Orphan Receptors Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) and Retinoid X Receptor (RXR) Activate and Bind the Rat Cholesterol 7α-Hydroxylase Gene (CYP7A)*

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The cholesterol 7α-hydroxylase gene (CYP7A) is transcriptionally regulated by a number of factors, including hormones, bile acids, and diurnal rhythm. Previous studies have identified a region from nucleotides (nt) −74 to −55 of the rat CYP7A promoter that enhanced bile acid repression of the SV40 early promoter, as assayed with a luciferase reporter gene in transiently transfected HepG2 cells. The rat CYP7A promoter/reporter activity was strongly stimulated by cotransfection with an expression plasmid encoding the nuclear hormone receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) in a dose-dependent manner. Site-directed mutagenesis in the region of nt −74 to −55 altered this stimulation. Recombinant COUP-TFII expressed in HepG2 or COS-1 cells were found to bind to nt −74 to −55 and nt −149 to −128 probes by electrophoretic mobility shift assay (EMSA) and by supershifting the corresponding band with COUP-TFII-specific antibodies. The region of nt −176 to −117 was previously mapped as a retinoid acid response region and was found to bind retinoid X receptor (RXR). EMSA supershift assays of wild-type and mutant oligomers using antibody against RXR revealed that the sequences between nt −145 and −134 were important for RXR binding. We conclude that COUP-TFII stimulates the transcriptional activity of the rat CYP7A promoter by binding to the sequences between nt −74 to −54 and nt −149 to −128. RXR may stimulate CYP7A gene transcription by binding to a direct repeat of the hormone response element separated by one nucleotide located at nt −146 to −134.

Cholesterol 7α-hydroxylase (EC 1.14.13.17) catalyzes the first and rate-limiting step in a pathway that converts cholesterol to bile acids. The catabolism of cholesterol to bile acids in the liver is the main mechanism for elimination of cholesterol from the body and thus plays an important role in maintaining cholesterol homeostasis (1). The gene CYP7A is transcriptionally regulated by a number of factors, including bile acids, cholesterol, hormones, and circadian rhythm (2–6).

The CYP7A promoter structure is typical of a DNA-dependent RNA polymerase II promoter in that, upstream of the TATA box, there are several cis-acting elements that regulate the transcriptional activity of this promoter (7–9). Transient transfection assay of chimeric CYP7A promoter/luciferase constructs in HepG2 cells revealed that the region from nt −416 to −32 of the rat CYP7A gene contained the promoter and regulatory domains conferring the activation of transcription by dexamethasone and retinoic acid and suppression by bile acids, phorbol esters, and insulin (2, 3, 9, 10). We have mapped two footprints that are protected from DNase I digestion using rat liver nuclear extracts (11). Footprint (Fp)1 A is located between nt −81 and −35, and FpB is located between nt −148 and −129. Nucleotide sequences in these two regions are highly conserved among homologous CYP7A genes of different species. These footprints contain liver-enriched transcription factors binding sites and hormone response elements. A putative bile acid response element (BARE) was mapped to the FpA region, which lead us to propose that a bile acid responsive nuclear receptor may be mediating bile acid response (11).

The molecular mechanism of bile acid repression is still unknown. Determining what factors interact with these BARE sequences is a first step in elucidating the nature of the bile acid response. A nuclear protein factor, which was found to bind a repeated sequence −65TCAAGTTCAAG-54 was named direct repeat binding protein (DRBP). Binding of DRBP to BARE was diminished when liver nuclear extract prepared from rats fed deoxycholate was used in EMSA (11). The sequence from nt −73 to −57 contains an imperfect direct repeat of the hormone response element (HRE, AGGTCA) separated by four nucleotides (DR4). According to the 3−4−5 rule for the binding specificity of the steroid/thyroid hormone family of transcription factors (12), a DR4 would be predicted to act as a thyroid hormone response element, hence “7αTRE” as the tentative name for this DR4 sequence (11). However, CYP7A promoter constructs containing these sequences did not respond to thyroid hormones in HepG2 cells (2). A number of nuclear factors have affinity for DR4 sequences, including TR, XONR, and LXR that form heterodimers with RXR (13, 14). COUP-TFII (or ARP-1) of orphan nuclear receptor family with promiscuous binding specificities also bind a DR4 sequence (15, 16).

The abbreviations used are: Fp, footprint; C/EBP, CAAT/enhancer binding protein; COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; DR, direct repeat sequence; DRBP, direct repeat binding protein; EMSA, electrophoretic mobility shift assay; HNF, hepaticocyte nuclear factor; HRE, hormone response element; LUC, luciferase; nt, nucleotide(s); RAR, retinoic acid receptor; RXR, retinoid X receptor; TRE, thyroid hormone response element; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; LSM, linker scanning mutation; IM, insertion mutant.

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COUP-TFII/RXR Activates Cholesterol 7α-Hydroxylase Gene

COUP-TFs are believed to influence developmental changes, which is supported by the report that the COUP-TFII promoter responds to differentiation signal, retinoic acid (17). Recently, we have mapped a complex retinoic acid response region located between −176 and −117 of the rat CYP7A promoter (10). Using transient transfection assay of CYP7A reporter activity in HepG2 cells, we have screened a number of known steroid/thyroid hormone nuclear receptors for their effects on gene transcription. COUP-TFII produced one of the strongest effects on transcription. Here, we provide evidence that orphan receptor COUP-TFII activates transcription of rat CYP7A by binding to nt −74 to −54 and to nt −149 to −128 and that an RXR homodimer may bind to a DR1 motif between −146 to −134.

EXPERIMENTAL PROCEDURES

Materials—DNA oligomers were synthesized by National Biosciences (Plymouth, MN) or Life Technologies, Inc. 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-32P)dCTP (3000 Ci/mol) and (α-35S)dATP (1200 Ci/mmol) Sequencing sequencing grade were obtained from ICN (Costa Mesa, CA) and DuPont NEN, respectively. The DNA purification systems used were the GeneClean kit, from BIO 101, Inc. (La Jolla, CA), and the Qiagen plasmid kit, acquired from Qiagen Inc. (Chatsworth, CA). Expression plasmids for the transcription factors were the generous gifts of Dr. W. Chen for pLen4S (HNF4) and p-LH3a (HNF3a), Dr. P. Johnson for pMEXC/EBP (C/EBPa) and pMEXCRP2.seq (LAP), and Dr. M.-J. Tsai for pTF3A (COUP-TFII). Antiserum against COUP-TFII was a kind gift of Dr. Tsai. Affinity-purified rabbit polyclonal antibodies against RXR (sc-773; cross-reacts with RXR α, β, β1, β2, γ1 and γ2 isoforms), RXR (sc-774; cross-reacts with RXR α, β, and γ isoforms), C/EBPa (sc-61), and erbAβ (sc-76) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other reagents were from Sigma.

Cell Lines—The human hepatoma cell line (HepG2, ATCC HB8065) and African green monkey kidney cells (COS-1, ATCC CRL1650) were obtained from the American Type Culture Collection, Rockville, MD. The cells were grown in a 1:1 mixture of Dulbecco’s modified Eagles medium and F12, (Life Technologies, Inc.) supplemented with 100 units/ml penicillin G-streptomycin sulfoxide (Celtex Corp., Hopkins, MN) and 10% (v/v) heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS).

Plasmid Construction—The sequences of the CYP7A promoter from nt −200 to +32 were amplified by polymerase chain reaction (PCR) using p-416/+32 (3) as the template. Primers were designed to introduce a 5′ SpeI and a 3′ XhoI site for cloning into the luciferase reporter vector pGL2-Basic (Promega) resulting in the plasmid p-200/+32. The mutations in the sequences from nt −74 to −54 (see Fig. 1A) were introduced by linking two fragments synthesized by PCR with mutagenic primers containing restriction sites at the 5′- or 3′-end and using p-416/+32 as a template. The region from nt −74 to −54 was replaced with a SpeI site (ACTAGT) by linking the nt −416 to −74 (SpeI and nt −533) SpeI/+32 fragments to generate p-416Δ−74/+54 SpeI. To delete the SpeI site, p-416Δ−74/+54 SpeI was digested with SpeI, treated with Mung bean nuclease, and then religated to generate deletion mutant p-416Δ−74/−54. p200Δ−74/−54 was obtained by PCR using p-416Δ−74/−54 as a template. p-416LSM−74/−71 (linter-scanning mutation) was constructed by ligating a nt −70 to +32 (SpeI/XhoI) fragment to Δ−416 Δ−74/−54SpeI digested with SpeI and XhoI. p-416Δ−74/−65 was isolated as a spontaneously arising deletion mutant. p-416LSM−59/−54, p-416LSM−70/−66, and p-416IM−64/−63 (insertion mutation) were constructed similarly to p-416LSM−74/−71. All promoter/reporter constructs were cloned into pGL2-Basic vector. All constructs were confirmed by sequencing with Sequenase 2.0 (U. S. Biochemicals, Corp., Cleveland, OH).

Transfection Assays—Confluent cultures of HepG2 cells grown in 12-well tissue culture plates were transfected with DNA by calcium phosphate method as described previously (3, 11). Luciferase activities were determined with the luciferase assay kit (Promega) according to manufacturer instructions using a Lumat LB9501 luminometer (Berthold Systems, Inc., Pittsburgh, PA). Luciferase activities were normalized for transfection efficiencies by dividing relative light units by β-galactosidase activity expressed from cotransfected pCMVβ plasmid (Clontech). β-Galactosidase activities were determined using O-ni- trophenyl-β-D-galactopyranoside as a substrate (18). 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, San Rafael, CA).

Preparation of Nuclear Extracts—Nuclei from HepG2 and COS-1 cells were isolated essentially as described (19, 20). Recombinant COUP-TFII was prepared by transfecting confluent HepG2 or subconfluent COS-1 cells in 100-mm tissue culture plates with 20 μg of pTFSA and 1 μg of pCMVβ. The monolayers were overlaid with DME/F12 containing 5% fetal calf serum after glycerol shock, and nuclei were harvested 40 h later. Transfection efficiency was monitored by β-galactosidase activity.

Electrophoretic Mobility Shift Assays (EMSA)—Double-stranded synthetic probes for EMSA were prepared by heating equal molar amounts of complementary synthetic oligomers to 95 °C in 2 × SSC (0.3 M NaCl, 0.03 mM Na2 citrate, pH 7.0) and allowing them to cool to room temperature. The resulting double-stranded fragments were designed with single-stranded 5′ overhangs for end-labeling by incorporating [α-32P]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 and purified through two G-50 spin columns. Binding reactions were initiated with the addition of 2 μg of nuclear extract to 100,000 cpm of oligomer probe dissolved in 20 μl of buffer containing 12 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 15% glycerol, and 2 μg of poly(dI-dC)poly(dI-dC). After incubation for the time indicated in the legends to Figs. 2–5, samples were run on 4% polyacrylamide gels, dried, and autoradiographed. EMSAs were quantitated with IP Lab Gel software (Signal Analytics Corp., Vienna, VA) in conjunction with a PhosphorImager 445Si (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Activation of CYP7A Promoter Activity by Recombinant COUP-TFII and Identification of a Negative Element—Co-transfection of the rat CYP7A promoter fragment from nt −416 to +32 fused to the luciferase gene (p416/+32) (Fig. 1A) with a plasmid encoding recombinant COUP-TFII (pTF3A) was found to strongly stimulate reporter activity (Fig. 1B). The dose-response curve resulting from co-transfacing with increasing amounts of pTF3A with p-200/+32 was indistinguishable from the stimulation curve of p-416/+32 (Fig. 1C) despite lower overall luciferase reporter activity (Fig. 1C) indicating the COUP-TFII activation elements were downstream of position nt −200. Deletion of nt −74 to −54 (Fig. 1A) reduced the stimulatory effect of COUP-TFII (Fig. 1B).

To define the sequences important for COUP-TFII activation, a number of deletions (Δ) and linker scanning mutations (LSM) were introduced into the nt −74 to −54 region. The dose-dependent stimulation of the promoter activity of the reporter plasmids by COUP-TFII fell into roughly three groups. In the first group, the wild-type promoter fragments (p-416/+32 and p-200/+32) showed the greatest stimulation of transcriptional activity, and the effect was positive at all points of the curve (Fig. 1B). The second group, which includes p-416LSM−74/−71, p-416LSM−70/−66, p-416Δ−74/−65, p-416LSM−59/−54, p-416Δ−74/−54, and p200Δ74/54 displayed considerably flatter dose-response curves (Fig. 1B). The mutations in this group destroyed one or both of the putative HRE half-sites, AGGTCA, either by completely deleting the sequence (p-416Δ−74/−65, p-416Δ−74/−54, and p200Δ74/54) or by replacing the sequence with an Spel restriction site, while preserving the relative spacing of sequences on either side of the mutation (p-416LSM−74/−71, p-416LSM−70/−66, and p-416LSM−59/−54) (Fig. 1A). Reporter plasmids with both HRE half-sites deleted (p-416Δ−74/−54 and p200Δ74/54) retained significant stimulation by COUP-TFII (approximately 3–4 fold), suggesting there may be additional COUP-TFII sites elsewhere in the promoter. Consistent with the hypothesis that the putative COUP-TFII activation elements were downstream...
**FIG. 1.** Effect of overexpressing recombinant COUP-TFII on transcriptional activity of the nt -416 to +32 fragment of the rat CYP7A promoter.

**A,** schematic representation of mutations introduced into the region of nt -74 to nt -54 of the rat CYP7A promoter, as described under "Experimental Procedures." Wild-type sequence from nt -81 to -48 is diagrammed on top. All the clones have the promoter sequences from nt -416 to +32 ligated into pGL2-Basic. Restriction sites are underlined, dashes in sequence represent deleted base pairs, and non-homologous sequences are indicated in lowercase letters. The DR4 is overlined, and the direct repeat described in (11) is boxed.

**B,** increasing mass of the expression plasmid pTF3A encoding COUP-TFII was cotransfected with CYP7A/luciferase reporter plasmids diagrammed in panel A. The total mass of plasmid was kept constant by the addition of vector without promoter. Confluent monolayers of HepG2 cells were transfected with 5 μg of the indicated reporter plasmids, various amounts of pTF3A, and 0.5 μg of pCMV-β as described under "Experimental Procedures" and then overlaid with DMEM/F12 medium without serum after glycerol shock. The cells were harvested 40 h after glycerol shock, and the luciferase activity was assayed and corrected for transfection efficiency with the β-galactosidase activity. The activation by COUP-TFII of the transcriptional activities were expressed as percentage of corrected luciferase activity without coexpressed COUP-TFII. The percentage activation differed from wild type p < 0.01 (p-200Δ-74/54; p-416Δ-74/65; p-416Δ-74/54; p-416Δ-74/54 Spe; p-416Δ-74/54). Error bars indicate standard deviation of the triplicate samples for each construct.

**C,** basal transcriptional activities of CYP7A promoter/luciferase reporter plasmids with mutations in the region from nt -74 to nt -54. Error bars indicate standard deviation of the triplicate samples for each construct.

*p* indicates statistical significance for difference from wild type p < 0.01. To control for variation in measured activities due to differences in individual plasmid preparations, relative activities of p-416/+32, p-416Δ-74/54Spe, p-416Δ-74/65, p-416Δ-74/54, p-416Δ-74/54CYP7A promoter/luciferase gene constructs were further assayed with three separate plasmid preparations transfected in triplicate in two different experiments as described under "Experimental Procedures." The results obtained were essentially the same as those shown in panel C.
of position nt −200, p-200Δ74/54 was stimulated to approximately the same extent as p-416Δ−74/−54, which has the same deletion but was 216 base pairs longer (Fig. 1B).

The third category was represented by an insertion mutant (IM), p-416IM−64/−63 that displayed an activation intermediate between the first and second groups. This plasmid, in which the HRE half-sites were intact but the spacing was increased from 4 to 5 base pairs (Fig. 1A), was strongly activated but to a somewhat lesser extent than the wild type, reaching a maximum of 1740 ± 99% versus 2960 ± 209%, respectively, at a transcription factor plasmid mass to reporter plasmid mass ratio of 0.2 (Fig. 1B).

The various mutations affected the basal activity. Mutant reporter plasmids p-416Δ−74/54, p-416LSM−74/−71, p-416LSM−59/−54, p-416Δ−74/65, and p200Δ74/54 had increased basal promoter activity relative to their parental plasmids (p-416/+32 and p-200/+32), as would be observed if a repressor binding site was mutated. With one exception, the basal promoter activity also fell into three groups, corresponding with the COUP-TFII responsiveness of the reporter plasmid (Fig. 1C). The promoter/reporter constructs with the lowest response to COUP-TFII had the highest basal activities (p-416Δ−74/54 and p-416LSM−74/−71). p-416IM−64/−63 basal transcriptional activity was not significantly higher than the wild type. The exception was p-416LSM−70/−66, which was activated by COUP-TFII to the same extent as p-416LSM−59/−54 and p-416Δ−74/65 but did not display the same transcriptional activation over p-416/+32. The activation of p-416Δ−74/−54 over p-416/+32 was not observed in the kidney cell line, COS-1.

Interaction of COUP-TFII with the CYP7A Promoter at nt −74 to −54—To determine if COUP-TFII activates transcriptional activity by directly binding to the sequence between nt −74 and −54, the ability of COUP-TFII to interact with the CYP7A sequences in vitro, was measured with the EMSA. Also, other promoter fragments from nt −200 to −74 were screened to determine if there were additional COUP-TFII binding sites.

Nuclear extracts were prepared from HepG2 cells transfected with pTF3A, an expression plasmid specifically encoding COUP-TFII. The gel shift patterns of the nt −72 to −59 and nt −65 to 54 oligomers are similar in HepG2 cells, regardless of COUP-TFII overexpression (Fig. 2B). Two extra bands were shifted with nt −74 to −53 probe that were enhanced by COUP-TFII overexpression (Fig. 2). The nt −74 to −53 included the putative DR4, which is overlined in Fig. 2A; the nt −72 to −59 or nt −65 to 54 oligomers do not. The diffuse nature of these bands was attributed to the low abundance of the nuclear factors.

Recombinant COUP-TFII expressed in the kidney cell line COS-1, bound the nt −74 to −53 (Fig. 2C), indicating the binding of COUP-TFII was not dependent on liver-specific factors. The increased band shift with COS-1 extracts was attributed to the higher activity of the MT2 promoter of pTF3A, which drives transcription of the recombinant gene encoding COUP-TFII, in COS-1 cells relative to HepG2 cells. This presumably results in higher levels of expression of recombinant COUP-TFII in this cell line. Overexpression of COUP-TFII was associated with a reduction in DRBP binding, even in probes unable to be shifted by COUP-TFII (nt −72 to −59 and −65/−54). The reduction in DRBP binding may be attributed to the reduced proportion of DRBP in the given mass of protein in COS-1 nuclear extracts overexpressed with recombinant COUP-TFII. Alternatively, if COUP-TFII interacts with DRBP in solution, the overexpressed COUP-TFII may be sequestering the DRBP and forms larger complexes with the probe nt −74/−53. COUP-TFII did not shift the probes lacking the COUP-

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**Fig. 2.** Effect of overexpressing recombinant COUP-TFII on the EMSA pattern of probes based on the sequence from nt −74 to −54. A, diagram of oligonucleotide probes. Shown are DR4 overlined, and the sequence, AGTTTAAAG, that has homology with nt −149 to −118 is boxed. B, EMSA with HepG2 cell extract. Shown is autoradiogram of EMSA performed with nuclear extracts prepared from HepG2 cells (HepG2) and HepG2 cells transfected with pTF3A (HepG2 + COUP-TFII) and oligomer probes based on the sequences from nt −74 to −55. Reactions were incubated at 30 °C for 15 min. Arrow indicates bands that change with overexpression of COUP-TFII. C, EMSA comparing the shift pattern of HepG2 cell nuclear extract with that of COS-1 cells. Experiment was performed as in panel B. Autoradiogram was overexposed relative to panel B to show fainter band shift with nt −72 to −59 and nt −65 to −54 probes. Arrow indicates band that increases with increased COUP-TFII expression.
The bands previously shown to bind nt 74 to 55 (Fig. 2C). It is apparent that COUP-TFII interacts with the DR4 of nt 74 to 55 and DRBP interacts with AGTTCAG sequence (Fig. 2A).

To further confirm that COUP-TFII binds to this sequence, antisera raised against COUP-TFII was added to an EMSA containing the nt 74 to 55 oligomer as a probe of nuclear extract from HepG2 cells. The band that increased in intensity when shifted by nuclear extracts from HepG2 overexpressing COUP-TFII was supershifted by the anti-COUP-TFII antibody (Fig. 3), indicating that COUP-TFII was interacting directly with the region. Extracts prepared from cells overexpressing recombinant C/EBPα, LAP, HNF4, or HNF3α did not produce any changes in the band-shift pattern (data not shown), and antisera directed against C/EBPα (potential binding site at nt 52 to 41) and c-erbA (a homologue of the thyroid receptor that is regulated by phosphorylation events) did not change the gel shift pattern (Fig. 3). Antibody against RAR and RXR did not supershift the nt 74 to 53 probe. However, when performed with extracts prepared from HepG2 cells treated with all-trans-retinoic acid, the ligand for RAR, the nt 74 to 54 probe was weakly supershifted with anti-RAR (data not shown). DRBP, the major nuclear factor that binds to the sequence was not affected by the antibody, indicating that the proteins responsible for that band are distinct from COUP-TFII and RXR. These results confirm that the COUP-TFII binds to DR4 motif, which does not bind thyroid hormone receptor. C/EBPα, LAP, HNF4, and HNF3α do not bind to this region of CYP7A promoter.

Interactions of COUP-TFII and RXR with the CYP7A Promoter at nt 149 to 128—The probe nt 149 to 128 shifted one band in HepG2 nuclear extracts. Antibody against COUP-TFII supershifted the oligomer and changed the pattern of bands as if a complex was disrupted (Fig. 4). The amount of probe supershifted was not increased with increasing amounts of antibody, indicating that antibody was in excess. Antibody against RXR supershifted a band in HepG2 nuclear extract treated with retinoic acid. The antibody directed against RAR did not supershift the probe even with extract prepared from HepG2 cells treated with all-trans-retinoic acid. Retinoic acid treatment may have increased the expression of endogenous RXR and showed the supershift with anti-RXR.

Once it had been determined that the antibody specific for RXR supershifted the nt 149 to 128 probe, nucleotides important for the binding of RXR were determined by EMSA with mutant oligonucleotides and the antibody against RXR. Double-stranded oligonucleotides were synthesized in which three bases were changed by transversions. Trinucleotide mutations in nt 145 to 134 completely abolished or reduced the supershift by antibody against RXR (Fig. 5). Mutations in nt 133/134 did not have any effect on supershift by antibody against RXR. These results indicate that RXR was interacting with the DR1 motif located from nt 146 to 134.

DISCUSSION

Cotransfection assays of COUP-TFII expression plasmid with wild-type and mutant CYP7A proximal promoter constructs and EMSA results indicate that COUP-TFII interacts with nt 74 to 53 and nt 149 to 128 of the rat CYP7A promoter. The region of nt 146 to 134 was shown by EMSA to bind RXR and contains HRE half-sites separated by one nucleotide (DR1), the preferred binding site for RXR homo-
supershifted probes are indicated with letters changed to C. Mutation from wild-type is indicated with lowercase with nt 2 G. For example, mutated according to the following rule: A to C, C to A, G to T, and T to interferes with vitamin D3, thyroid hormone, and retinoic acid many variations of GGTCA sequences in direct repeats and activator (24–27). COUP-TFII binds as a stable homodimer to deletion mutants (Fig. 1). However, several recent reports sup-
CYP7A promoter may explain the much higher activity of the all COUP-TFII expression plasmid to reporter plasmid ratios tried. The deletion of the strong proximal COUP-TFII site at nt –74 to –55 may allow enhancers in the promoter to interact more readily with the transcriptional machinery and resulting in higher transcriptional activity. COUP-TFII has been re-
ported to interact with TFIIB (28) and to affect the transcriptional activity by interacting with other transcription factors. For example, COUP-TFII is required for activation of the apolipoprotein A1 promoter by RXR (22). As the COUP-TFII and RXR both recognized the nt –149 to –128 probe, it is possible that COUP-TFII may interact with RXR. The heterodimer of COUP-TFII and RXR is known to bind a DRI motif and tran-
spress gene transcription (13).

Despite the finding that COUP-TF binds to a region responsive to bile acid repression, the role of COUP-TFII in the bile acid regulation of CYP7A is not clear and requires further study. The region from nt –65 to –54 can confer the bile acid response to SV40 promoter, but the probe does not bind COUP-
TFII in vitro (Fig. 2, B and C). However, the DRBP and COUP-
TFII apparently share binding sequence, the 3' COUP-TFII half-site overlaps with the DRBP binding site (Fig. 2A). COUP-TFII clearly influences cholesterol metabolism, in that this transcription factor has previously been shown to affect the transcription of the promoters for apolipoprotein AI (16), apolipoprotein AII, apolipoprotein B (29), apolipoprotein CIII (29, 30) and cholesteryl ester transfer protein gene (27).

The physiological role of the sequences from nt –74 to –54 may be to restrain CYP7A expression. This down-regulation may be necessary because of the cytotoxicity of bile acids and the need to preserve cholesterol for synthesis of steroid hor-
mones and membrane components. The exact interactions of the factors involved has yet to be defined; however, the data are consistent with a model in which COUP-TFII, RXR, and DRBP interact with and bind to a negative element in the region between nt –74 and –54 and a positive element from nt –149 to –118 (10) and lead to the regulation of the CYP7A gene transcription in response to signals from bile acids and hor-
mones. This work contributes to the increasing body of evidence indicating COUP-TF has a prominent role in transcriptional regulation of lipid metabolism and that factor binding to the nt –74 to –54 and nt –149 to –128 regions are important determinants of CYP7A transcription.

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![Fig. 5. EMSA supershift in the presence of RXR-specific antibod-
ies using probes based on the sequence from nt –149 to –128. EMSA was performed as in Fig. 4. Shown is the wild-type (WT) oligomer probe sequence on top with trinucleotide transversion mutations indicated below. Numbers over lanes correspond to the nucleotides mutated 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 changed to T, and nt –143 A changed to C. Mutation from wild-type is indicated with lowercase letters. Supershifted probes are indicated with arrow.](http://www.jbc.org/)

![Fig. 6. Diagram of CYP7A proximal promoter with DRBP, COUP-TFIIRXR and LAP binding sites indicated in relation to the transcription start site, D-site binding probe (DBP) and liver activating protein (LAP) binding sites. DBP and LAP (C/EBPβ) binding sites were determined previously (4, 23).](http://www.jbc.org/)
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