THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 33, Issue of August 17, pp. 30708–30716, 2001

*Printed in U.S.A.*

# The Negative Effects of Bile Acids and Tumor Necrosis Factor-α on the Transcription of Cholesterol 7α-Hydroxylase Gene (*CYP7A1*) Converge to Hepatic Nuclear Factor-4

A NOVEL MECHANISM OF FEEDBACK REGULATION OF BILE ACID SYNTHESIS MEDIATED BY NUCLEAR RECEPTORS*

Received for publication, April 12, 2001, and in revised form, June 11, 2001
Published, JBC Papers in Press, June 11, 2001, DOI 10.1074/jbc.M103270200

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**Bile acids regulate the cholesterol 7α-hydroxylase gene (*CYP7A1*), which encodes the rate-limiting enzyme in the classical pathway of bile acid synthesis. Here we report a novel mechanism whereby bile acid feedback regulates *CYP7A1* transcription through the nuclear receptor *hepatocyte nuclear factor-4* (HNF-4), which binds to the bile acid response element (BARE) at nt −149/−118 relative to the transcription start site. Using transient transfection assays of HepG2 cells with Gal4-HNF-4 fusion proteins, we show that chenodeoxycholic acid (CDCA) dampened the transactivation potential of HNF-4. Overexpression of a constitutive active form of MEKK1, an upstream mitogen-activated protein kinase (MAPK) module triggered by stress signals, strongly repressed the promoter activity of *CYP7A1* via the consensus sequence for HNF-4 embedded in the BARE. Similarly, MEKK1 inhibited the activity of HNF-4 in the Gal4-based assay. The involvement of the MEKK1-dependent pathway in the bile acid-mediated repression of *CYP7A1* was confirmed by co-transfecting a dominant negative form of the stress-activated protein kinase kinase, SEK, which abolished the effect of CDCA upon *CYP7A1* transcription. Treatment of transfected HepG2 cells with tumor necrosis factor α (TNF-α), an activator of the MEKK1 pathway, led to the repression of *CYP7A1* via the HNF-4 site in the BARE. TNF-α also inhibited the transactivation potential of HNF-4. Collectively, our results demonstrate for the first time that HNF-4, in combination with a MAPK signaling pathway, acts as a bile acid sensor in the liver. Furthermore, the effects of CDCA and TNF-α converge to HNF-4, which binds to the BARE of *CYP7A1*, suggesting a link between the cascades elicited by bile acids and pro-inflammatory stimuli in the liver.**

Cholesterol is an important precursor of several compounds that provide the physiological requirements in mammals. However, an excessive accumulation of this molecule represents a risk factor for the onset of cardiovascular diseases, which are the leading cause of death in industrialized countries. The elimination of cholesterol from the body is achieved mostly through its conversion to bile acids. Bile acid synthesis is regulated at the transcription level of the cholesterol 7α-hydroxylase gene (*CYP7A1*), which encodes the rate-limiting enzyme of the classical pathway (1–5). Bile acids exert a typical feedback repression of *CYP7A1* transcription via a so-called bile acid-responsive element (BARE[1]), a sequence located between nt −149 and nt −128 relative to the cap site of the gene (5).

Recent findings provide more insight into the biochemical and molecular mechanisms underlying the down-regulation of *CYP7A1* by bile acids. It has been shown that the bile acid receptor FXR (farnesoid X receptor, NR1H4 (6)) is implicated in this regulation, which is accomplished through a fine network involving other members of the nuclear receptor superfamily like the liver receptor homolog-1 (LRH-1, NR5A2), also known as CPF (*CYP7A1* promoter binding factor) or FTF ($\alpha_1$-fetoprotein transcription factor), and the small heterodimer partner (SHP, NR0B2) (7–11). Moreover, our recent observations provide evidence that the nuclear receptor hepatocyte nuclear factor-4 (HNF-4, NR2A1) binds to a repeat sequence separated by one nucleotide, called direct repeat 1 (DR1), which is embedded in the BARE of the *CYP7A1* promoter (12, 13). This DR1 motif is perfectly conserved in all the *CYP7A1* cloned from different species, and it corresponds to a phorbol-ester response sequence (PRS) (14) mediating the repression of *CYP7A1* elicited by PKC activators. Interestingly, a PKC-dependent signaling pathway has recently been implicated in the feedback reg-

* This research was supported in part by a grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BARE, bile acid responsive element; CBP, cAMP response element binding protein-binding protein; CDCA, chenodeoxycholic acid; *CYP7A1*, cholesterol 7α-hydroxylase gene; DBD, DNA-binding domain; DR1, direct repeat separated by one nucleotide; DR5, direct repeat separated by five nucleotides; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK1, MAPK kinase 1; MEKK1, MAPK kinase kinase 1; nt, nucleotide(s); PKC, protein kinase C; RARE, retinoic acid response element; SAPK, stress-activated protein kinase; SEK, stress-activated protein kinase kinase; TNF-α, tumor necrosis factor-α; FXR, farnesoid X receptor; LHR-1, liver receptor homolog-1; CPF, *CYP7A1* promoter binding factor; SHP, small heterodimer partner; CHO, Chinese hamster ovary cells; kb, kilobase(s); PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PRS, phorbol-ester response sequence; CMV, cytomegalovirus.

ulation of *CYP7A1* (15) by bile acids via the activation of the stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) cascade (16). We therefore hypothesized that this DR1 motif may also contribute to the inhibition of *CYP7A1* transcription by bile acids via a signal transduction pathway that dampens the HNF-4-mediated activation of *CYP7A1*.

We have obtained striking evidence that bile acids can down-regulate *CYP7A1* transcription by reducing the transactivation potential of HNF-4. We also show for the first time that TNF-α can mimic the effect of bile acids and that the intracellular cascades elicited by these two extracellular stimuli converge to HNF-4, which binds to the BARE of *CYP7A1*. We have also identified a mitogen-activated protein kinase (MAPK)-dependent signaling cascade that is responsible for the repression of *CYP7A1* transcription and HNF-4 activity. Our results highlight a novel mechanism of transcription regulation that can add a further level of control of *CYP7A1* expression in response to increased intrahepatic concentrations of bile acids. In view of the tight link between the signal transduction pathways elicited by bile acids and pro-inflammatory cytokines that emerged from this study, we propose that bile acids can act as stress-type extracellular cues affecting transcription machinery via specific MAPK cascades.

## EXPERIMENTAL PROCEDURES

*Materials*—All cell culture reagents were purchased from Life Technologies Italia S.r.l. (Milano, Italy). Restriction and modification enzymes, luciferin, and the plasmids pGL3-Promoter and pGL3-Basic were obtained from Promega Italia S.r.l. (Milano). The plasmids pSG424-VP16, containing the open reading frame of the DNA-binding domain (DBD) of the yeast transcription factor Gal4 (amino acids 1–147) fused to the activation domain of the herpes simplex virus protein VP16, and pSEK-DN (expressing a dominant negative form of the stress-activated protein kinase kinase in pMT2 vector), were kindly donated by Drs. Pier Giuseppe Pelicci, Pasquale De Luca, and Enrica Migliaccio (Istituto Europeo di Oncologia, Milano). The vectors pFR-Luc, containing the luciferase cDNA driven by five copies of the Gal4-binding element and a TATA box, and pFC-MEKK1 and pFC-MEK1, expressing the constitutive active forms of MEKK1 and MEK1, respectively, were from Stratagene (La Jolla, CA). The plasmid pBxG1, bearing the DBD of the yeast transcription factor Gal4 (Gal4-DBD), and its derivative pBxG1-HNF-4-(1–455), harboring the cDNA encoding the full-length HNF-4 fused in-frame with Gal4-DBD, were kindly provided by Dr. Iannis Talianidis (Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, Herakleion, Crete, Greece). The β-galactosidase expression vector pCMVβ was from CLONTECH (Palo Alto, CA). Oligonucleotides were synthesized by Genenco-Life Science (Firenze, Italy). The Sequenase 2.0 sequencing kit and radiolabeled compounds were purchased from Amersham Pharmacia Biotech (Milano). The Qiagen plasmid purification kit was purchased from Qiagen Gmbh (Hilden, Germany). The human hepatoblastoma cell line HepG2 and the African green monkey cell line CV-1 were from American Type Culture Collection (Manassas, VA). Chinese hamster ovary (CHO) cells were a kind gift of Dr. Marina Camera (Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, Milano). Tumor necrosis factor-α (TNF-α) was obtained from Sigma-Aldrich S.r.l. (Milano). General purpose chemicals were of the highest purity available.

*Plasmid Construction*—The plasmid p−118/−148tkLuc was made by ligating a double-stranded oligonucleotide carrying a copy of the rat *CYP7A1* bile acid response element-II (BARE-II) from nt −149 to nt −118 into the vector pFlashII (SynapSys, MA) digested with *Bam*HI. pGL3−149/−118SV was made by cloning the same DNA fragment into *Bgl*II-digested pGL3-Promoter. The vector pBxG1-HNF-4-(1–249) was made by digesting pBxG1-HNF-4-(1–455) with *Nhe*I and *Xba*I and religating the 4.2-kb fragment. The plasmids pGL3−376Luc, pGL3−376m10, pGL3−376m11, pGL3−376m2, pGL3−376m13, ph−339luc, and ph−135luc have been described elsewhere (4, 12, 17). Plasmids were verified by restriction digestion, sequenced, and purified with a Qiagen plasmid purification kit according to the manufacturer's instructions.

*Cell Cultures and Transient Transfections*—HepG2 cells were cultured in Dulbecco's modified Eagle's medium:F-12 (1:1) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units of penicillin G/ml, and 100 μg of streptomycin/ml, as previously described (18). Confluent cells cultured in 24-well cluster plates were transfected for 4 h by the calcium phosphate co-precipitation technique (4), with the addition of a total of 1.5 μg of plasmid DNA/well as indicated in each figure legend. Transfected cells were treated with the indicated concentrations of chenodeoxycholic acid (CDCA) or an equivalent amount of vehicle (0.1%, v/v ethanol) for 20 h in serum-free medium. Transfected cells treated with TNF-α (dissolved in PBS containing 0.1% fatty acid-free bovine serum albumin) were incubated for 40 h under the same conditions. In experiments with MAPKs, transfected cells were incubated in serum-free medium for 20 h.

CV-1 and CHO cells were plated in 24-well cluster plates ($10^5$ cells/well) the day before transfection in Dulbecco's modified Eagle's medium:F-12 (1:1) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units of penicillin G/ml, and 100 μg of streptomycin/ml. Transfections were performed exposing CV-1 cells and CHO cells to calcium phosphate co-precipitates for 16 and 6 h, respectively, at 37 °C in a humidified atmosphere of 5% $CO_2$. Co-precipitates contained a total of 1.5 μg of plasmid DNA as specified in each figure legend. Cells were then washed with phosphate-buffered saline and incubated for further 24 h at 37 °C in the presence of the indicated concentration of CDCA or an equivalent amount of vehicle (0.1% v/v ethanol) in medium containing 10% fetal calf serum stripped with dextran-coated charcoal.

*MTT Test*—Cytotoxicity of bile acids in cell cultures was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (19). Briefly, HepG2 cells were seeded in 24-well plates and allowed to grow to confluence. Cells were then treated with increasing concentrations of bile acids for 24 h in serum-free medium. The medium was removed, and cells were incubated with a solution containing 0.5 mg of MTT/ml of PBS at 37 °C for 3 h. The MTT solution was removed, and cells were overlaid with 500 μl/well isopropanol:dimethyl sulfoxide (9:1, v/v) for 15 min at 37 °C. Aliquots of 100 μl were read on a plate reader (model 3550, Bio-Rad, Milano) at 560 nm using a reference wavelength of 690 nm.

*Enzyme Assays*—Luciferase assays were performed using a luminometer (Lumat 9501, Berthold, Germany) as previously described (18). β-Galactosidase assays were carried out with chlorophenol red-β-D-galactopyranoside (Roche Molecular Biochemicals, Milano) as a substrate, and samples were analyzed in a plate reader (model 3550, Bio-Rad). Results are expressed as the ratio of luciferase activity over β-galactosidase activity and represent the mean ± S.D. values of triplicate samples. Each experiment was repeated at least twice. Statistical analyses were performed with unpaired Student's *t* test using MS Excel 98 for Macintosh (Microsoft, Redmon, WA).

## RESULTS

*Chenodeoxycholic Acid Represses CYP7A1 Transcription via the BARE at nt −149/−118*—In previous experiments we used the tauroconjugated form of chenodeoxycholic acid, which displays its effect on *CYP7A1* transcription in HepG2 cells only at high concentrations (*e.g.* 100 μM), because of the lack of the bile acid co-transport system in this cell line (5, 20). We tested the effect of unconjugated CDCA in concentration-response experiments using the plasmid p−149/−118tkLuc harboring the BARE of *CYP7A1* at nt −149/−118 cloned in front of the herpes simplex virus thymidine kinase (hsv-tk) minimal promoter and the luciferase reporter gene. As shown in Fig. 1, CDCA significantly decreased the promoter activity of p−149/−118tkLuc in a concentration-dependent fashion (5–25 μM). The ptkLuc vector did not respond to any of the tested concentrations, which demonstrates that the BARE is required to repress the transcription of the reporter system. Viability and cell functionality were checked by the MTT test, which indicated that CDCA manifested signs of toxicity only at concentrations above 25 μM (data not shown). Thus, in all subsequent experiments we never exceeded 25 μM, to avoid cell toxicity.

*CDCA Represses the Transactivation Potential of HNF-4*—Because the BARE at −149/−118 of *CYP7A1* contains a DR1 motif, which we have previously shown to bind HNF-4, we investigated whether this liver-enriched nuclear receptor plays an active role in the transcription regulation of this gene by bile acids. To this end, we constructed a chimeric HNF-4 fused with the DBD of the yeast transcription factor Gal4. This Gal4-

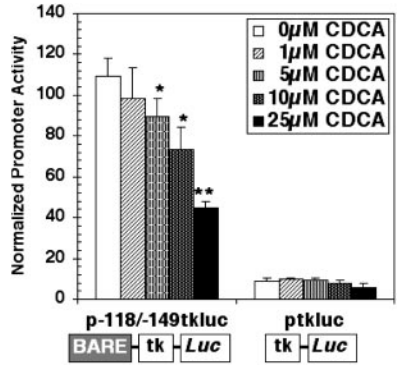


FIG. 1. **Unconjugated bile acids repress the transcription of *CYP7A1* via the bile acid responsive element at nt −149/−118.** Confluent cultures of HepG2 cells were transfected with 1.4 μg of luciferase plasmids and 100 ng of β-galactosidase expression vector (pCMVβ). Transfected cells were incubated in the presence of the indicated concentrations of CDCA or an equivalent amount of vehicle (0.1% ethanol) for 20 h. Results are expressed as ratio of luciferase to β-galactosidase and are the mean ± S.D. of experiments performed in triplicate. The *asterisks* (* and **) indicate statistical significance of treated samples *versus* controls at $p < 0.05$ and 0.01, respectively.

based assay allowed us to test the effect of CDCA on HNF-4 independently of the *CYP7A1* promoter context, thus avoiding the interference of other endogenous transcription factors binding to the BARE. The fusion of the entire HNF-4 open reading frame to Gal4-DBD increased the basal transcription activity more than 10-fold (Fig. 2*A*). Treatment of transfected HepG2 cells with 25 μM CDCA decreased the transcription activity of Gal4-HNF-4 by about 60%. The ablation of the HNF-4 C-terminal, which contains the ligand-binding domain of the nuclear receptor and the activation function-2, decreased the basal activity of the fusion protein (Fig. 2*A*, pGal4-HNF-4-(1–249)). Nonetheless, the amino acid sequence 1–249 of HNF-4 retained the responsiveness to CDCA.

To confirm the specificity of CDCA toward HNF-4, we also tested the chimeric transcription factor containing the activation domain of the viral protein VP16 fused to the Gal4-DBD. As shown in Fig. 2*B*, CDCA did not affect the transactivation potential of pGal4-VP16; we conclude that the effect of bile acids was specific to HNF-4 and was not due to a generic toxic effect.

*The Negative Effect of CDCA on the Transcription Activity of HNF-4 Is Liver-specific*—To investigate whether bile acids could affect the transactivation potential of HNF-4 in a non-liver environment, we carried out co-transfection experiments with the Gal4-based assay in the fibroblast cell line CV-1 and in the epithelial cell line CHO. Here, CDCA did not repress the transcription activity of any of the Gal4-HNF-4 constructs tested (Fig. 2, *C* and *D*). This result paralleled the lack of responsiveness of the *CYP7A1* promoter/luciferase fusion gene when transfected into a non-liver cell line, CV-1 (Fig. 2*C* and Ref. 5). Similar results were obtained with CHO cells transfected with the same plasmids (data not shown).

*MEKK1-dependent Signaling Pathway Mimics the Effect of Bile Acids*—Bile acids have been shown to decrease *CYP7A1* mRNA levels through activation of PKC-δ (15). We have shown that BARE coincides with the sequence conferring the negative responsiveness to activators of PKC such as phorbol esters (14). Because PKCs were previously shown to activate MAPKs in different cell systems (21, 22), we tested the possible involvement of MAPK family members in the regulation of *CYP7A1* transcription. Ectopic expression of a constitutive active form of MEKK1, a member of the MAPK family activated by stress stimuli, dramatically reduced the transcription activity of the rat *CYP7A1*/luciferase fusion gene (Fig. 3*A*, pGL3−376Luc). MEKK1 also repressed the transcription of the human *CYP7A1*/luciferase chimera (Fig. 3*C*). Remarkably, the overexpression of MEKK1 resulted in the repression of the promoter activity of BARE cloned in front of the heterologous SV40 promoter (Fig. 3*A*). It should be noted that in this case the effect was not as strong as with the native promoter, which suggests that activation of the MEKK1-dependent pathway may affect *CYP7A1* transcription via several promoter sequences. The construct containing only SV40 promoter in front of the luciferase gene did not respond to MEKK1. To verify whether other MAPK pathways may be involved in the regulation of *CYP7A1*, we tested the constitutive active form of MEK1, a member of the MAPK family activated by growth factors (23). The ectopic expression of MEK1 did not inhibit the *CYP7A1* promoter, either in its natural context (pGL3−376Luc) or with the BARE cloned in front of the heterologous SV40 promoter (Fig. 3*B*). The stimulation of the transcription activity observed with the latter construct was likely due to the effect of MEK1 on SV40 promoter, because the control vector lacking the BARE was stimulated to the same extent (Fig. 3*B*). Site-directed mutagenesis of the rat *CYP7A1* allowed us to map precisely the target sequence of MEKK1 cascade to the DR1 sequence, which is the binding site for HNF-4. Fig. 3*C* shows that substitution of the six nucleotides flanking the binding site of HNF-4 (pGL3−376m10) did not alter the response of the rat *CYP7A1* promoter to the activated form of MEKK1. Conversely, when the 5′-hexanucleotide of the DR1 sequence was mutated, thus destroying the HNF-4 binding site but preserving the LRH-1/CPF consensus sequence (Fig. 3*C*, pGL3−376m11), *CYP7A1* did not respond to MEKK1. As expected, the mutation of the 3′-hexanucleotide of the DR1 sequence also prevented the inhibition of *CYP7A1* transcription by MEKK1 (Fig. 3*C*, pGL3−376m2). To rule out the implication of LRH-1/CPF further we also tested a mutant in the 3′-hexanucleotide of the DR5 sequence, which is a retinoic acid response element (RARE) (12). The sequence of this mutant (Fig. 3*C*, pGL3−376m13) carries the T for G substitution at nt −128 of the rat *CYP7A1* promoter (wt −139 AGT**TCAAGGCCG**GGTAA −123, mutant m13 −139 AGT**TCAAGGCCt**ttgcc −123), which is a key nucleotide for LRH-1/CPF binding (24). As shown in Fig. 3*C*, pGL3−376m13 still responded to MEKK1, thus ruling out the involvement of LRH-1/CPF in this type of regulation. Finally, to confirm that the inhibition elicited by MEKK1 was solely mediated by HNF-4, we also tested a deletion of the human *CYP7A1* promoter (Fig. 3*C*, ph−135luc) that lacked the HNF-4 binding site but retained the LRH-1/CPF consensus sequence. This deletion mutant was not inhibited by MEKK1, whereas the construct containing the full DR1 sequence (Fig. 3*C*, ph−339luc) was strongly repressed. Collectively, these results prove that the MEKK1-dependent pathway mimics the inhibition of bile acids on *CYP7A1* promoter and its target is the HNF-4 binding site in the BARE.

*MEKK1 Down-regulates the Transcription Activity of HNF-4*—Because the MEKK1-dependent signaling pathway specifically mimics the effect of bile acids on *CYP7A1* promoter, we next asked whether MEKK1 could also regulate the activity of HNF-4, which binds to the BARE. For this purpose, we used the Gal4-based assay to test the effect of ectopic expression of constitutive active MEKK1. The results in Fig. 4*A* show that the transcription activity of HNF-4 was indeed down-regulated by MEKK1. The truncated form of HNF-4 (pGal4-HNF-4-(1–249)) was also negatively affected by MEKK1. Conversely, the Gal4-VP16 chimera was stimulated by MEKK1 (Fig. 4*B*), indicating that the negative effect was specific for HNF-4. Ectopic

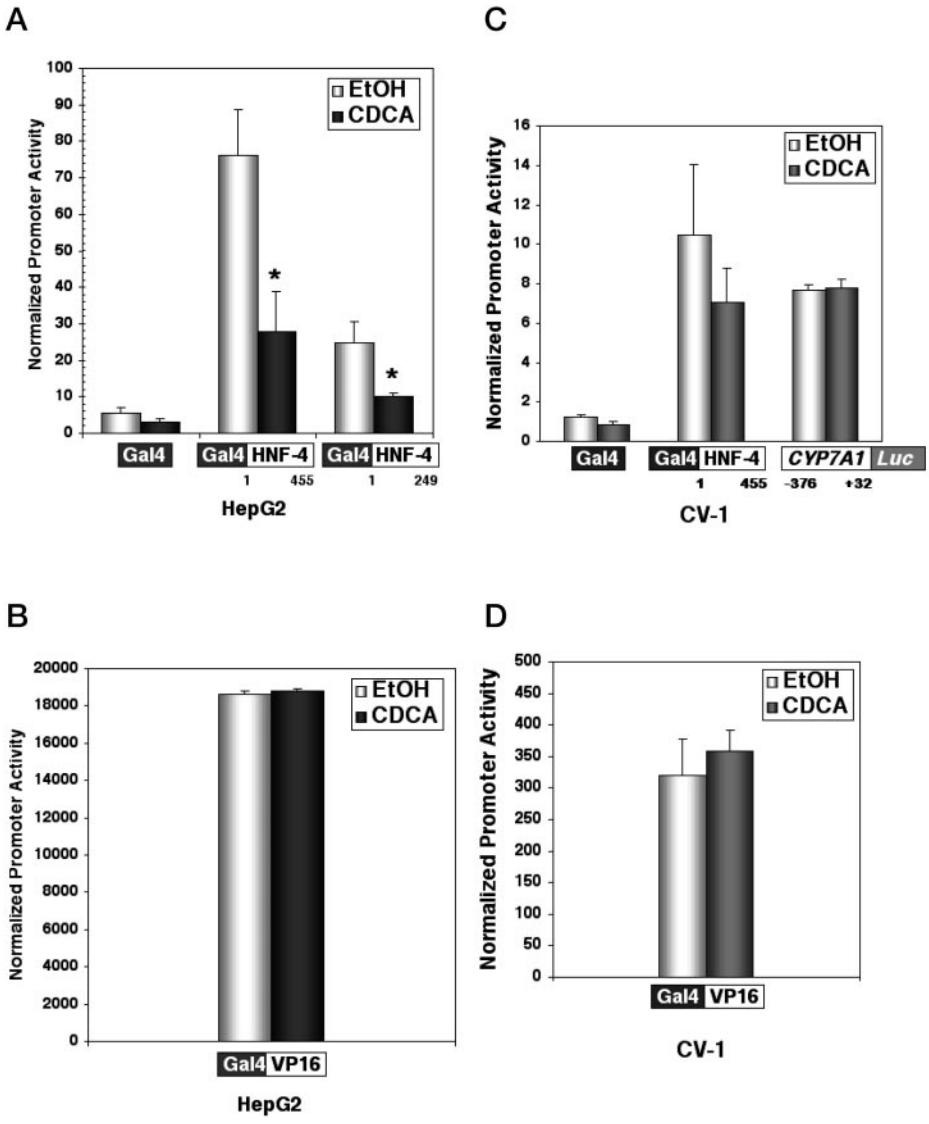


FIG. 2. **CDCA attenuates the transactivation potential of HNF-4 in the liver.** *A* and *B*, confluent cultures of HepG2 cells were transfected with 900 ng of pFR-Luc, 500 ng of expression plasmid for Gal4 fusion protein as indicated in the figure, and 100 ng of pCMVβ. *C* and *D*, CV-1, cells were transfected with 1.1 μg of pFR-Luc, 100 ng of expression plasmid for Gal4 fusion protein, and 300 ng of pCMVβ or, alternatively, with 1.2 μg of pGL3−376Luc (*CYP7A1–376/+32/Luc*) and 300 ng of pCMVβ as indicated. The legends at the *bottom of each graph* indicate that the cells were co-transfected with a vector carrying either Gal4DBD alone (*Gal4*) or Gal4DBD fused to full-length HNF-4 (*Gal4-HNF-4/1–455*) or Gal4-DBD fused to the first 249 amino acids of HNF-4 (*Gal4-HNF-4/1–249*) or Gal4-DBD fused to the activation domain of VP16 (*Gal4-VP16*). Transfected cells were treated with 25 μM CDCA (*A* and *B*) or 10 μM CDCA (*C* and *D*) or vehicle for 20 h. Results are expressed as a ratio of luciferase to β-galactosidase and are the mean ± S.D. of experiments performed in triplicate. The *asterisk* indicates statistical significance of treated samples *versus* controls at $p < 0.05$.

expression of constitutive active MEK1 did not repress the transactivation potential of HNF-4 (Fig. 4*C*). The stimulatory effect observed with this MAPK may be, at least in part, attributable to the backbone vector, because Gal4 alone was stimulated by MEK1. Similarly, Gal4-VP16 was stimulated by MEK1 (Fig. 4*D*).

*TNF-α Represses the Transcription of CYP7A1 via HNF-4 and BARE*—To demonstrate further that the activation of the MEKK1 pathway can reduce the transcription of *CYP7A1* and the activity of HNF-4, we tested the effect of TNF-α, a pro-inflammatory cytokine that induces the stress-activated family of MAPKs in different cell types (25–27). Treatment of HepG2 cells with TNF-α reduced transcription of the rat *CYP7A1*/luciferase fusion genes (Fig. 5*A*, pGL3−376Luc). The sequence spanning the BARE from nt −149 to nt −118, which contains the binding site for HNF-4 (DR1), conferred the responsiveness to TNF-α on the heterologous SV40 promoter (Fig. 5*A*, compare *pGL3-149/−118SV* to *pGL3-Promoter*). Interestingly, this effect was prevented when the first hexanucleotide of the HNF-4 binding site (wtDR1: **TGGACTTAGTTCA**) in the BARE was mutated (mutDR1: **ccacag**TAGTTCA) (Fig. 5*A*, pGL3−376Luc *versus* pGL3−376m11). It is worth mentioning that, in the mutant pGL3−376m11, the HNF-4 binding site is disrupted, whereas the consensus sequence for LRH-1 is intact; consequently, the effect of TNF-α cannot be mediated by this latter nuclear receptor. In similar experiments, the human *CYP7A1* promoter was repressed by TNF-α (Fig. 5*A*, ph−339luc) whereas the deletion down to nt −135, which lacks the first hexanucleotide of the HNF-4 binding site (DR1), did not respond to the cytokine (Fig. 5*A*, ph−135luc). Because HNF-4 is the transcription factor binding to the DR1 sequence in the BARE of *CYP7A1* promoter, we also tested the effect of TNF-α in the Gal4-based assay to assess whether this nuclear receptor was responsible for the effect of the cytokine on the *CYP7A1* transcription. As shown in Fig. 5*B*, TNF-α repressed the transcription activity of Gal4-HNF-4, either its full-length or its C-terminal deletion 1–249, which lacks the ligand-binding domain. Conversely, TNF-α did not affect the activity of Gal4-VP16 (Fig. 5*C*). On the basis of these results, we conclude that TNF-α impinges on *CYP7A1* transcription through HNF-4 that binds to BARE.

*Blockade of MEKK1 Pathway Prevents Bile Acid-mediated Repression of CYP7A1*—After determining that the MEKK1-dependent signal transduction pathway recapitulates the effect of bile acids on *CYP7A1* transcription through the repression of HNF-4 transcription activity, we wanted to prove the actual involvement of this signaling pathway in the cascade elicited by bile acids. To this end, we performed co-transfection exper-

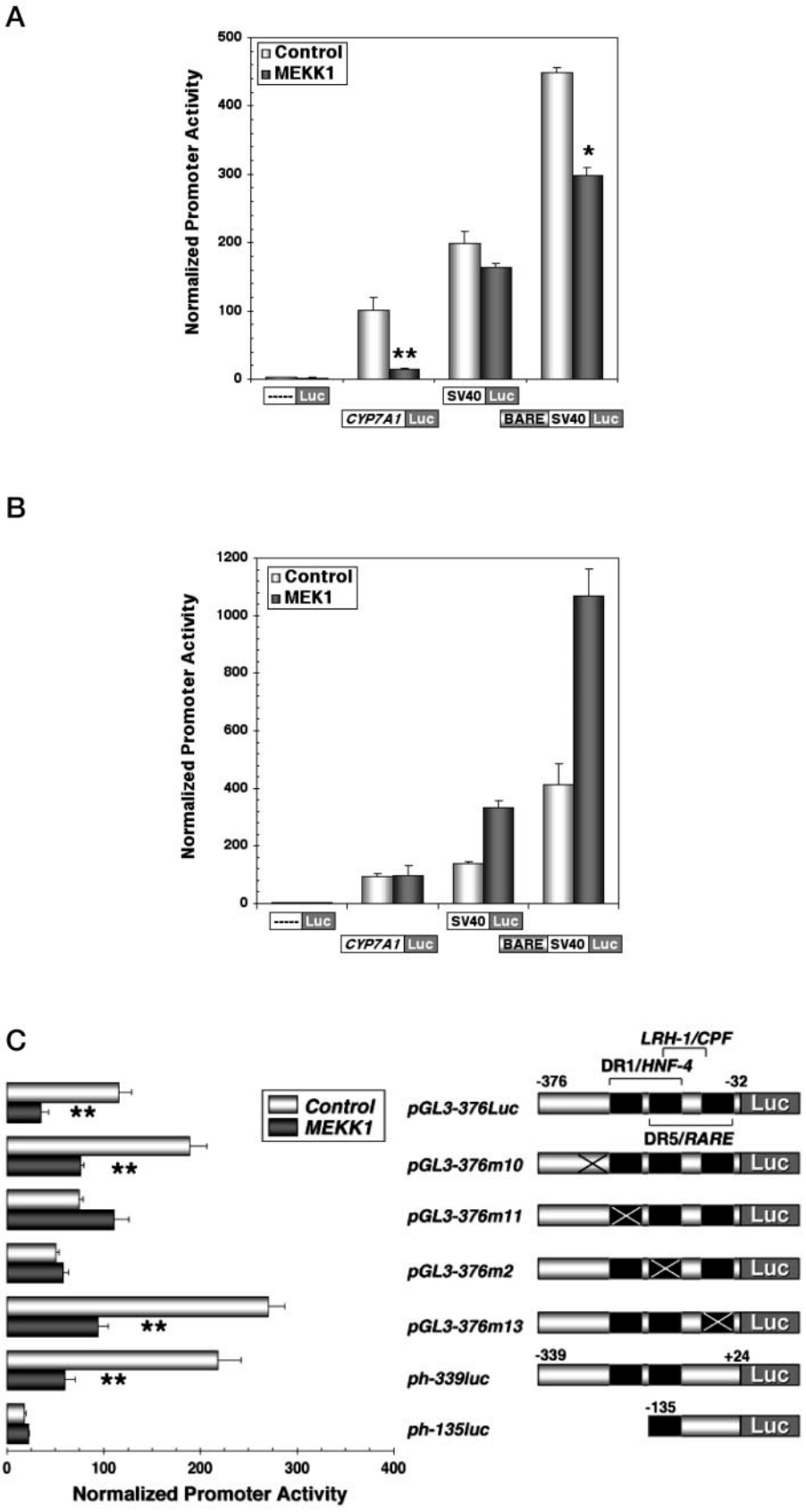


FIG. 3. **Effect of MAPKs on the transcription of *CYP7A1* promoter/luciferase chimeric genes.** Confluent cultures of HepG2 cells were transfected with 1.3 $\mu$g of luciferase plasmids, 75 ng of either pFC-MEKK1, an expression plasmid for a constitutive active form of MEKK1 (*A*), or pFC-MEK1, an expression plasmid for MEK1 (*B*), and 100 ng of pCMV$\beta$. Control samples were transfected with 75 ng of salmon sperm DNA as a carrier. SV40/Luc indicates that the luciferase gene is driven by SV40 promoter (pGL3-Promoter); *BARE-SV40/Luc* indicates that the luciferase gene is driven by one copy of the BARE at nt −149/−118 of *CYP7A1* in front of the SV40 promoter (pGL3-149/−118SV); *—Luc* is a promoterless luciferase vector (pGL3-Basic); *CYP7A1/Luc* indicates that the luciferase gene is driven by the rat *CYP7A1* promoter sequence at nt −376/+32 (pGL3−376Luc). *C*, HepG2 cells were transfected as in *A*; mutants of the rat *CYP7A1* upstream sequence were used to map the nucleotides mediating the effect of ectopic expression of constitutive active MEKK1. Mutant m10 was mutated in the six nucleotides immediately preceding the HNF-4 binding site (DR1 sequence); mutant m11 was mutated in the 5′-hexanucleotide of the DR1 leaving the LRH-1/CPF binding site intact (wt: −146 **TGGACTTAGTTCA**AGGCC**GGGTAA** −123 *versus* mut m11: −146 **ccacagTAGTTCATCA**AGGCC**GGGTAA** −123; note that DR1 and DR5 sequences are marked in *boldface* whereas the LRH-1 site is *underlined*); mutant m2 was mutated in the 3′-hexanucleotide of the DR1 thus destroying both HNF-4 and LRH-1/CPF binding sites (mut m2: −146 **TGGACTTctcTtA**ttGCC**GGGTAA** −123); mutant m13 was mutated in the 3′-hexanucleotide of the retinoic acid response element (*RARE/DR5* sequence) causing also the G to T mutation of the last nucleotide of the LRH-1/CPF binding site (mut m13 −146 **TGGACTTAGTTCA**AGGCC**tttgcc** −123). The plasmid ph−339luc contains the human *CYP7A1* sequence from nt −339 to +24, with a functional DR1/HNF-4 site; *ph−135luc* bears the human *CYP7A1* sequence from nt −135 to +24 where the DR1/HNF-4 site was deleted but the LRH-1/CPF site was still unaltered (*ph−339luc*: −144 **TGGACTTAGTTCA**AGGCCAGTTAC −121 *versus* ph−135luc −135 **TTCA**AGGCCAGTTAC −121. Note that the human *CYP7A1* upstream sequence does not contain a functional RARE/DR5. Results are expressed as a ratio of luciferase to $\beta$-galactosidase and are the mean ± S.D. of experiments performed in triplicate. The *asterisks* (* and **) indicate statistical significance of treated samples *versus* controls at $p <$ 0.005 and 0.05, respectively.

iments using a dominant negative mutant of SEK, a MAPK of the stress-activated pathway lying immediately downstream of MEKK1. CDCA repressed the *CYP7A1*/luciferase gene about 2-fold, but when dominant negative SEK was overexpressed, CDCA failed to repress the promoter activity of *CYP7A1* (Fig. 6). Therefore, the genetic approach that blocks the MEKK1

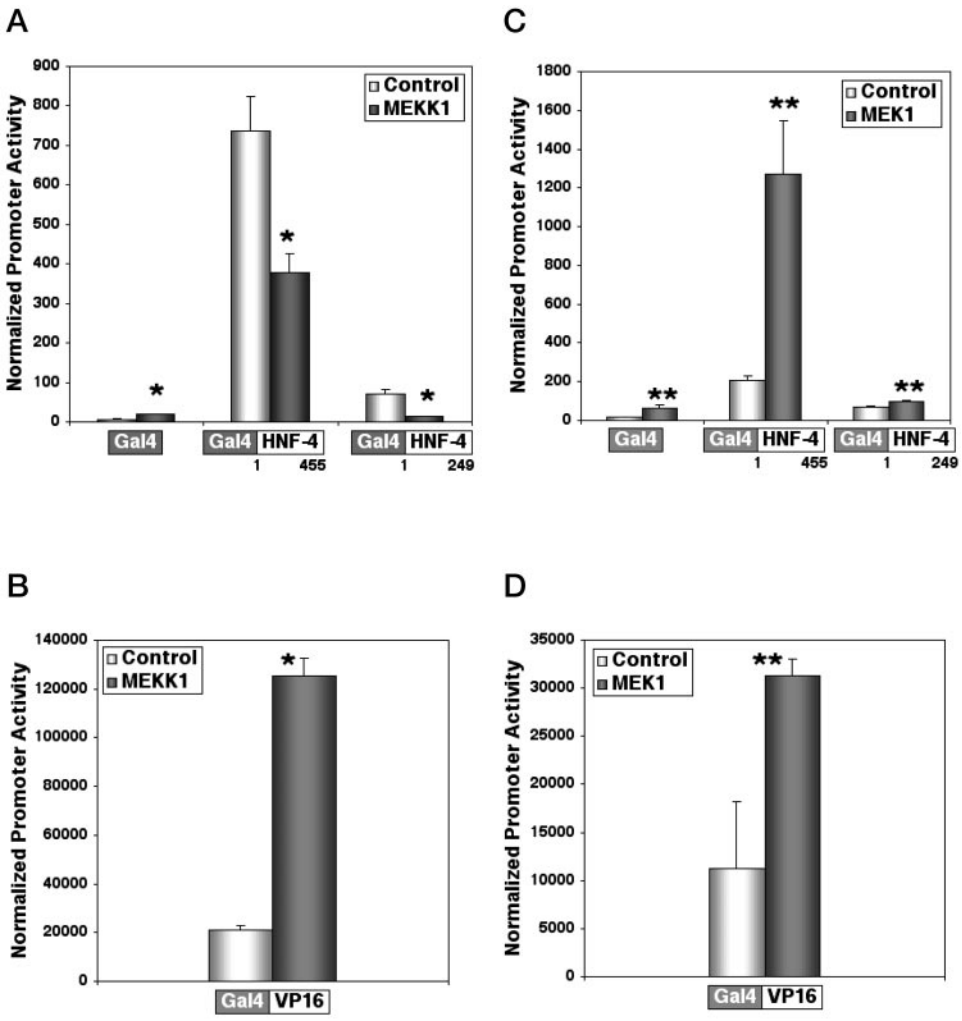


FIG. 4. **MEKK1 specifically inhibits the transactivation potential of HNF-4.** Confluent cultures of HepG2 cells were transfected with 1.3 μg of pFR-Luc, 75 ng of expression plasmid for Gal4 fusion protein as indicated in the figure, 75 ng of either pFC-MEKK1, an expression plasmid for a constitutive active form of MEKK1 (*A* and *B*), or pFC-MEK1, an expression plasmid for MEK1 (*C* and *D*), and 100 ng of pCMVβ. Control samples were transfected with 75 ng of salmon sperm DNA as a carrier. The legends at the *bottom of each graph* indicate that the cells were co-transfected with a vector carrying either Gal4-DBD alone (*Gal4*), Gal4-DBD fused to full-length HNF-4 (*Gal4-HNF-4/1–455*), Gal4-DBD fused to the first 249 amino acids of HNF-4 (*Gal4-HNF-4/1–249*), or Gal4-DBD fused to the activation domain of VP16 (*Gal4-VP16*). Results are expressed as a ratio of luciferase to β-galactosidase and are the mean ± S.D. of experiments performed in triplicate. The *asterisks* (* and **) indicate statistical significance of treated samples *versus* controls at $p < 0.005$ and 0.05, respectively.

pathway supports the idea that bile acids can regulate *CYP7A1* transcription by repressing HNF-4 transactivation potential via the MEKK1 signaling cascade.

## DISCUSSION

In this report we provide new insights into the regulation of *CYP7A1* and report a novel mechanism whereby bile acids affect the transcription of *CYP7A1*. By using a Gal4-based assay we show that bile acids inhibit the transactivation potential of HNF-4, a major transcription factor binding to the bile acid responsive element of *CYP7A1* (12). The region conferring the responsiveness to bile acids is within the first 249 amino acids of the nuclear receptor, which includes the ligand-independent activation function AF-1 in domain A/B and the DNA-binding domain. This region of HNF-4 contains several amino acids that can undergo post-translational modifications in response to extracellular stimuli. For example, protein kinase A was shown to inhibit the DNA-binding activity of HNF-4 via the phosphorylation of serines 133–134 (28). In preliminary experiments it was also shown that treatment of HepG2 cells with phorbol esters decreases the DNA-binding activity of HNF-4 to the BARE of *CYP7A1*.[2] More recently, it has also been reported that the transcription activity of HNF-4 can be modulated by acetylation of lysines 97, 99, 117, and 118 in the region containing the nuclear localization signal and the second zinc finger of the DNA-binding domain, via the histone acetyltransferase activity of CBP/p300 (29). Thus, it is possible that bile acids can modulate the transcription activity of HNF-4 by affecting the post-translational modification state of the receptor. Post-translational modifications of HNF-4 may alter the interactions with co-activators/co-repressors and the ability to recruit the pre-initiation complex on the TATA box of *CYP7A1* promoter. Alternatively, bile acids may induce post-translational modifications of co-activators or co-repressors interacting with HNF-4. Indeed, it has already been reported that extracellular signals can change the phosphorylation state and the activity of CBP/p300 and other transcription mediators (30–32). The observation that bile acids down-regulated the *CYP7A1* promoter- and Gal4-HNF-4-driven transcription only

[2] A. Sadeghpour, M. Crestani, and J. Y. L. Chiang, unpublished results.

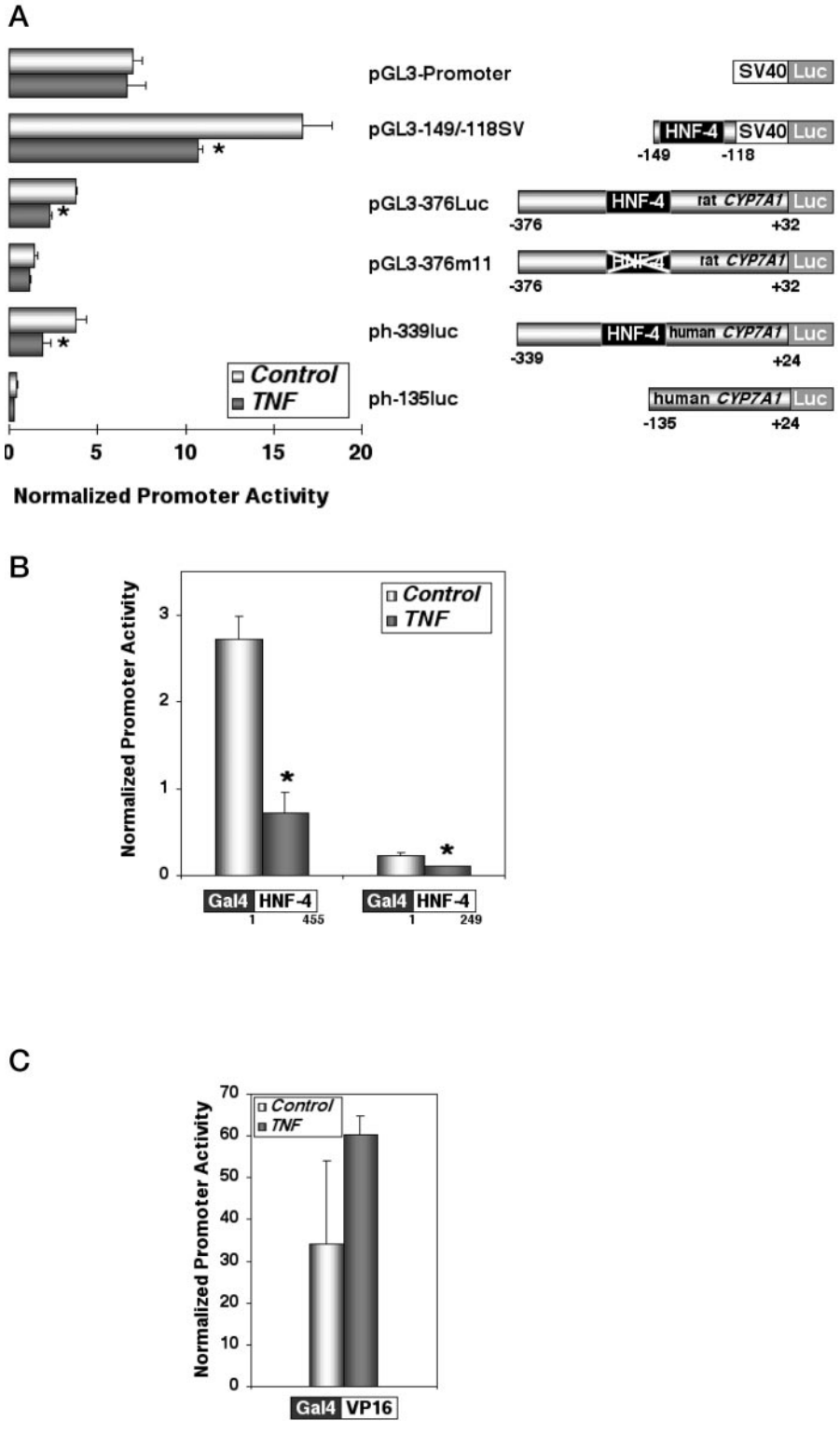


FIG. 5. **TNF-α inhibits the transcription of *CYP7A1* via HNF-4 binding to BARE.** *A*, confluent HepG2 cells were transfected with 1.2 μg of the indicated *CYP7A1* promoter/luciferase fusion genes and 300 ng of pCMVβ. Transfected cells were treated with 15 ng of TNF-α/ml or a corresponding amount of vehicle (PBS/0.1% fatty acid-free albumin) for 40 h in serum-free medium. *B* and *C*, confluent cultures of HepG2 cells were transfected with 700 ng of pFR-Luc, 300 ng of pCMVβ, and 500 ng of a vector carrying either Gal4-DBD fused to full-length HNF-4 (*Gal4-HNF-4/1–455*), Gal4-DBD fused to the first 249 amino acids of HNF-4 (*Gal4-HNF-4/1–249*), or Gal4-DBD fused to the activation domain of VP16 (*Gal4-VP16*). Transfected cells were treated as indicated in *A*. Results are expressed as ratio of luciferase to β-galactosidase and are the mean ± S.D. of experiments performed in triplicate. The *asterisk* indicates statistical significance of treated samples *versus* controls at $p < 0.05$.

in hepatic cells is intriguing, because it raises the possibility that some liver-specific factor other than HNF-4 may be required for the regulation by bile acids. Our current investigations are aimed at assessing whether a liver-enriched co-activator/co-repressor or specific subtypes of MAPK cascade members are involved in this phenomenon.

Our experimental evidence indicates that bile acids inhibit the activity of HNF-4 and the transcription of *CYP7A1* through a MAPK pathway. In particular, the MAPK cascade activated by stress signals is specifically involved in the regulation of *CYP7A1* by bile acids, as assessed in co-transfection assays with a constitutive active form of MEKK1 and with a dominant negative mutant of SEK, which prevented the effect of bile acids on *CYP7A1* promoter. Gupta *et al*. (16) reported that bile acids decrease *CYP7A1* mRNA levels by the PKC-dependent activation of the SAPK/JNK pathway, which causes the increase of SHP gene transcription and eventually the down-regulation of *CYP7A1* via interaction with LRH-1 (see discussion below). In this report, we show for the first time that the activation of the MEKK1-signaling cascade by bile acids depresses the activity of the nuclear receptor HNF-4 and ultimately the transcription of *CYP7A1*. Because SHP was shown

to interact with and dampen the transactivation potential of HNF-4 (33), it is possible that bile acids can also affect HNF-4 activity through JNK-mediated stimulation of the SHP gene.

It should be noted that del Castillo-Olivares and Gil (10) showed that mutation of the HNF-4 binding site did not impair the regulation of *CYP7A1* by bile acids. On the other hand, we also reported that the HNF-4 binding site was required for bile acid feedback regulation of *CYP7A1* (5). The reasons of this discrepancy are not clear but might be due to the different cellular systems used to perform these studies. At any rate, our results do not conflict with these data, because bile acids can affect the transcription of *CYP7A1* through both FXR/LRH-1 and HNF-4/MEKK1 pathways (see discussion below).

The other finding described in this report is that the pro-inflammatory cytokine TNF-α represses the transcription of *CYP7A1* by converging with bile acid signaling to HNF-4, which binds to the BARE (Fig. 7). It was previously shown that bacterial endotoxins and pro-inflammatory cytokines decrease the mRNA levels and activity of cholesterol 7α-hydroxylase in golden Syrian hamsters (34). More recently, Miyake and co-workers (35) have correlated the repression of *CYP7A1* mRNA levels by bile acids with the production of TNF-α and interleukin 1 by macrophages in the liver. Our results support these observations and, most importantly, reveal that HNF-4 is the target transcription factor mediating the effect of these pro-inflammatory cytokines on cholesterol 7α-hydroxylase gene. Moreover, given that the effects of bile acids and TNF-α converge to the same nuclear protein, one might speculate that bile acids themselves could be considered pro-inflammatory agents, which impair hepatic cellular functions and lead to the manifestation of certain liver diseases (*e.g.* cholestasis and cirrhosis) when they reach high intracellular concentrations. The fact that the treatment of mice with rosiglitazone, a peroxisome proliferator-activated receptor γ agonist with anti-inflammatory activity, prevented the repression of *CYP7A1* by a bile acid-rich diet (35) strongly argues for this possibility.

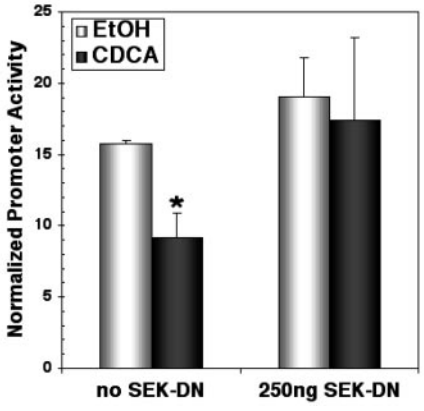


FIG. 6. **Blockade of MEKK1 signaling prevents bile acid-mediated repression of *CYP7A1* transcription.** Confluent cultures of HepG2 cells were transfected with 400 ng of pGL3−376Luc, a plasmid containing the sequence between nt −376 and +32 of the rat *CYP7A1* promoter, 250 ng of pMT2-SEK-DN, an expression vector for a dominant negative form of SEK, 750 ng of pMT2, and 100 ng of pCMVβ. Control samples (*no SEK-DN*) were co-transfected with 1 μg of empty expression vector pMT2. Transfected cells were treated with 10 μM CDCA or vehicle (0.1% ethanol) for 20 h in serum-free medium. Results are expressed as a ratio of luciferase to β-galactosidase and are the mean ± S.D. of experiments performed in triplicate. The *asterisk* indicates statistical significance of treated samples *versus* controls at $p < 0.05$.

Interestingly, TNF-α has been implicated as one of the factors contributing to insulin resistance, type-2 diabetes, and dyslipidemia (Ref. 36 and references therein). We also showed that HNF-4 is one of the target transcription factors mediating the negative effect of insulin on *CYP7A1* transcription (18). Mutations in HNF-4 can also lead to maturity-onset diabetes of the young type 1 (37). Also, Hayhurst *et al.* (38) developed a conditional HNF-4 knock-out mouse model that allowed assessment of the central role of this nuclear receptor in the maintenance of hepatocyte differentiation and lipid homeostasis. Consequently, it is conceivable that HNF-4 behaves as a master regulator of *CYP7A1* transcription, which can sense and mediate the effects of several extracellular cues converging to this key nuclear receptor.

Recently, several laboratories have found that bile acid feedback regulates *CYP7A1* transcription by a unique network involving the bile acid receptor FXR and the orphan nuclear receptors SHP and LRH-1/CPF (7–11, 16). Thus, one may wonder about the biological significance of the existence of multiple mechanisms for controlling *CYP7A1* transcription by bile acids in the liver. In this regard, it should be emphasized that these mechanisms are not mutually conflicting but may well coexist. Although experiments with knock-out mice emphasized the important role of FXR in bile acid-mediated feedback of *CYP7A1* transcription, some regulation of mRNA levels could

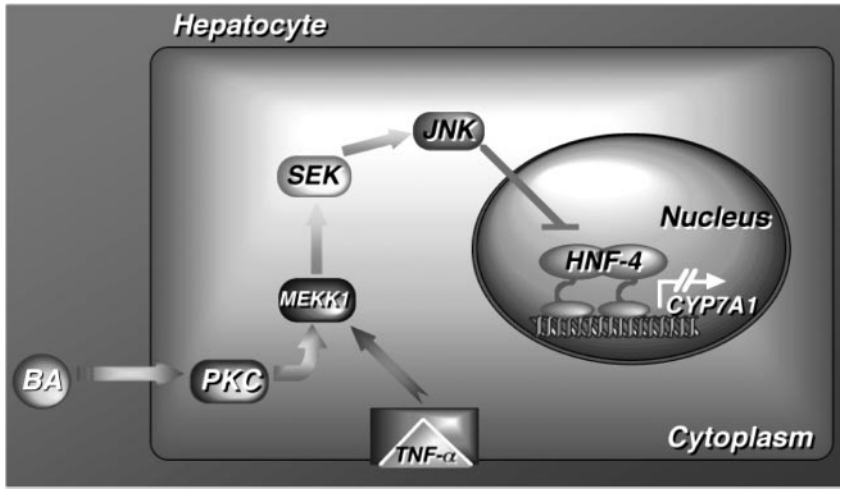


FIG. 7. **Model of *CYP7A1* regulation by bile acids and TNF-α.** The graphic shows the convergence of bile acid and TNF-α signaling cascades to HNF-4 and *CYP7A1* in the hepatocyte. Bile acids (*BA*) activate protein kinase C (*PKC*), which in turn activates the upstream MAPK MEKK1 (see Ref. 39). TNF-α binds to the cytokine receptor and activates MEKK1 (for simplification, intermediate steps are not shown). MEKK1 activates the downstream stress-activated protein kinases SEK1 and JNK. This signaling pathway decreases the transactivation potential of HNF-4 and ultimately the transcription rate of cholesterol 7α-hydroxylase gene (*CYP7A1*).

still be observed in *Fxr* null mice treated with bile acids (11). This can be explained by the results presented in this report. Also, ligand-bound FXR may affect HNF-4 transcription potential via stimulation of SHP transcription and its interaction with HNF-4; these two pathways may, therefore, cooperate in the regulation of gene transcription. In any case, the MEKK1/HNF-4 and FXR/SHP/LRH-1 pathways seem to act at different levels. The first pathway may effect short-term regulation, and as such it would only require the post-translational modifications that impair the competence of HNF-4 to transactivate *CYP7A1* transcription, allowing rapid adaptation to changes in intrahepatic bile acid concentrations. The second pathway displays the feature of long-term regulation, because it requires *de novo* synthesis of SHP to repress *CYP7A1* transcription promoted by LRH-1.

Thus, given the different nature of the mechanisms, these pathways may actually operate in combination to achieve the tight regulation of bile acid synthesis and to assure a prompt response of hepatic cells to excessive load of this class of molecules in the liver. Bile acids are amphipathic compounds: they exhibit both positive and negative effects, in that they are required to help the digestion and absorption of lipid-soluble components of the diet but can also be toxic if they reach high concentrations in certain tissues (*e.g.* the liver). It is possible that evolution has selected several levels of regulation of bile acid synthesis to provide the required amount of these molecules to the organism and at the same time to protect hepatic cells from their potentially harmful effects.

In conclusion, our results reveal a novel pathway of regulation of *CYP7A1* transcription by bile acids, which involves the nuclear receptor HNF-4. Moreover, TNF-α displays a similar effect that converges on the same transcription factor binding to the BARE of *CYP7A1* (Fig. 7). At present, it remains to be assessed whether bile acids impair the transactivation potential of HNF-4 via post-translational modifications of the receptor or by affecting the capacity of co-activators/co-repressors to interact with it. The next logical steps will be to determine the amino acids that are targeted by the bile acid- and TNF-α-induced signaling cascades and to study their effects on the interactions between HNF-4 and co-activators/co-repressors. The definition of the detailed molecular mechanisms underlying this type of regulation will help shed light on this fundamental biochemical problem and will contribute to the discovery of new molecules affecting bile acid and cholesterol metabolism.

*Acknowledgments*—We thank Dr. Pier Giuseppe Pelicci (Istituto Europeo di Oncologia, Milano, Italy) and Dr. Iannis Talianidis (IMBB-FORTH, Crete, Greece) for providing us with some of the plasmids used in this research. We also thank Prof. Norman B. Javitt (New York University, New York, NY) and Dr. Marina Camera (Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, Milano, Italy) for critically reviewing the manuscript.

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# The Negative Effects of Bile Acids and Tumor Necrosis Factor-α on the Transcription of Cholesterol 7 α-Hydroxylase Gene (*CYP7A1*) Converge to Hepatic Nuclear Factor-4: A NOVEL MECHANISM OF FEEDBACK REGULATION OF BILE ACID SYNTHESIS MEDIATED BY NUCLEAR RECEPTORS

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*J. Biol. Chem. 2001, 276:30708-30716.*
*doi: 10.1074/jbc.M103270200 originally published online June 11, 2001*

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