Title

Transcriptional regulation of human CYP3A4 basal expression by C/EBPα and HNF-3γ.

Authors

C. RODRÍGUEZ-ANTONA, R. BORT, R. JOVER, N. TINDBERG, M. INGELMAN-SUNDBERG, M. J. GÓMEZ-LECHÓN, and J. V. CASTELL

Institutional addresses

Departamento de Bioquímica, Facultad de Medicina, Universidad de Valencia, Avda. Blasco Ibáñez 13, E-46010 Valencia, Spain (C.R., R.B., R.J., J.V.C.)

Unidad de Hepatología Experimental, Centro de Investigación, Hospital Universitario La Fé, Avda. Campanar 21, E-46009 Valencia, Spain (M.J.G., J.V.C.)

Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institute, Nobels väg 13, 17177-Stockholm, Sweden (N.T., M.I.)

Running title

Regulation of human *CYP3A4* by C/EBPα and HNF-3γ.

Corresponding author

a) José V. Castell PhD MD

b) Unidad de Hepatología Experimental, Centro de Investigación, Hospital Universitario La Fé, Avda. Campanar 21, E-46009 Valencia, Spain

c) Tel: +34 96 1973048; Fax: +34 96 1973018

d) e-mail address: Jose.Castell@uv.es

Number of text pages: 36

Number of tables: 2

Number of figures: 6

Number of references: 40

Number of words in the abstract: 247

Number of words in the introduction: 636

Number of words in the discussion: 1296

Abbreviations

CYP, cytochrome P450; LETFs, liver-enriched transcription factors; 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; SDS, sodium dodecyl sulfate; TSA, trichostatin A.

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 key 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 non-hepatic 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 likely involving chromatin remodeling.

INTRODUCTION

The cytochromes P450 (CYP) are a superfamily of heme-containing enzymes which 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 inter-individual 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 most xenobiotic-metabolizing CYPs, 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 regulating the expression of *CYP* 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 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 *CYP* expression was first obtained in HepG2 cells, which showed augmented levels of *CYP 2B6, 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 which 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*[1], and recombinant promoter analysis has demonstrated that HNF-3 trans-activates rat *CYP2C6* and *CYP2C12* (Delesque-Touchard et al., 2000; Shaw et al., 1994). 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; Kaestner et al., 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

likely 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 α–α homodimer or 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, -104 to +6 (the PCR-primers used had at the 5´ end restriction enzymes sites for *Kpn*I or *Xho*I 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-*Hind*III, 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[1].

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-bp 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-bp 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-bp and the 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), and 2 units of Expand High Fidelity Taq polymerase (Roche) in a total volume of 50 µl. DNA was amplified for 30 cycles (denaturation at 94 ºC for 15 s, annealing at 55 ºC for 30 s, and extension at 72 ºC 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 Ultra Clean DNA Purification Kit (MO BIO Laboratories, Inc.). 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-bp 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 $MgCl_2$ by 94º C for 2 min and 55º C for 5 min. 2 units of Expand High Fidelity Taq polymerase (Roche) were added to the reaction mixture in a total volume of 50 µl. DNA was amplified for 10 cycles (denaturation at 94 ºC for 1 min, annealing at 50 ºC for 1 min, and extension at 72º C for 1 min). 0.2 µM of sense -163-FP and antisense +6-RP oligonucleotide primers were subsequently added to the reaction mixture, and DNA was amplified for additional 30 cycles (1 cycle = 94º C for 30 s, 55º C for 30 s, and 72º C for 30 s) with a final extension at 72 ºC 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. HepG2 cells were plated in Ham's F-12/Leibovitz L-15 (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 293 cells were maintained as monolayer cultures and grown in DMEM supplemented with 10% newborn calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin, 293 cells medium was supplemented with 3.5 g/l of glucose.

Plasmid DNAs were purified with Qiagen Maxiprep kit columns (QIAGEN) and quantified by absorbance at 260 nm and fluorescence using PicoGreen (Molecular Probes). The day before transfection, cells were plated in 35-mm diameter dishes with 1.5 ml of medium. 2 h before transfection medium was changed to DMEM/Nut F12 (Life Technologies) 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* 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 h (HepG2) or 20 h (HeLa). Then, the medium was replaced and 48 h after transfection firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega). In all experiments luciferase activity was normalized to transfection efficiency (*Renilla* 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 PBS, medium was replaced, and 48 h post-infection cells were harvested and frozen in liquid N_2.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts from HepG2 cells infected with C/EBPα or HNF-3γ adenovirus were prepared as described previously (Schreiber et al., 1989). Briefly, cells were scraped, washed with cold PBS, 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, twelve micrograms of nuclear extract were preincubated at 37 ºC 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), added to the reaction mixture, and incubated for 40 min at 37 ºC. 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 ºC, 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, prior to 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, USA) were added after the incubation with the labeled probe, and incubated for 45 min on ice. Gels were dried and exposed at –70ºC 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 mu, 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 previously described (Castell et al., 1997). The preparation of Ad-HNF-3γ was performed in a similar way[1]. 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 HepG2 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), contaminating genomic DNA was removed by incubation with DNase I Amplification Grade (Life Technologies), 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 of each deoxynucleotide triphosphate, 1 unit Taq DNA polymerase (Life Technologies), 0.2 µM 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 º C, amplification was performed by 30-35 cycles (94º C for 35 s, 60º C or 57º C, for C/EBPα or HNF-3γ, respectively, for 30 s; and 72ºC for 45 s) and a final extension of 72º C for 7 min. mRNA levels of *CYP3A4*, *CYP3A5*, *CYP3A7*, and β-actin were quantified by RT-PCR with the LightCycler Instrument (Roche) using the LightCycler-DNA Master SYBR Green I (Roche). 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-labelled IgG, using an Enhanced Chemiluminence Kit (Amersham Life Sciences).

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 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 (HepG2), 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 Figure 2*A*, 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 as *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. 2*B*). 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 HepG2 cell lines, respectively) giving relevance to a C/EBP responsive element located in the -163 to +6 fragment (Fig. 1). In HepG2 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 identical transfection conditions as 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. 2*B*). 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 HepG2 cells overexpressing C/EBPα.

Using a labeled probe matching the -163/ +6 region of the *CYP3A4* promoter (P1) different complexes were detected (Fig. 3*A*). Complexes 1 and 2 were specific as 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 since 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 isoform retarded the migration of both complexes 1 and 2 (Fig. 3*A*, lane

7).

These results were confirmed by EMSAs using labeled -115/-139 probe (P2). In this case non-specific complexes were absent probably due to the shortage of the probe, but again it was shown that C/EBPα binds the -121/-130 site (Fig. 3*A*, right panel). Both C/EBP isoforms α and β are abundant in liver and are known to form heterodimers and recognize the same DNA sequence (Shugart and Umek, 1997). Pre-incubation with specific C/EBP antibodies revealed that the formed complex largely corresponded to C/EBPα and to a less extent to endogenous C/EBPβ, in agreement with the high expression of C/EBPβ in HepG2 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. 3*B*).

The -1843/-1365 region of the *CYP3A4* promoter increased C/EBPα trans-activation in HepG2 cells, indicating that it contained functional C/EBPα sites (Fig. 2*B*). 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. 3*C* 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. 3*C* lane 5).

Expression of CYPs in Cells Infected 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 HepG2 cells, which have lost the expression of *CYP3A4* and other hepatic-specific genes (Fig. 4*A*) and HeLa cells, which are derived from cervix carcinoma cells and have no *CYP3A4* expression (Fig. 4*C*). In both cases, the cells were infected with Ad-C/EBPα, Ad-HNF-3γ or Ad-pAC, and 48 h post-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 4-fold the *CYP3A4* mRNA content of HepG2 cells, whereas Ad-HNF-3γ had no effect (Fig. 4*A*). Remarkably, infection of HepG2 cells with increasing amounts of Ad-HNF-3γ (0.75 to 4.5 MOI) simultaneously with 7.5 MOI of Ad-C/EBPα revealed a dose-dependent, 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 kb upstream 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 HepG2-infected cells. Figure 4*B* 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 non-hepatic HeLa cells, the *CYPs 3A4, 3A5,* and *3A7* mRNAs increased from undetectable levels to PCR-detectable levels, whereas the expression of the *CYPs 1A1, 1A2, 2B6, 2D6,* and *2E1* did not change (Fig. 4*C*). This demonstrated that the C/EBPα effect was specific for the CYP3A family and that it also occurred in non-hepatic cells. However, when C/EBPα and HNF-3γ were co-expressed in HeLa cells no difference in CYP3A´s expressions could be observed compared to 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 HepG2 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, that 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 added to the EMSA binding reaction supershifted complexes could only be detected with the -1710/-1738 labeled probe (Fig. 5*A* lane 14). As shown in Figure 5*B* the specific complex of the -1710/-1738 probe had a relatively high affinity (100 excess of unlabeled probe was required for complete competition) (Fig. 5*B* 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. This data support a direct effect of HNF-3γ in *CYP3A4* promoter.

As a further test to 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, PXR, CAR and RXR-α, which are important for *CYP3A4* expression. No changes in the expression of these factors could be detected in HepG2 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 (Cirillo et al., 2002; Crowe et al., 1999). To investigate whether this mechanism could be responsible for the cooperative effect observed between C/EBPα and HNF-3γ, we treated HepG2 cells overexpressing C/EBPα and/ or HNF-3γ with trichostatin A (TSA), a compound which 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 13-fold the C/EBPα activatory effect (compare bars 2 and 6 in Fig. 6) and clearly abolished HNF-3γ cooperative effect (bars 6 and 8 of Fig. 6 are not significantly different,

while 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 CYPs (Delesque-Touchard et al., 2000; Jover et al., 1998; Ourlin et al., 1997). However, their role in the regulation of the constitutive expression of *CYP3A4* in hepatocytes, which is much higher than in non-hepatic 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 3*B*). The similar results obtained in hepatic and non-hepatic cell lines transfected with 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. 2*B*). 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 non-hepatic cells (Fig. 2*B*), 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 non-disturbing 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 HepG2 cells (4-fold for 7.5 MOI) while 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 co-expressed, evidenced that the intrinsic levels of C/EBPα in HepG2 cells were insufficient to bring about the HNF-3γ cooperative effect. Low levels of C/EBPα in HepG2 cells have been described previously (Jover et al., 1998), Fig. 4*A*.

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 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), and this 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, PXR, CAR and RXR-α, which could be indirect mediators.

In the non-hepatic 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

convination with C/EBPα. The later was in contrast with the findings in the hepatic HepG2 cells, but was consistent with the lack of C/EBPα effect in the distal binding sites of the *CYP3A4* promoter when the luciferase reporter assays were carried out in HeLa cells (Fig. 2*B*). We have shown that the HNF-3γ cooperative effect occurs through a distal site which is located near C/EBPα sites that are non 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 due to a drastic decrease in similarity. However, sequence analysis of the *CYP3A5* distal promoter, with identical conditions as those described for *CYP3A4* in Methods, located a C/EBP site at positions -1621/-1630 and two overlapping HNF-3 sites between positions -1740/-1755, similarly to *CYP3A4*.

The results obtained with TSA (Fig. 6), an inhibitor of hystone 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 HepG2 cells (compare bars 1 and 5 in Fig. 6). The relevant results are that cells overexpressing C/EBPα, increased the *CYP3A4* mRNA levels 13-fold when treated with TSA, but that 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.

C/EBPα and HNF-3γ play important roles in the constitutive expression of human CYPs. C/EBPα regulates the expressions of *CYP2B6*, *CYP2D6* and *CYP2C9* (Jover et al., 1998), and the expression of several *CYP2Cs* are regulated by HNF-3γ (Delesque-Touchard et al., 2000; Shaw et al., 1994). 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 CYPs. 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 patho-physiological situations. For example, during inflammatory processes C/EBPα and CYP3A4 expression decrease (Donato et al., 1998; Welm et al., 2000). It has also been described that diet and hormonal status can 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 inter-individual 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 close proximity between 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.

ACKNOLEDGMENTS

C. Rodríguez-Antona was recipient of a fellowship of Generalitat Valenciana. The authors thank E. Belenchón and C. Corchero for their technical assistance.

REFERENCES

Antonson P and Xanthopoulos KG (1995) Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). *Biochem Biophys Res Commun* **215**:106-113.

Burgess-Beusse BL and Darlington GJ (1998) C/EBPalpha is critical for the neonatal acute-phase response to inflammation. *Mol Cell Biol* **18**:7269-7277.

Castell JV, Hernández D, Gómez-Foix AM, Guillén I, Donato T and Gómez-Lechón MJ (1997) Adenovirus-mediated gene transfer into human hepatocytes: analysis of the biochemical functionality of transduced cells. *Gene Ther* **4**:455-464.

Cereghini S (1996) Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J* **10**:267-282.

Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M and Zaret KS (2002) Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* **9**:279-289.

Clark K, Halay E, Lai E and Burley S (1993) Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature* **364**:412-420.

Crowe A, Sang L, Li K, Lee K, Spear B and Barton M (1999) Hepatocyte nuclear factor 3 relieves chromatin-mediated repression of the alpha-fetoprotein gene. *J Biol Chem* **274**:25113-25120.

Delesque-Touchard N, Park SH and Waxman DJ (2000) Synergistic action of hepatocyte nuclear factors 3 and 6 on CYP2C12 gene expression and suppression by growth hormone-activated STAT5b. Proposed model for female specific expression of CYP2C12 in adult rat liver. *J Biol Chem* **275**:34173-37182.

Donato MT, Gomez-Lechon MJ, Jover R, Nakamura T and Castell JV (1998) Human hepatocyte growth factor down-regulates the expression of cytochrome P450 isozymes in human hepatocytes in primary culture. *J Pharmacol Exp Ther* **284**:760-767.

Forrester LM, Henderson CJ, Glancey MJ, Back DJ, Park BK, Ball SE, Kitteringham NR, McLaren AW, Miles JS, Skett P and Wolf CR (1992) Relative expression of Cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Bichem. J.* **281**:356-368.

Gómez-Foix AM, Coats WS, Baque. S, Alam T, Gerard RD and Newgard CB (1992) Adenovirus mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen metabolism. *J Biol Chem* **267**:25129-25134.

Gonzalez F and Lee Y (1996) Constitutive expression of hepatic cytochrome P450 genes. *FASEB J* **10**:1112-1117.

Guengerich FP (1993) Cytochrome P450 enzymes. *American Scientist* **81**:440-447.

Hashimoto H, Toide K, Kitamura R, Fujita M, Tagawa S, Itoh S and Kamataki T (1993) Gene structure of CYP3A4, an adult-specific form of cytochrome P450 in human livers, and its transcriptional control. *Eur J Biochem* **218**:585-595.

Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, L. PN and Kolchanov NA (1998) Databases on Transcriptional Regulation: TRANSFAC, TRRD, and COMPEL. *Nucleic Acids Res* **26**:364-370.

Imae M, Inoue Y, Fu Z, Kato H and Noguchi T (2000) Gene expression of the three members of hepatocyte nuclear factor-3 is differentially regulated by nutritional and hormonal factord. *J Endocrinol* **167**:R1-R5.

Jounaidi Y, Guzelian P, Maurel P and Vilarem M (1994) Sequence of the 5'-flanking region of CYP3A5: comparative analysis with CYP3A4 and CYP3A7. *Biochem Biophys Res Commun* **205**:1741-1747.

Jover R, Bort R, Gomez-Lechon MJ and Castell JV (1998) Re-expression of C/EBP alpha induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS Lett* **431**:227-230.

Kaestner KH, Hiemisch H, Luckow B, and Schutz G (1994) The HNF-3 family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. *Genomics* **20**:377-385.

Kaestner K, Hiemisch H and Schutz G (1998) Targeted disruption of the gene encoding hepatocyte nuclear factor 3gamma results in reduced transcription of hepatocyte-specific genes. *Mol Cell Biol* **18**:4245-4251.

Kaestner KH, Katz J, Liu Y, Drucker DJ and Schutz G (1999) Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. *Genes Dev* **13**:495-504.

Legraverend C, Eguchi H, Strom A, Lahuna O, Mode A, Tollet P, Westin S and Gustafsson J (1994) Transactivation of the rat CYP2C13 gene promoter involves HNF-1, HNF-3, and members of the orphan receptor subfamily. *Biochemistry* **33**:9889-9897.

Li AP (1995) Substrates of human hepatic cytochrome P450 3A4. *Toxicology* **104**:1-8.

Osada S, Yamamoto H, Nishihara T and Imagawa M (1996) DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *J Biol Chem* **271**:3891-3896.

Ourlin J, Jounaidi Y, Maurel P and Vilarem M (1997) Role of the liver-enriched transcription factors C/EBP alpha and DBP in the expression of human CYP3A4 and CYP3A7. *J Hepatol* **26**:54-62.

Rodriguez-Antona C, Donato MT, Boobis A, Edwards RJ, Watts PS, Castell JV and Gomez-Lechon MJ (2002) Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica* **32**:505-520.

Schreiber E, Matthias P, Muller MM and Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17**:6419.

Shaw PM, Weiss MC and Adesnik M (1994) Hepatocyte nuclear factor 3 is a major determinant of CYP2C6 promoter activity in hepatoma cells. *Mol Pharmacol* **46**:79-87.

Shim E, Woodcock C and Zaret K (1998) Nucleosome positioning by the winged helix transcription factor HNF3. *Genes Dev* **12**:5-10.

Shugart EC and Umek RM (1997) Transcription factors and cell differentiation: the cell paradigm, in *Transcription factors in Eukaryotes* (Papavassiliou A ed), Landes Bioscience., Austin, Texas, USA.

Smith C and Hager G (1997) Transcriptional regulation of mammalian genes in vivo. *J Biol Chem* **272**:27493-27496.

Sund N, J., Vatamaniuk MZ, Casey M, Ang SL, Magnuson MA, Stoffers DA, Matschinsky FM and Kaestner KH (2001) Tissue-specific deletion of Foxa2 in pancreatic beta cells results in hyperinsulinemic hypoglycemia. *Genes Dev* **15**:1706-1715.

van Holde K (1997) Chromatin estructure and regulation of gene expression. Minireview series. *J Biol Chem* **272**:26073.

Wang JC, Stafford JM, Scott DK, Sutherland C and Granner DK (2000) The molecular physiology of hepatic nuclear factor 3 in the regulation of gluconeogenesis. *J Biol Chem* **275**:14717-14721.

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR and Darlington GJ (1995) Impaired energy homeostasis in C/EBP alpha knockout mice. *Science* **269**:1108-1112.

Waxman DJ, Lapenson DP, Aoyama T, Gelboin HV, Gonzalez FJ and Korzekwa K (1991) Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochromes P450s. *Arch Biochem Biophys* **290**:160-166.

Welm AL, Mackey SL, Timchenko LT, Darlington GJ and Timchenko NA (2000) Translational induction of liver-enriched transcriptional inhibitory protein acute phase response leads to repression of CCAAT/enhancer binding protein alpha mRNA. *J Biol Chem* **275**:27406-27413.

Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, Meinhardt T, Prüß M, Reuter I and Schacherer F (2000) TRANSFAC: an integrated system for gene expression regulation. *Nucleic Acids Res*:316-319.

Yamashita T, Hashimoto S, Kaneko S, Nagai S, Toyoda N, Suzuki T, Kobayashi K and Matsushima K (2000) Comprehensive gene expression profile of a normal human liver. *Biochem Biophys Res Commun.* **269**:110-116.

Yoshida M, Horinouchi S and Beppu T (1995) Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. *BioEssays* **17**:423-430.

FOOTNOTES

This work has been supported by the European Union, BIOTECH Contract BIO4-CT96-0052 and BIOMED Contract BMH4-CT86-0254 (Eurocyp). C. Rodríguez-Antona was recipient of a fellowship from the Generalitat Valenciana.

José V. Castell, Unidad de Hepatología Experimental, Centro de Investigación, Hospital Universitario La Fé, Avda. Campanar 21, E-46009 Valencia, Spain

[1] R. Bort, R. Jover, C. Rodríguez-Antona, M.J. Gómez-Lechón, and J.V. Castell, manuscript in preparation.

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

TABLE 2

Oligonucleotide PCR primers for cDNA amplification.

Gene	Forward primer sequence 5´to 3´	Reverse primer sequence 5´to 3´	Annealing T. (ºC)	Fragment size (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

FIGURE LEGENDS

Figure 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 (gray squares) and HNF-3 (black squares). 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.

Figure 2. Basal activity and transactivation by C/EBPα and HNF-3γ of *CYP3A4* promoter constructs in HeLa and HepG2 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. 48 h after transfection cells were scraped, lysed, and both firefly and *Renilla* luciferase activities were measured. *A.* Basal activity of *CYP3A4* promoter deletion constructs. 1 µg of these constructs and 0.1 µg of the plasmid pRL-CMV, as a transfection efficiency control, were transfected with calcium phosphate in HeLa and HepG2 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 HepG2 cells. The insertless plasmid pAC/CMVpLpA was added, to have a constant amount of total expression vector, and 0.1µg of the plasmid pRL-CMV was used to control transfection efficiency. Values represent firefly/ *Renilla* luciferase activity ratios divided by protein content. Bars represent the mean ± S.D. of four independent experiments.

Figure 3. Binding of C/EBPα to *CYP3A4* promoter and functionality of the sites. For electrophoretic mobility shift assays (EMSAs) double stranded DNAs matching *CYP3A4*

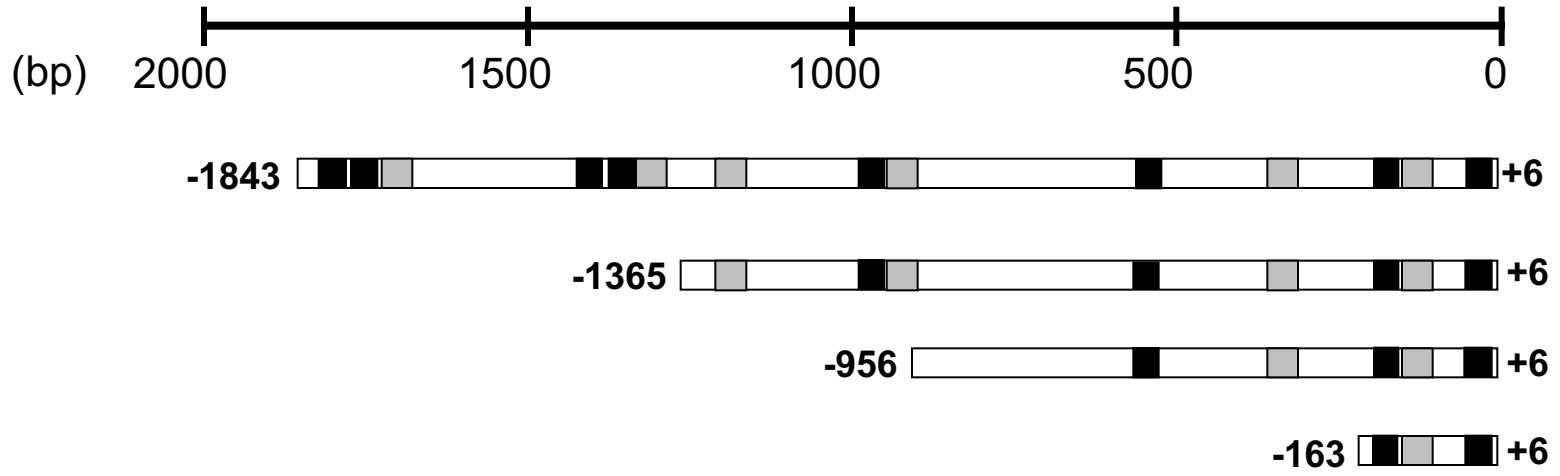
promoter sequence were labeled and incubated with nuclear extracts of HepG2 cells overexpressing C/EBPα. Unlabelled oligonucleotides were incubated at 50-fold molar excess for competition. *A*, Binding of C/EBPα to the proximal *CYP3A4* promoter. EMSAs were performed using labeled P1 (-163 to +6) in the left panel or P2 (-115 to –139) in the right panel. 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. 0.5μg of these constructs 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. 48 h after transfection cells were scraped, lysed, and both firefly and *Renilla* luciferase activities were measured. Values represent firefly/ *Renilla* 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. is nuclear extract, IS is itself, U is an oligonucleotide with an unrelated sequence, P2m is a -115 to -139 oligonucleotide with the central nucleotides of the putative C/EBPα binding site at -121/-130 mutated. FP is free probe, NS is non-specific complex, C1 and C2 are specific complexes, C/EBP is a DNA/C/EBPα complex and SS is supershifted complex.

Figure 4. Effect of C/EBPα and HNF-3γ adenoviral vectors on *CYP* expression. Cells were infected with Ad-C/EBPα and/ or Ad-HNF-3γ and 48 h after infection cells were harvested. Total RNA was isolated and CYP and β-actin mRNA contents were measured by RT-PCR as described in Methods. For quantitative measurements the *CYP* 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 HepG2 *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 immunoblottin 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 HepG2 *CYP3As* expression**.** HepG2 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 stands for adenoviral-untreated cells and Ad-pAC stands for Ad-pAC treated cells. *C*, CYP 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 CYPs 1A1, 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.

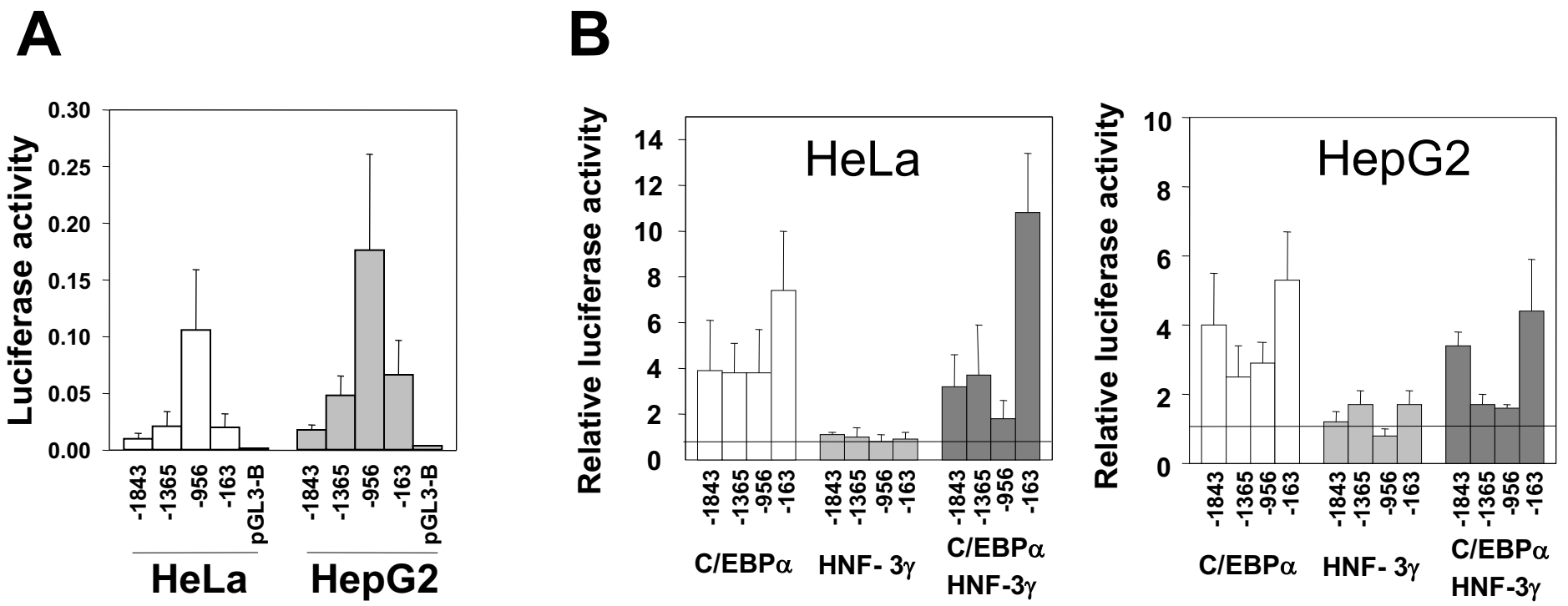
Figure 5. Binding of HNF-3γ to *CYP3A4* Promoter. *A*, HNF-3γ binds only one of the eight putative HNF-3 sites identified in *CYP3A4* promoter. Double stranded oligonucleotides matching *CYP3A4* promoter sequence between positions: -13/-37, -178/-202, -557/-581, -983/-1007, -1424/-1448, -1436/-1460 and -1710/-1738 and that contained the eight putative HNF-3 sites shown in Figure 1, were labeled and incubated with nuclear extracts of HepG2 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, that 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 were used for competition: IS is itself, U is a oligonucleotide with an unrelated sequence, HNF-3c is a consensus HNF-3 binding sequence. FP is free Probe, NS is non-specific complex, HNF-3 is a DNA/HNF-3γ complex, SS is supershifted complex.

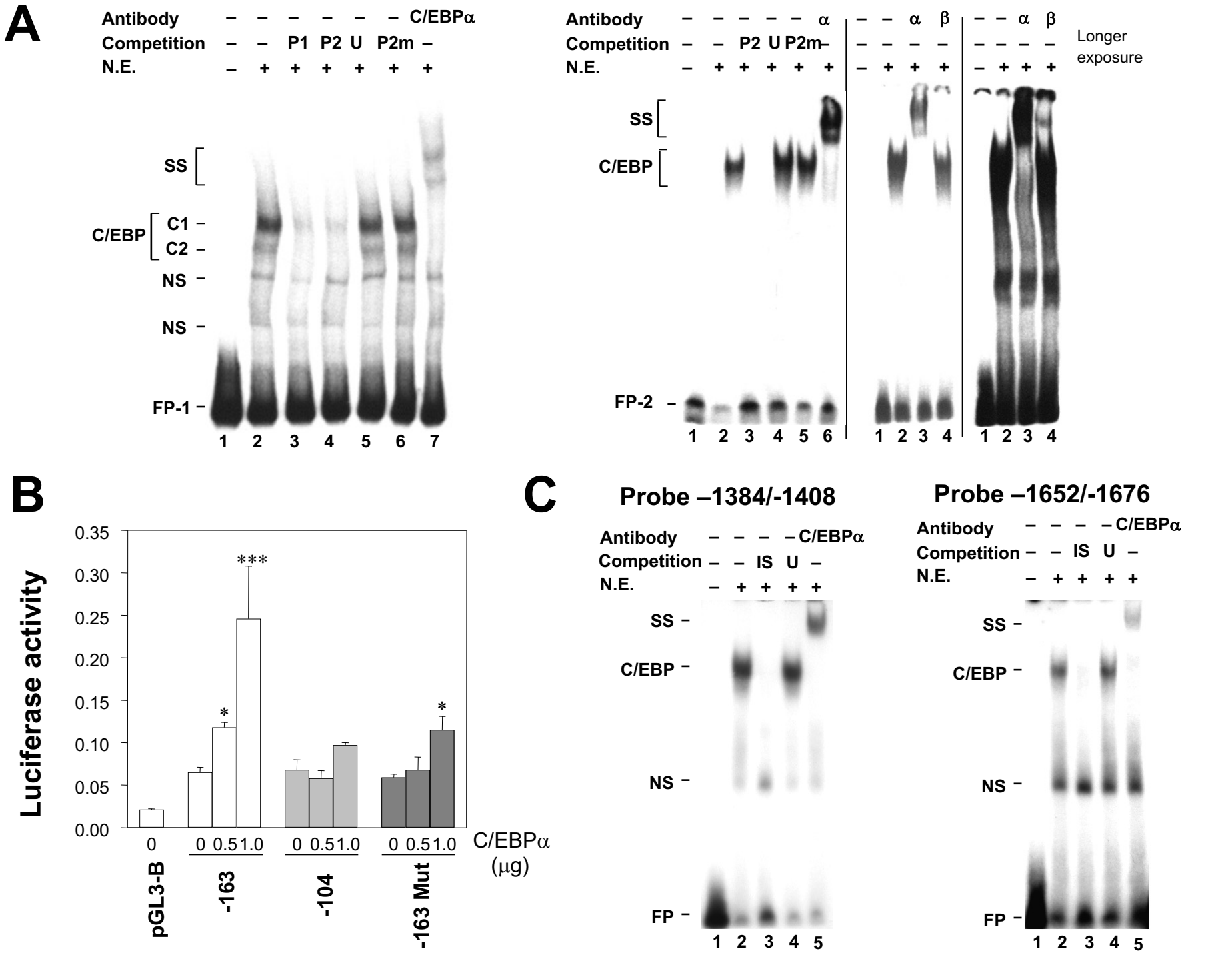
Figure 6. Effect of trichostatin A on C/EBPα and HNF-3γ -dependent activation of *CYP3A4*. HepG2 cells were infected with 7.5 MOI Ad-C/EBPα and/ or 4.5 MOI Ad-HNF-3γ adjusting the final adenoviral dose to 12 MOI with Ad-pAC. 24 h after infection cells were treated with the deacetylase inhibitor trichostatin 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.

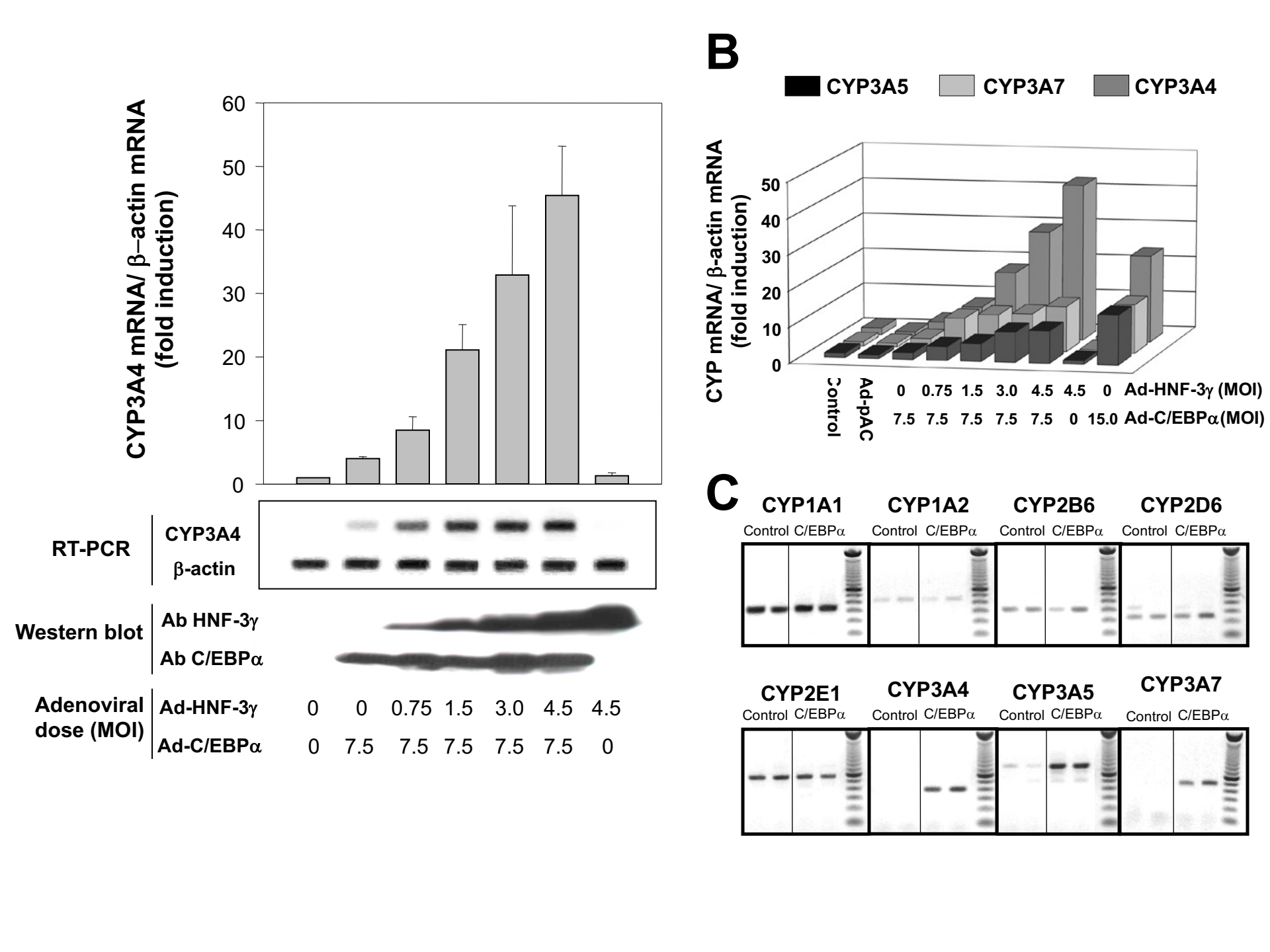
CYP3A4

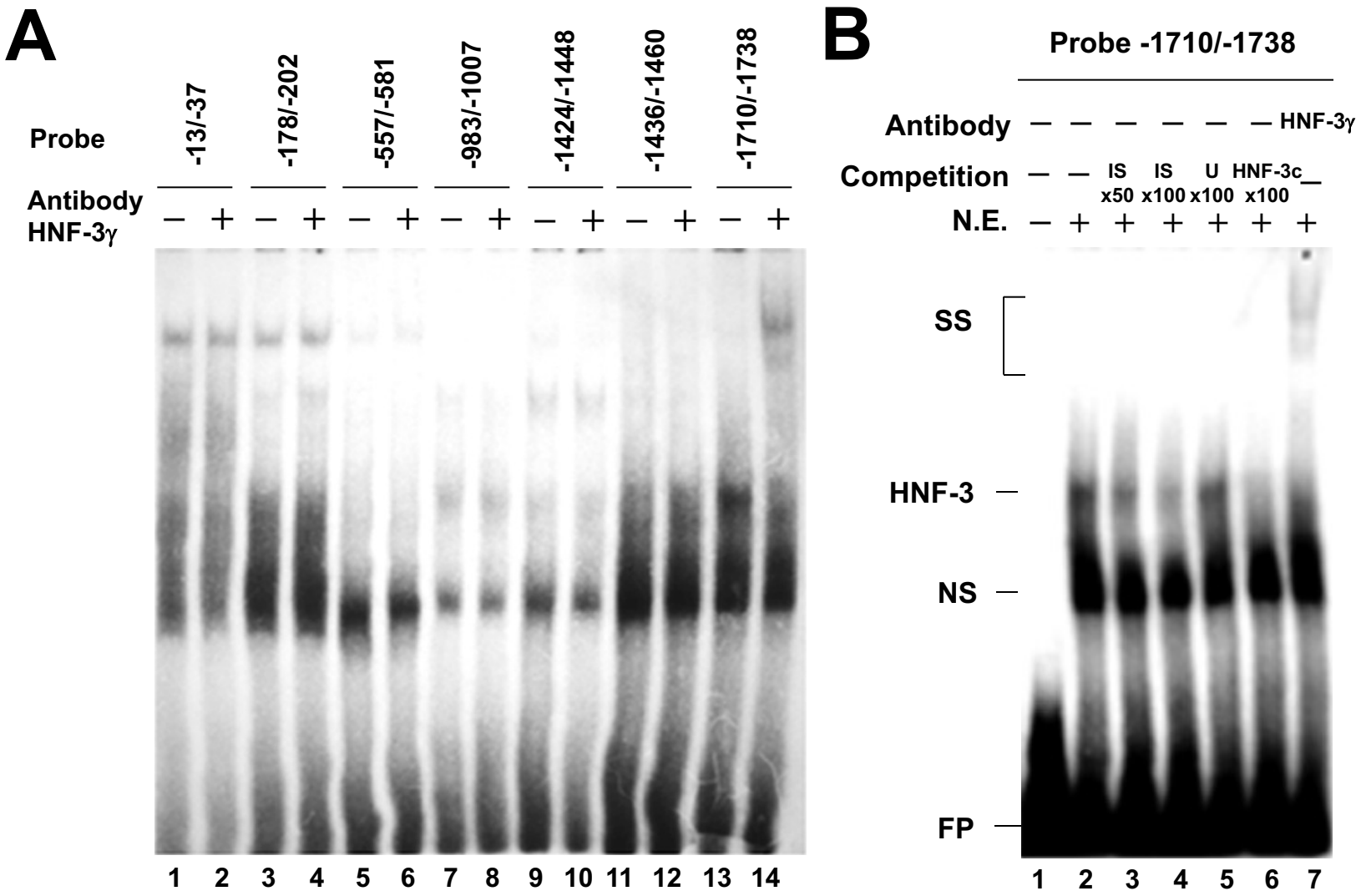


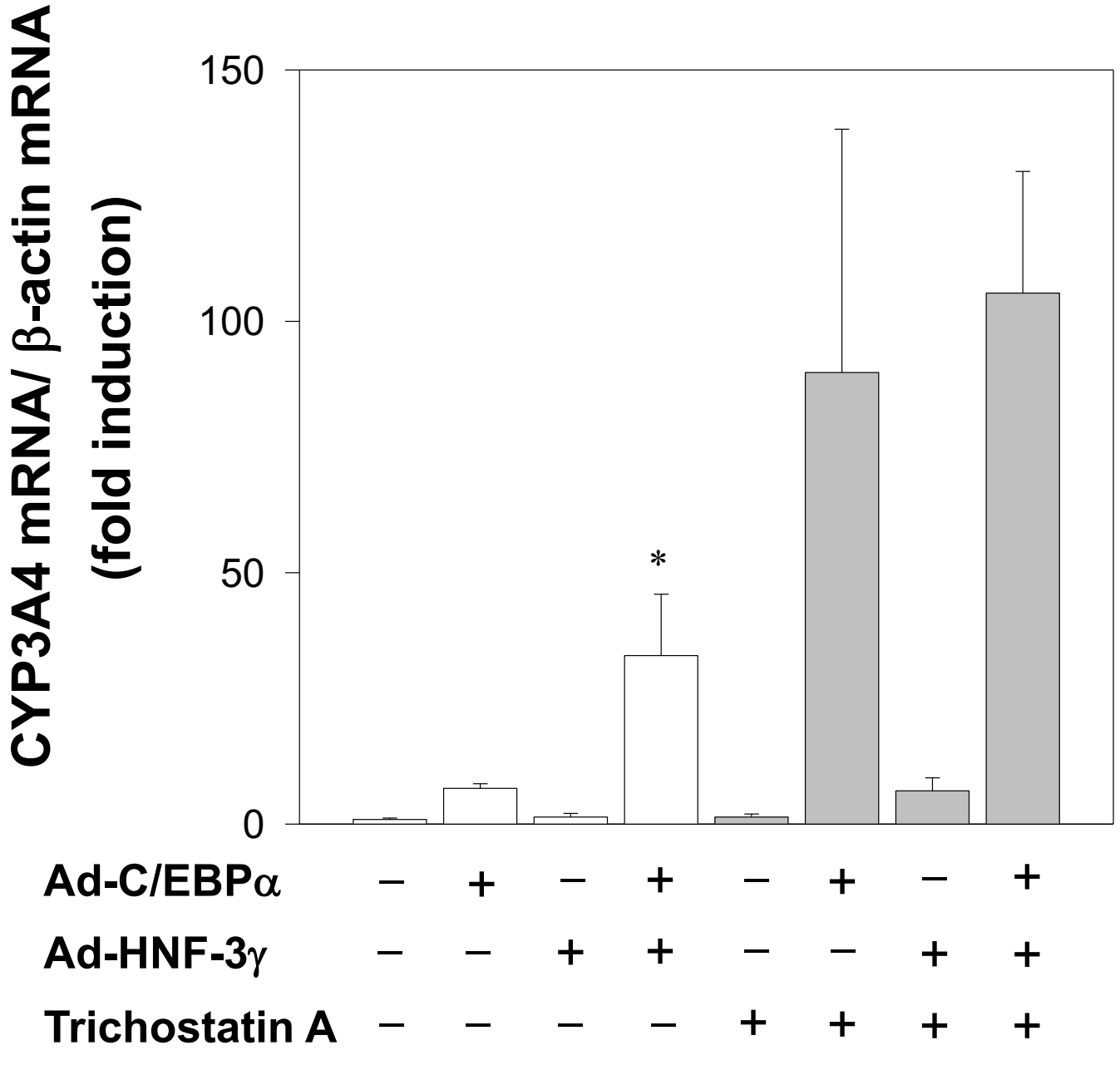
CONSENSUS SITE	POSITION	CONSENSUS SITE	POSITION
C/EBP	-121/ -130	**HNF-3**	-22/ -30
C/EBP	-315/ -324	**HNF-3**	-184/ -192
C/EBP	-964/ -973	**HNF-3**	-567/ -575
C/EBP	-1202/ -1211	**HNF-3**	-989/ -997
C/EBP	-1393/ -1402	**HNF-3**	-1432/ -1440
C/EBP	-1659/ -1668	**HNF-3**	-1446/ -1454
		HNF-3	-1718/ -1726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


A
Luciferase activity
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-1843
-1365
-956
-163
pGL3-B
-1843
-1365
-956
-163
pGL3-B
HeLa
HepG2
B
Relative luciferase activity
HeLa
14
12
10
8
6
4
2
0
-1843
-1365
-956
-163
-1843
-1365
-956
-163
-1843
-1365
-956
-163
C/EBPα
HNF- 3γ
C/EBPα
HNF-3γ
Relative luciferase activity
HepG2
10
8
6
4
2
0
-1843
-1365
-956
-163
-1843
-1365
-956
-163
-1843
-1365
-956
-163
C/EBPα
HNF- 3γ
C/EBPα
HNF-3γ


A
Antibody – – – – – – C/EBPα
Competition – – P1 P2 U P2m –
N.E. – + + + + + +
SS
C/EBP
C1
C2
NS
NS
FP-1
1 2 3 4 5 6 7
Antibody – – – – – α – – α β – – α β
Competition – – P2 U P2m – – – – – – – –
N.E. – + + + + + – + + + – + + +
Longer exposure
SS
C/EBP
FP-2
1 2 3 4 5 6 1 2 3 4 1 2 3 4
B
Luciferase activity
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
***
*
*
0 0 0.5 1.0 0 0.5 1.0 0 0.5 1.0
C/EBPα (μg)
pGL3-B
-163
-104
-163 Mut
C
Probe –1384/-1408
Antibody – – – – C/EBPα
Competition – – IS U –
N.E. – + + + +
SS
C/EBP
NS
FP
1 2 3 4 5
Probe –1652/-1676
Antibody – – – – C/EBPα
Competition – – IS U –
N.E. – + + + +
SS
C/EBP
NS
FP
1 2 3 4 5


CYP3A4 mRNA/ β–actin mRNA
(fold induction)
60
50
40
30
20
10
0
RT-PCR
CYP3A4
β-actin
Western blot
Ab HNF-3γ
Ab C/EBPα
Adenoviral dose (MOI)
Ad-HNF-3γ 0 0 0.75 1.5 3.0 4.5 4.5
Ad-C/EBPα 0 7.5 7.5 7.5 7.5 7.5 0
B
CYP3A5
CYP3A7
CYP3A4
CYP mRNA/ β-actin mRNA
(fold induction)
50
40
30
20
10
0
Control
Ad-pAC
0 0.75 1.5 3.0 4.5 4.5 0 Ad-HNF-3γ (MOI)
7.5 7.5 7.5 7.5 7.5 0 15.0 Ad-C/EBPα(MOI)
C
CYP1A1
CYP1A2
CYP2B6
CYP2D6
Control C/EBPα
CYP2E1
CYP3A4
CYP3A5
CYP3A7


A
Probe
-13/-37
-178/-202
-557/-581
-983/-1007
-1424/-1448
-1436/-1460
-1710/-1738
Antibody
HNF-3γ
– + – + – + – + – + – + – +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
Probe -1710/-1738
Antibody – – – – – – HNF-3γ
Competition – – IS x50 IS x100 U x100 HNF-3c x100
N.E. – + + + + + +
SS
HNF-3
NS
FP
1 2 3 4 5 6 7


CYP3A4 mRNA/ β-actin mRNA
(fold induction)
150
100
50
0
*
Ad-C/EBPα – + – + – + – +
Ad-HNF-3γ – – + + – – + +
Trichostatin A – – – – + + + +