HNF4 and COUP-TFII interact to modulate transcription of the cholesterol 7α-hydroxylase gene (*CYP7A1*)

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Abstract **The gene for cholesterol 7α-hydroxylase (*CYP7A1*) contains a sequence at nt −149 to −118 that was found to play a large role in determining the overall transcriptional activity and regulation of the promoter. Hepatocyte nuclear factor 4 (HNF4) and chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) synergistically activate transcription of the *CYP7A1* promoter. Transactivation of *CYP7A1* by HNF4 in the human hepatoma cell line, HepG2, was enhanced by cotransfection with COUP-TFII or the basal transcription element binding protein (BTEB). HNF4 prepared from rat liver nuclear extracts bound to oligomers homologous to the nt −146 to −134 sequences in electrophoretic mobility shift assays (EMSA), which corresponded to a conserved region containing a direct repeat of hormone response elements spaced by one nucleotide (DR1). The sequences surrounding this DR1 were found to be essential for the HNF4 transactivation. In vitro-translated COUP-TFII was found to bind the adjacent sequences from nt −139 to −128 (DR0), but COUP-TFII interacted with this region at a much lower affinity than to the COUP-TFII-site at nt −72 to −57 (DR4). Mutations at nt −139 to −128 or nt −72 to −57 reduced the COUP-TFII and HNF4 synergy; however, these COUP-TFII-binding sequences were not absolutely required for the cooperative effect of HNF4 and COUP-TFII on transactivation. These results indicated that the observed transactivation was the result of protein/protein interactions facilitated by the juxtaposition of the binding elements.**—Stroup, D., and J. Y. L. Chiang. **HNF4 and COUP-TFII interact to modulate transcription of the cholesterol 7α-hydroxylase gene (*CYP7A1*). *J. Lipid Res.* 2000.** 41: **1–11.**

Supplementary key words bile acid synthesis • hepatocyte nuclear factor 4 • chicken ovalbumin upstream promoter transcription factor II • orphan receptors • transcriptional regulation • cholesterol metabolism

The catabolism of cholesterol to bile acids in the liver is the main mechanism for cholesterol elimination from the body, and thus plays an important role in maintaining cholesterol homeostasis (1). Cholesterol 7α-hydroxylase (EC 1.14.13.17) catalyzes the first and rate-limiting step in the neutral bile acid synthesis pathway. Transcription of the gene (*CYP7A1*) for this enzyme is specific to the post-partum liver and regulated by a number of factors, including bile acids, hormones, and circadian rhythm (2–6). Mice in which *Cyp7A1* has been inactivated have a severe phenotype; 85% of the pups do not survive past 18 days and exhibit impaired bile, lipid, and vitamin metabolism (7, 8).

Because this tightly regulated gene is so important for health and is expressed in a strictly liver-specific manner, the *CYP7A1* promoter has been the subject of intense study. Transcription factor binding sites for BTEB (9), C/EBPα, C/EBPβ, DBP (5, 10), COUP-TFII (11), HNF3α (12), and HNF4α (13) have been mapped. Of particular interest are HNF4 and COUP-TFII because of the important roles they play in regulating gene transcription in eukaryotic cells, such as defining tissue-specific expression, embryogenesis, and response to certain effectors (14).

HNF4 is expressed in the liver, intestine, and kidney (15, 16), and the homodimer recognizes a direct repeat (DR) of 5′AGGTCA 3′ separated by one nucleotide (DR1) as a binding site. Transcripts for this factor are detected very early in development (17) and death in utero results from disruption of HNF4 (18). For these reasons and because HNF4 is known to regulate the expression of other liver-enriched transcription factors, such as HNF1 (19), it is believed that HNF4 activity is required very early in the cascade of transcriptional events that lead to the differentiation of endodermal cells. Defects in the HNF4 gene locus have been associated with maturity-onset diabetes of the young (MODY1) (20) and late-onset non-insulin-dependent diabetes mellitus (NIDDM) (21).

Like HNF4, COUP-TF-gene disruptions are lethal early

Abbreviations: BTEB, basal transcription element binding protein; C/EBP, CAAT/enhancer binding protein; COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; *CYP7A1*, cholesterol 7α-hydroxylase gene; DMEM, Dulbecco's modified Eagle's medium; DBP, D-site binding protein; DR, direct repeat of the hormone response element; EMSA, electrophoretic mobility shift assay; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); HNF, hepatocyte nuclear factor; HRE, hormone response element; LSM, linker scanning mutation; LUC, luciferase; nt, nucleotide; PEPCK, phospho*enol*pyruvate carboxykinase; RXR, retinoid X receptor.

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in embryogenesis (22). The members of this family of factors can interact with DRs with a variety of spacings (23). This promiscuous binding specificity confers COUP-TFs with the ability to interfere with a number of other nuclear receptors by competing for the HRE, including: estrogen receptor (24), peroxisome proliferator activated receptor (25), retinoic acid receptor (26), thyroid hormone receptor, and vitamin D3 receptor (27). COUP-TFII, which has an intrinsic transcriptional activation activity (28), can influence a promoter's response to a signal in more ways than just passively repressing signals by competition. In fact, several mechanisms for active repression (silencing, quenching, and transrepression) have been proposed (29) where COUP-TFs can influence basal or activated transcription by protein–protein interaction with either the preinitiation complex via TFIIB (28) or other transcription factors (22). This multifaceted ability of the COUP-TFs may determine the ultimate effect a given signal has on a given cell type (30).

HNF4 is among the nuclear receptors shown to be influenced by COUP-TFs. The effect of COUP-TFs on the transcriptional activation activity of HNF4 depends on the promoter. COUP-TFs negatively effect gene transcription by interfering with HNF4 on the apolipoprotein gene promoters: apoA-I (31), apoA-II (32), apo-B (32), and apoC-III (32, 33). However, HNF4 and COUP-TFs act synergistically on the promoters for the phospho*enol*pyruvate carboxykinase (PEPCK) (34) and HNF1 (19) genes, and this cooperative activation effect on transcription may be tissue-specific. COUP-TFII binding to the HNF1 promoter is not required for COUP-TFII to act as an auxiliary cofactor for HNF4-mediated activation of transcription. HNF4 and COUP-TF interact through the ligand binding domain of HNF4 (19), which corresponds to the same region identified as the interaction site for the transcriptional coactivator, CREB-binding protein (CBP) (35). COUP-TFII and HNF4 have been previously described to act cooperatively on the rat *CYP7A1* promoter (13) or competitively (36) on the human *CYP7A1* promoter.

Transactivation by recombinant HNF4 of *CYP7A1* promoter–luciferase reporter constructs in HepG2 cells is disrupted by mutations in nt −149 to −118 and in vitro-expressed HNF4 binds to this sequence in the EMSA (13). This element has at least three imperfect hormone response elements (HRE), including a DR1 and an overlapping DR5. RXR and an unidentified partner can bind to the DR1, and this binding is increased by retinoic acid treatment (11). COUP-TFII, also, interacts with the nt −149 to −118 sequence, as shown by supershift in DNA/nuclear protein complexes with COUP-TFII specific antibodies (11) and with COUP-TFII overexpressed in COS1 cells (36). The exact sequences important for COUP-TFII binding to this site have not been determined, nor has it been determined whether COUP-TFII actually interacts with the DNA instead of associating with HNF4 by protein/protein interaction.

The HNF4 response element at nt −146 to −129 is completely conserved over evolution and mutations in this sequence interfere with the transcriptional response of *CYP7A1* to bile acids, phorbol esters and retinoic acid (2, 13, 37). Here, we show HNF4 was the predominant factor binding to this element, and endeavored to identify other factors that influence HNF4 in the *CYP7A1* promoter context. COUP-TFII is one such factor. This paper focuses on the effect of COUP-TFII on HNF4 transactivation. To this end, we determined how mutations in the *CYP7A1* promoter changed the effect of simultaneous expression of these two transcription factors on transcriptional activity of luciferase promoter reporter constructs.

EXPERIMENTAL PROCEDURES

Materials

Oligomeric DNA was synthesized by Life Technologies/GIBCO-BRL (Gaithersburg, MD). DNA restriction and modifying enzymes, reporter lysis buffer, luciferase assay system, and the reporter vectors pGL2-Basic and pGL2-Promoter were purchased from Promega (Madison, WI). The radioactive isotopes α(^{32}P)dCTP (3000 Ci/mol) and α(^{35}S)-dATP (1200 Ci/mmol) sequencing grade were obtained from DuPont/NEN (Boston, MA). Plasmids were purified with Nucleobond Plasmid Purification Kit (Clontech Lab., Palo Alto, CA). Expression plasmids for the transcription factors were generous gifts from Drs. W. Chen, Rockefeller University, NY, for pLen4S (HNF4); Dr. Fuji-Kuriyame, Tohoku University, Sendai, Japan, for pRSVBTEB (BTEB); and Dr. M-J. Tsai, Baylor University, TX, for pTF3A (COUP-TFII). Antiserum against COUP-TFII was kind gift from Dr. Tsai; anti-Arp-1 was from Dr. S. Karathanasis, Wyeth-Ayerst Research, PA; and anti-HNF4 was from Dr. F. Sladek, University of California, Riverside, CA. Affinity-purified rabbit polyclonal antibodies against RAR (sc-773; cross-reacts with RAR α1, α2, β1, β2, γ1, and γ2 isoforms), RXR (sc-774; cross-reacts with RXR α, β, and γ isoforms), cJun, LAP (sc-746), PPARα (sc-1985) and C/EBPα (sc-61) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other reagents were from Sigma Chemical Company (St.Louis, MO).

Cell line

The human hepatoma cell line (HepG2, ATCC #HB8065) was obtained from the American Type Culture Collection, Rockville, MD, and cultivated in a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 (GIBCO-BRL, Grand Island, NY) supplemented with 100 units/ml penicillin G–streptomycin sulfate (Celox Corp., Hopkins, MN) and 10% (v/v) heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS).

Plasmids

Construction of plasmids p-416/+32, p416Δ74/54, pGL2h298, and p-200/+32 were described previously (11). The construction of p416LSM146/141, p416LSM139/134, and p416LSM133/128 were described previously (2). The plasmid pcDNA3.1/HISC::COUP-TF was constructed by ligating the EcoRI fragment of pTF3A (27) containing the cDNA for human COUP-TFII into the EcoRI site of pcDNA3.1/HIS.C (Stratagene, La Jolla, CA). pcDNA3-HNF4 was constructed by moving the BamHI fragment containing the HNF4 cDNA from pLENS4 (14) to pcDNA3 (Stratagene, La Jolla, CA).

Preparation of nuclear extracts

Nuclei from Sprague-Dawley rat liver and HepG2 tissue culture cells were isolated and nuclear proteins were extracted es-

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sentially as described in Latchman (38) and Sierra, Tian, and Schibler (39).

Transfection assays

Confluent cultures of HepG2 cells grown in 12-well tissue culture plates were transfected with DNA by calcium phosphate method as previously described (40, 41). The ratio of reporter plasmid to expression plasmids encoding transcription factors was 1 to 0.2, with 2.5 μg of reporter plasmid per well. These conditions were chosen to give near maximal linear response to the cotransfected transcription factors as determined by dose–response curves for COUP-TFII (11), HNF4 (13), and BTEB (9). Luciferase activities were determined with the luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions using a Lumat LB9501 luminometer (Berthold Systems, Inc., Pittsburgh, PA) to measure initial total light emission for 10 sec. Luciferase activities were normalized for transfection efficiencies by dividing relative light units by β-galactosidase activity expressed from cotransfected pCMVβ plasmid (Clonetech Lab. Palo Alto, CA). β-Galactosidase activities were determined using *o*-nitrophenyl-β-d-galactopyranoside as a substrate (42). The average of the corrected luciferase activity from cell extracts is given and error bars indicate the standard deviation of activity from triplicate samples. All transfections were repeated at least two times. Statistical significances were analyzed by Student's *t*-test, using Sigma Plot software (Jandel Scientific).

Purification of nuclear proteins

After dialysis against GSB (12 mm HEPES, pH 7.9, 50 mm KCl, 1 mm EDTA, 1 mm dithiothreitol, and 15% glycerol), 5 mg of nuclear extract was loaded on to a 10 ml heparin-Sepharose column (Sigma Chemical Co. St. Louis, MO) equilibrated with the same buffer. The column was washed with 100 volumes each with GSB containing 250 mm, 350 mm, and 650 mm KCl. The fractions containing protein were assayed by EMSA using nt −149 to −118 as the labeled oligomer probe. Fractions with shifting activity were further purified by binding to a column prepared by immobilizing 40 nmoles multimerized double-stranded nt −149 to −118 oligomer on CNBr-activated agarose (Sigma Chemical Co. St. Louis, MO). The shifting activity was eluted with GSB containing 250 mm KCl, and the process was repeated. Native molecular weights were determined separating samples through a standardized Superdex-200 HR 10/30 FPLC column (Pharmacia Biotech, Piscataway, NJ) equilibrated with GSB.

Electrophoretic mobility shift assay (EMSA)

Double-stranded synthetic probes for EMSA were prepared by heating equal molar amounts of complementary synthetic oligomers to 100°C in 2× SSC (0.3 m NaCl, 0.03 m Na_3 citrate, pH 7.0), holding at 88°C for 10 min, then allowing the hybridization mix to cool to ambient temperature in a heating block. The resulting double-stranded fragments were designed with single-stranded 5′ overhangs for end-labeling by incorporating α(^{32}P)dCTP (3000 Ci/mol) with the Klenow fragment of DNA polymerase I. Oligonucleotides blunted with non-labeled dNTPs were used as cold competitors in EMSA. Labeled fragments were isolated from a 15% polyacrylamide gel. DNA binding reactions were initiated by the addition of 100,000 cpm of oligomer probe to proteins preincubated with 1 μg poly(dI−dC)·poly(dI−dC) and 40 pmol single-stranded synthetic oligomer dissolved in 1× GSB, and the 20 μl reactions were incubated for 30 min at 25°C. Samples were run on 4% polyacrylamide gels, dried and autoradiographed (43). EMSA were quantitated with IP Lab Gel software (Signal Analytics, Corp., Vienna, VA) in conjunction with a Molecular Dynamics Phosphorimager 445Si.

RESULTS

Activation of *CYP7A1* promoter/luciferase reporter constructs with recombinant transcription factors

A number of *CYP7A1* promoter constructs with mutations were tested for their ability to be transactivated by HNF4, COUP-TFII and BTEB at ratios found to stimulate transcription of the reporter constructs proportional to the mass of expression plasmid used, as determined previously (13) for HNF4, (11) for COUP-TFII and (9) for BTEB. The wild-type reporter construct, p −416/+32, encoding the rat *CYP7A1* sequences from nt −416 to +32, showed greater luciferase activity with both HNF4 and COUP-TFII expressed together than either transcription factor separately (**Fig. 1A**). The CMV promoter, as measured by β-galactosidase reporter activity, was not affected by cotransfection with COUP-TFII or HNF4 individually or in combination. COUP-TFII strongly transactivated all rat *CYP7A1* promoter constructs, especially when the DR4 at nt −74 to −57, a previously identified COUP-TFII activation site (11), was intact. The construct, p416LSM146/141, in which the 5′ HRE half-site of the DR1 was mutated, was not stimulated by HNF4 and activation by COUP-TFII was not potentiated by overexpression of HNF4. The plasmid p416LSM139/134 that has the 3′ half-site of the DR1 mutated could not respond to HNF4, but some potentiation was recovered. The DR1 was intact on p416LSM133/128, but could not respond to overexpressed HNF4. However, the HNF4 and COUP-TFII coexpression stimulated the promoter to a greater extent than COUP-TFII alone. The plasmid p416Δ74/54 could respond to HNF4 and, though p416Δ74/54 had a weakened response to COUP-TFII alone as expected after deleting the COUP-TFII activation element, it robustly responded to the combined expression of HNF4 and COUP-TFII. This cooperative effect could have been due to protein/protein interactions between HNF4 and COUP-TFII without the need for COUP-TFII binding to the promoter, as was described previously (19), or to other sequences being able to substitute for the strong COUP-TFII-activation site at nt −74 to −54. It is unlikely that COUP-TFII-response elements reside in the sequences from nt −416 to −201 in that removal of these sequences did not reduce the COUP-TFII transactivation (11) and (Fig. 1B). When both the elements at nt −149 to −128 and nt −74 to −54 are deleted, the activity is not significantly different between HNF4 and COUP-TFII expressed together and COUP-TFII overexpressed singly (data not shown). The lack of potentiation with p416LSM146/141 indicated that there were no other sites on the nt −416 to +32 promoter fragment that can substitute for the nt −146 to −134.

The robust synergy of HNF4 and COUP-TFII on the luciferase activity of p416Δ74/54, in spite of the lack of a strong COUP-TF site, led to us to test whether HNF4 and COUP-TFII would transactivate the human *CYP7A1* gene, which does not bind COUP-TFII (Arp-1) at nt −71 to −53 (44). COUP-TFII and HNF4 did not apparently interfere with each other on the human nt −298 to +24 *CYP7A1* promoter/reporter construct (pGLh298). In fact, though

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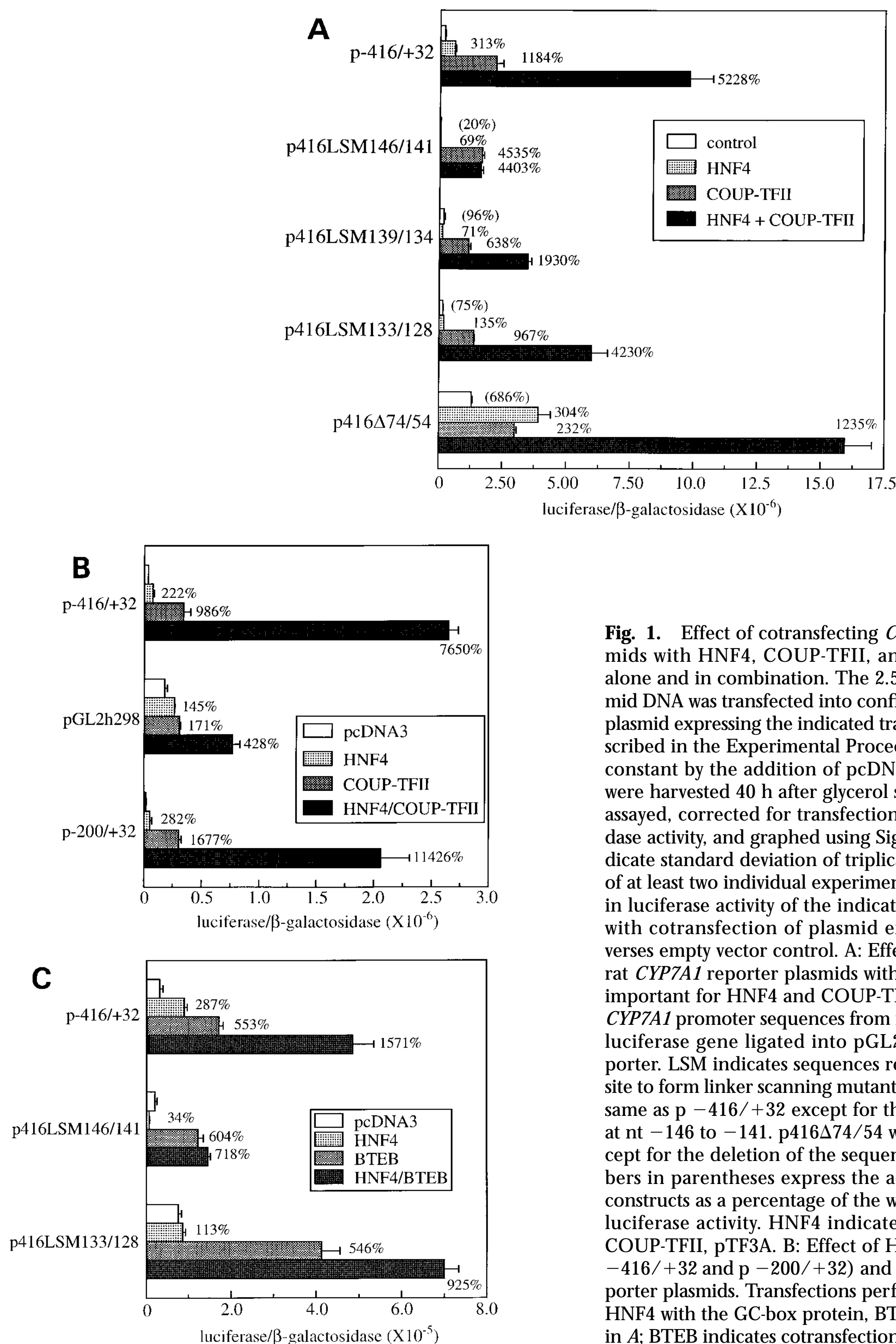

Fig. 1. Effect of cotransfecting *CYP7A*/luciferase promoter plasmids with HNF4, COUP-TFII, and BTEB expression plasmids, alone and in combination. The 2.5 μg of promoter/reporter plasmid DNA was transfected into confluent HepG2 cells with 0.5 μg of plasmid expressing the indicated transcription factor per well, as described in the Experimental Procedures. Total DNA mass was kept constant by the addition of pcDNA3 without an insert. The cells were harvested 40 h after glycerol shock; the luciferase activity was assayed, corrected for transfection efficiency with the β-galactosidase activity, and graphed using Sigma Plot software. Error bars indicate standard deviation of triplicate samples, data representative of at least two individual experiments. Percentages indicate change in luciferase activity of the indicated promoter/reporter plasmid with cotransfection of plasmid expressing transcription factor verses empty vector control. A: Effect of HNF4 and COUP-TFII on rat *CYP7A1* reporter plasmids with mutations in sequences found important for HNF4 and COUP-TFII. p-416/+32 contains the rat *CYP7A1* promoter sequences from nt −416 to +32 upstream of the luciferase gene ligated into pGL2-Basic, which serves as the reporter. LSM indicates sequences replaced with an *Nhe*I restriction site to form linker scanning mutants, i.e., p416LSM146/141 was the same as p −416/+32 except for the substitution of the sequences at nt −146 to −141. p416Δ74/54 was the same as p −416/+32 except for the deletion of the sequences from nt −74 to −54. Numbers in parentheses express the activity of the mutant promoter constructs as a percentage of the wild type p −416/+32 corrected luciferase activity. HNF4 indicates cotransfection with pLen4S; COUP-TFII, pTF3A. B: Effect of HNF4 and COUP-TFII on rat (p −416/+32 and p −200/+32) and human (pGL2h298) *CYP7A1* reporter plasmids. Transfections performed as in *A*. C: Interaction of HNF4 with the GC-box protein, BTEB. Transfections performed as in *A*; BTEB indicates cotransfection with pRSVBTEB.

the overall effect was smaller in magnitude than with the rat gene, coexpression resulted in an increase larger than that expected by merely addition of the two effects (Fig. 1B). The luciferase activity was not repressed by the addition of COUP-TFII with HNF4 at any of the ratios of transcription factors to reporter plasmid assayed. This would indicate that the HNF4 binding at nt −146 to −128 was not antagonized by the COUP-TFII, even without the strong activating activity of COUP-TFII through the DR4 element at nt −74 to −57.

To determine whether the synergy was specific for COUP-TFII, another, unrelated, transcription factor that

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was found to have affinity for the nt −140 to −118 region was tested. BTEB, a GC-box binding protein we have shown to interact with nt −140 to −118 and nt −101 to −82 (9), also acted synergistically with HNF4 (Fig. 1C). Like COUP-TFII, plasmids with mutations in nt −146 to −140, did not show synergy with coexpression of BTEB with HNF4, but the plasmid with mutations in nt −133 to −128 was coactivated in spite of no transactivation with HNF4 alone. The BTEB site at nt −101 to −82 was intact and thus available for BTEB-binding in this clone (p416LSM133/128).

The transfection data suggested that, as the HNF4-response element covered more than the expected 13 bp, factors binding to adjacent sites may be required cofactors for HNF4 transactivation. The effect of HNF4 on the *CYP7A1* promoter was context-dependent like other previously identified HNF4 elements. Consistent with this was the finding that HNF4 overexpression does not transactivate the nt −149 to −118 when positioned upstream of the SV40 promoter (data not shown). To examine how the context of the HNF4-response element influenced the ability of HNF4 to transactivate the *CYP7A1* promoter, the sequences important for DNA binding were determined with HNF4 purified from rat liver and in vitro expressed COUP-TFII.

EMSA using proteins purified from rat liver nuclear extracts and in vitro translated proteins

Nuclear extracts from rat liver were dialyzed against GSB and bound to a heparin column equilibrated with the same buffer, and eluted off with a step gradient into three fractions: 0.10 to 0.25 m KCl, 0.25 to 0.35 m KCl, and 0.35 to 0.65 m KCl. The majority of the nt −149 to −118 oligomer shifting activity eluted of the heparin column in the 0.35 to 0.65 m KCl step. Western blotting of the 0.35 to 0.65 m KCl fractions indicated that they contained material that reacted with antibodies directed against HNF4 and COUP-TFII. The 0.35 to 0.65 m KCl fraction of the heparin column was further purified through a DNA-affinity column composed of immobilized nt −149 to −118 double-stranded oligomer. HNF4 antigens eluted from the immobilized nt −149 to −118 oligomer column

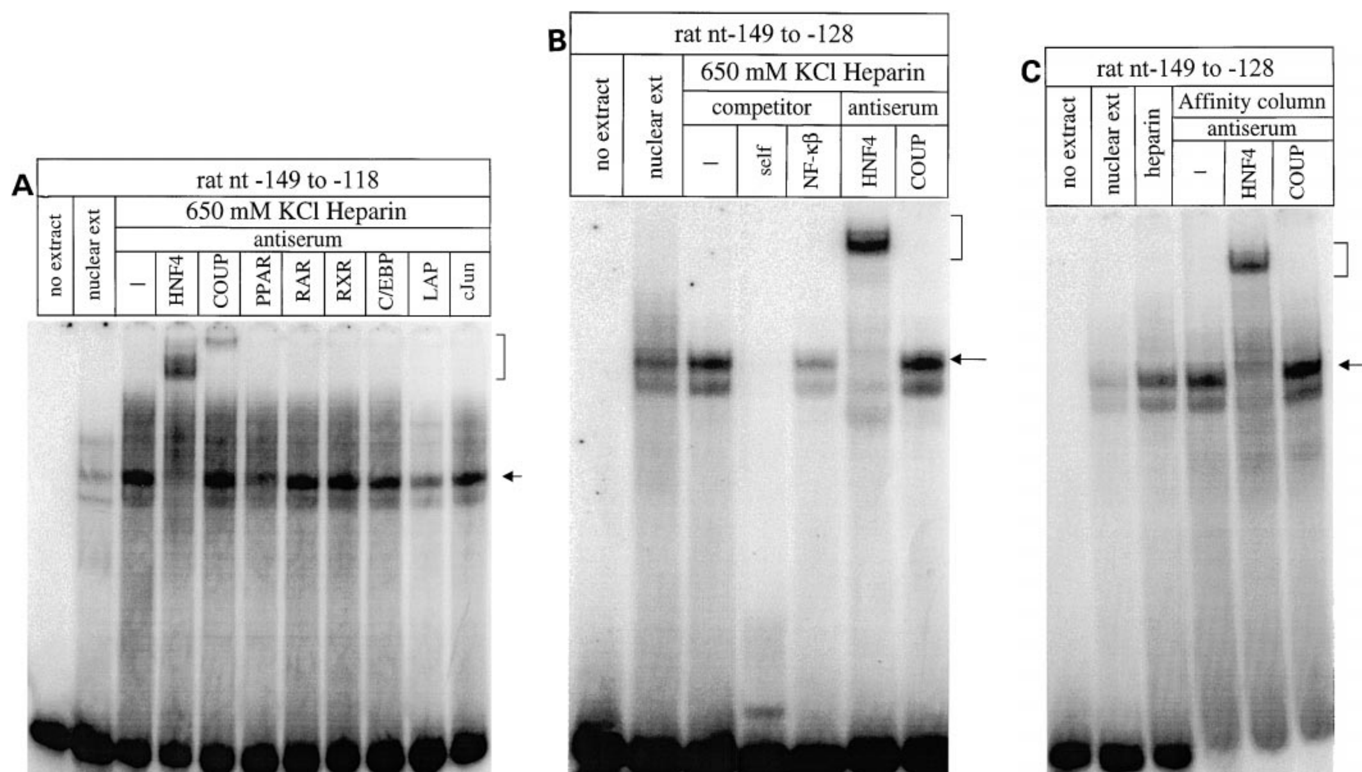

Fig. 2. Antibody supershift EMSA using partially purified rat liver nuclear extracts. Arrow indicates band associated with HNF4 shifting activity and bands dependent on the addition of antibody indicated with a bracket. A: The nt −149 to −118 probe was incubated with 5 μg of nuclear extracts that were fractionated as described in the Experimental Procedures; no extract, probe mixed with buffer salts and dI:dC; nuclear ext, nuclear extract; 650 mM Hep, material eluted with 350 to 650 mm KCl from a heparin column; Antiserum, antigen to which serum was raised is indicated over each lane, 1 μl antiserum was added to each reaction containing probe and material from the 650 mm KCl eluent. B: Antibody supershift EMSA performed as in *A*, using an oligonucleotide probe homologous to the rat *CYP7A1* promoter sequence from nt −149 to −128, prepared by digesting the nt −149 to −118 probe with *Msp*I before labeling; competitor, unlabeled competitor added to a 1 to 10 ratio with the radiolabeled probe; −, no competitor; self, unlabeled nt −149 to −118; NF-Kβ, the binding sequence for the transcription factor NF-Kβ: gatcAGTTGAGGGGACTTTCCCAGGCgatc was used as an unrelated control competitor. C: Antibody supershift EMSA performed as in *B*, proteins and serum are as indicated: heparin, 350 to 650 mm KCl from a heparin column; large type Affinity column, material eluted with 250 mm KCl off of a immobilized nt −149 to −118 DNA-affinity column; −, no antiserum; HNF4, antiserum directed against HNF4; COUP, antiserum directed against COUP-TFII.

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when the column was washed with GSB/250 mm KCl. The purification did not achieve homogeneity, as determined by silver staining after SDS-PAGE; however, COUP-TFII was not detected in HNF4-containing fractions eluted from the DNA-affinity column by Western blotting (data not shown).

The nt −149 to −118 probe bound to the 0.35 to 0.65 m KCl heparin fractions could be supershifted by antibody against HNF4 and COUP-TFII, but not PPAR, RAR, RXR, C/EBP, LAP or cJun (**Fig. 2A**). Several bands were apparent in the EMSA when the nt −149 to −118 probe was shifted by the nuclear extracts, but the most prominent band, indicated by an arrow in Fig. 2, could be supershifted by the addition of HNF4 antibody. From this it was concluded that the main nt −149 to −118 shifting complex included HNF4 or an HNF4-related protein. The COUP-TFII supershift was lost when the sequences from nt −127 to nt −118 were removed from the nt −149 to −118 probe, but HNF4 binding was unaffected (Fig. 2B). The variable, faster migrating band was associated with an HNF4-related degradation product because the activity could be shifted with antibody directed against HNF4 and the band increased over time in storage. Further purification of the extract by DNA-affinity column resulted in a loss of the supershift of COUP-TFII, but not the supershift with the HNF4 antibody (Fig. 2C). Using DNA affinity-purified material, the shifting activity identified as HNF4 was reduced by mutation in the sequences from nt −145 to −134 (**Fig. 3A**). The protein had a somewhat greater affinity for the probe with the mutations at nt −136 to −134 than the other mutations in the DR1, though the binding to nt −136/−134 probe was significantly less than the binding to mutations outside the DR1. Concluding that mutations in the 3′ HRE of the DR1 allowed some, although weakened, HNF4 interaction with its binding site would be consistent with the observation of synergy between HNF4 and COUP-TFII in spite of the mutation in the 3′ HRE of the HNF4 binding element (p416LSM139/134 of Fig. 1A).

HNF4 and COUP-TFII can interact in solution (19), raising the possibility that COUP-TFII did not interact directly with the nt −149 to −118 probe, which would be an attractive explanation for the lack of competition for binding. However, COUP-TFII and HNF4 protein/protein interaction are believed not to survive the EMSA conditions (35), so we investigated whether COUP-TFII could

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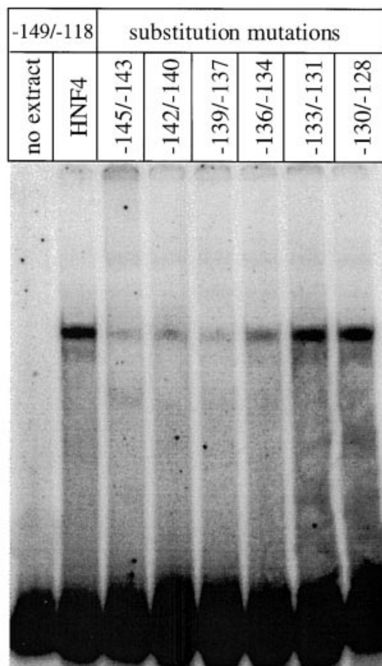
A.

-149 CTATGGACTTAGTTCAAGGCCGGGTAATGCTA -118



B.

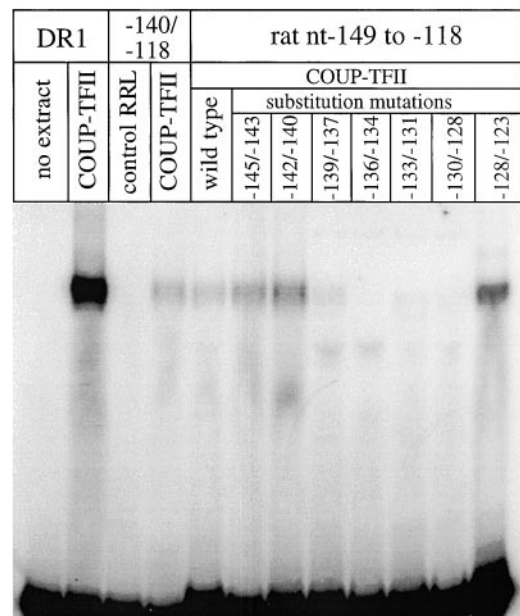


Fig. 3. Changes in gel shift pattern resulting from transversion mutations in the sequence from nt −149 to −118. Wild type (WT) oligomer probe corresponds to the rat *CYP7A1* sequence from nt − 149 to −118. Numbers over lanes correspond to the nucleotides mutated away from wild-type sequence according to the following rule: A to C, C to A, G to T, and T to G. For example, −145/−143 corresponds to the nt −149 to −128 probe with nt −145 G changed to T, nt −144 G to T and nt −143 A changed to C. A: The material eluted with 650 mm KCl from heparin was bound to a DNA-affinity column and eluted with 250 mm KCl in GSB as described in the Experimental Procedures. The eluent was dialyzed and subjected to EMSA as above. B: TNT coupled reticulocyte lysates programed with pcDNA3.1-HIS::COUP (COUP-TFII) were reacted with indicated probes. Wild-type and substitution mutations are the same as in *A* and *B*, −140/−118, probe homologous with the rat *CYP7A* sequence from nt −140 to −118 with GATC overhangs for labeling; DR1, known COUP-TFII binding site gatcctaggggtcaaaggtcaaatggaagatc, the HRE half-sites are underlined (53); control RRL, TNT lysate programmed with empty vector.

bind directly to the DNA sequence at nt −149 to −118 in the absence of HNF4 to explain the supershift with antibody directed against COUP-TFII (Fig. 2A). COUP-TFII was expressed in vitro and reacted with wild-type and mutant probes based on the sequences from nt −149 to −118. The nt −140 to −118 probe (which lacks the 5′ half site of the DR1) and the nt −149 to −118 probes with mutations in the sequences from nt −145 to −140 were shifted by the in vitro-expressed COUP-TFII, but probes with mutations from nt −139 to −128 did not (Fig. 3B). These sequences do not correspond to either the DR1 or DR5, but to a previously unrecognized DR0. In vitro-expressed HNF4 and COUP-TFII could bind in the absence of the other and were found to have distinct shifts (data not shown). The migration of the shifting complex formed by in vitro-expressed HNF4 and the nt −149 to −118 probe was indistinguishable from that formed by HNF4 derived from rat liver (data not shown).

The COUP-TFII binding to the rat and human nt −149 to −118 was considerably weaker than to the rat nt −74 to −53. In an EMSA optimized for COUP-TFII binding, increasing the mass of the rat nt −74 to −53 probe resulted in a rapid increase in bound probe, whereas the amount of bound nt −149 to −118 probe barely increased with increasing amounts of added probe (**Fig. 4A**). Though the equilibrium was further to the left than that of nt −74 to −53 (Fig. 4B), COUP-TFII binding to the nt −149 to −118 probe was sequence specific (Fig. 3B).

COUP-TFII–HIS did not shift the human wild-type nt −71 to −51 probe (Hwt), but interacted strongly with the corresponding sequences of the rat probe (R − 74/−53) (**Fig. 5**). Introducing a G into the human sequence so that the HRE 5′ half site of the DR4 present in the rat sequence was created, allowed detectable but weak interaction of COUP-TFII with the probe (HmG1). Deletion of that G in the rat sequence (RmG2) resulted in the reduction, but not the loss, of the ability of COUP-TFII to shift the probe. Mutating the T at position nt −60 of the rat to the C found in the human sequence prevented binding altogether (RmT9). Apparently, the binding of COUP-TFII to nt −74 to −57 was more sensitive to changes in the 3′ HRE half-site (Fig. 5), whereas, the 5′ HRE half site was more important for determining HNF4 binding to nt −146 to −134 (Fig. 3A). **Figure 6** shows the alignment of the rat and human sequences in the nt −149 to −118 and nt −74 to −55 regions. The rat nt −149 to −128 sequence was found to be similar to the human, except for one nucleotide at the extreme 3′ nucleotide (nt −128 G→A) which mutates the sequence towards the consensus HRE (Fig. 6).

DISCUSSION

In summary, we found that the synergistic effect of HNF4 and COUP-TFII on the transcription of *CYP7A1* required the sequences from nt −146 to −141. HNF4 was the predominant factor from rat liver nuclear extracts that bound to this DR1 element located at nt −146 to −134 and COUP-TFII had affinity for a DR0 found at nt −139 to −128. In spite of apparently overlapping binding sites, HNF4 and COUP-TFII were shown not to interfere with one another in cotransfection assays, which was consistent with the strong binding of HNF4 to nt −146 to −134 and weak binding of COUP-TFII to nt −139 to −128, as determined by EMSA. Deletion of a previously mapped COUP-TFII-response element at nt −72 to −57 dramatically re-

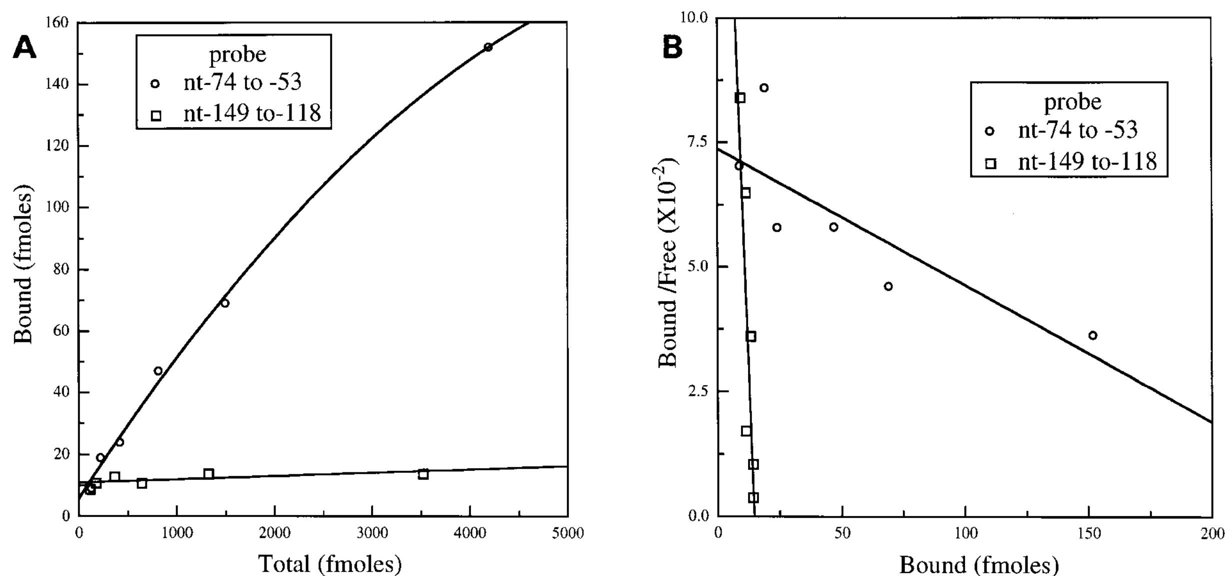


Fig. 4. EMSA of COUP-TFII binding to the rat nt −149 to −118 and nt −74 to −53 sequences. Relative affinities of COUP-TFII for the rat sequences nt −149 to −118 and nt −74 to −53 were estimated by EMSA. A constant amount of in vitro expressed COUP-TFII-HIS was reacted with sequential 2-fold dilutions of labeled probes based on the rat nt −74 to −53 sequences and nt −149 to −118. Samples were counted in triplicate. A: Graph of total fmoles of probe plotted against fmoles shifted. B: Data plotted as bound probe verses ratio of bound to free probe.

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probe	IR1	Hwt	HmG1		R-74/-53		RmG2		RmT9		R-65/-54	
COUP	+	+	−	+	−	+	−	+	−	+	−	+

IR1 gatcAGGTCAATGACCTgatc

R-74/-53 gatcTTTGGTCACTCAAGTTCAAGTTgatc

Hwt gatcTTTG -TCAACCAAGCTCAAGTTgatc

HmG1 gatcTTTGGTCAACCAAGCTCAAGTTgatc

RmG2 gatcTTTG -TCACTCAAGTTCAAGTTgatc

RmT9 gatcTTTGGTCACTCAAGCTCAAGTTgatc

R-65/-54 gatcTCAAGTTCAAGTgatc

Fig. 5. EMSA of COUP-TFII binding to the rat and human nt −74 to −53 sequences. Top: EMSA with in vitro-expressed COUP-TFII. Indicated probes were incubated with 5 μl in vitro transcription/translation reaction programed with either the empty expression vector pcDNA3.1HISC (−) or with pcDNA3.1HISC::COUP-TFII (+). Arrow indicates shifted complex attributed to COUP-TFII. Bottom: Sequence of probes used in EMSA; IR1 stands for inverted repeat of AGGTCA separated by one nucleotide; R − 74/−53, oligomer was homologous to the rat *CYP7A1* sequence from nt −74 to −53; Hwt, human wild-type sequence from nt −71 to −51; HmG1, probe was the same as human sequence (Hwt) with the insertion of a G in between nt −68 and −67 to mutate the human sequence toward the rat at that position; RmG2, probe was the same as the rat sequence (R − 74/−53) with the deletion of the G at nt −70 to mutate the sequence toward the human sequence; RmT9, rat sequence with the T at nt −60 transitioned to a C to mutate toward human sequence; and R − 65/−54, rat sequence from nt −65 to −54. Lower case letters are non-homologous single-stranded ends added to the synthetic oligomers for labeling; arrows under sequence indicate position and direction of HRE repeats; Xs are placed between rat and human probes where the sequences diverge; and mutated bases in HmG1, RmG2 and RmT1 are underlined.

duced the activation by COUP-TFII alone, but did not prevent the transactivation by HNF4 alone nor the enhancement of HNF4 transactivation of *CYP7A1* by cotransfection with COUP-TFII. We conclude that HNF4 requires auxiliary factors to mediate transactivation of *CYP7A1* gene transcription and, though COUP-TFII binding to the adjacent site may not be required for the observed

-149 -145 -142 -139 -136 -133 -130 -128 -123 -118

RAT CTAT GGA CTT AGT TCA AGG CCG GGTAATGCTA

HUMAN CTGT GGA CTT AGT TCA AGG CCA GTTACTACCA

-147 HNF4 COUP-TFII -116

COUP-TFII

RAT -74 TTTGGTCACTCAAGTTCAAG -55

HUMAN -71 TTTG -TCAACCAAGCTCAAG -53

Fig. 6. Proposed recognition sites for HNF4 and COUP-TFII on the *CYP7A1* proximal promoter. Boxes indicate the sequences important for binding of indicated transcription factors, arrows underline the imperfect HRE half-sites, x indicates mismatch between rat and human. Top, rat sequences from nt −149 to −118 and human nt −147 to −116; bottom, rat sequences from nt −74 to −55 and human nt −71 to −53; showing lack of DR4 in human sequences.

synergy between HNF4 and COUP-TFII, the juxtaposition of the binding elements facilitates the protein/protein interactions that result in transcriptional transactivation.

Previous work has implicated HNF4 in regulating *CYP7A1* through the completely conserved DR1 at nt −146 to −130 (13, 37). Because mutations in this DR1 disrupt the response of *CYP7A1* to phorbol esters, bile acids, and retinoic acid, HNF4 might be the terminal transducer for all these effectors. The transcriptional activation activity of HNF4 does, indeed, respond to a wide variety of signals. HNF4 transactivating activity is modulated by protein kinase A (PKA) (45) and possibly fatty acyl-CoA thioesters (46), indicating an active role for HNF4 beyond ontogeny. Phosphorylation of serines in the DNA-binding domain of HNF4 by PKA interfered with the ability of the transcription factor to bind its recognition site (45); whereas tyrosine phosphorylation is required for DNA binding (47).

However, many factors can bind to a DR1 sequences to which HNF4 also can potentially bind, including COUP-TFII, RAR/RXR, and PPAR/RXR (48). It was, therefore, necessary to examine in detail the relative binding of the different factors to these sequences in order to correctly understand their relative contribution to the expression of *CYP7A1.* HNF4 prefers a T at position 1 of the DR1 (48), such as is found at nt −149 to −128 of the *CYP7A1* gene. However, the sequence diverges from the consensus DR1 at positions 4 (T to A) and 6 (A to T). We show here that this divergence did not prevent high affinity binding of HNF4, but apparently prevented COUP-TFII from recognizing the sequence.

COUP-TFII or other factors such as BTEB (9) or the human homolog of the nuclear receptor Ftz-F1, CPF (9, 49), can bind to the sequences overlapping the DR1 and these sequences were found to be important for the ability of HNF4 to transactivate *CYP7A1* transcription in HepG2

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cells. HNF4 has been described as being able to interact directly with TFIIB (50), so it is likely that HNF4 can activate transcription effectively on its own, in spite of lacking the glutamine and proline-rich N-terminal transcriptional activation domain found in COUP-TFII. However, the HNF4 binding element at nt −149 to −118 alone cannot confer HNF4 responsiveness to the SV40 promoter. This was consistent with previous reports that HNF4 requires multiple copies of binding elements in order to activate transcription. This requirement can be overcome by the presence of auxiliary elements (32, 33, 48), in the case of *CYP7A1*, the overlapping COUP-TFII-binding element can fulfill this role.

The strong COUP-TFII element previously mapped at nt −72 to −57 on the rat promoter could possibly function as an auxiliary site for HNF4 transactivation, such as what is found in the PEPCK promoter (34). Or, in contrast, the nt −72 to −57 element could be analogous to the 6βB-B site at nt −139 to −118 of the *CYP3A1* promoter which, when occupied by COUP-TFII, prevents transcriptional activation by HNF4 binding to the 6βB-A site at nt −105 to −86 (51). *CYP7A1* differs from both of these promoters in that deletion of the DR4 element did not effect activation by HNF4 alone positively or negatively, and the mutant reporter constructs still exhibited synergistic activation by HNF4 and COUP-TFII. This was true, also, for the human *CYP7A1* construct, which lacks the DR4 COUP-TFII motif in the proximal promoter, further supporting the idea that COUP-TFII binding to the DR0 at nt −139 to −128 was important for the observed synergy between HNF4 and COUP-TFII.

The HNF4/COUP-TFII interaction on the *CYP7A1* mutant promoters apparently shares features with the HNF1 promoter. As the effect of the mutations in the DR0 COUP-TFII site could be overcome by elevated levels of COUP-TFII, COUP-TFII binding to the promoter may not be absolutely required for the synergistic interaction of HNF4 and COUP-TFII. COUP-TFII does not bind to the HNF1 promoter, but acts synergistically with HNF4 by aligning the AF2 activation domain of HNF4 in a more productive confirmation by protein/protein interaction (19). In the case of rat *CYP7A1*, the COUP-TFII site overlapping the HNF4 binding element allows COUP-TFII to act as an auxiliary factor for HNF4. The juxtaposition likely facilitates the interaction of the factors. The synergy observed in p416LSM139/134 and p416LSM133/128 may be via the COUP-TFII site at nt −71 to −57, through residual binding of nt −139 to −128 or via COUP-TFII interacting with another factor, such as Sp1. COUP-TF has been shown to activate transcription of the NGFI-A gene promoter via an Sp1 site by interacting with the Sp1 binding to its element (52). *CYP7A1* does have an Sp1 site at nt −101 to −82 (9).

In vitro-expressed COUP-TFII interacts with a DR0 at −139 to −128, but with a much lower affinity compared to the DR4 at the rat nt −74 to −53. This DR4 motif was shown to be lacking in the human promoter, accounting for the reduced stimulation of the human gene by COUP-TFII relative to the rat gene promoter. The previous assumption was that COUP-TFII competed with HNF4 for binding to nt −144 to −132 on the human *CYP7A1* gene promoter (36), but cotransfection of HNF4 and COUP-TFII resulted in the same synergy as with rat *CYP7A1*, with the nt −74 to −57 DR4 intact or deleted. Here, we demonstrated that COUP-TFII actually interacts with a HRE six base pairs downstream of the sequences important for HNF4 binding, which is also conserved in human. This would correspond to one half-turn of the DNA double helix, suggesting that COUP-TFII may not compete with HNF4 because it interacts with the opposite face of the helix. This would be consistent with one binding conformation postulated by Cooney, et al. (53). However, in light of the low affinity of binding of COUP-TFII for this sequence, these data, in themselves, did not rule out the observation that HNF4 out-competes COUP-TFII for occupation of this element, and the observed synergy was due exclusively to protein/protein interactions. This interpretation was consistent with the observation that mutation of the COUP-TFII site does not change the HNF4 binding site which still exhibited synergy, but does not explain why overexpression of HNF4 cannot transactivate the promoter construct to the same extent as wild-type. Additionally, binding of COUP-TFII to the nt −149 to −118 probe was not eliminated by addition of purified HNF4 when the probe was limiting and immunoprecipitation of COUP-TFII by antibody directed against HNF4 was increased by the addition of the nt −149 to −118 oligomer probe, consistent with both factors binding the same molecule (data not shown). Moreover, though the HNF4 and COUP-TFII binding elements in nt −146 to −128 are highly conserved between the rat and human *CYP7A1* promoter, the human promoter was not transactivated to the same extent as the rat promoter. Therefore, though the issue of whether or not COUP-TFII binding to the promoter is required for the cooperation with HNF4 was not rigorously answered, the simplest interpretation was that an additional factor binding to the nt −139 to −128 was required for the transactivational activity under baseline conditions, but this can be overcome with the overexpression of COUP-TFII or BTEB. It is clear that HNF4 requires the cooperation of other factors to exert its transactivational effect on the *CYP7A1* promoter.

Transcription is the interpretation of the vocabulary of transcription factors (repertoire) assembled in the grammar of the promoter context. Promoter context exerts profound influences on the activity of the transcription factors. This is especially true for the nuclear hormone receptors because of competition for similar DNA binding sequences, dimeric partners and for protein/protein interaction sites on the transcriptional machinery. HNF4 is known to be affected by all these influences, in addition to posttranslational modification. As the sequences from nt −147 to −128 are conserved over evolution, suggesting the importance of this composite element to the proper expression of *CYP7A1*, we conclude that many effectors may act through the nt −147 to −128 by altering the interactions between this cohort of transcription factors.

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We would like to thank Mrs. Rhonda Kimmel for excellent technical support and Dr. Maurizio Crestani for constructing pcDNA3-HNF4. We are grateful to Dr. W. Chen, R. Evans, Dr. S. Karathanasis, F. Sladek. and M-J. Tsai for providing materials for this research. This research was supported by NIH grant awards GM31584 and DK44442. D. S. is a recipient of a Public Health Service Individual National Research Service Award, HL09156-01.

Manuscript received 1 February 1999 and in revised form 5 August 1999.

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