Transcriptional activation of the cholesterol 7α-hydroxylase gene (*CYP7A*) by nuclear hormone receptors

Maurizio Crestani,* Azita Sadeghpour,* Diane Stroup,* Giovanni Galli,† and John Y. L. Chiang[1,*]

Department of Biochemistry and Molecular Pathology,* Northeastern Ohio Universities College of Medicine, PO Box 95, Rootstown Ohio 44272-0095; and Institute of Pharmacological Sciences,† School of Pharmacy, University of Milan, via Balzaretti 9, 20133 Milan, Italy

Abstract The gene encoding cholesterol 7α-hydroxylase (*CYP7A*), the rate-limiting enzyme in bile acid synthesis, is transcriptionally regulated by bile acids and hormones. Previously, we have identified two bile acid response elements (BARE) in the promoter of the *CYP7A* gene. The BARE II is located in nt −149/−118 region and contains three hormone response element (HRE)-like sequences that form two overlapping nuclear receptor binding sites. One is a direct repeat separated by one nucleotide DR1 (−146-TGGACTtAGTTCA-134) and the other is a direct repeat separated by five nucleotides DR5 (−139-AGTTCAaggccGGG TAA-123). Mutagenesis of these HRE sequences resulted in lower transcriptional activity of the *CYP7A* promoter/reporter genes in transient transfection assay in HepG2 cells. The orphan nuclear receptor, hepatocyte nuclear factor 4 (HNF-4)[1], binds to the DR1 sequence as assessed by electrophoretic mobility shift assay, and activates the *CYP7A* promoter/reporter activity by about 9-fold. Cotransfection of HNF-4 plasmid with another orphan nuclear receptor, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), synergistically activated the *CYP7A* transcription by 80-fold. The DR5 binds the RXR/RAR heterodimer. A hepatocyte nuclear factor-3 (HNF-3) binding site (−175-TGTTTGTTCT-166) was identified. HNF-3 was required for both basal transcriptional activity and stimulation of the rat *CYP7A* promoter activity by retinoic acid. Combinatorial interactions and binding of these transcription factors to BAREs may modulate the promoter activity and also mediate bile acid repression of *CYP7A* gene transcription.—Crestani, M., A. Sadeghpour, D. Stroup, G. Galli, and J. Y. L. Chiang. **Transcriptional activation of the cholesterol 7α-hydroxylase gene (*CYP7A*) by nuclear hormone receptors. *J. Lipid Res.* 1998.** 39: **2192–2200.**

Supplementary key words bile acid response element • gene transcription and regulation • nuclear hormone receptor • bile acid synthesis • cytochrome P450 • cholesterol 7α-hydroxylase

Bile acid biosynthesis is a pivotal pathway in maintaining the balance between cholesterol supply and disposal in the body (1, 2). The first and rate-limiting reaction of this metabolic pathway is the 7α-hydroxylation of cholesterol, which is catalyzed by cholesterol 7α-hydroxylase, a product of a liver-specific cytochrome P450 gene (*CYP7A*) (3). Many lines of evidence suggest that cholesterol 7α-hydroxylase is regulated mainly at the transcriptional level by a wide array of stimuli including bile acids, hormones, and second messengers (4–8). The expression of cholesterol 7α-hydroxylase activity is developmentally regulated (9, 10) and shows a strong diurnal rhythm (11, 12). Several transcription factors have been shown to regulate *CYP7A* expression. These include the diurnally regulated albumin D-site binding protein (DBP) (11, 12), C/EBP and LAP (12), hepatocyte nuclear factor (HNF)-3 (8, 13), and HNF-4 (14). In previous studies we identified regions important for the regulation of *CYP7A* gene transcription by bile acids and hormones (7, 8, 15–17). Hydrophobic bile acids are potent feedback inhibitors of *CYP7A* transcription and mediate their effects through two regions in the rat *CYP7A* promoter which we named bile acid response element (BAREs) (15, 17). These BARE sequences are highly conserved in different species and contain several direct repeats (DR) of hormone response elements (HRE) (AGGTCA) of various nucleotide spacing, i.e., a DR4 (−72/−57) in BARE-I (−74/−54), and overlapping DR1 (−146/−134) and DR5 (−139/−123) in BARE-II (−149/−123). The DR4 was recently identified as a binding site for chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) which strongly stimulated the transcription of the *CYP7A* (18). The DR4 also binds

Abbreviations: HNF-4, hepatocyte nuclear factor-4; HNF-3, hepatocyte nuclear factor-3; HNF-1, hepatocyte nuclear factor-1; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; ARP-1, apolipoprotein A-I regulatory protein; RARE, retinoic acid response element; DR, direct repeat; RAR, retinoic acid receptor; RXR, retinoid X receptor; C/EBP, CAAT/enhancer binding protein; LAP, liver activating protein; BARE, bile acid response element; PRS, phorbol ester response sequence; at-RA, all-*trans* retinoic acid; EMSA, electrophoretic mobility shift assay; HRE, hormone response element; CMV, cytomegalovirus; kb, kilobase; nt, nucleotide; Luc, luciferase; HDL, high density lipoprotein; PPARγ, peroxisome proliferator activated receptor γ; CREB, cAMP response element binding protein; CBP, CREB binding protein.

[1]To whom correspondence should be addressed.

the liver orphan receptor (LXR)/retinoid X receptor (RXR) (19), but the role of LXR on transcriptional regulation of the *CYP7A* has not been demonstrated. LXR is activated by oxysterols and has been suggested to play a role in regulating cholesterol metabolism (19). We found that all-*trans* retinoic acid (at-RA) could enhance the expression of the rat *CYP7A* and the retinoic acid response element (RARE) was mapped to the −176/−117 region of the rat *CYP7A* promoter (7, 16). Retinoid receptors and COUP-TFII are important regulators of morphogenesis, development, differentiation, and metabolism (reviewed in refs 20, 21). COUP-TFs have also been shown to play a critical role in organogenesis (22).

As both BARE-I and BARE-II are important in basal transcriptional activity and in mediating bile acid repression of the *CYP7A*, transcription factors binding to these response elements are likely to play important roles in bile acid feedback mechanism. To understand the mechanism of regulation of *CYP7A* gene transcription in bile acid synthesis and cholesterol homeostasis, it is important to identify and characterize transcription factors that bind to the identified BAREs. In this report, we identified HNF-4 and the RAR/RXR heterodimer as transcription factors binding to the BARE-II by site-directed mutagenesis of HRE sequences and transient transfection assays of *CYP7A* promoter/luciferase reporter chimeric genes in HepG2 cells, and by electrophoretic mobility shift assays (EMSA) of oligonucleotide probes with in vitro translated nuclear receptors.

EXPERIMENTAL PROCEDURES

Materials

Restriction and modification enzymes, the plasmids containing the luciferase reporter gene pGL2-Basic and pGL2-Promoter, the Luciferase Assay System and the TNT® Coupled Reticulocyte Lysate System were purchased from Promega (Madison, WI). AmpliTaq DNA polymerase was from Perkin-Elmer Applied Biosystems (Foster City, CA). The mammalian expression vector pcDNA3 was obtained from Invitrogen (Carlsbad, CA). pCMVβ was from Clontech (Palo Alto, CA). The Sequenase kit was acquired from Amersham Life Science (Cleveland, OH). The radiochemicals [α^{32}P]dCTP (3000 Ci/mmol) and [α^{35}S]dATP (1200 Ci/mmol) Sequenase grade were from ICN (Costa Mesa, CA) and Amersham International (England, UK), respectively. The oligonucleotides for site-directed mutagenesis of the rat *CYP7A* promoter were synthesized by M-Medical (Florence, Italy) whereas the double-stranded oligonucleotides for EMSAs were from Life Technologies (Gaithersburg, MD) and their sequences are listed in the corresponding figures. The human hepatoblastoma cell line, HepG2, was obtained from ATCC (Rockville, MD). Tissue culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD). The Nucleobond AX plasmid purification kit was from Macherey-Nagel (Düren, Germany). The expression plasmids for RARα, RXRα, in the pCMX backbone were generous gifts from Dr. Ronald M. Evans (The Salk Institute, San Diego, CA). pLen4S (HNF-4) was obtained from Dr. W. Chen (The Rockefeller University, New York, NY), pTF3A (COUP-TFII) was donated by Dr. M-J. Tsai (Baylor College of Medicine, Houston, TX).

Plasmid construction

The transversion mutations of the putative hormone response element in the region spanning nt −174 to −120 were generated by double polymerase chain reaction with the megaprimer system described by Barik (23). In some cases, the mutations were designed to avoid the creation of known transcription factor binding sites. The wild-type sequence from −376/+32 region and the corresponding mutants were subcloned into pGL2-Basic vector cut with *Kpn*I and *Xho*I. The HNF-4α expression vector, pCMV-HNF-4, was made by cutting pLen4S with *Bam*HI to release HNF-4 cDNA and subcloning the 2.8 kb insert into pcDNA3 digested with *Bam*HI. All the plasmids were verified by restriction analysis and sequencing, and were purified with Nucleobond columns according to the manufacturer's instructions.

Cell culture and DNA transfection

HepG2 cells were grown in 48-well cluster plates as described previously (7). HepG2 cells were grown to confluence for 6–7 days. Transfection experiments were performed with a modification of the calcium phosphate–DNA coprecipitation method (7) using 750 ng of reporter vector/well and 50 ng of pCMVβ to normalize for differences in transfection efficiency. HepG2 were exposed to transfection cocktails for 4 h and 16 h, respectively. In cotransfection experiments, the total quantity of plasmid was kept constant by adding an equal amount of empty expression vector. After transfection, cells were treated with the indicated concentrations of all-*trans* retinoic acid or an equivalent amount of ethanol. The concentration of ethanol never exceeded 0.1% of the total volume. Cells were harvested with lysis buffer (40 mm Tricine, pH 7.8, 50 mm NaCl, 2 mm EDTA, 1 mm $MgSO_4$, 5 mm DTT, 1% Triton X-100) and luciferase and β-galactosidase were assayed as described (7). Results are expressed as the ratio of luciferase activity versus β-galactosidase activity and are the mean ± standard deviations of triplicate samples. Each experiment was repeated at least twice.

Preparation of nuclear extracts

HepG2 cells were grown to confluence (5–6 days) in 10-cm dishes and nuclear extracts were prepared according to Dent and Latchman (24).

Electrophoretic mobility shift assay

Double-stranded synthetic oligonucleotides carrying the 5′-overhang nucleotides GATC were annealed by heating at 95°C in 2× SSC buffer (0.3 m NaCl, 0.03 m sodium citrate, pH 7.0) and slowly cooling to room temperature. The oligonucleotides were labeled by filling-in with [α^{32}P]dCTP using the Klenow fragment of DNA polymerase I and were purified through 15% polyacrylamide gels. RARα, RXRα, and HNF-4 were synthesized in vitro using the TNT coupled transcription/translation system programmed with the expression vectors pCMX-RARα, pCMX-RXRα, and pCMV-HNF-4, respectively. Binding reactions were carried out by preincubating 3–6 μl of in vitro synthesized receptors or 2–5 μg of nuclear extracts in binding buffer (12 mm HEPES, pH 7.9, 50 mm KCl, 1 mm EDTA, 1 mm dithiothreitol, 15% glycerol) containing 2 μg of poly(dI-dC)poly(dI-dC) on ice for 15–30 min. In experiments with receptors translated in vitro, 40 pmol of single-stranded unrelated oligonucleotide was added to decrease the nonspecific binding of proteins present in the reticulocyte lysate. Labeled double-stranded oligonucleotides (20–40 fmol) were added and incubated for 15 min at room temperature. In competition experiments, competitors were added along with probe. The total volume of the reactions was 20 μl. Samples were electrophoresed through a 4% non-denaturing polyacrylamide gel in 0.5× TBE (45 mm Tris-borate, 1 mm EDTA) at 160–200 V for 2 h at room temperature, dried, and quantitated

with IP Lab Gel software (Signal Analytical Corp., Vienna, VA) in conjunction with a PhosphorImager 445Si (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Statistical analysis was performed with Student's *t* test using Excel 5.0 software (Microsoft, Redmond, WA).

RESULTS

Site-directed mutagenesis of the rat *CYP7A* promoter

Our previous results have defined a BARE-II in a DNase I footprinted region (nt −149 to −118) of the rat *CYP7A* promoter (17). This sequence also mediates the stimulation by retinoic acid and inhibition by phorbol ester (16). To further characterize the function of nucleotide sequences in BARE-II, we sequentially mutated five hexameric HRE-like sequence motifs in plasmid p-376/Luc construct containing the nucleotide sequence from −376 to +32 of the rat *CYP7A* gene and studied the effects of mutations on *CYP7A* transcriptional activity by transient transfection of these chimeric constructs into HepG2 (**Fig. 1**). As shown in Fig. 1, mutations of the hexameric HREs half-sites in DR1 and DR5 (p-376m11, m2, and m12) reduced basal transcriptional activity of these chimeric genes to a much lower level than that of the wild-type promoter chimeric construct (p-376Luc), indicating that these DR1 and DR5 sequences are important for the basal level of *CYP7A* transcription. Mutation of a HRE sequence at −174/−163, (p-376m6 and m7) also reduced promoter activity. In contrast, mutations of -163AGCCT CTTCT-154 (p-376m8 and m9) resulted in the stimulation of reporter activity by 2- to 3-fold. This sequence is similar to a consensus sequence for a universal silencer element (ANCCTCTCT) found in many genes (25). Mutation of a 3′ half-site of a DR5, -128GGGTAA-123 (p-376m13), also stimulated the reporter gene activity. These results suggest that the transcription factor binding to the adjacent DR1 is more active than the one binding to the overlapping DR5 in enhancing basal transcriptional activity. Moreover, the factor(s) binding to the DR5 may interfere with those bound to the DR1. This mutagenesis analysis suggests that the HRE-like sequences at nt −175/−166 and −146/−123 are important in activating transcriptional activity of the rat *CYP7A* promoter.

Identification of a retinoic acid response element (RARE)

Previously we mapped a RARE in −176/−117 region of the rat *CYP7A* (16); the location of this RARE was further refined by assaying the stimulatory effects of all-*trans*-retinoic acid on the *CYP7A*/Luc reporter activity of wild-type and mutant constructs, shown in Fig. 1. In this experiment, 10 μm RA, instead of 1 μm used normally, was used to maximize the difference beween plasmids in their response to RA. Mutations of the HRE-half sites in the DR5 (p-376m2, m12 and m13) completely abolished stimulatory effect of retinoic acid, indicating that the DR5 (−139-**AGTTCA**aggcc**GGGTAA**-123) is a retinoic acid response element. However, mutation of an upstream HRE half-site, TGTTCT (p-376m6), also abolished the stimulatory effect by at-RA suggesting that this HRE half-site is essential for the response to at-RA. Interestingly, a much stronger stimulation of about 15-fold by at-RA was observed when the 5′ half-site of the DR1, TGGACT (p-376m11), was mutated. This DR1 (**TGGACT**t**AGTTCA**) shares its 3′ half-site with the 5′ half-site of the DR5 (RARE). This suggests that DR1 is not a RARE and destruction of the 5′ half-site of the DR1 may enhance retinoic acid receptor binding to the DR5 sequence. When the *CYP7A*/luciferase gene was transfected into CV-1, neither at-RA alone nor in

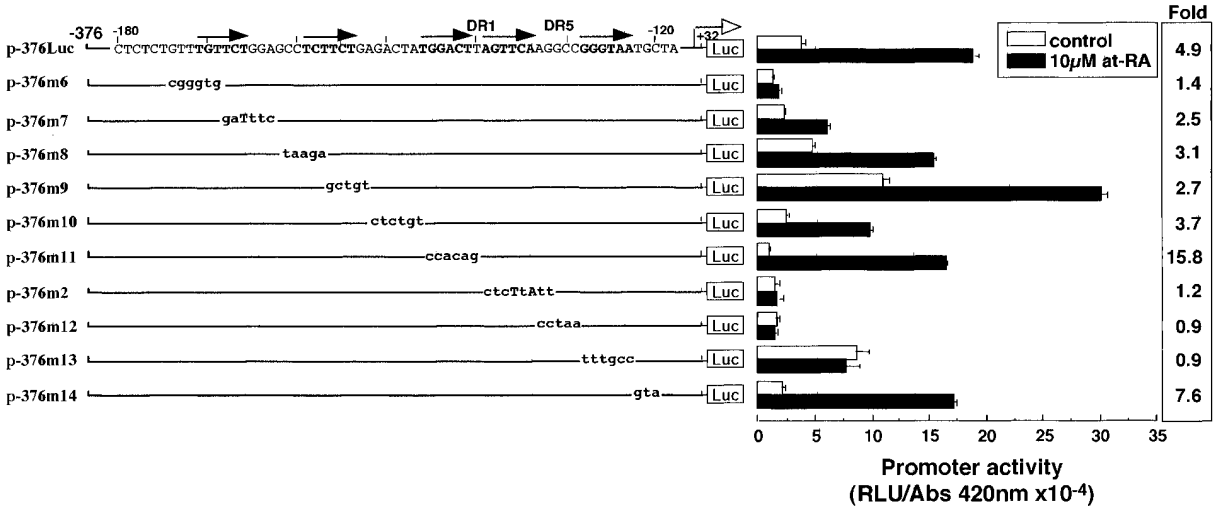


Fig. 1. Basal transcriptional activity and effect of retinoic acid on the rat *CYP7A*/luciferase chimeric gene constructs. The wild-type rat *CYP7A*/luciferase chimeric reporter plasmid p-376Luc (HRE indicated in bold types) and its derivative mutant constructs (mutations indicated in lower case) were transfected into HepG2 culture as described under Experimental Procedures. Transfected samples were incubated in the presence of ethanol (0.1% final) (open bars) or the indicated concentration of at-RA (filled bars). Results are expressed as relative luciferase unit (RLU) divided by β-galactosidase activity (expressed as Abs 420 nm) and are representative of at least three separate experiments each performed in triplicate. Fold stimulation by at-RA for each mutant is shown in the right panel.

combination with retinoid receptors cotransfected affected the transcription of *CYP7A* (data not shown). This suggests that transactivation of the *CYP7A* by retinoic acid is liver-specific. Neither the human nor the hamster *CYP7A*/luciferase genes responded to at-RA (data not shown).

Both RXR and RAR are required for binding to the DR5 sequence

We next assessed the interaction of in vitro translated retinoid receptors with the rat *CYP7A* promoter by means of EMSA. With the probe spanning the sequence −149/−118, we detected a band-shift only in the presence of both RXR and RAR (**Fig. 2**, lane 5), but not with RAR or RXR alone (Fig. 2, lanes 3 and 4). The band resulting from the binding of the RXR/RAR heterodimer was lost in the presence of a large excess of an unlabeled, competing oligonucleotide containing a consensus DR5 (**AGGTCAn_5AGGTCA**) (Fig. 2, lanes 6, 7). No binding of retinoid receptors was detected when the 3′ half-site of the DR5 was deleted but the DR1 motif was still intact (probe B, Fig. 2, lanes 9, 10, 11). On the other hand, the combination of RXR and RAR, but not RAR or RXR alone, could bind to a probe with the 5′ half-site of the DR1 mutated, but in which the DR5 remained intact (probe C, Fig. 2, lanes 13, 14, 15). Taken together, the results of transfection assays and electrophoretic mobility shift assays reveal that the RXR/RAR heterodimer binds to the DR5 motif and stimulates the transcriptional activity of the *CYP7A*. The RXR/RAR heterodimer prefers binding to a DR5 with the polarity of RXR occupying the 5′ half-site (26). Although RAR/RAR and RXR/RXR homodimers and RAR/RXR heterodimer (RAR binds to the 5′-site) are known to bind a typical DR1, they do not bind to this DR1 motif of the *CYP7A*.

Identification of an HNF-3 binding site

As the sequence about 35 nt upstream of the RARE was necessary for mediating the effect of retinoic acid, we wanted to test whether retinoid receptors or other nuclear receptors could bind to this region containing a HRE-like half-site. The results of gel–shift experiments with the probe spanning nt −180 to −151 incubated in the presence of in vitro synthesized RAR and/or RXR or HNF-4 show no band-shift (data not shown). However, nuclear extracts from HepG2 cells contained factors that shifted several bands (**Fig. 3**). These band-shifts were sequence-specific as addition of an excess of unlabeled probe could compete out the binding. When the competitor was the oligonucleotide containing the same mutation as in p-376m6, no competition of binding was observed (Fig. 3A). The sequence −177 TCTGTTTGTTCT −166 is similar to an HNF-3 binding site in the tyrosine aminotransferase gene (27) and contains the HRE-like half-site TGTTCT. An unlabeled oligonucleotide probe of the known HNF-3 binding

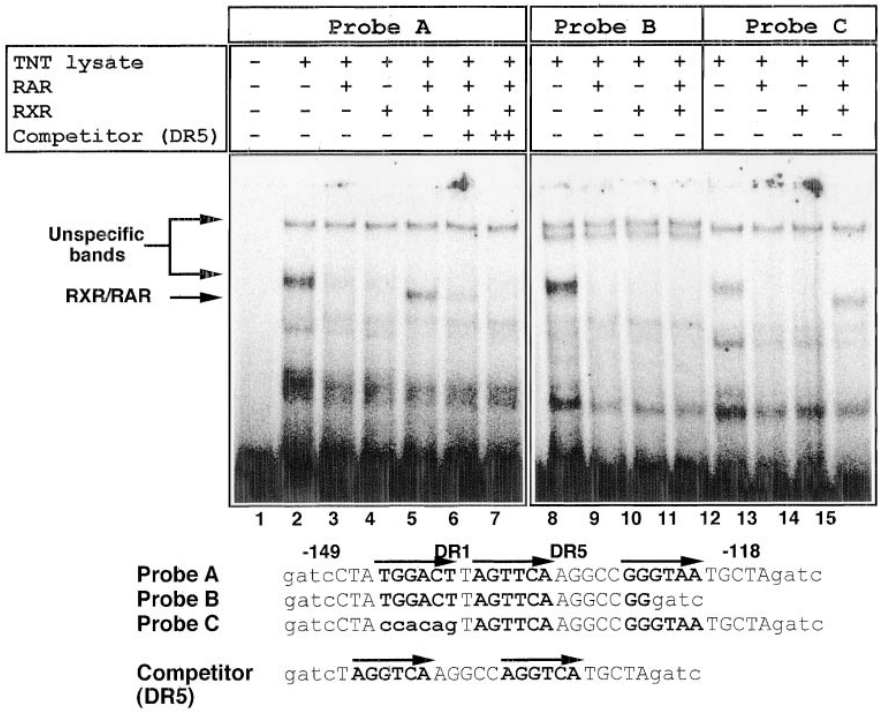


Fig. 2. Identification of an RXR/RAR binding site in BARE-II of the rat *CYP7A* by EMSA. Binding of retinoid receptors was analyzed by EMSA. Double-stranded oligonucleotides were labeled with Klenow as described in Experimental Procedures and incubated with in vitro translated receptors as indicated in the figure. Competition was carried out by adding 10-fold (+) or 30-fold (++) molar excess of an unlabeled double-stranded oligonucleotide bearing a canonical DR5/RARE. Putative DR1 and DR5 sequences are indicated in bold characters. The mutated site on the "probe C" is indicated with bold lower case characters. GATC overhangs at both ends of the probes are in lower case. Positions relative to the transcription start site are shown on top of the oligonucleotide sequences.

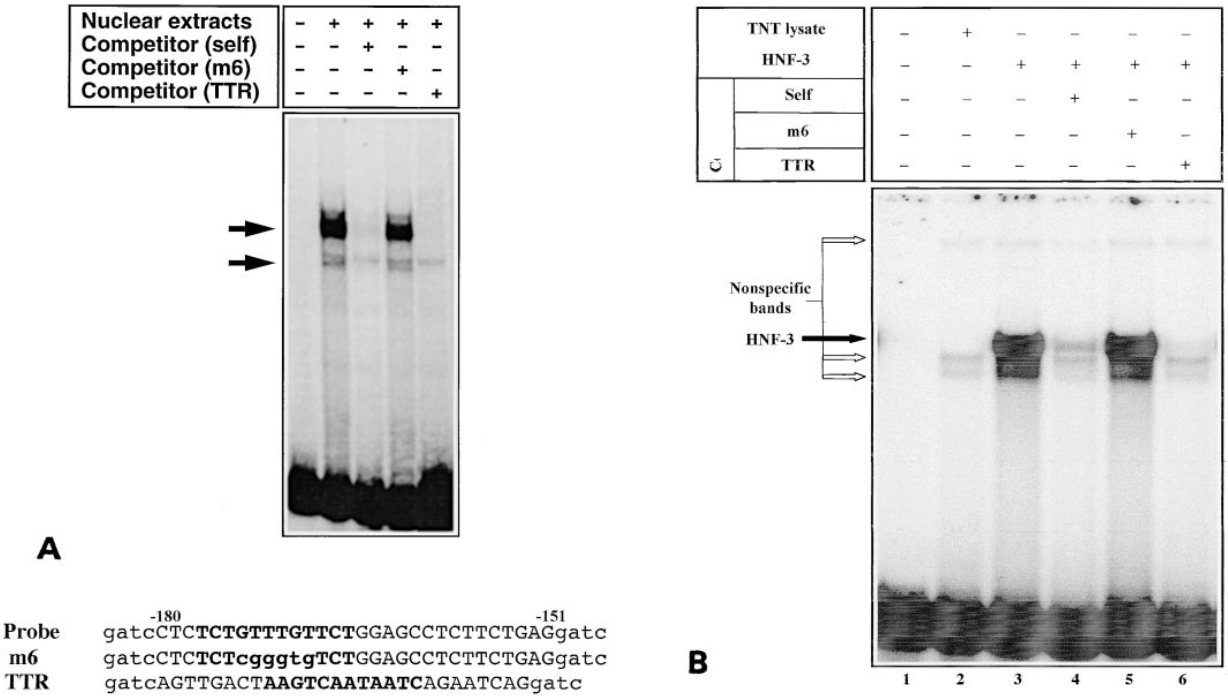


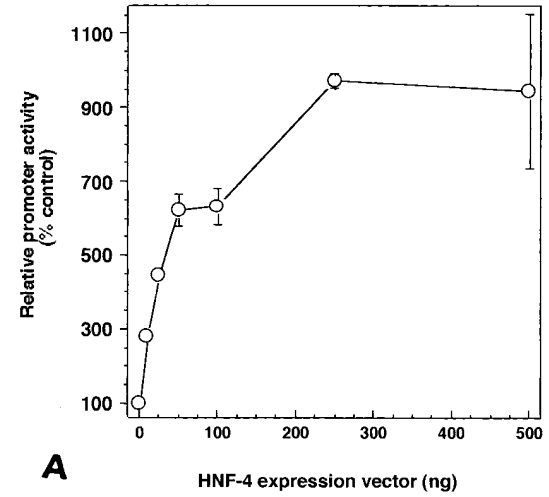
Fig. 3. Identification of an HNF-3 binding site by EMSA. Panel A: HepG2 nuclear extracts or panel B: in vitro translated HNF3α were incubated with the probe indicated on the bottom of the panel (putative binding site in the probe is in bold characters) under the conditions described in Experimental Procecures. Competitors were unlabeled probe, an oligonucleotide carrying the same mutation as p-376m6 (m6) (see Fig. 1) or an oligonucleotide bearing the HNF-3 consensus sequence at −110/−85 of the transthyretin gene (TTR), and were added at 100-fold molar excess. Arrows indicate the major bands specifically competed for by competitors.

sequence of the transthyretin gene (TTR) competed out all but one of the shifted bands (Fig. 3A). The cluster of bands shifted with probe −180/−151 resembles the one obtained with HNF-3α, β and γ isoforms (28). To further prove HNF-3 binding to this region, in vitro translated HNF-3α was used instead of crude nuclear extract and the same competition assays were repeated (Fig. 3B). When the pcDNA3 expression vector was used as a control, three unidentified bands were shifted (Fig. 3B, lane 2). EMSA with in vitro synthesized HNF-3α showed a strongly shifted band (Fig. 3B, lane 3). Unlabeled probe (Fig. 3B, lane 4) or probe containing the known HNF-3 binding sequence of transthyretin gene (TTR) (Fig. 3B, lane 6), could compete out the probes. When the probe bearing the same mutations as in p-376m6 was added as competitor, it did not compete out the probe (Fig. 3B, lane 5). It is clear that the −180/−151 region binds HNF3 which is required for both basal transcriptional activity of p-376/Luc and stimulation of promoter activity by retinoic acid (Fig. 1).

HNF-4 binds to the DR1 sequence in the −149/−118 region and competes with retinoid receptors for partially overlapping binding sites

Another transcription factor that may bind to the DR1 sequence is HNF-4, a liver-enriched transcription factor of the orphan nuclear receptor family. HNF-4 binds exclusively as a homodimer to a DR1 element (29). In cotransfection assays in HepG2 or CV-1 (data not shown) cultures, increasing amounts of HNF-4 expression vector enhanced the transcriptional activity of the *CYP7A* promoter/luciferase gene by 9-fold (**Fig. 4A**). All *trans*-retinoic acid or HNF-4 stimulated the wild-type reporter gene activity by 3- to 4-fold. However, the *CYP7A*/luciferase gene could not be further stimulated by combination of retinoic acid and HNF-4 (Fig. 4B). Mutation of the 5′ half-site (TGGACT) of the DR1 element completely abolished the transactivation by HNF-4 but the response to retinoic acid was enhanced (Fig. 4B, p-376m11). Conversely, mutation of the 3′ half-site (AGT TCA) of the DR1 element, which is also the 5′ half-site of the DR5 element (RARE), abrogated the activation by both HNF-4 and retinoic acid (Fig. 4B, p-376m2). It is noteworthy that also the basal activity of these two mutants was lower than that of the wild-type promoter, suggesting that binding of HNF-4 is important for the basal transcriptional activity of *CYP7A*.

EMSA with in vitro synthesized HNF-4 shows a clear shift of the probe nt −149/−118 (**Fig. 5**, probe A, lane 3) that could be competed out by addition of a large excess of unlabeled oligonucleotide containing a consensus binding site for HNF-4 (DR1, **AGGTCAtAGGTTA**) (Fig. 5, lanes 4, 5). No band-shift was detectable with the labeled probe with a mutation in the 5′ half-site of the DR1 element (Fig. 5, probe B, lane 8). Taken together, the results of transient transfection assays and DNA–protein interaction analysis demonstrate that the DR1 element (**TG GACTtAGTTCA**) is a functional binding site for HNF-4. Retinoid receptors and HNF-4 may compete for binding to partially overlapping DR1 and DR5 motifs in the −149/−118 region of the rat *CYP7A*.



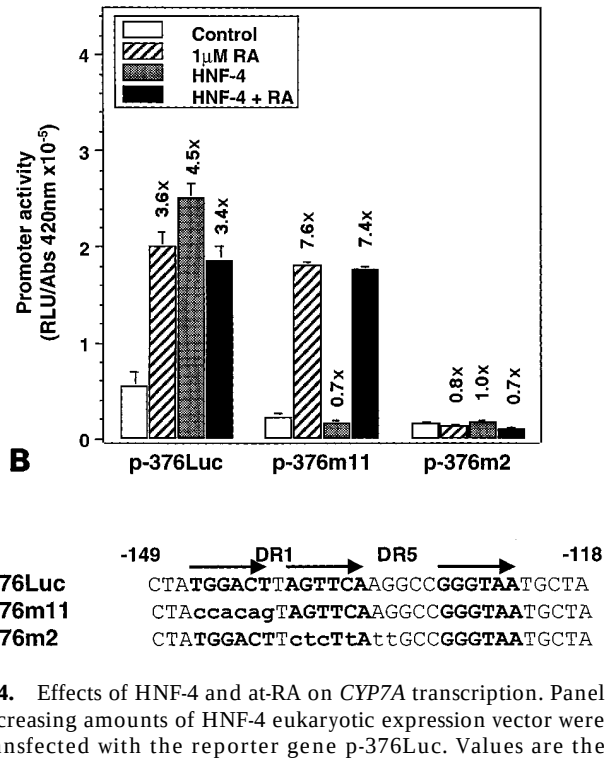


Fig. 4. Effects of HNF-4 and at-RA on *CYP7A* transcription. Panel A: increasing amounts of HNF-4 eukaryotic expression vector were cotransfected with the reporter gene p-376Luc. Values are the mean ± standard deviations of triplicate samples and are expressed as percentage of the samples cotransfected with the empty expression vector pcDNA3. The graph is representative of two independent experiments. Panel B: p-376Luc and the mutants in the putative DR1 sequence, p-376m11 and p-376m2, were cotransfected into HepG2 cells with 250 ng of pcDNA3 or pCMV-HNF-4 and treated with ethanol or at-RA as indicated. Results are expressed as luciferase divided by β-galactosidase activity and represent the mean ± standard deviations of triplicate samples. The graph is a representation of three separate experiments.

HNF-4 and COUP-TFII synergistically activate *CYP7A*

As both HNF-4 and COUP-TFII (18) can interact with the DR1 sequence in this region of the rat *CYP7A* promoter, we performed cotransfection experiments to study the effect of the combined overexpression of COUP-TFII and HNF-4 on the *CYP7A*. In HepG2 cultures, HNF-4 or COUP-TFII alone could strongly stimulate the reporter

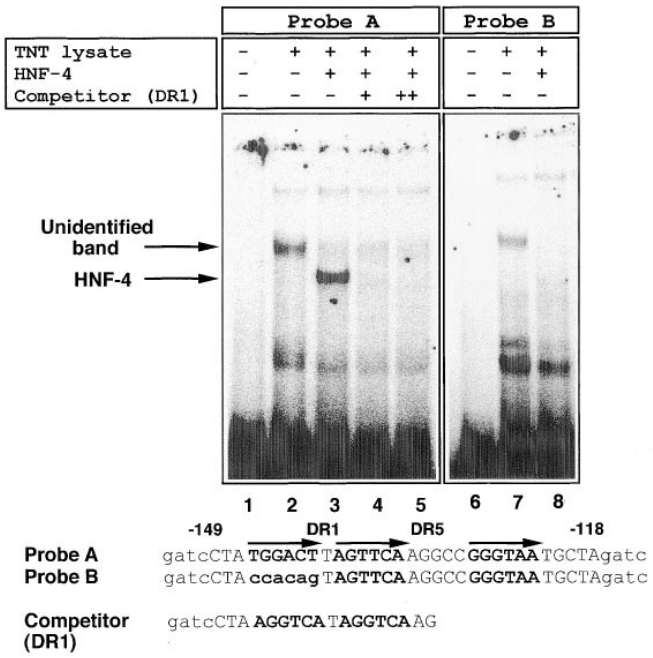


Fig. 5. Identification of an HNF-4 binding site in BARE-II of the rat *CYP7A* promoter by EMSA. HNF-4 was in vitro translated with the reticulocyte lysate system and an aliquot of the receptor was incubated either with probe A (nt −149/−118) or with a mutant in the upstream half-site of the DR1 element as shown in the figure. Where indicated, a 10-fold (+) or 100-fold (++) molar excess of the cold competitor carrying a perfect consensus sequence for HNF-4 was added to the binding reaction.

activity of the *CYP7A*/luciferase gene by 9-fold (**Fig. 6**). When both COUP-TFII and HNF-4 were cotransfected, the activity was stimulated about 80-fold (Fig. 6). To rule out a possible contribution of the downstream COUP-TFII binding site to this synergy, we also performed a transfection using a mutant in the downstream COUP-TFII binding site (18). The results obtained with this mutant were identical to those with the wild-type plasmid (data not shown), providing evidence that COUP-TFII can synergize with HNF-4 on this region of the rat *CYP7A* promoter.

DISCUSSION

Our results underscored the importance of the −149/−118 sequence in the transcriptional regulation of the *CYP7A* by hormones and bile acids. In particular, we have recently mapped a bile acid response element and a phorbol ester response sequence to this region (16, 17). In this report, HNF-4 and RXR/RAR were identified as the transcription factors that bind to this BARE sequence. An understanding of the arrangement of liver-specific transcription factors on the promoter may provide possible mechanisms for regulation of *CYP7A* transcription during development and under pathophysiological conditions.

The HNF-4 binding site in the BARE-II of *CYP7A* is a DR1 which is completely conserved in the rat, human, hamster, rabbit, and mouse gene. This HNF-4 binding site

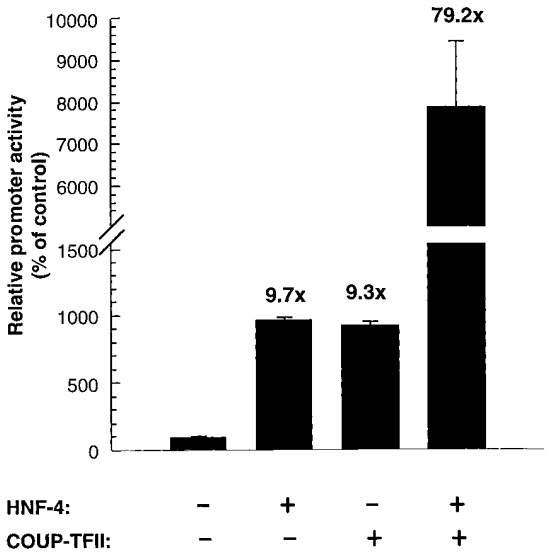


Fig. 6. Synergistic effect of HNF-4 and COUP-TFII on CYP7A. HepG2 cultures were cotransfected with p-376Luc (750 ng) and the combinations of HNF-4 (250 ng) and COUP-TFII (150 ng) eukaryotic expression vectors as indicated. The fold stimulation is indicated on top of each bar. Results are expressed as mean ± standard deviation of triplicate samples and the graph is representative of three independent experiments.

overlaps with a downstream DR5 which binds RXR/RAR heterodimer. However, the interaction of HNF-4 and RXR/RAR to their binding sites are mutually exclusive in the rat gene. HNF-4 may have higher affinity or preference over RXR/RAR on binding to BARE-II. It is interesting that HNF-3 is required for both basal level of expression and regulation of *CYP7A* gene transcription by retinoic acid in the liver. The HNF-3 site (-177-TCTGTTTGTTCT-166) identified is unique in that it contains an HRE half-site which may play a role in mediating response to hormones and other stimuli.

HNF-4 and COUP-TFII synergistically stimulate *CYP7A* transcription by 80-fold. This is similar to the synergistic stimulation of HNF-1 promoter by HNF-4 and COUP-TFII (30). COUP-TFII may act as an auxiliary cofactor for HNF-4 and both COUP-TFII and HNF-4 can interact with TFIIB(31, 32). COUP-TFII also could attenuate the stimulatory effect of retinoic acid. This is consistent with its negative role on transcription of many genes by interferring with other transactivators (33). It is likely that binding of HNF-4 to the DR1 in BARE-II may bring it into juxtaposition with the downstream BARE-I and facilitate a direct interaction of HNF-4 with COUP-TFII which binds the DR4 in BARE-I (**Fig. 7**). This will allow interaction of these liver-enriched transactivators with general transcription machinery and other coactivators or corepressors that regulate *CYP7A* gene transcription. HNF-4 has been recently shown to interact with CREB binding protein, CBP, a transcriptional coactivator of many transcription factors (34).

HNF-4 binds to DNA as a homodimer and is required for activation of liver-specific genes involved in lipid metabolism, such as apoA-I, A-II, B-100 and C-III (35). Multiple loci on chromosomes 3, 5, and 11 were linked to regulation of *CYP7A* mRNA level in response to atherogenic diet (36). These loci coincide with the loci controlling the high density lipoprotein (HDL) level in response to atherogenic diet. Interestingly, apoA-I, the major apolipoprotein in HDL particles, is also regulated by HNF-4 (28). Our finding is consistent with the suggestion that apoA-I and *CYP7A* might be coordinately regulated (37). HNF-4 may play a major role in regulating cholesterol homeostasis.

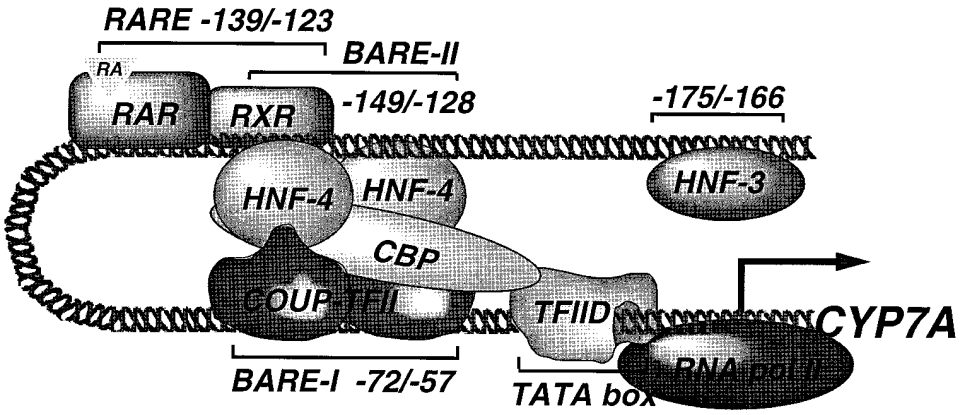


Fig. 7. Model of regulation of *CYP7A* by HNF-4, COUP-TFII, HNF-3, and RAR/RXR. HNF-4 binds to the DR1 at nt −146/−134 as a homodimer and can interact with COUP-TFII homodimer bound to the DR4 at nt −72/−57. The TC-rich region (nt −117/−99) bends the DNA and loops the upstream BARE-II over the BARE-I, and facilitates the interaction between these orphan nuclear receptors. HNF-4 may recruit coactivator CBP which directly interacts with TATA box binding protein complexes TFIID and general transcription factors (i.e., TFIIB, not shown), and enhance the rate of transcription of RNA ploymerase II. The RARE is partially overlapped with the HNF-4 site. Note the polarity of binding of retinoic receptors to the DR5 (i.e., RXR binds to the 5′ half-site and RAR binds to the 3′ half-site). HNF-3 binds to the region at −175/−166 of the rat *CYP7A* promoter, transactivates the basal level of gene transcription, and may also enhance transactivation by these orphan nuclear receptors.

Moreover, defects of HNF-4α gene have been found in maturity-onset diabetes of the young (MODY) (38, 39) and in late-onset non-insulin-dependent diabetes mellitus (NIDDM) (40). Liver genes involved in lipid metabolism and regulated by HNF-4 are likely candidate genes in polygenic disease of diabetes.

The finding that HNF-4 and COUP-TFII are major transcription factors binding to the BAREs suggests that these orphan nuclear receptors may play roles in mediating bile acid response. The endogenous ligands for HNF-4 and COUP-TFII are not known. Recent study based on indirect evidence suggested that fatty acyl-CoA thioesters were the ligands of HNF-4α and could activate or repress gene transcription depending on the chain length and degree of saturation of fatty acids (41). Many endogenous steroids and fatty acids have been identified as ligands for orphan receptors, such as peroxisome proliferator-activated receptor γ (PPARγ) (42, 43). These findings are consistent with our hypothesis that hydrophobic bile acids or their metabolites might activate bile acid receptors and exert their negative effects by binding to hormone response elements in BARE of the *CYP7A* gene (15). We have previously identified that RXR bound to the DR1 sequence in BARE-II (18); however, the RXR partner has not been identified.

Bile acids have been shown to stimulate protein kinase C isoforms and may be involved in down-regulation of *CYP7A* transcription by bile acids (44). It is possible that this signal transduction pathway may lead to phosphorylation of transcription factors and interfere with their interaction and binding to BAREs. This study suggests that combinatorial interaction of nuclear receptors binding to BAREs in response to physiological stimuli may determine the levels of expression of cholesterol 7α-hydroxylase activity in the liver during development and under different pathophysiological conditions (45). We are currently studying the roles of and mechanisms by which these orphan nuclear receptors regulate *CYP7A* transcription in response to bile acid feedback. Unraveling the mechanisms of feedback regulation of *CYP7A* expression by bile acids may reveal potential targets for new therapeutics for diseases related to bile acid metabolism such as atherosclerosis, diabetes, cholestasis, and gallstone disease.

We would like to thank Mrs. Rhonda Kimmel and Mr. Gottardo Baffi for their excellent technical support. We are grateful to Drs. R. Evans, M-J. Tsai, and W. Chen for kindly providing us with their plasmids.This work was supported by NIH grants GM31584 and DK44442 to JYLC; a National Research Service Award (HL09156) to D.S.; and a grant from the Italian Ministry of University and Scientific Research to G.G.

Manuscript received 10 July 1998.

REFERENCES

1. Russell, D. W., and K. D. R. Setchell. 1992. Bile acid biosynthesis. *Biochemistry.* **31:** 4737–4749.
2. Chiang, J. Y. L., and Z. R. Vlahcevic. 1996. The regulation of cholesterol conversion to bile acids. *In* Advances in Molecular and Cell Biology. JAI Press, Inc., Greenwich, London. 269–316.
3. Li, Y. C., D. P. Wang, and J. Y. L. Chiang. 1990. Regulation of cholesterol 7α-hydroxylase in the liver. *J. Biol. Chem.* **265:** 12012–12019.
4. Hylemon, P. B., E. C. Gurley, R. T. Stravitz, J. S. Litz, W. M. Pandak, J. Y. L. Chiang, and Z. R. Vlahcevic. 1992. Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. *J. Biol. Chem.* **267:** 16866–16871.
5. Pandak, W. M., Y. C. Li, J. Y. L. Chiang, E. J. Studer, E. C. Gurley, D. M. Heuman, Z. R. Vlahcevic, and P. B. Hylemon. 1991. Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholate and cholesterol in the chronic biliary diverted rat. *J. Biol. Chem.* **266:** 3416–3421.
6. Stravitz, R. T., P. B. Hylemon, D. M. Heuman, L. R. Hagey, C. D. Schteingart, H-T. Ton-Nu, A. F. Hoffman, and Z. R. Vlahcevic. 1993. Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. *J. Biol. Chem.* **268:** 13987–13993.
7. Crestani, M., D. Stroup, and J. Y. L. Chiang. 1995. Hormonal regulation of the cholesterol 7α-hydroxylase gene (*CYP7*). *J. Lipid. Res.* **36:** 2419–2432.
8. Wang, D-P., D. Stroup, M. Marrapodi, M. Crestani, G. Galli, and J. Y. L. Chiang. 1996. Transcriptional regulation of the human cholesterol 7α-hydroxylase gene (*CYP7A*) in HepG2 cells. *J. Lipid Res.* **37:** 1831–1841.
9. Ness, G. C. 1994. Developmental regulation of the expression of genes encoding proteins involved in cholesterol homeostasis. *Am. J. Med. Genet.* **50:** 355–357.
10. Smith, J. L., S. R. Lear, and S. K. Erickson. 1995. Developmental expression of elements of hepatic cholesterol metabolism in the rat. *J. Lipid Res.* **36:** 641–652.
11. Lavery, D. J., and U. Schibler. 1993. Circadian transcription of the cholesterol 7α-hydroxylase gene may involve the liver-enriched bZIP protein DBP. *Genes & Dev.* **7:** 1871–1884.
12. Lee, Y-H., J. A. Alberta, F. J. Gonzalez, and D. J. Waxman. 1994. Multiple, functional DBP sites on the promoter of the cholesterol 7α-hydroxylase P450 gene, *CYP7*. *J. Biol. Chem.* **269:** 14681–14689.
13. Molowa, D. T., W. S. Chen, G. M. Cimis, and C. P. Tan. 1992. Transcriptional regulation of the human cholesterol 7α-hydroxylase gene. *Biochemistry.* **31:** 2539–2544.
14. Cooper, A. D., J. Chen, M. J. Botelho-Yetkinler, Y. Cao, T. Taniguchi, and B. Levy-Wilson. 1997. Characterization of hepatic-specific regulatory elements in the promoter region of the human cholesterol 7α-hydroxylase gene. *J. Biol. Chem.* **272:** 3444–3452.
15. Chiang, J. Y. L., and D. Stroup. 1994. Identification of a putative bile acid-responsive element in cholesterol 7α-hydroxylase gene promoter. *J. Biol. Chem.* **269:** 17502–17507.
16. Crestani, M., A. Sadeghpour, D. Stroup, G. Galli, and J. Y. L. Chiang. 1996. The opposing effects of retinoic acid and phorbol esters converge to a common response element in the promoter of the rat cholesterol 7α-hydroxylase gene (*CYP7A*). *Biochem. Biophys. Res. Commun.* **225:** 585–592.
17. Stroup, D., M. Crestani, and J. Y. L. Chiang. 1997. Identification of a bile acid response element in the cholesterol 7α-hydroxylase gene (*CYP7A*). *Am. J. Physiol.* **273:** G508–G517.
18. Stroup, D., M. Crestani, and J. Y. L. Chiang. 1997. Orphan receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and retinoid X receptor activate and bind the rat cholesterol 7α-hydroxylase gene (*CYP7A*). *J. Biol. Chem.* **272:** 9833–9839.
19. Lehmann, J. M., S. A. Kliewer, L. B. Moore, T. A. Smith-Oliver, B. B. Oliver, J-L. Su, S. S. Sundseth, D. A. Winegar, D. E. Blanchard, T. A. Spencer, and T. M. Wilson. 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J. Biol. Chem.* **272:** 3137–3140.
20. Kastner, P., M. Mark, and P. Chambon. 1995. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life. *Cell.* **83:** 859–869.
21. Chambon, P. 1996. A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10:** 940–954.
22. Pereira, F. A., Y. Qiu, M-J. Tsai, and S. Y. Tsai. 1995. Chicken ovalbumin upstream promoter transcription factor (COUP-TF): expression during mouse embryogenesis. *J. Steroid Biochem. Mol. Biol.* **53:** 503–508.
23. Barik, S. 1993. PCR Protocols: Current Methods and Applications. B. A. White, editor. Humana Press, Totowa, NJ. 277–286.

24. Dent, C. D., and D. S. Latchman. 1993. *In* Transcription Factors: a Practical Approach. D. S. Latchman, editor. IRL Press at Oxford University Press, New York, NY. 1–26.
25. Ogbourne, S., and T. M. Antalis. 1998. Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. *Biochem. J.* **331:** 1–14.
26. Mangelsdorf, D. J., K. Umesono, and R. M. Evans. 1994. The Retinoids: Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. 2nd ed. Raven Press Ltd, New York. 319–349.
27. Rigaud, G., J. Roux, R. Pictet, and T. Grange. 1991. In vivo footprinting of rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. *Cell.* **67:** 977–986.
28. Harnish, D. C., S. Malik, E. Kilbourne, R. Costa, and S. K. Karathanasis. 1996. Control of apolipoprotein A-I gene expression through synergistic interactions between hepatocyte nuclear factors 3 and 4. *J. Biol. Chem.* **271:** 13621–13628.
29. Jiang, G., L. Nepomuceno, K. Hopkins, and F. M. Sladek. 1995. Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. *Mol. Cell. Biol.* **15:** 5131–5143.
30. Ktistaki, E., and I. Talianidis. 1997. Chicken ovalbumin upstream promoter transcription factors act as auxiliary cofactors for hepatocyte nuclear factor 4 and enhance hepatic gene expression. *Mol. Cell. Biol.* **17:** 2790–2797.
31. Malik, S., and S. Karathanasis. 1995. Transcriptional activation by the orphan nuclear receptor ARP-1. *Nucleic Acids Res.* **23:** 1536–1543.
32. Malik, S., and S. K. Karathanasis. 1996. TFIIB-directed transcriptional activation by the orphan nuclear receptor hepatocyte nuclear factor 4. *Mol. Cell. Biol.* **16:** 1824–1831.
33. Cooney, A. J., S. Y. Tsai, B. W. O'Malley, and M-J. Tsai. 1992. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.* **12:** 4153–4163.
34. Yoshida, E., S. Aratani, H. Itou, M. Miyagishi, M. Takiguchi, T. Osumu, K. Murakami, and A. Fukamizu. 1997. Functional association between CBP and HNF4 in trans-activation. *Biochem. Biophys. Res. Commun.* **241:** 664–669.
35. Sladek, F. M. 1994. Hepatocyte Nuclear Factor 4 (HNF4). *In* Liver Gene Expression. F. Tronche, and M. Yaniv, editors. R. G. Landes Co., Austin, TX. 207–230.
36. Machleder, D., B. Ivandic, C. Welch, L. Castellani, K. Reue, and A. J. Lusis. 1997. Complex genetic control of HDL levels in mice in response to an atherogenic diet. *J. Clin. Invest.* **99:** 1406–1419.
37. Dueland, S., D. France, S-L. Wang, J. D. Trawick, and R. A. Davis. 1997. Cholesterol 7α-hydroxylase influences the expression of hepatic apoA-I in two inbred mouse strains displaying different susceptabilities to atherosclerosis and in hepatoma cells. *J. Lipid Res.* **38:** 1445–1453.
38. Yamagata, K., N. Oda, P. J. Kaisaki, S. Menzel, H. Furuta, M. Vaxillaire, L. Southam, R. D. Cox, G. M. Lathrop, V. V. Boriraj, X. Chen, N. J. Cox, Y. Oda, H. Yano, M. M. LeBeau, S. Yamada, H. Nishigori, J. Takeda, S. S. Fajans, A. T. Hattersley, N. Iwasaki, T. Hanson, O. Pedersen, K. S. Polonsky, R. C. Turner, G. Velho, J-C. Chevre, P. Froguel, and G. I. Bell. 1996. Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). *Nature.* **384:** 455–458.
39. Yamagata, K., H. Furuta, N. Oda, P. J. Kaisaki, S. Menzel, N. J. Cox, S. S. Fajans, S. Signorini, M. Stoffel, and G. I. Bell. 1996. Mutations in the hepatocyte nuclear factor-4α gene in maturity-onset diabetes of the young (MODY1). *Nature.* **384:** 458–460.
40. Hani, E. B., L. Suaud, P. Boutin, I-C. Chevre, E. Durand, A. Philippi, F. Demenais, N. Vionnet, H. Furuta, G. Velho, G. I. Bell, B. Laine, and P. Froguel. 1998. A missense mutation in hepatocyte nuclear factors-4α, resulting in a reduced transactivation activity, in human late-onset non-insulin-depedent diabetes mellitus. *J. Clin. Invest.* **101:** 521–526.
41. Hertz, R., J. Magenheim, I. Berman, and J. Bar-Tana. 1998. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4α. *Nature.* **392:** 512–516.
42. Forman, B. M., P. Tontonoz, J. Chen, R. P. Brun, B. M. Spiegelman, and R. M. Evans. 1995. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell.* **83:** 803–812.
43. Forman, B. M., J. Chen, and R. M. Evans. 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Proc. Natl. Acad. Sci. USA.* **94:** 4312–4317.
44. Stravitz, R. T., Y-I. Rao, Z. R. Vlahcevic, E. C. Gurley, W. D. Jarvis, and P. B. Hylemon. 1996. Hepatocellular protein kinase C activation by bile acids: implications for regulation of cholesterol 7α-hydroxylase. *Am. J. Physiol.* **271:** G293–G303.
45. Chiang, J. Y. L. 1998. Regulation of bile acid synthesis. *Front. Biosci.* **3:** 176–193.