Transcriptional regulation of the human cholesterol 7α-hydroxylase gene (*CYP7A*) in HepG2 cells

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Abstract A stable HepG2 cell line harboring a human cholesterol 7α-hydroxylase (*CYP7A*) minigene/luciferase reporter gene construct was selected for studying transcriptional regulation of *CYP7A* gene promoter. Insulin and phorbol 12-myristate-13-acetate (PMA) strongly repressed the promoter activity as measured with luciferase activity expressed in the cells. The promoter activity of the 5′ progressive deletion/luciferase reporter gene constructs was studied in a transient transfection assay in HepG2 cells. PMA represses the promoter activity and the response elements were localized in the -184/-151 and -134/-81 regions. Insulin also represses the promoter activity and response element was mapped in the -298/-81 region. Surprisingly, glucocorticoid receptor (GR) strongly inhibited promoter activity in the presence of dexamethasone, and response elements were localized in the -298/-151 and the -150/+24 regions. Thyroid hormone receptor also repressed promoter activity and response elements were localized in the -150/+24 and upstream regions. Cotransfection of *CYP7A* chimeric constructs with an expression vector carrying liver-enriched transcription factor HNF3α stimulated the reporter gene activity, but cotransfection with GR plasmid interfered with the HNF3α-stimulated activity possibly through competition for binding to overlapping GR/HNF3 binding sites. Thus, human cholesterol 7α-hydroxylase gene promoter is strongly repressed by insulin, PMA, and steroid/thyroid hormones and results in the low level of cholesterol 7α-hydroxylase expression in the human liver.—**Wang, D-p., D. Stroup, M. Marrapodi, M. Crestani, G. Galli, and J. Y. L. Chiang.** Transcriptional regulation of the human cholesterol 7α-hydroxylase gene (*CYP7A*) in HepG2 cells. *J. Lipid Res.* 1996. **37:** 1831–1841.

Supplementary key words cytochrome P450 • hormonal regulation • gene transcription • nuclear receptors • hepatocyte nuclear factor • HNF 3α • phorbol esters • thyroid hormones • glucocorticoid • insulin

The conversion of cholesterol to bile acids in the liver is a major pathway for disposal of cholesterol from the body (1). Cholesterol 7α-hydroxylase is the first and rate-limiting enzyme in bile acid biosynthetic pathway that converts cholesterol to bile acids in the liver. This enzyme activity is regulated primarily at the level of gene transcription by bile acids returning to the liver via portal vein (1). Hydrophobic bile acids strongly repressed, but hydrophilic bile acids had little effect on the expression of cholesterol 7α-hydroxylase mRNAs (2, 3). Thyroid hormones and glucocorticoid are required for the expression of cholesterol 7α-hydroxylase mRNAs in primary cultures of rat hepatocytes (4, 5). Liver-enriched hepatocyte nuclear factor 3 (HNF 3) strongly stimulated the human *CYP7A* promoter activity in HepG2 cells (6). The expression of this gene follows a robust diurnal rhythm and is stimulated by DBP, a liver-enriched, diurnally regulated transcription factor (7, 8).

Most studies on the regulation of cholesterol 7α-hydroxylase were carried out in the rat model. However, species differences in bile acid synthesis and cholesterol 7α-hydroxylase activity have been reported (9–11). Cholesterol 7α-hydroxylase activity is increased by cholestyramine and repressed by bile acids in humans (12). As the purified human enzyme has K_m and V_{max} similar to those of the rat enzyme (13), the low cholesterol 7α-hydroxylase activity expressed in the human liver apparently is due to the low level of gene transcription. In spite of the high sequence homology in the proximal promoters among different species, many consensus response elements are not well conserved (11). Therefore, different *cis*-regulatory elements in the human gene and/or different amounts of transcription factors present in the liver might regulate the human *CYP7A* gene expression

Abbreviations: *CYP7A*, cholesterol 7α-hydroxylase gene, according to the recommended nomenclature (Nelson, D. R., et al. *Pharmacogenetics.* January, 1996); HNF, hepatocyte nuclear factor; GR, glucocorticoid receptor; GRE, glucocortcoid response element; TR, thyroid hormone receptor; TRE, thyroid hormone response element; Luc, luciferase gene; PMA, phorbol 12-myristate-13-acetate; nt, nucleotide; Dex, dexamethasone; T_4, thyroxine.

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by somewhat different mechanisms. To test this hypothesis, transcriptional regulation of the human *CYP7A* gene 5′-upstream sequence by physiological regulators needs to be studied in a suitable transfection assay system in culture.

HepG2 cells synthesize and secrete normal primary bile acids (14) and have been adopted as a model for studying transcriptional regulation of cholesterol 7α-hydroxylase gene by transient transfection assay (15, 16). The expression and regulation of cholesterol 7α-hydroxylase mRNAs in HepG2 cells by bile acids, hormones and cholesterol have been reported by several laboratories (2, 14, 15). We found that rat *CYP7A* gene promoter is stimulated by glucocorticoid, cAMP, and retinoic acids, and repressed by bile acids, insulin and phorbol esters (16). Most response elements conferring the regulation by these physiological regulators in the rat *CYP7A* promoter are localized in a region from the transcription start site (+1) upstream to about nt -416 (2, 16–19). We suggest that interactions among these positive and negative regulatory factors with overlapping *cis*-regulatory elements in the rat *CYP7A* gene promoter determine the level of gene transcription in the liver under different physiological conditions (16).

In the present study, we report the isolation of a stable HepG2 cell line harboring a human *CYP7A* minigene/luciferase reporter gene for studying transcriptional regulation of the gene by hormones. Promoter/reporter chimeric genes were constructed for transient transfection assay of the promoter activity to identify promoter regions responsive to regulation by thyroid hormones, glucocorticoid, phorbol esters, insulin, and the liver-enriched transcription factor HNF3α. It is evident from our results that the human *CYP7A* promoter is regulated quite differently from the rat gene in the HepG2 transfection assay system.

MATERIALS AND METHODS

Materials

HepG2 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). Dexamethasone (Dex) and L-thyroxine (T_4) were from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 salt solution, fetal calf serum, and other tissue culture reagents were from GIBCO/BRL (Grand Island, NY) or Celox (Hopkins, MN). Restriction enzymes and other modifying enzymes were purchased from BRL (Bethesda, MD) or Promega (Madison, WI). [α-^{32}P]-dCTP and [α-^{35}S]-dATP were obtained from DuPont/New England Nuclear (Boston, MA). Luciferase reporter gene vectors pGL2, pGL3, and the reagents for luciferase assays were purchased from Promega. Geneclean DNA purification kit was from BIO 101 (La Jolla, CA). Qiagen DNA purification columns were from Qiagen (Chatsworth, CA). Oligonucleotides were synthesized by National Biosciences (Plymouth, MI).

Isolation of stable cell lines harboring human *CYP7A* minigene

The 2.7 kb *Hind* III/*Bgl* II fragment containing sequences from nt -371 (*Hind* III) to +2377 (*Bgl* II) site of intron 2 of the human *CYP7A* gene was removed from a human *CYP7A* genomic clone pHG7α5.0 (20) by restriction enzyme digestion. The pcDNA3 vector (Invitrogen, San Diego, CA) was modified by removing the Xba I site and digested with *Hind* III and *Bgl* II to remove the CMV promoter. The resulting 4.7 kb vector was ligated to the 2.7 kb *Hind* III to *Bgl* II fragment of the *CYP7A* gene. The 2.8 kb *Spe* I/*Sma* I fragment containing the luciferase gene and SV40 polyA signal from pFlash I vector (SynapSys, Berlington, MA) was inserted into the *Xba* I site (+1712) in the exon 2 of the *CYP7A*/pcDNA3 plasmid. The resulting plasmid pCSH-1 (Fig. 1) contained the human *CYP7A* minigene and luciferase re-

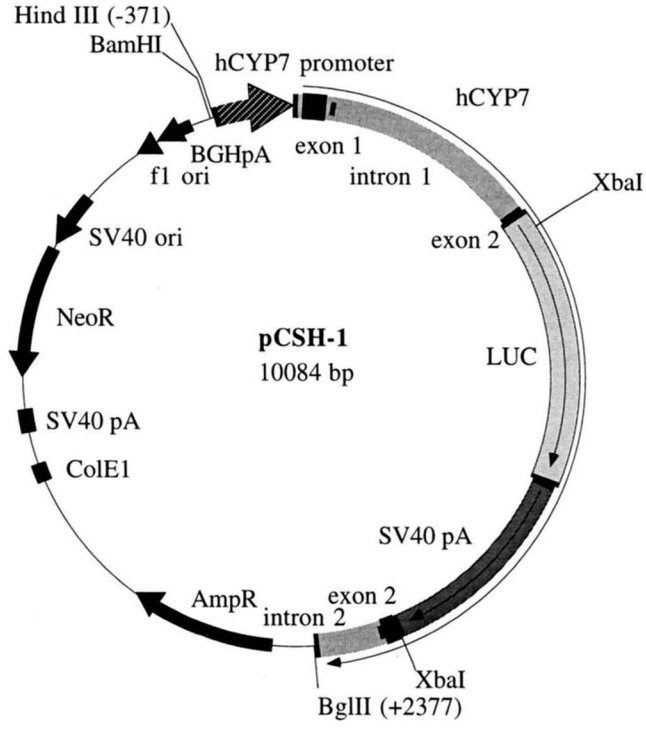


Fig. 1. Human *CYP7A* minigene/luciferase chimeric gene construct. The human *CYP7A* minigene constructed for stable transfection contains sequences from -371 (*Hind* III site) to +2377 (*Bgl* II site) of intron 2. The heavy stripped arrow represents the promoter and direction of transcription. The luciferase gene (LUC) and SV40 polyA signal derived from pFLASH I vector are inserted into *Xba* I site of exon 2. Light lines with solid arrows are derived from pcDNA3 vector. NeoR, neomycin resistant gene; BGHpA, bovine growth hormone polyadenylation signal.

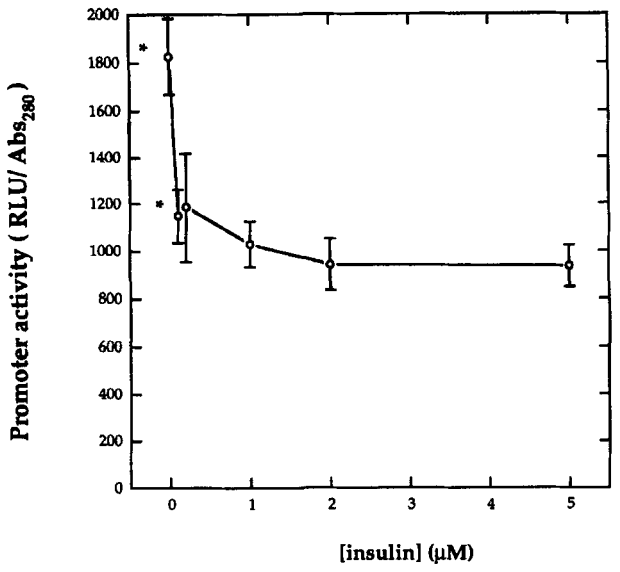


Fig. 2. Effect of insulin on the *CYP7A* transcription in stable HepG2 cell line. Stable HepG2 cell line 2.2.1 harboring the human *CYP7A* minigene/luciferase chimeric construct was selected as described in Methods. Confluent cultures of the stable HepG2 cells were treated with insulin at the concentrations indicated. Cells were harvested 40 h after treatment and cell extracts were used to determine luciferase activity (RLU/Abs 280). Representative data shown are the averages of triplicate assays in one experiment. Error bars indicate standard deviation from the mean; (*) indicates statistically significant difference between activities in the control and 0.1 μM insulin-treated cells ($P \leq 0.01$).

porter gene linked to the neomycin resistant gene. The pCSH-1 was transfected into HepG2 cells and selected for resistance to G418 essentially according to the method described in Current Protocols of Molecular Biology (21). Confluent HepG2 cells were split 1/15 into 4 × 100 cm dishes containing 12 ml DMEM/F12 medium supplemented with 10% fetal bovine serum, 100 IU penicillin, and 100 μg streptomycin 24 h prior to transfection. Cells were transfected with 25 μg DNA/well using Ca^{2+} phosphate coprecipitation method and incubated in complete medium for 48 h. Cells were split 1/15 (5×10^5 cells/plate) into complete medium plus 400 μg G418/ml. Media were changed every 5 days and isolated colonies were transferred to 12-well plates after 17 days. Cultures were maintained at 200 μg/ml G418. The stable cell lines were screened for luciferase activity. A stable cell 2.2.1 that expressed a moderate luciferase activity was selected for testing the effects of bile acids and hormones.

Construction of human *CYP7A* promoter/luciferase reporter chimeric genes

A 1.9 Kb 5′-flanking region of human *CYP7A* gene was obtained by polymerase chain reaction (PCR) amplification using a human *CYP7A* clone pHG7α5.0 as a template (20). The 5′-primer used in PCR reaction was 5′-CGGGGTACCTGAGATTTGGATGGGGACACA-3′, and the 3′-primer was 5′-TAGGAAGGGAAAGATTAGTGAGCTCGCCAT-3′. *Kpn* I (-1877) and *Xho* I (+24) sites were introduced into the 5′- and 3′-primers, respectively, for generating cloning sites. The 1.9 Kb PCR product (-1877 to +24) was digested with *Kpn* I and *Xho* I, and ligated to *Xho* I–*Kpn* I-digested luciferase reporter gene vector pGL2basic. The nucleotide sequence of this chimeric construct, pLUC-1877/+24, was confirmed by DNA sequencing. The pLUC-1723 was constructed by restriction digestion of pLUC-1877 with *Eco*R V and *Xho* I and ligated into *Sma* I and *Xho* I digested pGL2-basic vector. Another construct, pLUC-298, was a spontaneously arising mutant identified by DNA sequencing. The pLUC-785 and pLUC-371 were generated by restriction digestion of pLUC-1877 with *Spe* I and *Hind* III, respectively, blunted with Klenow fragment of DNA polymerase I and then digested with *Xho* I. These fragments were cloned into pGL2-basic vector cut with *Mlu* I, blunted with Klenow, and digested with *Xho* I. The construct pLUC-150 was obtained by PCR using a human *CYP7A* gene plasmid (pHG7α5.0) as the template for amplification using primers to introduce a *Kpn* I site in the 5′-end (-150) and Xho I site (+24) in the 3′-primer. The PCR product was digested with *Kpn* I and *Xho* I, and cloned into pGL2basic plasmid cut

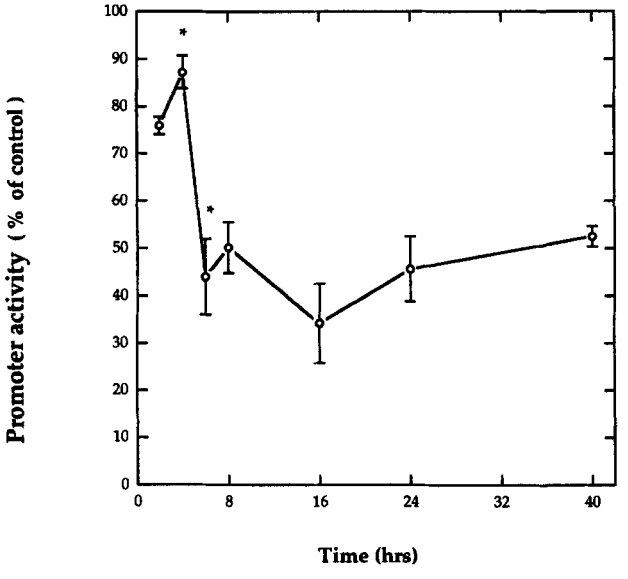


Fig. 3. Effect of PMA on the *CYP7A* transcription in stable 2.2.1 HepG2 cell. Confluent cultures of stable HepG2 cells were treated with PMA (1 μM). Cells were harvested at the time intervals indicated for the determination of luciferase activity in cell extracts. Activities are expressed as the percentage of luciferase activity in HepG2 cells without PMA treatment. Representative data shown are the averages of triplicate assays of one determination. Error bars indicate standard deviation from the mean; (*) indicates statistically significant difference ($P \leq 0.001$).

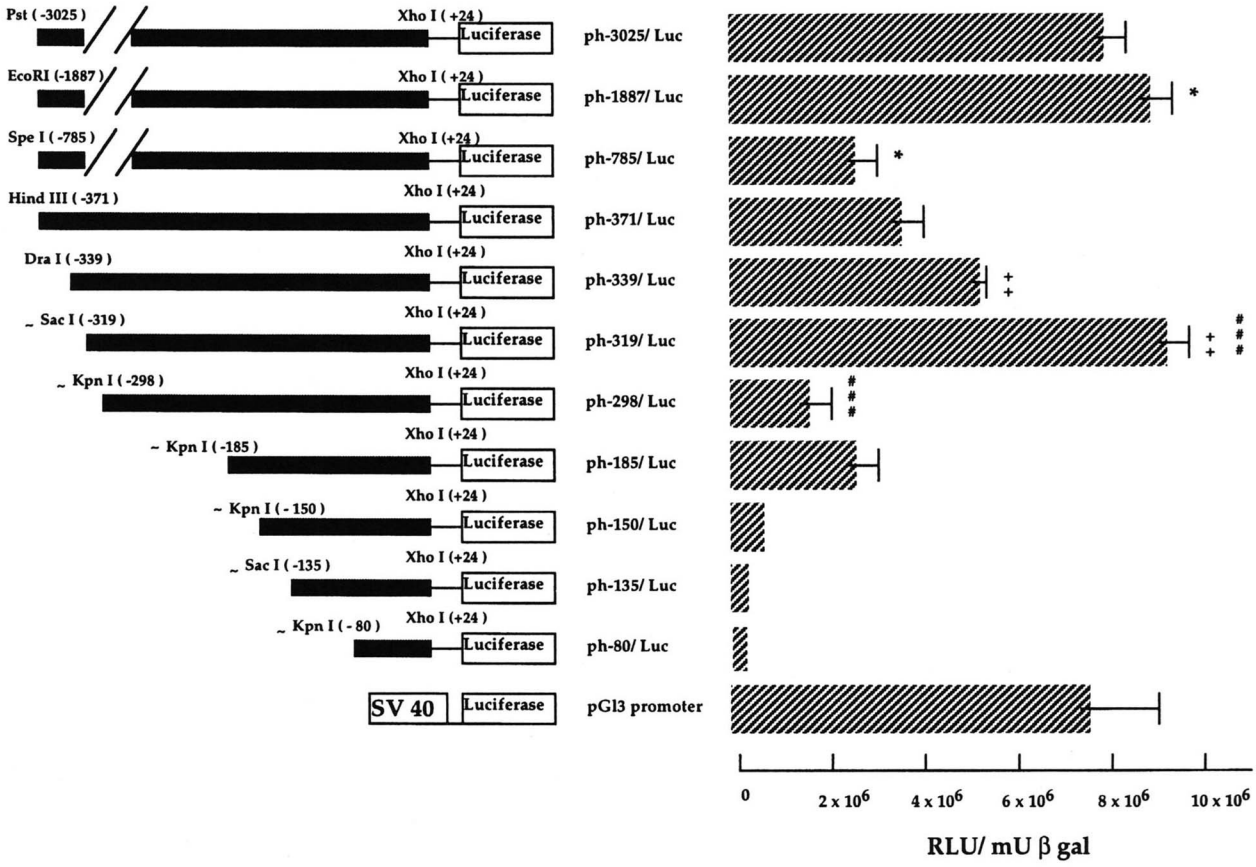


Fig. 4. Basal activity of the human *CYP7A*/luciferase chimeric gene constructs. Confluent HepG2 cells were transfected with 2.5 μg of the chimeric gene plasmids indicated. All chimeric constructs were made using pGL3 reporter gene vector as described in Methods. Constructs with (~) mean that the 5′ restriction sites indicated are generated by PCR primer. These constructs were designed for deletion of some putative sequence motifs. The pGL3promoter vector was included as a reference for SV40 promoter activity in each assay. Transcription activities of chimeric gene constructs are expressed as the luciferase activity expressed in the HepG2 cell extracts (RLU/mU of β-galactosidase activity). Representative data shown are the averages of triplicate assays in one experiment. Error bar represents standard deviation from the mean. Statistics of *t*-test were done using statistic softwares; (*, ++, or ###) indicates statistically significant differences between two plasmids (all $P \leq 0.001$).

with the same enzymes. All the constructs were confirmed by sequencing. Chimeric genes were also constructed using a pGL3 basic reporter vector. This new vector features a number of modification from the original pGL2 vector by eliminating Ap1, Ap2, and TGT3 sites in the luciferase gene, and other modifications to increase luciferase activity by about 100-fold. The ph-1887Luc was obtained by linking a *EcoR* I (-1887) to *Hind* III (-371) fragment to a PCR product containing *Hind* III/*Xho* I(+24), blunt-ended at the *EcoR* I site, and cloned into *Nhe* I (blunt)/*Xho* I cut pGL3 basic vector. The ph-3025Luc was constructed by linking a *Pst* I (-3025) to *Hind* III fragment to a *Hind* III/*Xho* I fragment. The ph-785Luc (*Spe* I/*Xho* I), and ph-371Luc (*Hind* III/*Xho* I), ph-298Luc (*Kpn* I/*Xho* I) and ph-150Luc (*Kpn* I/*Xho* I) were also obtained by cloning the fragments into *Nhe* I (blunt)/*Xho* I or *Kpn* I/*Xho* I cut pGL3 basic vector. In addition, we constructed ph-80/Luc, ph-135/Luc, ph-185/Luc and ph-319/Luc by PCR using ph-371/Luc as the template for amplification with 5′ primers with a *Kpn* I (or *Sac* I) restriction site. The 3′-primer used was the same as described above to introduce a *Xho* I site at +24. The PCR products were digested with *Kpn* I or *Sac* I and *Xho* I, and cloned into *Kpn* I (or *Sac* I) and *Xho* I sites of pGL3 basic vector. The ph-339/Luc was obtained by restriction digestion of ph-371/Luc with *Dra* I and *Xho* I and ligated into a pGL3 basic vector cut with *Nhe* I, blunted with Klenow and digested with *Xho* I.

Transient transfection assays of human *CYP7A*/luciferase chimeric genes in HepG2 cells

HepG2 cells were cultured to confluence and used for transfection assay as described previously (2, 16). Viabil-

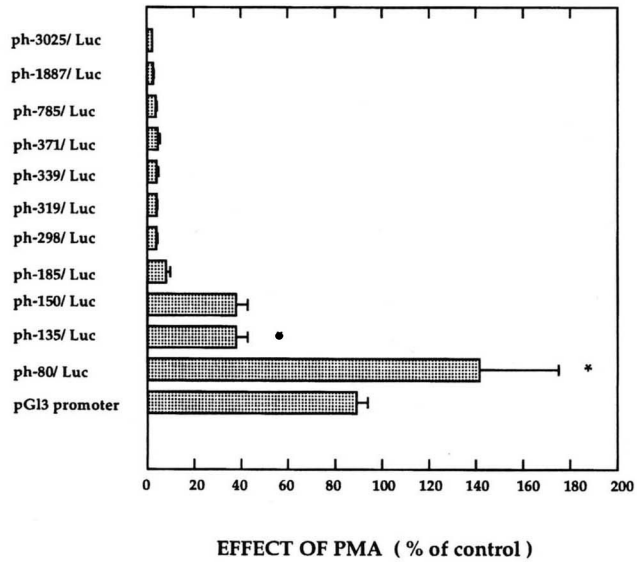


Fig. 5. Identification of the PMA response elements in human *CYP7A*. Confluent HepG2 cells were transfected with 2.5 μg of chimeric gene constructs in pGL3 vector and treated with 1 μM PMA after glycerol shock. Cells were harvested 16 h after the treatment. Transcription activities were determined and expressed as the percentage of luciferase activity in HepG2 cells without the treatment with PMA (7.02, 6.56, 1.22, 5.39, 4.60, 4.68, 4.04, 60.63, 0.15, 0.13, 0.02, and 7.41 × 10^6 RLU/mU β-gal for ph-3025Luc, ph-1887Luc, ph-785Luc, ph-371Luc, ph-339luc, ph-319Luc, ph-298Luc, ph-185Luc, ph-150Luc, ph-135Luc, ph-80Luc, and pGL3-promoter, respectively). Representative data shown are the averages of triplicate assays in one experiment. Error bars represent standard deviation from the mean; (*) indicates statistically significant difference between two plasmids ($P \leq 0.006$).

ity of the cells was assessed by Trypan Blue exclusion, which was higher than 95% in all of HepG2 cell cultures treated with different reagents. In each transfection assay, test plasmid (2.5 μg/well) was cotransfected with β-galactosidase plasmid (pCMV β-gal, 5 μg/well) as an internal standard for the adjustment of transfection efficiency. The luciferase activity expressed in HepG2 cells was measured using assay reagents from Promega and expressed as relative light units (RLU) /mU of β-galactosidase activity or per OD_{280} for the stable cell line (22). Each transfection experiment was performed at least three times using at least two different plasmid preparations. Data presented in each figure are the representation of a typical experiment of triplicated assays of each plasmid.

In co-transfection experiments, appropriate amounts of expression plasmid containing cDNA for glucocorticoid receptor (6RGR, from K. Yamamoto), thyroid hormone receptor (TRα, from R. Evans), or HNF3α (pLH3α from Dr. W. Chen) was cotransfected with 2.5 μg testing plasmids. The amount of DNA in each assay was kept constant by adding pGL2-basic plasmid.

RESULTS

Insulin and phorbol esters repress the human *CYP7A*/luciferase reporter gene activity in a stable HepG2 cell line

The *CYP7A* minigene we constructed contains a 2748 bp gene sequence from nt -371 (*Hind* III) to nt +2377(*Xba* I) in intron 2 (**Fig. 1**). The rationale for choosing this segment of *CYP7A* gene for the design of minigene construct are: the *Hind* III restriction site is conserved in rat, human, hamster, and mouse *CYP7A* gene; the sequences from transcription start site to *Hind* III site are highly conserved among different species; most of the response elements conferring regulation by physiological regulators are found in this upstream region; and introns may increase transcriptional efficiency of eukaryotic genes (23).

Our criteria for the selection of stable cell line are: the high level of luciferase activity expressed; the response of stable cells to known physiological regulators, such as bile acids, phorbol esters and insulin; and the integration of luciferase and *CYP7A* minigenes in the genomic

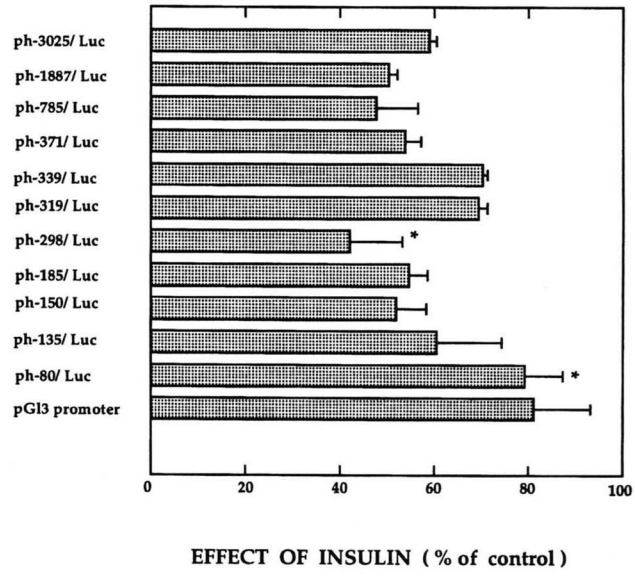


Fig. 6. Identification of the insulin response elements in human *CYP7A*. Confluent cultures of HepG2 cells were transfected with 2.5 μg plasmid of human *CYP7A* deletion mutants in pGL3 vectors. Cultures were treated with 1 μM insulin and cells were harvested 40 h later. Transcriptional activities are expressed as the percentage of luciferase activities in untreated HepG2 cells (these control values are 9.42, 10.56, 2.94, 4.02, 6.36, 10.89, 1.17, 0.77, 0.64, 0.12, 0.05, 8.27 × 10^6 RLU/mU β-gal for ph-3025Luc, ph-1887Luc, ph-785Luc, ph-339Luc, ph-319Luc, ph-298Luc, ph-185Luc, ph-150Luc, ph-135Luc, ph-80Luc, and pGL3- promoter, respectively). Representative data shown are the averages of triplicate assays in one experiment. Error bars represent the standard deviation from the mean; (*) indicates statistically significant difference between two plasmids ($P \leq 0.009$).

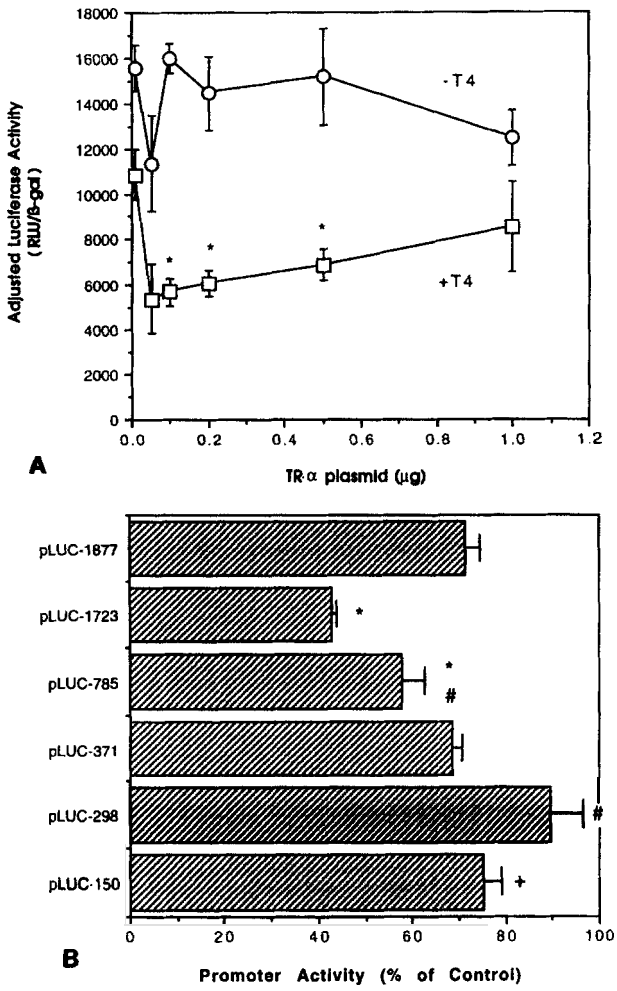


Fig. 7. Effects of thyroid hormone on human *CYP7A/Luc* transcription in HepG2 cells. A: Different amounts of thyroid hormone receptor α (TRα) expression plasmid were cotransfected with 2.5 μg of plasmid pLUC-1877 (in pGL2 vector) into confluent cultures of HepG2 cells grown in serum-free media with (□) or without (○) T_4 (1 μM). Cells were harvested 40 h after the treatment. Luciferase activities of HepG2 cell extracts were expressed as RLU/mU β-galactosidase activity; (*) indicates statistically significant difference between each assay with T_4 vs. without T_4. B: Confluent HepG2 cells were cotransfected with TRα expression plasmid (0.05 μg) and 2.5 μg plasmid of deletion mutants in pGL2 vector. Cells were treated with 1 μM T_4 and harvested 40 h later for the determination of promoter activity. Luciferase activities of HepG2 cell extracts cotransfected with chimeric plasmid and TRα plasmid are expressed as the percentage of the control without cotransfection of TRα (control activities are 40.06, 62.05, 47.57, 16.41, 42.93, and 11.01×10^3 RLU/mU β-gal, for pLuc-1877, pLuc-1723, pLuc-785, pLuc-371, pLuc-298, and pLuc-150, respectively). Representative data shown are the averages of triplicate assays in one experiment. Error bars represent the standard deviations from the means; (*) and (#) indicate statistically significant differences with $P \leq 0.01$ and 0.004, respectively, for the activity of two plasmids; (+) is for the activity difference between T_4 treated and non-treated control ($P \leq 0.001$).

DNA. The stable cell line HepG2/2.2.1 met all these criteria, although the luciferase activity expressed was intermediate among 12 positive cell lines selected. The luciferase activity expressed under the control of this human minigene in the stable HepG2 cell line was inhibited by hydrophobic bile acids taurodeoxycholic and taurochenodeoxycholic acid in a dose-dependent manner, but less hydrophobic taurocholic acid and hydrophilic tauroursodeoxycholic acid had less or no effect on the activity (data not shown). Another cell line expressed about 4-fold higher activity but was not repressed by bile acids.

We demonstrated previously that insulin repressed the transcriptional activity of the rat *CYP7A* in HepG2 cells, therefore, we further tested the effect of insulin on the human *CYP7A* transcription in the stable HepG2 cells. As shown in **Fig. 2,** insulin repressed the human *CYP7A*/Luc activity by about 50% at a concentration as low as 0.1 μM.

We reported previously that the phorbol ester, PMA, repressed the rat *CYP7A* transcription in transient transfection assay (16). As shown in **Fig. 3,** 1.0 μM PMA suppressed the human *CYP7A* transcription by about 70%, after the treatment of the cells with PMA for 16 h. Prolonged treatment with PMA reduced its effect to about 50%.

Transient transfection assay of human *CYP7A*/luciferase chimeric genes in HepG2 cells

To identify regions of human *CYP7A* promoter responsive to potential regulators, the human *CYP7A* 5′-upstream flanking region was linked to the luciferase reporter gene of pGL3 vector and the 5′-progressive deletion constructs were made for transient transfection assays in HepG2 cells. **Figure 4** shows the luciferase activities of these chimeric reporter gene constructs in transient transfection assay in HepG2 cells. The luciferase activities were strongly reduced when sequences upstream of nt -150 were removed. Both negative response elements (nt -339 to -320 and -371 to -340) and positive response elements (nt -1887 to -786, -298 to -151) were localized in the upstream regions. The longer constructs, ph-1887Luc and ph-3025Luc, had activities as high as the shorter construct ph-319Luc. A putative HNF3 site (16) may be located in the -319/-299 fragment to explain a much higher activity in the ph-319Luc than in ph-298Luc plasmid. This pattern of promoter activity is quite different from that of the rat *CYP7A* gene in that the rat -344 (*Hind* III)/+32 fragment has the highest activity and the promoter activities of longer constructs were progressively reduced (16, 17).

Insulin, PMA, and steroid/thyroid hormones repress the human *CYP7A* promoter activity

As shown in **Fig. 5,** PMA repressed the promoter activities of all constructs except the shortest fragment, ph-80Luc. PMA repressed the ph-135Luc and ph-150 by about 60%. It is apparent that PMA responsive elements

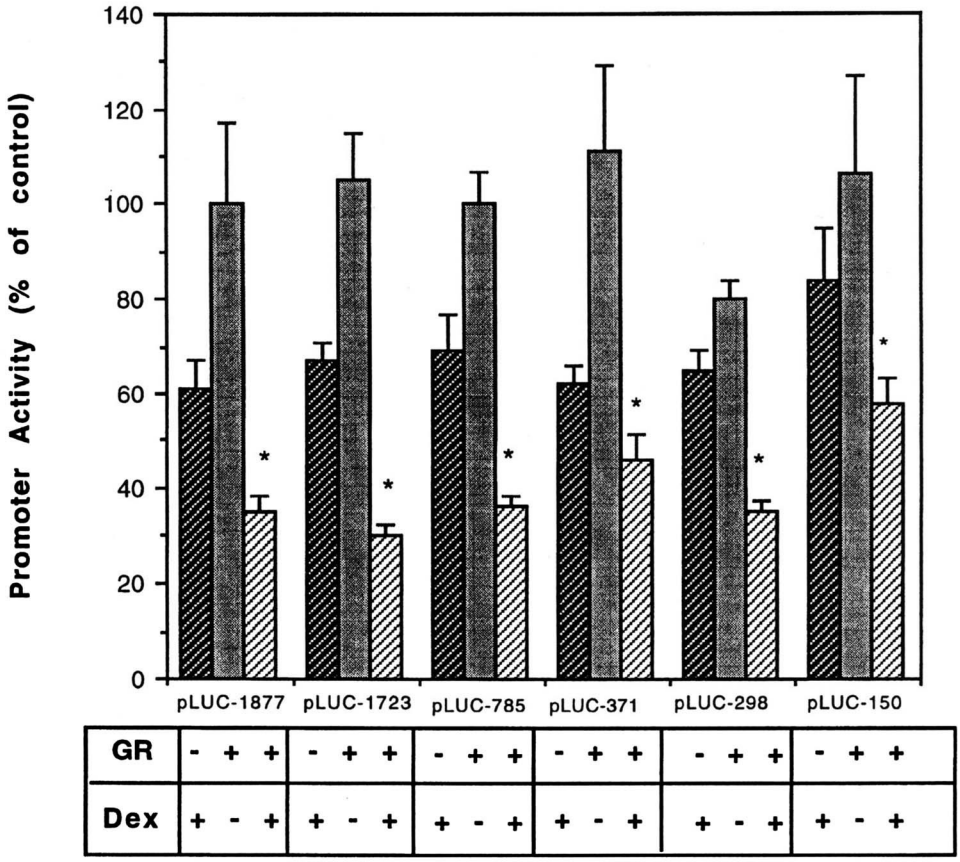


Fig. 8. Effects of glucocorticoid hormones on human *CYP7A* transcription. Confluent cultures of HepG2 cells were transfected with GR expression plasmids (0.05 μg) and plasmids (2.5 μg) containing deletion constructs of human *CYP7A* in pGL2 vector. Cells were grown in serum-free media with or without dexamethasone (0.1 μM) as indicated. Cells were harvested 40 h later for the determination of luciferase activity (RLU/mU β-galactosidase). Luciferase activities were expressed as the percentages of the control HepG2 cells which were not treated with Dex but cotransfected with GR; (*) indicate statistic difference of assays with GR+Dex vs. without GR and Dex, all $P \leq 0.003$. The promoter activities for non-treated HepG2 cells transfected with chimeric constructs (RLU/mU β-gal activity) are 48.60, 56.84, 49.32, 34.747, 59.17, and 8.39×10^3, for pLuc-1877, pLuc-1723, pLuc-785, pLuc-371, pLuc-298, and pLuc-150, respectively.

are located in the nt -184/-151 and in the -134/-81 fragment. Insulin repressed the activity of ph-298Luc by about 60% (**Fig. 6**). An insulin response element is located in the nt -298/-81 fragment. The pGL3-promoter vector was not affected significantly by PMA or insulin.

We reported previously that dexamethasone (Dex) strongly stimulated the rat *CYP7A* promoter activity and thyroid hormone did not have much effect (16). Thyroid hormone (T_4) did not affect the promoter activity of pLUC-1877 transiently transfected into confluent cultures of HepG2 cells (**Fig. 7A**). However, significant repression of promoter activity was observed when these transiently transfected HepG2 cells were also co-transfected with expression plasmid carrying thyroid hormone receptor α (TRα). The negative thyroid hormone response elements (TRE) were mapped in the -1723/-785, the -786/-299 and also in the -150/+24 regions (Fig. 7B). In these regions, several HRE-half sites are present.

Dex (0.1 μM) repressed the promoter activity by about 20 to 40%, when added into HepG2 cells transfected with *CYP7A/Luc* plasmids. Apparently endogenous GR in HepG2 cells may mediate the repression by Dex. Overexpression of glucocorticoid receptor in the HepG2 cells further repressed the activity of pLUC-1877 by 65% (**Fig. 8**). GR repressed the activity in a dose-response manner; at the pLUC-1877 to GR expression plasmid mass ratio of 25 (0.1 μg GR plasmid), activity was almost completely abolished (data not shown). Without the addition of Dex, cotransfection of GR plasmid (0.05 μg) with deletion constructs (2.5 μg) did not affect the activity. Upon the addition of Dex, the transcription of these constructs were repressed by 50 to 70%.

We reported previously that retinoic acids strongly stimulated the rat *CYP7A/Luc* gene expression (16), however, we found that retinoic acids did not affect the human *CYP7A/Luc* expression (data not shown).

Effect of co-transfection of liver-enriched transcription factor HNF3α on human *CYP7A* promoter activity

Liver-enriched transcription factors such as HNF1, HNF3, HNF4, DBP, and C/EBP are known to regulate liver-specific transactivation of genes involved in lipoprotein and lipid metabolisms (24). Molowa et al. (6) previously reported that the -432/-220 fragment of the human *CYP7A* gene could confer a 11-fold stimulation of promoter activity by HNF3. These investigators suggested that three putative HNF3 consensus sequences at nt -316, -288, and -255 were HNF3 binding sites as demonstrated by DNase I footprinting assay. They suggested that HNF3 are essential for transactivation of human *CYP7A* promoter. Co-transfection with HNF3α expression plasmid (1:1 mass ratio) stimulated the activity of pLUC-371 by about 10-fold (**Fig. 9A**), but had very little effect on the pLUC-150 (data not shown).

Ligand-activated glucocorticoid receptor represses the human *CYP7A* promoter activity by interfering with HNF3

As negative GREs are localized in the -298/-151 region and GRE half-sites (TGTTCT, ref. 25) were found to overlap with HNF3 sites (26) in the same region (**Fig. 10**), we hypothesize that the GR may repress the human *CYP7A* transcription by competing for the overlapping GR/HNF3 binding sites. We studied the effect of cotransfection of GR or HNF3α on the HNF3α or GR-stimulated transcription of the human *CYP7A* promoter, respectively. Figure 9A shows that the luciferase activity of pLUC-371 stimulated by overexpression of HNF3α (plasmid mass ratio of 1:1 of pLUC-371 to HNF3α) could be repressed by cotransfection with GR plasmid in a dose-dependent manner, in the presence of 0.1 μM Dex. As low as 0.1 μg of GR plasmid repressed the activity by about 80%. On the other hand, the luciferase activity of pLUC-371 repressed by overexpression of GR (0.1 μg) could be stimulated by cotransfection with HNF3α plasmid in the amount exceeding 0.1 μg (Fig. 9B).

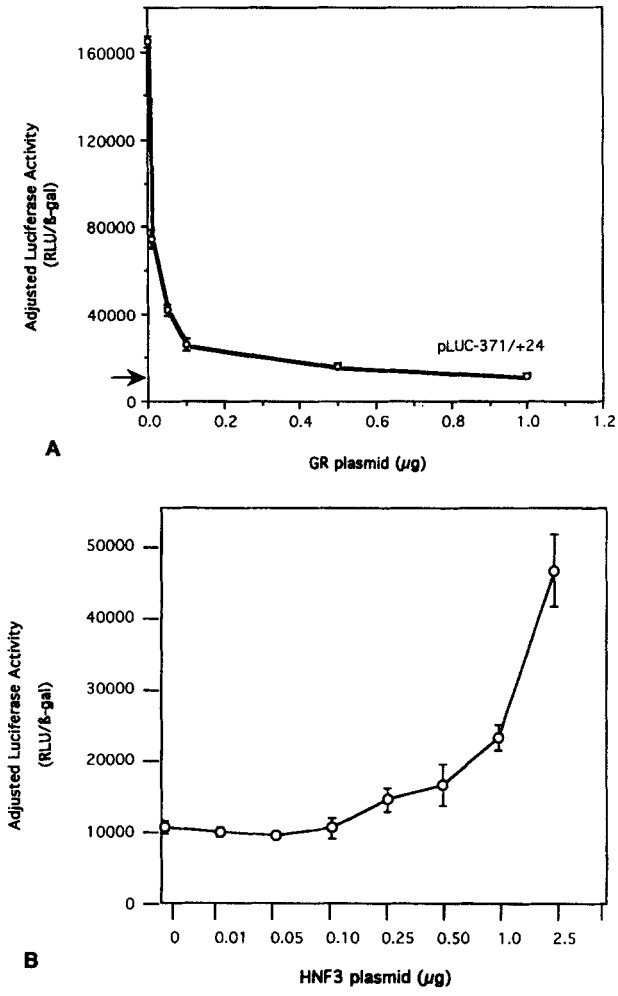
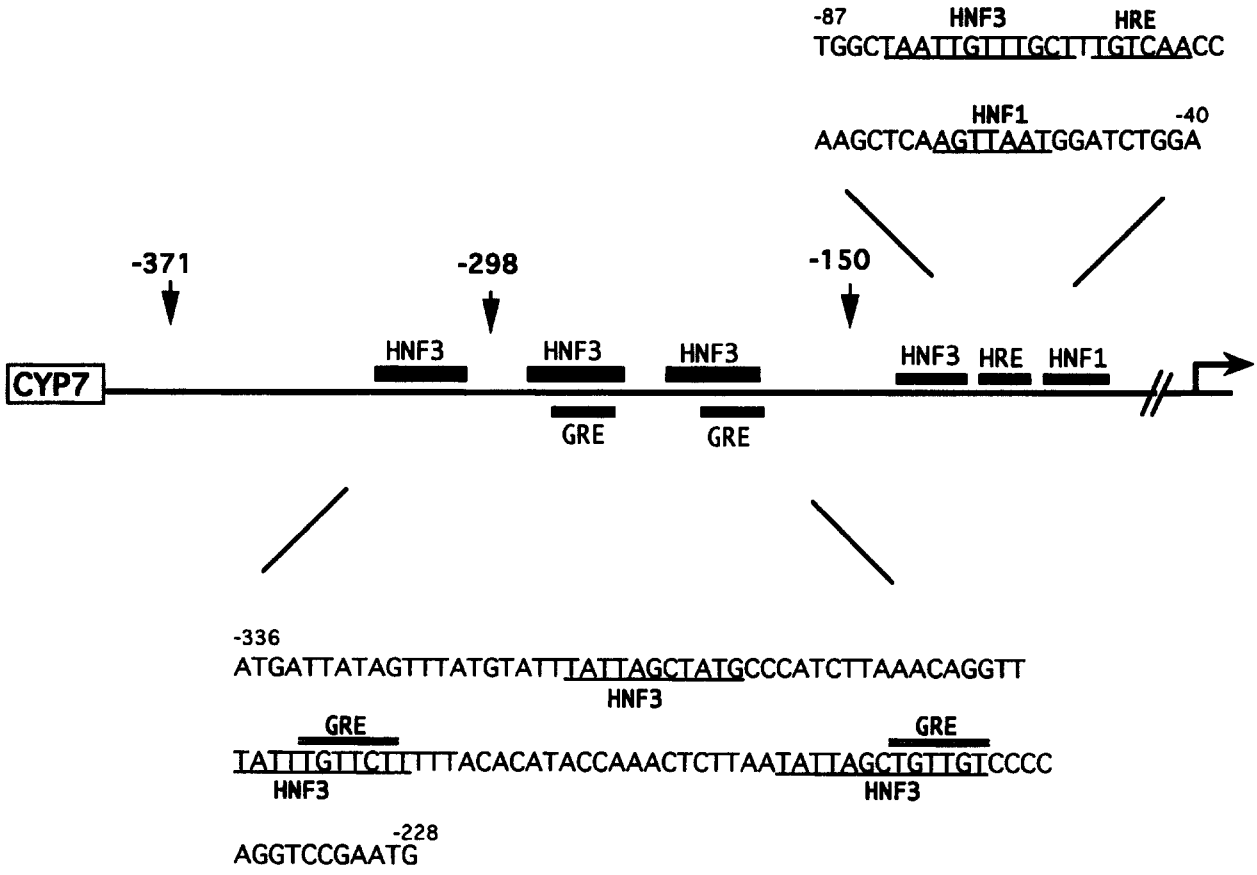


Fig. 9. Effects of cotransfection with HNF3 and GR on the transcriptional activity of *CYP7A* gene. A: Confluent HepG2 cells were cotransfected with pLUC-371 (2.5 μg), HNF3α expression plasmid (2.5 μg), and different amounts of GR expression plasmid as indicated. Cultures were incubated in serum-free medium containing 0.1 μM Dex for 40 h and harvested for the assay of luciferase activity expressed in cell extracts. The arrow indicates the control activity of pLUC-371, without cotransfection with HNF3 or GR expression plasmid. B: Confluent HepG2 cells were cotransfected with pLUC-371 (2.5 μg), GR expression plasmid (0.1 μg), and different amounts of HNF3α expression plasmids as indicated. Cells were cultured in serum-free medium containing 0.1 μM Dex for 40 h and harvested for the assay of luciferase activity expressed in cell extracts. Representative data shown are the averages of triplicate assays in one experiment. Error bar represents the standard deviation from the mean.

DISCUSSION

The stably transfected HepG2 cell line we isolated responded to known physiological regulators such as insulin and PMA. In stable cell lines, genes are integrated into chromosomes, but in the transient transfection assay, transcription was tested with naked DNA fragments. The chromosomal structures are known to influence gene transcription and regulation. Therefore, this stably transfected HepG2/2.2.1 cell line should be useful for the high throughput screening of drugs affecting the human *CYP7A* gene transcription.

The expression of luciferase activities that reflect the promoter activity of the human *CYP7A*/luciferase chimeric genes using the luciferase vector pGL3 were



HNF3 consensus: 5' TATTGA(C/T)TT(A/T)G 3'

1/2 GRE consensus: 5' TGTTCT 3'

Fig. 10. HNF3 binding sites and GREs in the human *CYP7A* gene promoter. The consensus sequences for HNF3 binding and GRE half sites are indicated in the -371 fragment of the human *CYP7A* promoter.

very high in the transient transfection assays in HepG2 cells. This system is more suitable for studying transcriptional repression than the chloramphenical acetyl-CoA transferase (CAT) assay which suffers from low sensitivity and is laborious. The transient transfection assay reported by Molowa et al. (6) using CAT reporter gene showed that the -276/+29 fragment had almost no activity. This is surprising as the proximal promoter is essential for tissue-specific expression of basal transcription activity of the eukaryotic gene. The patterns of promoter activities of the 5′-deletion constructs of the rat and human *CYP7A* genes are quite different, indicating a species difference in the regulation of these two homologous genes. This prompted us to investigate hormonal regulation of the human *CYP7A* gene in detail.

It is somewhat unexpected that glucocorticoid receptor strongly repressed the human *CYP7A* gene but stimulated the rat *CYP7A* gene promoter activity. Analysis of the nucleotide sequences in the -298/-151 region of the human gene identified a perfect GRE half-site TGTTCT (25) and a modified GRE (TGTTGT) in previously identified HNF3 sites (6). Many composite GREs have been identified as negative GREs which have a TGTTCT half-site overlapping with another tranactivator binding site, i.e., AP-1 site (25, 27). The cotransfection results are consistent with the model that GR competes with the HNF3α for the overlapping binding site, thus represses the transcriptional activity of the gene.

The finding that thyroid hormone inhibits *CYP7A* promoter activity is in contrast to the report that thyroid

hormone stimulates the expression of cholesterol 7α-hydroxylase mRNA in hypophysectomized rats (28, 29). The stabilization of mRNA by thyroid hormone may be more than compensating the down-regulation of gene transcription. TR can function both as a transcription activator or repressor (30, 31). The silencing function of TR may be mediated through its interaction with the general transcription factor TFIIB (32) or by forming a heterodimer with RXR and preventing the activating function of RXR (33). Similar mechanisms may explain the repressive effect of TR on human *CYP7A* gene expression.

The inhibitory effects of PMA and insulin on human *CYP7A* promoter were much stronger than on the rat gene. The suppression of *CYP7A* promoter activity by PMA mimics the inhibitory effect of bile acids which are known to bind and activate protein kinase C isoforms (34, 35). Stravitz et al. (36) recently suggested that bile acids might activate PKC isoforms which presumably repressed *CYP7A* gene expression by phosphorylation of a transcription factor. The strong repression of human *CYP7A* promoter activity by insulin may be physiologically significant in the regulation of cholesterol homeostasis. Cholesterol 7α-hydroxylase activity is known to be inhibited by insulin and stimulated in diabetic rats (37, 38). The negative insulin response sequence (IRS, TGTTTTC) is known to bind HNF3 which may function as an accessary factor in insulin repression of glucocorticoid stimulated-transcription of phosphoenolpyruvate carboxykinase (PEPCK) and insulin-like growth factor binding protein-1 (IGFBP-1) gene (39, 40). Similar interactions of GR, HNF3, and insulin response factor with the IRS/GR/HNF3 motif in the -293/-273 region may also regulate the *CYP7A* gene transcription. Inhibition of the *CYP7A* gene transcription by insulin may lead to hypercholesterolemia in insulin resistance and metabolic syndromes in humans.

It is now apparent that the cholesterol 7α-hydroxylase gene is regulated by a complex mechanism involving multiple factors. The most significant finding of this study is that the human *CYP7A* gene is resistant to the stimulation by hormones and is more responsive to repression by insulin and PMA. This may explain the much lower level of cholesterol 7α-hydroxylase activity and mRNA expression in the human than in the rat liver. The rat *CYP7A* gene may be unique in that it can be stimulated by many hormones, including glucocorticoid, retinoic acids, and cAMP. This species difference in the transcription regulation of the *CYP7A* gene may contribute to different cholesterol metabolism and homeostasis observed in different animals.

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