase of another transcription factor that would bind *CYP3A4* promoter and cooperate with C/EBPα. EMSA analysis revealed that HNF-3γ binds the *CYP3A4* promoter at a distal site (−1718/−1730), supporting the idea that HNF-3γ exerts its cooperative effect through a direct mechanism. The similarity of the DNA binding domain of HNF-3 with that of linker histones (Clark et al., 1993) enables HNF-3 proteins to modify nucleosome positioning and facilitate the binding of other transcription factors (Crowe et al., 1999). The HNF-3γ site is located 50 nucleotides upstream of a C/EBPα binding site (−1659/−1668), and it is likely that HNF-3γ could affect C/EBPα binding. This effect cannot occur in the luciferase reporter plasmids lacking the characteristic chromatin structure of genomic DNA (Smith and Hager, 1997), which explains that the cooperative effect was not detected in these assays. Supporting the notion of the direct effect of HNF-3γ, the overexpression of HNF-3γ did not enhance the expression of other hepatic transcription factors such as HNF-4α, pregnane X receptor, constitutive androstane receptor, and retinoid X receptor-α, which could be indirect mediators.

In the nonhepatic HeLa cells, the adenoviral overexpression of C/EBPα increased the *CYP3A4* mRNA content to detectable levels, but HNF-3γ showed no effect, either alone or in combination with C/EBPα. The latter was in contrast with the findings in the hepatic Hep G2 cells but was consistent with the lack of C/EBPα effect in the distal binding sites

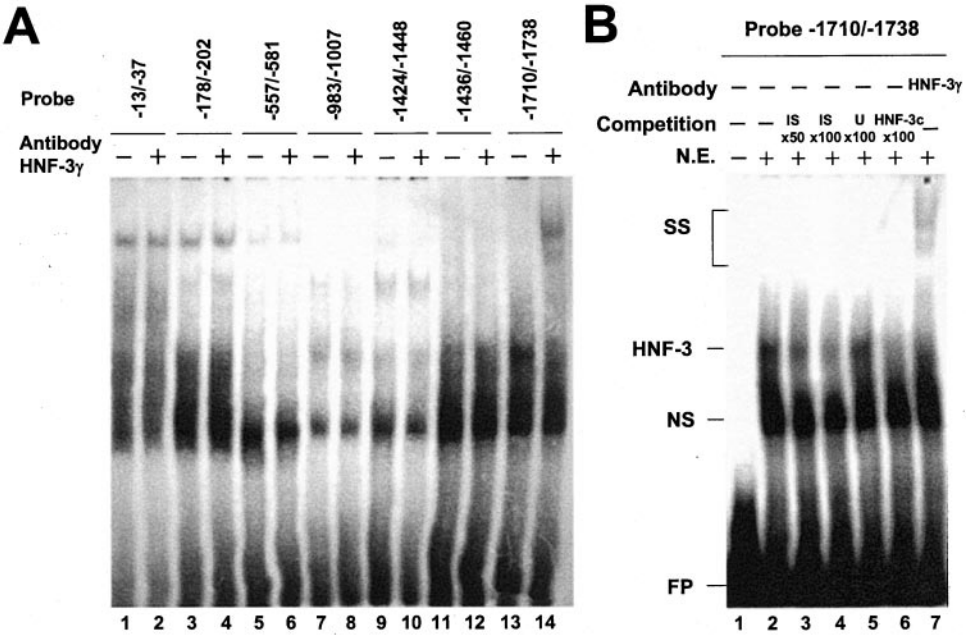


Fig. 5. Binding of HNF-3γ to *CYP3A4* promoter. A, HNF-3γ binds only one of the eight putative HNF-3 sites identified in *CYP3A4* promoter. Double-stranded oligonucleotides matching *CYP3A4* promoter sequence between positions: −13/−37, −178/−202, −557/−581, −983/−1007, −1424/−1448, −1436/−1460, and −1710/−1738 and those that contained the eight putative HNF-3 sites shown in Fig. 1 were labeled and incubated with nuclear extracts of Hep G2 cells overexpressing HNF-3γ. During the binding reaction, a specific HNF-3γ antibody was added in the indicated samples. B, HNF-3γ binds *CYP3A4* distal promoter with high affinity. The oligonucleotide −1710/−1738, which contains the overlapping −1718/−1726 and −1722/−1730 putative HNF-3 binding sites, was labeled, and 50- or 100-fold molar excess of unlabeled probes was used for competition: IS, itself; U, oligonucleotide with an unrelated sequence; HNF-3c, consensus HNF-3 binding sequence; FP, free Probe; NS, nonspecific complex; HNF-3, DNA/HNF-3γ complex; SS, supershifted complex.

of the *CYP3A4* promoter when the luciferase reporter assays were carried out in HeLa cells (Fig. 2B). We have shown that the HNF-3γ cooperative effect occurs through a distal site that is located near C/EBPα sites that are not active in HeLa cells.

The up-regulation of *CYP3A4* expression by the cooperation of C/EBPα and HNF-3γ was also detected in *CYP3A5* and *CYP3A7* genes (Fig. 4, B and C), indicating that similar binding sites for C/EBPα and HNF-3γ should be found in their promoters. In the case of *CYP3A7*, the proximal C/EBPα site had one nucleotide change with respect to *CYP3A4*, and the distal C/EBPα and HNF-3γ sites were identical. In the case of CYP3A5 (which shows the lowest response), the proximal C/EBPα site had a lower similarity with the consensus sequence than those of *CYP3A4* and *CYP3A7*. The promoter of *CYP3A5* could not be successfully aligned with *CYP3A4* at distal positions because of a drastic decrease in similarity. However, sequence analysis of the *CYP3A5* distal promoter, with conditions identical to those described for *CYP3A4* under *Materials and Methods*, located a C/EBP site at positions −1621/−1630 and two overlapping HNF-3 sites between positions −1740/−1755, similar to *CYP3A4*.

The results obtained with TSA (Fig. 6), an inhibitor of histone deacetylases able to change chromatin conformation to a more relaxed state and more accessible to transcription factors, is consistent with the proposed model for the cooperative effect between C/EBPα and HNF-3γ. It is known that TSA can alter the expression of some genes (Yoshida et al., 1995), but TSA treatment by itself did not modify the levels of *CYP3A4* in Hep G2 cells (Fig. 6, compare bars 1 and 5). The relevant results are that cells overexpressing C/EBPα increased the *CYP3A4* mRNA levels 13-fold when treated with TSA, but cells treated with TSA had lost the response to the cooperative effect of HNF-3γ. This is in agreement with the requirement of cellular chromatin structure to detect HNF-3γ effect and suggests that the modification of chromatin structure is a common mechanism for TSA and HNF-3γ. However, further studies are required to fully understand the molecular mechanism involved.

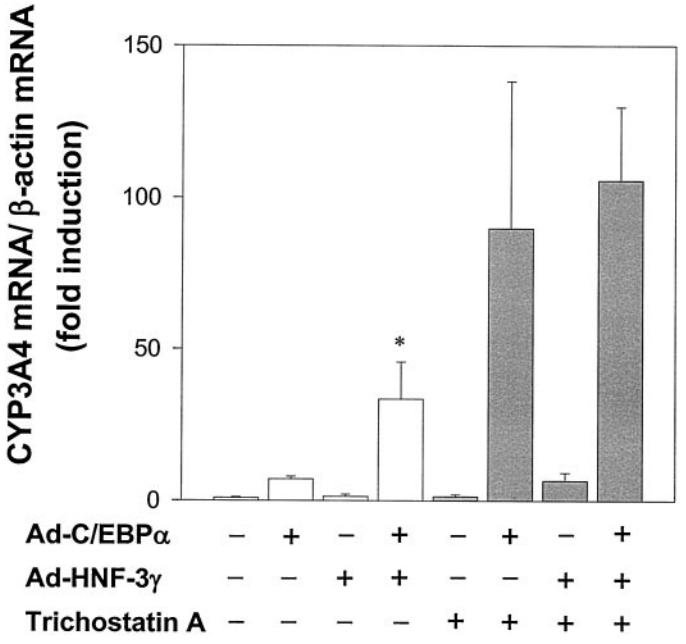


Fig. 6. Effect of trichostatin A on C/EBPα- and HNF-3γ–dependent activation of *CYP3A4*. Hep G2 cells were infected with 7.5 MOI Ad-C/EBPα and/or 4.5 MOI Ad-HNF-3γ, adjusting the final adenoviral dose to 12 MOI with Ad-pAC. Twenty-four hours after infection, cells were treated with the deacetylase inhibitor trichostatin A at 3 μM for 24 h. Total RNA was isolated, mRNA levels of *CYP3A4* and β-actin were determined by RT-PCR, and *CYP3A4* mRNA values were normalized, dividing by their respective β-actin mRNA values. The results were expressed as fold induction relative to the levels in cells infected with Ad-pAC. Data are the mean of four independent experiments ± S.D. Significantly different (*, $p < 0.05$) from cells treated with Ad-C/EBPα.

C/EBPα and HNF-3γ play important roles in the constitutive expression of human P450s. C/EBPα regulates the expressions of *CYP2B6*, *CYP2D6*, and *CYP2C9* (Jover et al., 1998), and the expression of several *CYP2C*s are regulated by HNF-3γ (Shaw et al., 1994; Delesque-Touchard et al., 2000). We now have found that the highest expression of *CYP3A4*, *CYP3A5*, and *CYP3A7* was obtained in hepatic cells expressing a combination of C/EBPα and HNF-3γ, a mechanism that may also operate in other P450s. Because of the important roles played by C/EBPα and HNF-3γ in the constitutive expression of human *CYP3A4*, variations in the expression of C/EBPα and HNF-3γ could ultimately be responsible of the different expression levels of *CYP3A4* found in humans. In this context, the levels of C/EBPα and HNF-3γ proteins are known to change in the liver under several pathophysiological situations. For example, during inflammatory processes, C/EBPα and CYP3A4 expression decrease (Donato et al., 1998; Welm et al., 2000). Diet and hormonal status have also been described to greatly alter HNF-3γ expression in liver (Imae et al., 2000). Further studies could determine whether variations in C/EBPα and HNF-3γ expression could be involved in CYP3A4 intra- and interindividual variability.

In conclusion, we have localized binding sites for C/EBPα and HNF-3γ in *CYP3A4* promoter, and by reporter assays we have shown their relevance for gene expression. By use of adenoviral expression vectors, we have found a synergistic effect between C/EBPα and HNF-3γ in the expression of hepatic *CYP3A* genes. Finally, the proximity of C/EBPα and HNF-3γ distal sites and the abolishment of HNF-3γ action by a deacetylase inhibitor suggest that HNF-3γ facilitates C/EBPα action by modification of the chromatin structure of *CYP3A4* promoter.

Acknowledgments

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