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Down-regulation of Cholesterol 7α-Hydroxylase (*CYP7A1*) Gene Expression by Bile Acids in Primary Rat Hepatocytes Is Mediated by the c-Jun N-terminal Kinase Pathway*

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Cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the neutral pathway of bile acid biosynthesis, is feedback-inhibited at the transcriptional level by hydrophobic bile acids. Recent studies show that bile acids are physiological ligands for farnesoid X receptor (FXR). Activated FXR indirectly represses *CYP7A1* transcription through induction of small heterodimer protein (SHP-1). In this study, we provide evidence that bile acids rapidly down-regulate *CYP7A1* transcription via activation of the JNK/c-Jun pathway. Furthermore, we demonstrate that SHP-1 is also a direct target of activated c-Jun. In primary rat hepatocyte cultures, taurocholate (TCA) strongly activated JNK in a time- and concentration-dependent manner. Tumor necrosis factor-α, a potent activator of JNK, also rapidly activated JNK and down-regulated *CYP7A1* mRNA levels. Overexpression of dominant-negative JNK1 or a transactivating domain mutant of c-Jun significantly blocked the ability of TCA to down-regulate *CYP7A1* mRNA. In contrast, overexpression of wild-type c-Jun (c-Jun^{wt}) enhanced the repression of *CYP7A1* by TCA. Moreover, overexpression of c-Jun^{wt} resulted in increased SHP-1 promoter activity. Mutation of a putative AP-1 (c-Jun) element suppressed c-Jun-mediated activation of the SHP-1 promoter construct. These results indicate that the bile acid-activated JNK pathway plays a pivotal role in regulating *CYP7A1* levels in primary rat hepatocytes.

The metabolism of cholesterol to bile acids represents a major pathway for its elimination from the body and accounts for ~50% of total output in humans. Bile acid biosynthesis can proceed via either the "neutral" (classic) or the "acidic" (alternative) pathway (1). The end products of cholesterol degradation via these pathways are the two primary bile acids, cholic and chenodeoxycholic acid.

It is well established that bile acids undergoing enterohepatic circulation feedback repress their own biosynthesis (2). In experimental animals and in humans, the expansion of the bile acid pool by bile acid feeding suppresses bile acid biosynthesis, whereas the depletion of the bile acid pool by biliary diversion or bile acid sequestrant feeding increases bile acid biosynthesis. The first and rate-limiting enzyme in the neutral bile acid biosynthetic pathway, cholesterol 7α-hydroxylase (CYP7A1),[1] is the most highly regulated step in this feedback inhibitory loop (3); however, other genes encoding bile acid biosynthetic enzymes are also under dynamic control by the bile acid pool (4–8). Studies from our laboratory have demonstrated that bile acids repress *CYP7A1* at the level of gene transcription and that the degree of repression paralleled both the concentration and relative hydrophobicity of bile acids added to the diets of intact animals (9–11) and to primary rat hepatocyte cultures (12).

Bile acids have been shown to bind and activate the orphan nuclear receptor, farnesoid X receptor (FXR) (13–15). FXR binds to DNA as a heterodimer with retinoid X receptor (RXR), recognizing an inverted hexanucleotide repeat separated by a single base (an IR-1 motif) (16). Although the activated FXR/RXR heterodimer repressed *CYP7A1* promoter activity (14, 17, 18), no IR-1 element has been identified in the *CYP7A1* promoter. Recent studies show that activated FXR induces the expression of small heterodimer partner 1 (SHP-1) protein, an orphan nuclear receptor that lacks a DNA-binding domain (19, 20). It has been proposed that elevated SHP-1 protein levels prevent activation of *CYP7A1* transcription by heterodimerizing with LRH-1 (CPF or FTF), a positive transcription factor required for maximal *CYP7A1* transcription. Finally, studies with FXR "knock-out" mice showed that these animals were defective in bile acid regulation of a number of genes involved in bile acid biosynthesis and transport, including *CYP7A1* (7). However, several genes regulated by bile acids were not altered in the FXR null mice.

In the current study, we provide evidence for a "cell signaling model" of bile acid regulation of *CYP7A1*. We have previously reported that bile acids activate different isoforms of protein kinase C (PKC) in a time (minutes)- and concentration (10–100 μM)-dependent manner (21, 22). Moreover, activation of PKC by phorbol esters repressed *CYP7A1* transcriptional activity (22). It is well accepted that activated PKC contributes to the

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[1] The abbreviations used are: *CYP7A1*, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; RXR, retinoid X receptor; SHP-1, small heterodimer partner 1; LRH-1, liver receptor homolog-1; CPF, *CYP7A1* promoter binding factor; FTF, α1-fetoprotein transcription factor; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; TNFα, tumor necrosis factor α; TCA, taurocholate; DCA, deoxycholate; MKK4, mitogen-activated protein kinase kinase 4; AP-1, activator protein-1; *CYP8B1*, sterol 12α-hydroxylase; GST, glutathione *S*-transferase; CMV, cytomegalovirus; MEK, mitogen-activated protein kinase/ERK kinase; dn, dominant-negative; bp, base pair(s); BARE, bile acid-responsive element; IR-1, inverted repeat-1.

activation of downstream mitogen-activated protein kinase signaling cascades, including the extracellular-signal regulated (ERK) pathway and the c-Jun N-terminal kinase (JNK) pathway (23). Indeed, it has been shown *in vitro* and *in vivo*, that both bile acids and phorbol esters can activate the PKC/Raf-1/ERK1,2 pathway and regulate expression of the low density lipoprotein receptor (*LDLR*) gene and the early growth response genes (24, 25). Other studies have shown that tumor necrosis factor α (TNFα), which activates the JNK signaling pathway, can repress the mRNA and activity of CYP7A1 (26, 27). Thus, evidence exists supporting the hypothesis that activation of signaling cascades may also be important in the regulation of *CYP7A1* and other genes by bile acids. However, it is unclear which signaling pathways activated by bile acids may be important in the regulation of *CYP7A1* and how the bile acid-activated signaling pathways interact with FXR to regulate overall bile acid biosynthesis.

The studies reported herein demonstrate that bile acids rapidly down-regulate *CYP7A1* gene transcription via a JNK/c-Jun-dependent mechanism. We also provide evidence that the SHP-1 promoter is a direct target of activated c-Jun, suggesting "cross-talk" occurs between the bile acid-activated JNK signaling cascade and FXR in sensing bile acid levels in primary rat hepatocytes.

EXPERIMENTAL PROCEDURES

Primary Rat Hepatocyte Cultures—Primary rat hepatocyte monolayer cultures were prepared from male Harlan Sprague-Dawley rats (200–300 g) using the collagenase-perfusion technique of Bissell and Guzelian (28). Hepatocytes were plated on collagen-coated culture dishes in serum-free William's E medium containing penicillin (100 units/ml), dexamethasone (0.1 μM), thyroxine (1 μM), and insulin (100 nM). Before plating, cells were judged to be greater than 90% viable using trypan blue exclusion. Cells were routinely incubated for 22 h at 37 °C in humidified 5% CO_2, and either prewarmed bile acids or TNFα was added in the indicated concentrations.

JNK Activity Assay—After treatments, hepatocytes (1.3×10^6 cells/35-mm dish) were washed with ice-cold phosphate-buffered saline followed by homogenization in cold lysis buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 0.05% SDS, 0.05% sodium deoxycholate, 1% Triton X-100, 5 mM NaF, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μM Microcystin-LR, and 40 μg/ml each of pepstatin A, aprotinin, and leupeptin). Cell supernatants (500 μg) were incubated with 1 μg of anti-JNK1 antibody (Santa Cruz Biotechnology) at 4 °C for 2–3 h. The immune complexes were isolated by the addition of Protein A-agarose beads. The immunoprecipitates were recovered by centrifugation and washed (10 min) sequentially with lysis buffer, phosphate-buffered saline, and kinase assay buffer (25 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 0.1 mM Na_3VO_4, and 0.1% (v/v) 2-mercaptoethanol). JNK activity was determined by incubating the washed immunoprecipitates in a reaction mixture containing 40 μl of kinase assay buffer, 0.1 mM ATP, 1 μM Microcystin-LR, 10 μCi of [γ-^{32}P]ATP, and 10 μg of recombinant GST-c-Jun (amino acids 1–169) for 20 min at 37 °C. Reactions were terminated by adding 5× SDS-polyacrylamide gel electrophoresis sample buffer and boiling for 5 min. Phosphorylated GST-c-Jun was resolved in 10% SDS-polyacrylamide gel electrophoresis, the gels were dried and autoradiographed, and the radioactivity incorporated in GST-c-Jun determined by laser scanning the autoradiograms.

ERK Activity Assay—This assay was performed similarly to the JNK activity assay described above. After treatments, cell extracts were incubated with 1 μg each of rabbit anti-ERK1 and anti-ERK2 antibody (Santa Cruz Biotechnology), and the immunoprecipitates were washed as described above. ERK activity was determined in a reaction mixture identical to that described for JNK, except for the substrate (0.5 mg/ml myelin basic protein). Reactions were terminated by spotting 30 μl of supernatant reaction mixture onto phosphocellulose discs and washing the discs four times (10 min each) in 180 mM phosphoric acid. The radioactivity incorporated in the substrate was determined by scintillation counting.

Poly-L-lysine-conjugated Adenovirus Infection/Transfection of Hepatocytes—Expression vectors encoding for dominant-negative JNK1 (pCMV-dnJNK1), dominant-negative MKK4 (pCMV-dnMKK4), or wild-type c-Jun (pCMV-c-Jun^{wt}) (29) were introduced using conjugated DNA-adenovirus complexes (29–32). Freshly isolated hepatocytes at 50% confluency were cultured for 4 h prior to transfections. Using polystyrene tubes, 1 μg (35-mm dish) or 7 μg (100-mm dish) of either control plasmid (pCMV) or the plasmid encoding for the gene of interest was incubated with poly-L-lysine-coupled virus in HEPES-buffered saline (HBS; 20 mM HEPES, pH 7.3, 150 mM NaCl) solution in the dark for 30 min at room temperature. After 30 min, additional poly-L-lysine (Sigma Chemical Co., M_r 29,300) (1.3 μg of poly-L-lysine/μg of DNA) in HBS was added, and the complexes were incubated for another 30 min. The DNA-conjugated virus was added to the hepatocytes at a multiplicity of infection of 300. The cells were incubated for 22–24 h to allow for the expression of the gene products and experimental additions were made thereafter.

Infection of Primary Hepatocytes with Adenovirus Encoding TAM67—Primary rat hepatocytes were plated on 150-mm culture dishes for 24 h under culture conditions as described above. After 24 h, the cultures were infected with unpurified recombinant adenovirus encoding for dominant-negative c-Jun mutant (TAM67) (33) at a multiplicity of 1–10 plaque-forming units/cell. All experiments were compared with control (null) virus infection. After 3 h of infection, the virus was removed and replaced with fresh medium, and the cells were allowed to incubate for an additional 24 h. Experimental additions were made 24 h after the media change.

Quantitation of CYP7A1 mRNA—Total RNA was prepared from cultured hepatocytes using the guanidinium thiocyanate CsCl centrifugation. *CYP7A1* mRNA levels were determined by RNase protection assay as described previously (34). Rat cyclophilin mRNA was used as an internal control.

Transient Transfections and Dual Luciferase Reporter Assay—The rat SHP promoter firefly luciferase construct (bases −441 to +19) was kindly provided by Dr. Steven A. Kliewer (Glaxo Wellcome, NC). FXR expression plasmid (pCMV-rFXR) was a gift from Dr. Gregorio Gil (Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University). Site-directed mutagenesis of the putative AP-1 binding site in the rat SHP promoter luciferase construct was performed using the Stratagene QuikChange site-directed mutagenesis kit with the ΔratAP1 (bases −298 to −256, 5′-CCCTGTTTATACACTTGtcagATCCGATAAAGGGCATCCAGGC-3′) primer. The mutated construct was sequenced prior to use to verify DNA sequence fidelity. Primary rat hepatocytes were seeded into 24-well plates (8×10^4 cells/well) in growth medium (William's E supplemented with 10% fetal calf serum, 0.1 μM dexamethasone, and 1 μM thyroxine) and incubated for 6 h. Hepatocytes were transfected using the Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Each well received 100 ng of the rSHP-luciferase construct, 2 ng of pRL-TK construct (a plasmid containing the herpes simplex thymidine kinase promoter upstream from the *Renilla* luciferase gene), to normalize for transfection efficiencies, and 30 ng of either pCMV-lacZ (control promoter plasmid), pCMV-rFXR, or pCMV-c-Jun^{wt}. After 16 h, the medium was changed to serum-free medium, and the cells were treated with taurocholate (TCA) (50 μM) for 24 h prior to harvesting. Dual luciferase output (Dual Luciferase Reporter Assay System, Promega) was quantified with a luminometer (Lumat LB9501, Berthold), and the results are expressed as an index of relative light units.

Statistical Analyses—Data were analyzed by Student's *t*-test. Level of significance was set at $p < 0.05$.

RESULTS

Taurine-conjugated Bile Acids and TNFα Stimulate JNK Activity in Primary Cultures of Rat Hepatocytes—Bile acids have been reported to induce the expression of inflammatory cytokines such as TNFα and interleukin-1 in macrophages (27). TNFα has been reported to down-regulate *CYP7A1* in the liver (26, 27). In numerous cell lines, TNFα has been shown to potently activate the JNK cascade (35). In this context, we wanted to determine whether bile acids might also activate the JNK cascade in primary cultures of rat hepatocytes and down-regulate *CYP7A1*. To achieve this, primary rat hepatocytes were incubated with taurine-conjugated bile acids (50 μM) for 90 min prior to cell harvest. The two isoforms of JNK (JNK1 and JNK2) were then immunoprecipitated, and the JNK kinase activity was determined using GST-c-Jun as the substrate as described under "Experimental Procedures." Fig. 1*A* shows that the addition of TCA to hepatocytes increased JNK activity by ~4-fold. TCA caused a time-dependent increase in JNK

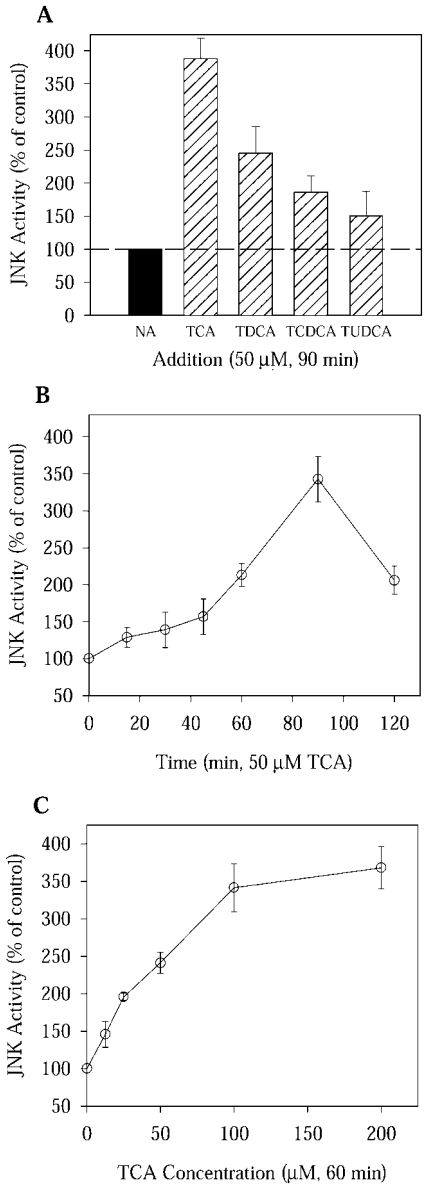

FIG. 1. **Effect of different taurine-conjugated bile acids on JNK1 activity in primary rat hepatocytes.** *A*, JNK activity in response to taurine-conjugated bile acids (50 μM for 90 min) (*NA*, no addition). *B*, time course of TCA (50 μM) activation of JNK. *C*, effect of TCA concentration (60-min incubations) on JNK activity. All values are means ± S.E.; n = 3–6.

activity with peak activation occurring at 90 min followed by a rapid decline in activity (Fig. 1*B*). A dose-response curve indicated that concentrations of as low as 12.5 μM TCA resulted in detectable JNK activation and that maximal stimulation of JNK activity occurred between concentrations of 100 and 200 μM (Fig. 1*C*). Taurodeoxycholate and taurochenodeoxycholate, the two other relatively hydrophobic bile acids, also stimulated JNK activity by ~1.5- to 2-fold, whereas tauroursodeoxycholate, a hydrophilic bile acid, had virtually no effect. It should be noted that the degree of JNK activation by different bile acids paralleled their ability to repress *CYP7A1* mRNA levels in primary rat hepatocytes (36).

Treatment of hepatocytes with TNFα (2 ng/ml) also caused a rapid increase (~2-fold) in JNK activity (Fig. 2*A*). Moreover, TNFα also rapidly (2 h) down-regulated (70–80%) *CYP7A1* mRNA levels in primary rat hepatocytes (Fig. 2*B*). *CYP7A1* mRNA levels rebounded with longer incubations, suggesting that TNFα did not induce apoptosis under our culture conditions.

MEK1 Inhibitor Does Not Block the Bile Acid-mediated Down-regulation of CYP7A1 Expression in Primary Hepatocytes—Previous reports in the literature have shown that bile acids can sequentially activate the ERK1/2 cascade (Raf-1/MEK1,2/ERK1,2) (24, 25). In addition, data from our laboratory showed that both hydrophobic and hydrophilic bile acids could rapidly activate the ERK cascade in primary rat hepatocytes (37). Studies were therefore carried out to determine if the ERK cascade was involved in the bile acid-dependent down-regulation of *CYP7A1*. Primary rat hepatocytes were pretreated (30 min) with PD98059 (50 μM), a specific inhibitor of MEK1 activation, which prevents activation of downstream ERK1 and ERK2. Next, DCA (50 μM) was added to the cells for 20 min, and ERK1/ERK2 activity was determined. As shown in Fig. 3*A*, pretreatment with PD98059 prevented activation of the ERK pathway by DCA. Furthermore, PD98059 failed to block the down-regulation of *CYP7A1* mRNA by DCA (50 μM, 6 h) (Fig. 3*B*). PD98059 alone had no effect on *CYP7A1* mRNA levels. This result indicates that activation of the ERK pathway does not result in the feedback repression of *CYP7A1* by bile acids in primary rat hepatocytes.

Bile Acid-dependent Down-regulation of CYP7A1 mRNA Is Mediated by the JNK Pathway—The data presented above suggest that the JNK pathway may be important in the down-regulation of *CYP7A1* by bile acids and TNFα. To test this hypothesis, we employed dominant-negative mutants of MKK4 (kinase upstream of JNK1,2) and JNK1 to block the ability of TCA to activate the endogenous JNKs. A plasmid expressing either dominant-negative MKK4 (pCMV-dnMKK4), dominant-negative JNK1 (pCMV-dnJNK1), or the control plasmid (pCMV) was transfected into hepatocytes using a poly-L-lysine-conjugated adenovirus system. After 24-h incubation, hepatocytes were treated with 50 μM TCA for 90 min, and the cells were harvested. As expected, in control plasmid-infected cells, TCA treatment stimulated JNK1 activity (Fig. 4*A*). However, stimulation of JNK1 activity by TCA was almost completely blocked in cells overexpressing the dominant-negative mutants of MKK4 or JNK1. We next tested the ability of TCA to down-regulate *CYP7A1* mRNA in hepatocytes expressing the dominant-negative JNK1 protein. As shown in Fig. 4*B*, the dominant-negative JNK1 significantly blocked the ability of TCA to down-regulate *CYP7A1* mRNA, suggesting a role for the JNK pathway in the rapid bile acid-mediated repression of *CYP7A1*.

Stimulation of the JNK pathway by extracellular stimuli results in the activation of the immediate-early transcription factor c-Jun. To assess the role of c-Jun in bile acid-mediated repression of *CYP7A1* transcription, we transfected hepatocytes with a wild-type c-Jun expression construct (pCMV-c-Jun^{wt}) using a poly-L-lysine-conjugated adenovirus system. In parallel experiments, hepatocytes were also infected with a recombinant adenovirus to express dominant-negative c-Jun (TAM67). TAM67 is deleted in the N-terminal domain (amino acids 3–122) of c-Jun and is incapable of being transactivated by JNK. Hepatocytes were treated 24 h after infection with either media control or 50 μM TCA, and the effect of TCA on *CYP7A1* mRNA levels was determined 18 h later. TCA treatment of control-infected hepatocytes repressed *CYP7A1* mRNA levels by ~60%. This TCA-mediated down-regulation of *CYP7A1* mRNA levels was further enhanced in cells overexpressing wild-type c-Jun (Fig. 5*A*). In contrast, overexpression of TAM67 resulted in an increase of ~2- to 3-fold in the basal levels of *CYP7A1* mRNA as compared with cells infected with a control (null) virus (Fig. 5*B*). Moreover, expressing a dominant-negative c-Jun also significantly blocked the ability of TCA to down-regulate *CYP7A1* mRNA (Fig. 5*C*). These experiments

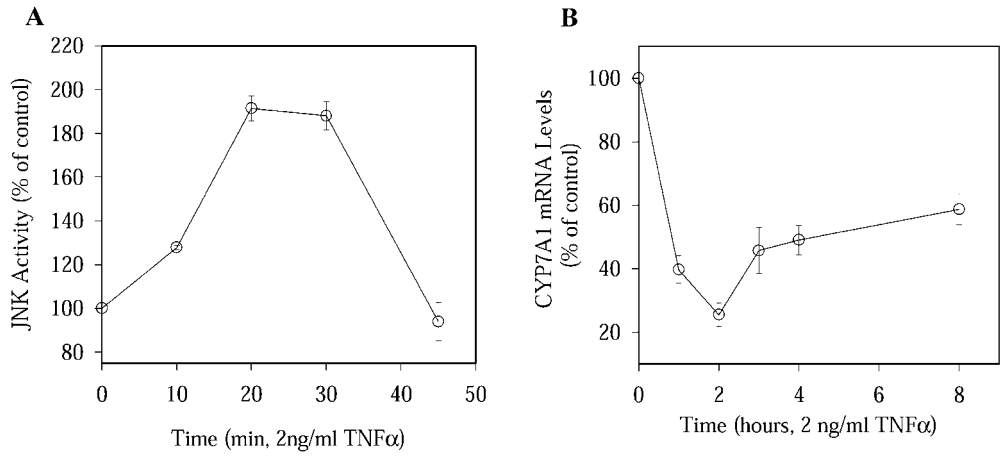

FIG. 2. **Effect of TNFα on JNK1 activity and *CYP7A1* mRNA levels in primary rat hepatocytes.** *A*, time course of TNFα (2 ng/ml) activation of JNK. *B*, effect of TNFα (2 ng/ml) on *CYP7A1* mRNA levels. TNFα was added to hepatocyte cultures at 48 h, total RNA was isolated at time points indicated, and *CYP7A1* mRNA was quantitated as described under "Experimental Procedures." All values are means ± S.E.; $n = 3$.

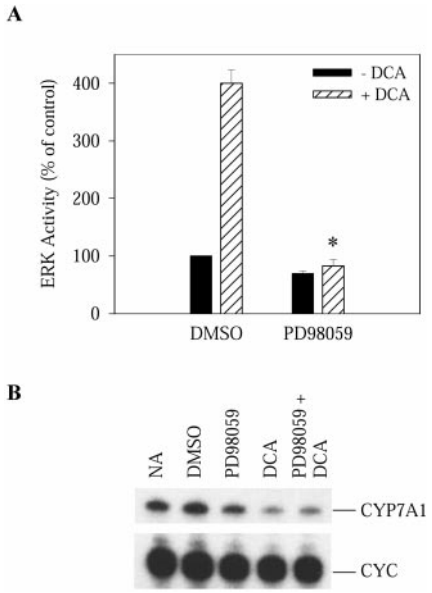

FIG. 3. **Effect of inhibition of the ERK pathway on ERK activity and DCA-mediated repression of *CYP7A1* mRNA levels.** *A*, cultured hepatocytes were treated as indicated with a vehicle control of dimethyl sulfoxide (*DMSO*), the MEK1 inhibitor, PD98059 in DMSO (50 μM for a 30-min pretreatment), and/or DCA (50 μM for 20 min), or left untreated. The cells were then harvested and assayed for ERK activity. Values are mean ± S.E.; $n = 3$. *, $p < 0.001$. Statistical significance was calculated between DCA-treated samples. *B*, cultured hepatocytes were pretreated with PD98059 as described above, followed by treatment with DCA (50 μM) for 6 h. Total RNA was then isolated and subjected to RNase protection assay for *CYP7A1* and cyclophilin (*CYC*) mRNAs.

strongly support a role for c-Jun as the rapid mediator of *CYP7A1* regulation by bile acids.

How Do Both c-Jun and FXR Down-regulate CYP7A1 Gene Expression?—Previous work has demonstrated that bile acids down-regulate *CYP7A1* gene expression by activating the nuclear hormone receptor FXR (14, 17, 18). It was recently shown that the mechanism by which activated FXR repressed *CYP7A1* transcription is through induction of the gene encoding SHP-1 protein (19, 20). The data presented above suggests that bile acids regulate *CYP7A1* transcription by activating the transcription factor c-Jun. This observation raised the question whether in addition to FXR, SHP-1 expression was also up-regulated by activated c-Jun. To test this hypothesis, the proximal 500–600 bp of the mouse, rat, and human SHP-1 promoters were examined for potential c-Jun (AP-1) binding sites. A highly conserved AP-1 binding site was identified ~250–300 bp upstream of the transcription initiation site in the SHP-1 promoter of all three species (Fig. 6*A*). To test if the SHP-1 promoter was regulated by c-Jun, primary rat hepatocytes were co-transfected with an expression plasmid for wild-type c-Jun (pCMV-c-Jun^{wt}) and a luciferase reporter construct under the control of the rat SHP-1 promoter. In a parallel experiment, an FXR expression plasmid (pCMV-rFXR) was co-transfected with the SHP-1 luciferase promoter construct as a positive control for these studies. Following transfection, cells were incubated with TCA (50 μM) for 24 h and harvested, and luciferase activity was measured. As shown in Fig. 6*B*, overexpression of FXR induced SHP-1 promoter activity ~ 2-fold in the presence of TCA. Similarly, TCA treatment of cells transfected with the c-Jun expression plasmid also resulted in an increase of ~2-fold in SHP-1 reporter activity. Mutating the AP-1 site eliminated the c-Jun responsiveness of the SHP promoter. These data provide strong evidence that bile acids stimulate SHP-1 transcription in an AP-1-dependent manner in primary rat hepatocytes.

DISCUSSION

The conversion of cholesterol to bile acids via the neutral pathway is regulated at the level of *CYP7A1* gene expression. Several groups have demonstrated the feedback inhibition of *CYP7A1* transcription in response to increasing concentrations of bile acids (9–12, 36). Moreover, it was shown that hydrophobic bile acids are more potent repressors of *CYP7A1* mRNA than hydrophilic bile acids (11, 36). In total, these data suggest that bile acids can regulate their own biosynthesis and that they may do so by either binding to a specific nuclear hormone receptor and/or by activating one or more signaling cascades in the liver.

A major breakthrough in understanding the regulation of gene expression by bile acids came with the discovery that FXR is activated by bile acids (13–15). It was shown that both conjugated and unconjugated bile acids could bind to and activate FXR at physiological concentrations (10–100 μM). Studies with FXR "knock-out" mice revealed that a number of genes involved in cholesterol homeostasis are regulated by FXR, namely, *CYP7A1*, *CYP8B1*, intestinal bile acid binding protein (*I-BABP*), canalicular bile salt excretory pump (*BSEP*), phospholipid transfer protein (*PLTP*), and hepatic basolateral transporter *NTCP* (7). FXR can regulate gene expression by either a direct or an indirect mechanism. FXR induces gene expression by binding to an FXR-response element (IR1-motif) in the promoters of target genes (16). In contrast, the bile

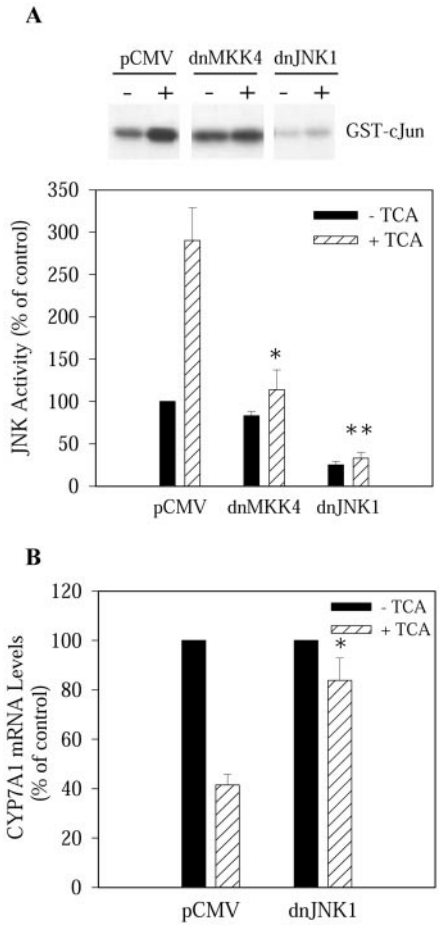

FIG. 4. **Effect of dominant-negative mutants of MKK4 and JNK1 on JNK activity and effect of dnJNK1 on *CYP7A1* mRNA levels.** *A*, hepatocytes were transfected with either control plasmid (pCMV), dominant-negative MKK4 plasmid (pCMV-dnMKK4), or dominant-negative JNK1 plasmid (pCMV-dnJNK1) for 24 h to allow for expression of dominant-negative gene products. After 24 h, the cells were treated with TCA (50 μM, 90 min) or left untreated. The cells were then harvested and assayed for JNK activity. *Top panel*, representative autoradiogram depicting JNK activity in transfected hepatocytes in the absence (−) and in the presence (+) of TCA as assayed by incorporation of labeled ATP in GST-c-Jun. *Lower panel*, quantitative results of JNK activity assay. Values are mean ± S.E.; $n = 3$. **, $p < 0.025$; *, $p < 0.001$. Statistical significance was calculated between TCA-treated control (*pCMV*) *versus* TCA-treated dnMKK4 or dnJNK1. *B*, cultured hepatocytes were transfected as described above, followed by treatment with TCA (50 μM) for 18 h. Total RNA was then isolated and subjected to RNase protection assay for *CYP7A1* and cyclophilin mRNAs. Values are mean ± S.E.; $n = 4$. *, $p < 0.025$. Statistical significance was calculated between TCA-treated pCMV and dnJNK1.

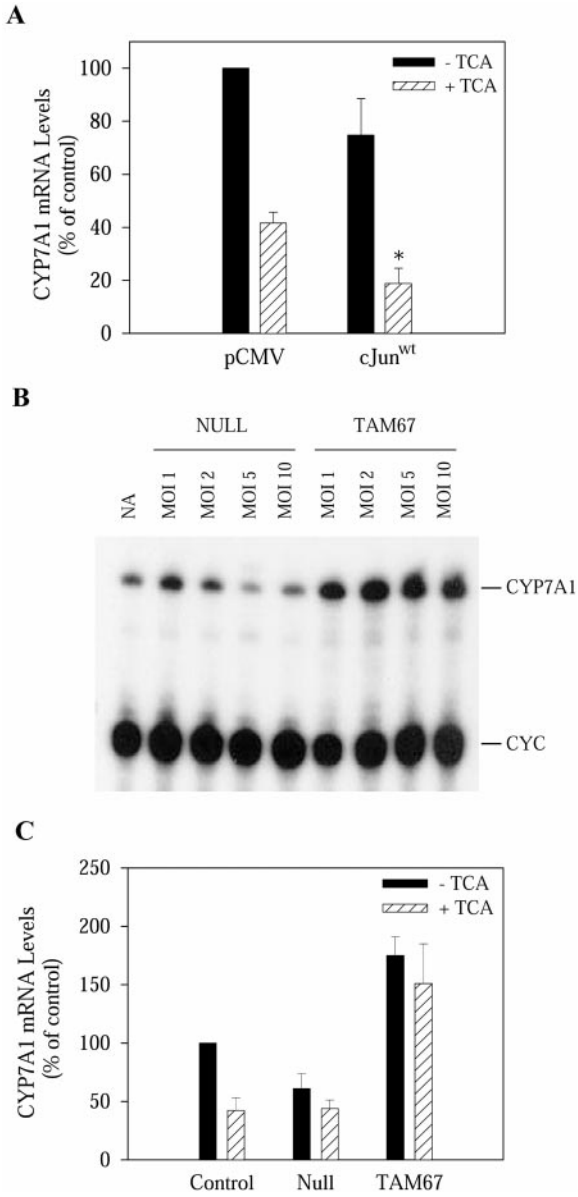

FIG. 5. **Effect of overexpression of wild-type c-Jun and TAM67 on *CYP7A1* mRNA levels.** *A*, hepatocytes were transfected with either control plasmid (pCMV) or a plasmid encoding for wild-type c-Jun (pCMV-c-Jun^{wt}) for 24 h to allow for overexpression of c-Jun. After 24 h, the cells were treated with TCA (50 μM, 18 h) or left untreated. Total RNA was then isolated and subjected to RNase protection assay for *CYP7A1* and cyclophilin mRNAs. Values are mean ± S.E.; $n = 3$. *, $p < 0.05$. Statistical significance was calculated between TCA-treated samples. *B*, recombinant adenovirus encoding a dominant-negative c-Jun mutant (*TAM67*) or adenovirus without expression vector (*NULL*) in the indicated multiplicity of infection (*MOI*) were added to cultured hepatocytes for 24 h. Total RNA was isolated and subjected to RNase protection assay for *CYP7A1* and cyclophilin (*CYC*) mRNAs. *C*, hepatocytes were treated with either the null virus or the TAM67 adenovirus as described above. Following infection, TCA (50 μM) was added to the hepatocytes for 6 h, and total RNA were isolated and subjected to RNase protection assay for *CYP7A1* and cyclophilin (*CYC*) mRNAs. Values are mean ± S.E.; $n = 4$.

acid-responsive elements (BAREs) in the promoters of the *CYP7A1* and *CYP8B1* genes lack a functional FXR binding site (17, 18). Recent studies reveal that FXR induces the expression of the SHP-1 protein (19, 20). SHP-1 represses *CYP7A1* transcription by inhibiting the activity of LRH-1, a positive transcription factor that binds to the BARE region in the *CYP7A1* promoter. Consistent with this model are the findings that FXR null mice fail to feedback repress *CYP7A1* and are defective in the bile acid induction of SHP-1.

In the current study, we provide evidence that feedback repression of *CYP7A1* by bile acids is also mediated by activation of the JNK signaling cascade. We show that the JNK cascade is rapidly activated by bile acids, specifically TCA, in a time- and concentration-dependent manner. Additionally, the rank order of potency for bile acid induction of the JNK pathway mirrors their ability to repress *CYP7A1* mRNA levels in primary hepatocytes (36). Interestingly, hydrophobic bile acids have been recently shown to induce the expression of cytokines TNFα and interleukin-1 in macrophages (27). Induction of these cytokines has been correlated with the down-regulation of *CYP7A1* mRNA and activity in intact animals (26, 27). In the current investigation, we demonstrate that activation of the JNK signaling pathway by TNFα rapidly down-regulates *CYP7A1* mRNA levels in primary rat hepatocytes. The role of the JNK pathway in mediating the inhibitory effects of bile

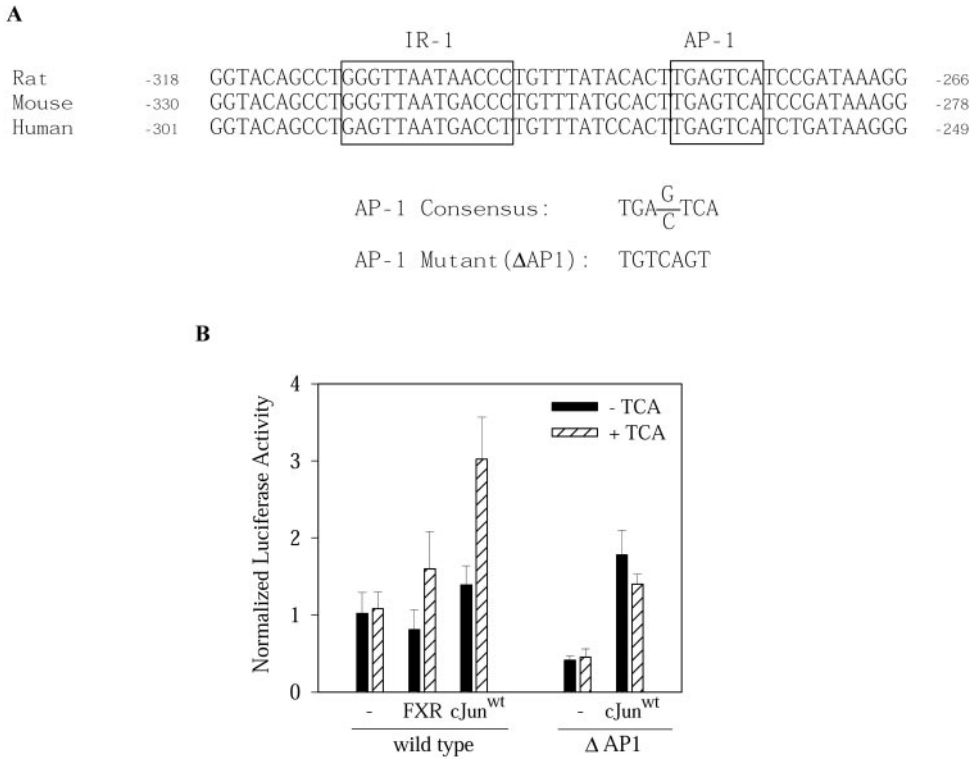

FIG. 6. **Wild-type c-Jun and FXR activate the rat SHP-1 promoter.** *A*, alignment of the proximal regions of the rat, mouse, and human SHP-1 promoters. The conserved FXR binding site (*IR-1*) and the putative AP-1 binding site are *boxed*. Shown *below the figure* are the AP-1 site consensus sequence and the sequence of the mutated AP-1 site in the rat SHP-1 promoter. *B*, hepatocytes were transfected with the luciferase reporter plasmid containing the proximal promoter of the rat (−441 to +19) SHP-1 gene or with the corresponding reporter plasmid in which the AP-1 site had been mutated (Δ*AP1*) in combination with expression plasmids for either FXR, c-Jun^{wt}, or lacz (−) (control promoter plasmid). Following transfection, cells were treated with 50 μM TCA for 24 h and harvested for measurement of luciferase activity. Values are mean ± S.E. of at least two experiments performed in triplicate.

acids on *CYP7A1* expression is supported by the observation that overexpression of dominant-negative JNK1 blocked the ability of TCA to activate JNK and to down-regulate *CYP7A1* mRNA (Fig. 4).

In human colon carcinoma cell line HCT116 and in human adenocarcinoma cells, treatment with bile acids enhanced the phosphorylation of c-Jun, a transcription factor substrate of JNK and a component of the classic AP-1 heterodimer (38, 39). In these cells, bile acids stimulated gene transcription by enhancing the AP-1 transcriptional activity and DNA binding to promoter constructs. In contrast, in the present study, overexpression of wild-type c-Jun enhanced the repression of *CYP7A1* by TCA (Fig. 5*A*). Moreover, overexpression of TAM67, a non-transactivable mutant of c-Jun, significantly blocked the ability of TCA to repress *CYP7A1* mRNA. Interestingly, the basal levels of *CYP7A1* mRNA were 2- to 3-fold higher in TAM67-overexpressing cells compared with null virus-infected cells (Fig. 5*B*). Together, these observations indicate that activated c-Jun acts as a repressor of *CYP7A1* mRNA in primary rat hepatocyte cultures.

Previous reports in the literature and the data presented in this study suggest that bile acids entering the hepatocyte activate both FXR and the cell signaling cascades. It is of interest to note that both FXR and the JNK pathway are activated by similar concentrations of bile acids (10–100 μM). Therefore, how does bile acid-activated FXR and -activated c-Jun both interact to coordinately regulate *CYP7A1* gene expression in the hepatocyte? Our data indicate that activated c-Jun induces SHP-1 promoter activity (Fig. 6*B*). In this regard, a consensus AP-1 binding site was identified in close proximity to the FXR binding site in the mouse, rat, and human SHP-1 promoters. Mutations in the AP-1 binding site abolished bile acid responsiveness of the rat SHP-1 promoter. Thus, we believe that activation of the JNK/c-Jun pathway by bile acids results in the induction of SHP-1 expression. SHP-1, in turn, interacts with LRH-1 and indirectly represses *CYP7A1* transcription (Fig. 7).

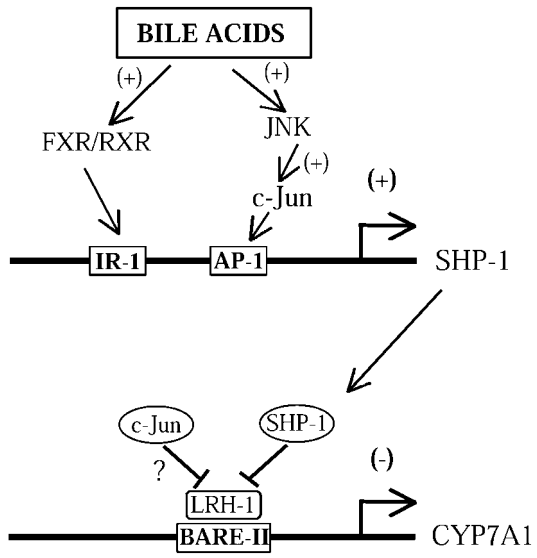

FIG. 7. **Model for regulation of *CYP7A1* gene expression by FXR and JNK signaling cascade.** This model predicts that bile acids entering the hepatocyte activate both the FXR and the JNK signaling cascade. Activated FXR and c-Jun in turn enhance SHP-1 transcription by binding to the IR-1 and AP-1 elements in the SHP-1 promoter, respectively. Elevated SHP-1 protein levels repress *CYP7A1* transcription by interacting with LRH-1, a positive transcription factor that binds to the BARE-II region in the *CYP7A1* promoter. We speculate that c-Jun might also directly interact with LRH-1 to repress *CYP7A1* transcription. See text for details.

However, we do not exclude additional mechanisms of c-Jun-mediated bile acid repression of *CYP7A1*. It is possible that activated c-Jun might also directly interact with LRH-1 or another positive transcription factor required for *CYP7A1* gene expression. Finally, it is possible that LRH-1 might be a substrate of activated JNK. In this regard, it has been recently reported that both mitogen-activated protein kinase kinase 4 (MKK4) and JNK phosphorylate RXR, altering its functional properties (40).

In conclusion, our combined results suggest that bile acids activate the JNK/c-Jun cascade in primary rat hepatocytes. Activation of this protein kinase cascade is involved in the down-regulation of *CYP7A1* by bile acids. We hypothesize that activation of this pathway provides the hepatocytes with another "sensing mechanism" for detecting bile acid levels as well as stress signals. Together, FXR and the JNK/c-Jun signaling pathway allow the hepatocytes great flexibility in responding to both intra- and extracellular signals for maintaining bile acid homeostasis in the liver.

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REFERENCES

1. Vlahcevic, Z. R., Pandak, W. M., and Stravitz, R. T. (1999) *Gastroenterol. Clin. North Am.* **28,** 1–25
2. Chiang, J. Y. L. (1998) *Front. Biosci.* **3,** D176–D193
3. Russell, D. W., and Setchell, K. D. (1992) *Biochemistry* **31,** 4737–4749
4. Vlahcevic, Z. R., Eggertsen, G., Bjorkhem, I., Hylemon, P. B., Redford, K., and Pandak, W. M. (2000) *Gastroenterology* **118,** 599–607
5. Ghosh, S., Natarajan, R., Pandak, W. M., Hylemon, P. B., and Grogan, W. M. (1998) *Am. J. Physiol.* **274,** G662–G668
6. Vlahcevic, Z. R., Jairath, S. K., Heuman, D. M., Stravitz, R. T., Hylemon, P. B., Avadhani, N. G., and Pandak, W. M. (1996) *Am. J. Physiol.* **270,** G646–G652
7. Sinal, C. J., Tohkin, M., Miyata. M., Ward, J. M., Lambert. G., and Gonzalez, F. J. (2000) *Cell* **102,** 731–744
8. Gupta, S., Stravitz, R. T., Pandak, W. M., Muller, M., Vlahcevic, Z. R., and Hylemon, P. B. (2000) *Hepatology* **32,** 341–347
9. Horton, J. D., Cuthbert, J. A., and Spady, D. K. (1995) *J. Biol. Chem.* **270,** 5381–5387
10. Jelinek, D. F., Andersson, S., Slaughter, C. A., and Russell, D. W. (1990) *J. Biol. Chem.* **265,** 8190–8197
11. Pandak, W. M., Vlahcevic, Z. R., Heuman, D. M., Redford, K. S., Chiang, J. Y., and Hylemon, P. B. (1994) *Hepatology* **19,** 941–947
12. Twisk, J., Lehmann, E. M., and Princen, H. M. (1993) *Biochem. J.* **290,** 685–691
13. Wang, H., Chen, J., Hollister, K., Sowers, L. C., and Forman, B. M. (1999) *Mol. Cell* **3,** 543–553
14. Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lustig, K. D., Mangelsdorf, D. J., and Shan, B. (1999) *Science* **284,** 1362–1365
15. Parks, D. J., Blanchard, S. G., Bledsoe, R. K., Chandra, G., Consler, T. G., Kliewer, S. A., Stimmel, J. B., Willson, T. M., Zavacki, A. M., Moore, D. D., and Lehmann, J. M. (1999) *Science* **284,** 1365–1368
16. Forman, B. M., Goode, E., Chen, J., Oro, A. E., Bradley, D. J., Perlmann, T., Noonan, D. J., Burka, L. T., McMorris, T., Lamph, W. W. (1995) *Cell* **81,** 687–693
17. Chiang, J. Y., Kimmel, R., Weinberger, C., and Stroup, D. J. (2000) *J. Biol. Chem.* **275,** 10918–10924
18. del Castillo-Olivares, A., and Gil, G. (2000) *Nucleic Acids Res.* **28,** 3587–3593
19. Lu, T. T., Makishima, M., Repa, J. J., Schoonjans, K., Kerr, T. A., Auwerx, J., and Mangelsdorf, D. J. (2000) *Mol. Cell* **6,** 507–515
20. Goodwin, B., Jones, S. A., Price, R. R., Watson, M. A., McKee, D. D., Moore, L. B., Galardi, C., Wilson, J. G., Lewis, M. C., Roth, M. E., Maloney, P. R., Willson, T. M., and Kliewer, S. A. (2000) *Mol. Cell* **6,** 517–526
21. Stravitz, R. T., Rao, Y. P., Vlahcevic, Z. R., Gurley, E. C., Jarvis, W. D., and Hylemon, P. B. (1996) *Am. J. Physiol.* **271,** G293–G303
22. Stravitz, R. T., Vlahcevic, Z. R., Gurley, E. C., and Hylemon, P. B. (1995) *J. Lipid Res.* **36,** 1359–1369
23. Robinson, M. J., and Cobb, M. H. (1997) *Curr. Opin. Cell Biol.* **9,** 180–186
24. Kumar, A., Chambers, T. C., Cloud-Heflin, B. A., and Mehta, K. D. J. (1997) *J. Lipid Res.* **38,** 2240–2248
25. Brady, L. M., Beno, D. W., and Davis, B. H. (1996) *Biochem. J.* **316,** 765–769
26. Feingold, K. R., Spady, D. K., Pollock, A. S., Moser, A. H., and Grunfeld, C. (1996) *J. Lipid Res.* **37,** 223–228
27. Miyake, J. H., Wang, S. L., and Davis, R. A. (2000) *J. Biol. Chem.* **275,** 21805–21808
28. Bissell, D. M., and Guzelian, P. S. (1980) *Ann. N. Y. Acad. Sci.* **349,** 85–98
29. Auer, K. L., Contessa, J., Brenz-Verca, S., Pirola, L., Rusconi, S., Cooper, G., Abo, A., Wymann, M. P., Davis, R. J., Birrer, M., and Dent, P. (1998) *Mol. Biol. Cell* **9,** 561–573
30. Park, J. S., Boyer, S., Mitchell, K., Gilfor, D., Birrer, M., Darlington, G., El Deiry, W., Firestone, G. L., Munger, K., Band, V., Fisher, P. B., and Dent, P. (2000) *J. Biol. Chem.* **275,** 18–28
31. Park, J. S., Qiao, L., Gilfor, D., Yang, M. Y., Hylemon, P. B., Benz, C., Darlington, G., Firestone, G., Fisher, P. B., and Dent, P. (2000) *Mol. Biol. Cell* **11,** 2915–2932
32. Cristiano, R. J., Smith, L. C., Kay, M. A., Brinkley, B. R., and Woo, S. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11548–11552
33. Brown, P. H., Alani, R., Preis, L. H., Szabo, E., and Birrer, M. J. (1993) *Oncogene* **8,** 877–886
34. Hylemon, P. B., Gurley, E. C., Stravitz, R. T., Litz, J. S., Pandak, W. M., Chiang, J. Y., and Vlahcevic, Z. R. (1992) *J. Biol. Chem.* **267,** 16866–16871
35. Davis, R. J. (2000) *Cell* **103,** 239–252
36. Stravitz, R. T., Hylemon, P. B., Heuman, D. M., Hagey, L. R., Schteingart, C. D., Ton-Nu, H. T., Hofmann, A. F., and Vlahcevic, Z. R. (1993) *J. Biol. Chem.* **268,** 13987–13993
37. Stravitz, R. T., Vlahcevic, Z. R., and Hylemon, P. B. (1999) in *Falk Symposium 108: Bile Acids and Cholestasis* (Paumgartner, G., Stiehl, A., Gerok, W., Keppler, D., and Leuschner, U., eds) Chapter 3, pp. 39–50, Academic Publishers, Boston
38. Qiao, D., Chen, W., Stratagoules, E. D., and Martinez, J. D. (2000) *J. Biol. Chem.* **275,** 15090–15098
39. Zhang, F., Subbaramaiah, K., Altorki, N., and Dannenberg, A. J. (1998) *J. Biol. Chem.* **273,** 2424–2428
40. Lee, H. Y., Suh, Y. A., Robinson, M. J., Clifford, J. L., Hong, W. K., Woodgett, J. R., Cobb, M. H., Mangelsdorf, D. J., and Kurie J. M. (2000) *J. Biol. Chem.* **275,** 32193–32199

MECHANISMS OF SIGNAL TRANSDUCTION:

Down-regulation of Cholesterol 7α -Hydroxylase (*CYP7A1*) Gene Expression by Bile Acids in Primary Rat Hepatocytes Is Mediated by the c-Jun N-terminal Kinase Pathway

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