Hormonal regulation of the cholesterol 7α-hydroxylase gene (CYP7)

Maurizio Crestani,[1] Diane Stroup, and John Y. L. Chiang[2]

Department of Biochemistry and Molecular Pathology, Northeastern Ohio Universities College of Medicine, 4209 State Route 44, P. O. Box 95, Rootstown, OH 44272-0095

Abstract The transcriptional regulation of the rat cholesterol 7α-hydroxylase gene (CYP7) by hormones and signal transduction pathways was studied by transient transfection assay of the promoter activity. HepG2 cells were transfected with deletion mutants of the CYP7 upstream region linked to the luciferase reporter gene. The transcription of CYP7/luciferase chimeric genes was higher in confluent than in subconfluent cultures of HepG2 cells. Glucocorticoid receptors, in the presence of dexamethasone, up-regulated the CYP7 gene through two regions located between -3262 and -2803, and between -344 and -222, respectively. Thyroid hormones did not have any effect on the promoter activity. Insulin inhibited the promoter activity through sequences located between -344 and -222, and abolished the stimulation by dexamethasone. Hence, the insulin effect was dominant over that of glucocorticoids. Treatment of transfected HepG2 cells with phorbol 12-myristate 13-acetate (PMA), a known activator of protein kinase C (PKC), resulted in a time-dependent inhibition of the CYP7 promoter activity. The negative phorbol ester-response sequences were mapped between -344 and -222, and between -200 and -161, respectively. The CYP7 promoter activity was induced nearly 5-fold by all-*trans*-retinoic acid through sequences in the region from -200 to -129. Finally, cyclic AMP and protein kinase A (PKA) stimulated the expression of the CYP7/luciferase gene through multiple sequences in the distal and proximal regions, and both positive and negative response regions were mapped. Our results revealed that the -416 fragment of the rat CYP7 gene confers the activation by glucocorticoids and retinoic acid, and inhibition by insulin, phorbol esters and cAMP. It appears that this proximal promoter may contain a pleiotropic domain that regulates the effects of multiple signals.—**Crestani, M., D. Stroup, and J. Y. L. Chiang.** Hormonal regulation of the cholesterol 7α-hydroxylase gene (CYP7). *J. Lipid Res.* 1995. **36:** 2419–2432.

Supplementary key words cholesterol 7α-hydroxylase • HepG2 cells • gene regulation • hormones • second messengers

Cholesterol 7α-hydroxylase (EC 1.14.13.17) is the first and rate-limiting enzyme in bile acid synthesis, the major metabolic pathway for cholesterol disposal from the body (1). The activity of this enzyme is regulated principally at the transcriptional level by bile acids returning to the liver through enterohepatic circulation (2), cholesterol (3, 4), steroid/thyroid hormones (5, 6), insulin (7), and diurnal rhythm (8). The gene encoding cholesterol 7α-hydroxylase (CYP7) has been cloned from the rat (9–11), human (12–14), hamster (15), and mouse (16). Primary cultures of rat (17, 18) and pig (19) hepatocytes and HepG2 cells (20) have been used to study bile acid synthesis in general and the regulation of the CYP7 gene in particular. Most intermediates in the bile acid biosynthetic pathway have been detected in HepG2 cells (21, 22) and the defects in this metabolic pathway reported previously were due to culture conditions (20). Several authors have shown that HepG2 cells exhibit a more adult or terminally differentiated phenotype when grown to confluence (23, 24). Interestingly, at this stage HepG2 cultures do not display any defect in bile acid synthesis (25) and CYP7 mRNA levels can be regulated by hydrophobic bile acids (26, 27) and other signals (glucocorticoid hormones, lipoproteins) (26). Therefore, this cell line is considered a suitable model to study the regulation of bile acid synthesis (21, 22, 26, 28, 29). Recently, results from several laboratories have shed light on the mechanisms of transcriptional regulation of the CYP7 gene. In transient transfection experiments using primary rat hepatocytes (30) and HepG2 cells (27),

Abbreviations: CYP7, cholesterol 7α-hydroxylase gene; BARE, bile acid responsive element; kb, kilobase(s); DRBP, Direct Repeat Binding Protein; Luc, luciferase gene; CAT, chloramphenicol acetyltransferase; GRE, glucocorticoid response element; GR, glucocorticoid receptor; HNF3, hepatocyte nuclear factor 3; HNF4, hepatocyte nuclear factor 4; PCR, polymerase chain reaction; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PRS, PMA responsive sequence; RAR, retinoic acid receptor; RXR, retinoid X receptor; IRS, insulin responsive sequence; AP-1, activator protein 1; C/EBP, CCAAT/enhancer binding protein; DBP, albumin D-site binding protein; NF-κB, nuclear factor κB; PEPCK, phosphoenolpyruvate carboxykinase.

[1]On leave from Istituto di Scienze Farmacologiche, Università degli Studi di Milano, Facoltà di Farmacia, via Balzaretti 9, 20133 Milano, Italy.

[2]To whom correspondence should be addressed.

it has been shown that bile acids can down-regulate the transcriptional activity of rat CYP7 promoter/reporter genes. Molowa et al. (12) have identified three HNF-3 binding sites in the human gene required for transactivating the CYP7 gene. Chiang and Stroup (31) have mapped a putative bile acid responsive element (BARE) in the proximal promoter region of the CYP7 gene between nt -73 and -55, and identified a bile acid responsive protein. Ramirez et al. (32), using a conditionally transformed hepatocyte cell line and transgenic mice, have demonstrated that both bile acid- and cholesterol-mediated regulation of cholesterol 7α-hydroxylase occurs at the transcriptional level. In addition, the same authors have shown that an enhancer located 7 kb upstream of the transcription start site may be important for the liver-specific expression of the CYP7 gene. Other laboratories have also shown that DBP, a liver-enriched transcription factor, can bind and transactivate the rat CYP7 gene (8, 33), providing evidence that DBP is a regulator of the circadian expression of CYP7. Nevertheless, the molecular mechanisms of regulation of the CYP7 gene by physiological factors, such as hormones and signal transduction pathways, have not been studied. In order to understand the mechanisms of gene regulation, it is necessary to identify *cis*-regulatory elements in the gene that are responsive to known physiological stimuli.

In the present study, we investigated the effect of known physiological regulators on the transcriptional activity of the CYP7 gene and defined the regions containing the response elements that mediate these effects. For this purpose, we transfected HepG2 cells with rat CYP7 promoter/luciferase chimeric genes and mapped promoter regions controlling the regulation by glucocorticoid hormones, insulin, cyclic AMP, retinoic acid, and phorbol esters. The results suggest that hormones and second messengers affect the transcription rate of the rat CYP7 gene mainly through the region 416 nt upstream of the transcription start site. We propose that the rat CYP7 gene may be finely regulated by distinct signals converging to consensus sequences clustered in the proximal promoter.

EXPERIMENTAL PROCEDURES

Materials

DNA restriction and modifying enzymes, Reporter Lysis Buffer, Luciferase Assay System, and the reporter vectors pGL2-Basic, pGL2-Promoter, pGL2-Control, and pSV40 β-galactosidase were purchased from Promega (Madison, WI). The vectors pFlashII, pCMVβ, and pBluescript II KS+ were obtained from Synapsys (Burlington, MA), Clontech (Palo Alto, CA) and Stratagene Cloning Systems (La Jolla, CA), respectively. Tissue culture media were purchased from Celox (Hopkins, MN); fetal calf serum and other media supplies were from GIBCO-BRL (Grand Island, NY). Plasticware for tissue culture was obtained from Corning (New York, NY) and Sarstedt (Newton, NC). The human hepatoblastoma cell line HepG2 was supplied by American Type Culture Collection (Rockville, MD). The mammalian expression vectors for the glucocorticoid receptor (6RGR) and for the catalytic subunit of the cyclic AMP-dependent protein kinase (pCEV) were gifts from Dr. Keith R. Yamamoto (34) and Dr. G. Stanley McKnight (35), respectively. Sequenase was purchased from USB (Cleveland, OH). [^{35}S]dATP (1200 Ci/mmol) sequencing grade was obtained from DuPont/NEN (Boston, MA). The GeneClean Kit was from BIO 101 (La Jolla, CA). The Qiagen Plasmid Kit was acquired from Qiagen Inc. (Chatsworth, CA). Oligonucleotides for the polymerase chain reaction were synthesized by National Biosciences (Plymouth, MN). Insulin, dexamethasone, L-thyroxine, all-*trans*-retinoic acid, dibutyryl cyclic AMP, and phorbol 12-myristate 13-acetate (PMA) were supplied by Sigma (St. Louis, MO).

Construction of rat CYP7 promoter/luciferase reporter genes

The rat CYP7/luciferase chimeric gene p-3644Luc was made by ligation of the 3.2kb *Sac*I-*Hin*dIII fragment, spanning the upstream region between nt -3644 and -345, to a 380bp *Hin*dIII-*Xba*I fragment, generated by polymerase chain reaction (36), spanning the sequence between nt -344 and +36. The *Xba*I site was filled in with Klenow and the 3.6kb *Sac*I-*Xba*I fragment was subcloned into the pGL2-Basic vector cut with *Xho*I, filled in with Klenow, and digested with SacI. The deletion clones p-3262Luc, p-2803Luc, p-2772Luc, p-2092Luc, p-1573Luc, p-1335Luc, and p-778Luc were generated by digesting p-3644Luc with StuI, ScaI, SmaI, AflII, XbaI, Tth111I, and NcoI, respectively, at the positions indicated in Fig. 1 and subcloning them in the multiple cloning site of the pGL2-Basic vector. The clone p-593Luc was obtained by cutting p-1573Luc with EcoRI (nt -592) and BglII. The EcoRI-BglII fragment was subcloned into pGL2-Basic cut with MluI, filled in with Klenow, and digested with BglII. The plasmid p-416Luc was made by PCR using the SacI fragment of the rat CYP7 gene as a template. The 5′-primer was 5′-ATACTTCTGGAGGTTCATTT-3′ and the 3′-primer (L1), bearing a XhoI restriction site (underlined), was 5′-agatggctcGAGACTCTTTGCCTAGCAAA-3′ (capital letters indicate gene sequence). The DNA fragment was digested with XhoI and cloned into SmaI-XhoI sites of pGL2-Basic. p-344Luc was obtained by HindIII digestion of p-416Luc and by subcloning the HindIII frag-

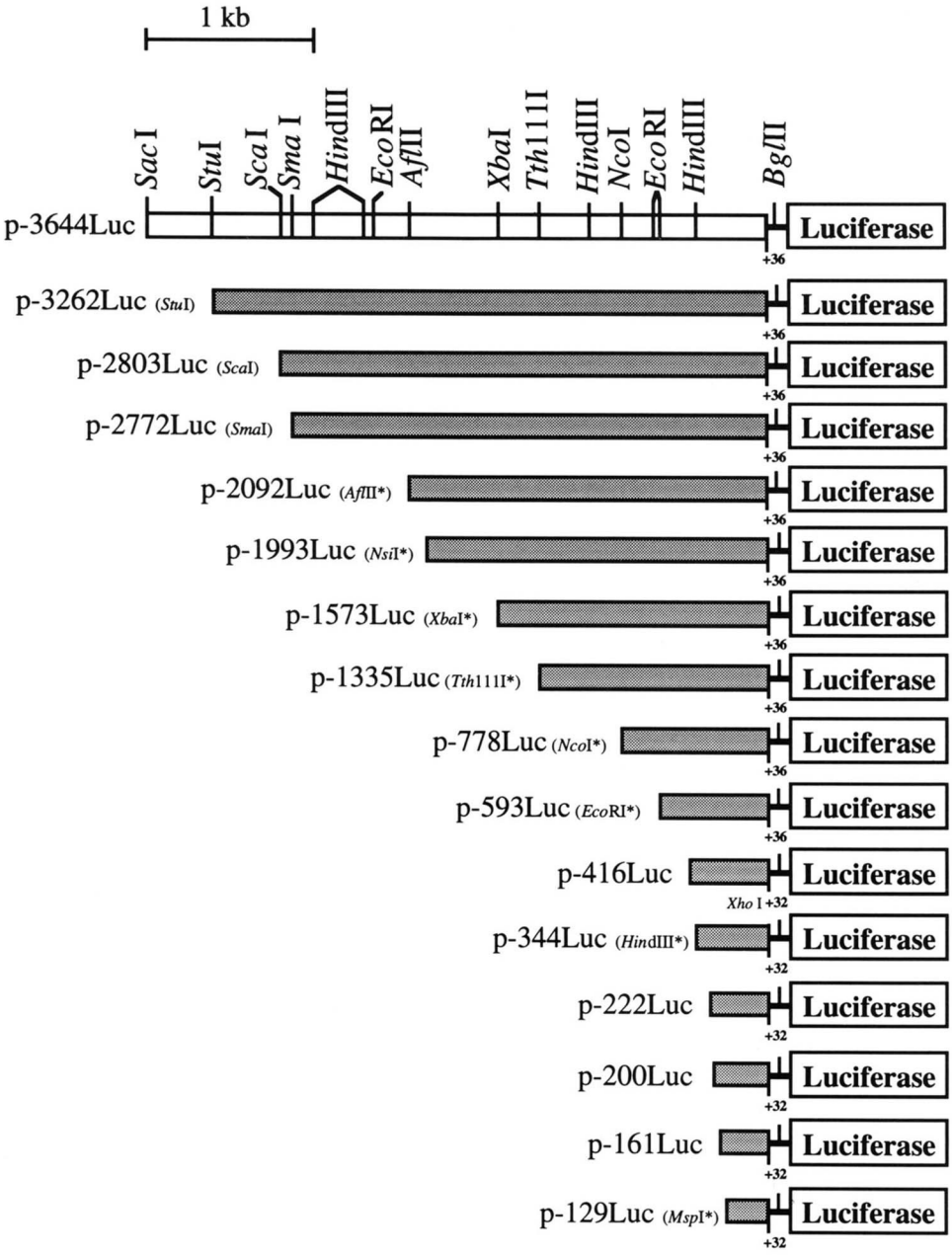

Fig. 1. Construction of rat CYP7/luciferase chimeric genes. The restriction map of p-3644Luc is shown on top. The deletion clones were generated either by restriction digestion or by PCR as described under Experimental Procedures. The numbers indicate the position relative to the transcription start site. The enzymes used to cut the parental p-3644Luc are shown in parentheses. The asterisks indicate that the 5'-end was filled in with Klenow before subcloning into pGL2-Basic.

ment into the HindIII site of pGL2-Basic. p-222Luc was generated by PCR using the 5′-primer 5′-CAGCACATGAGGGACAG-3′ and the 3′-primer L1. The resulting DNA was cut with XhoI and subcloned into SmaI-XhoI sites of pGL2-Basic. p-200Luc was made by PCR with the 5′-primer 5′-ggggtacCAGCTTATCGAGTATTGC-3′, containing a KpnI site, and the 3′-primer L1. The DNA was digested with KpnI-XhoI and subcloned into pGL2-Basic cut with the same enzymes. p-161Luc was obtained by PCR with the 5′-primer 5′-CTCTTCTGAGACTATGGA-3′ and the 3′-primer L1. The fragment, cut with XhoI, was subcloned into the SmaI-XhoI digested multiple cloning site of pGL2-Basic. The plasmid p-129Luc was obtained by MspI digestion of p-200Luc,

followed by treatment with Klenow and digestion with XhoI. The resulting fragment was subcloned in compatible restriction sites of pGL2-Basic.

All plasmids were verified by restriction digestion analysis and sequencing, and were purified either by double banding in cesium chloride gradients or with Qiagen columns according to the manufacturer's instructions.

Cell culture and DNA transfection

HepG2 cells were grown between passage 5 and 50 in our laboratory in Dulbecco's modified Eagle's medium (DMEM)–F-12 (Ham) 1:1, containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU penicillin G/ml, and 100 μg streptomycin sulfate/ml, at 37°C in a humidified incubator in the presence of 5% CO_2. Cells were seeded in 6-well cluster plates at $0.7 \times 105/cm^2$ (subconfluent cultures) or $1.3 \times 10^5/cm^2$ (confluent cultures) with 2.5 ml of medium and grown for 24 h (subconfluent cultures) or 6 days (confluent cultures). In the latter case, cells were refed with fresh medium every 2 days. Transfection experiments were carried out with the calcium phosphate–DNA coprecipitation technique (37), adding 5 μg of test plasmid and 0.5 μg of pCMVβ or 1 μg of pSV40 β-galactosidase as internal standards for the adjustment of transfection efficiency. In cotransfection experiments, 0.05–2 μg of either 6RGR or pCEV expression plasmid was added to the cells and pBluescript II KS+ was used as a carrier to keep the total amount of plasmid constant at 7 μg/well. Four hours after the addition of DNA, cells were shocked 90 sec with 15% glycerol in TBS (25 mM Tris, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4), washed twice with TBS and refed with serum-free medium with the indicated amount of hormones or vehicle. Transfected cells were incubated 24–40 h.

Cell harvest and reporter enzyme assays

Cells were washed twice with phosphate-buffered saline (PBS), lysed, and harvested with Reporter Lysis Buffer (200 μl) following the manufacturer's instructions. Luciferase activity was assayed by mixing luciferase assay reagent (100 μl) with cell extracts (20 μl) and measuring total light emission at room temperature during the initial 10 sec of the reaction (0–10 sec integral) with a luminometer (Lumat LB9501, Berthold). β-Galactosidase activity was determined as described (38) using *o*-nitrophenyl-β-SD-galactopyranoside as a substrate. Luciferase activity was normalized for variation in transfection efficiency by dividing relative light units (RLU) by β-galactosidase activity.

Statistical analysis

Statistically significant differences were tested by means of Student's t test. Analysis was performed using SigmaPlot software (Jandel Scientific, San Rafael, CA).

RESULTS

Effect of cell density on the promoter activity of CYP7/luciferase chimeric genes

To test the influence of cell density on the promoter activity of the CYP7 upstream sequence, we transfected subconfluent (24 h old) and confluent (6 days old) HepG2 cells with CYP7 promoter/luciferase chimeric genes. As shown in **Fig. 2,** all the deletion clones containing the upstream sequence from nt -3644 to -416 were more active in confluent cultures than in subconfluent cultures. However, when the sequence was deleted down to nt -222, the trend was reversed (Fig. 2). This suggests that the sequence between nt -416 and -222 may confer the developmentally dependent expression of the CYP7 gene. The activity of the SV40 early promoter (pGL2-Promoter) was not affected by cell density. Moreover, p-344Luc and p-416Luc displayed the highest promoter activities among the regions we tested, which

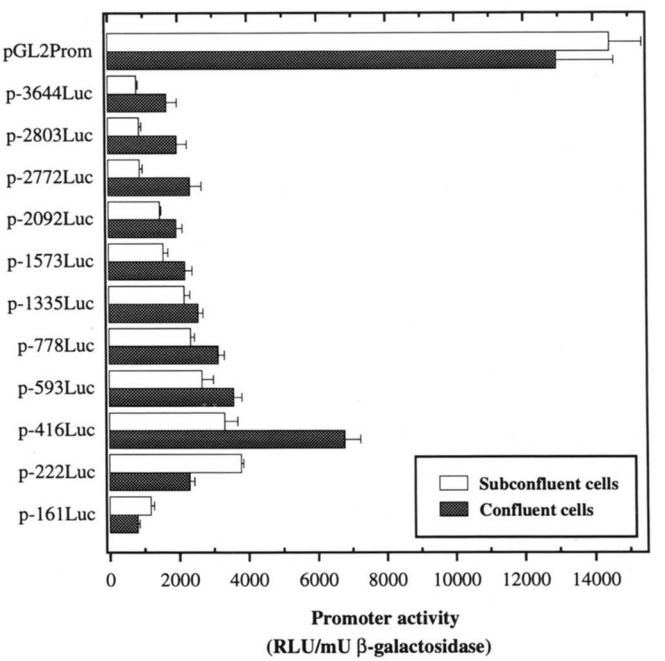

Fig. 2. Effect of cell density on the promoter activity of rat CYP7/luciferase chimeric genes transfected into HepG2 cells. Subconfluent and confluent HepG2 cultures were transfected as described under Experimental Procedures and incubated for 40 h in serum-free medium. At the end of the experiment, cells were harvested and reporter enzyme activities were determined as described. Results are expressed as the ratio of luciferase and β-galactosidase activity and represent the mean ± SD of three independent determinations.

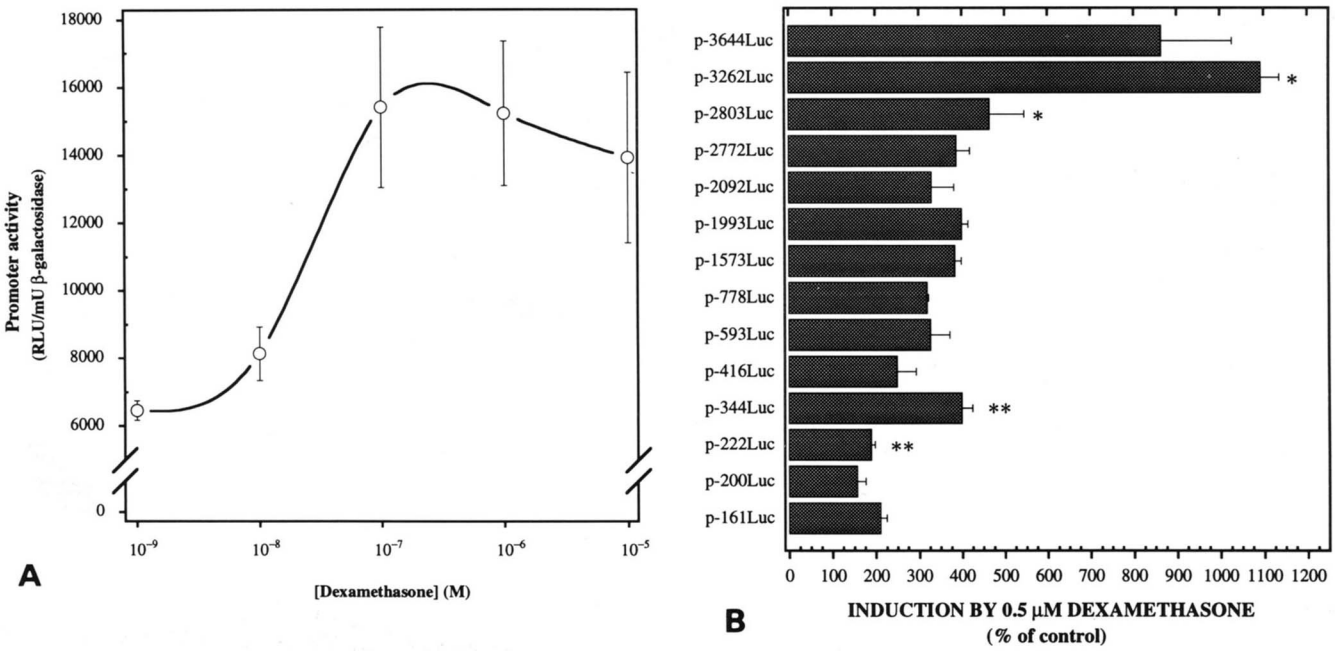

Fig. 3. Effect of dexamethasone on the promoter activity of the CYP7 gene in HepG2 cells. Panel A, confluent HepG2 cultures were transfected with p-3644Luc and incubated 40 h in serum-free medium containing increasing concentrations of dexamethasone. The results are expressed as the ratio of luciferase and β-galactosidase activity and represent the mean ± SD of three independent determinations. Panel B, confluent HepG2 cells were cotransfected with the indicated rat CYP7/luciferase chimeric genes and 6RGR, the mammalian expression vector for the glucocorticoid receptor, as described under Experimental Procedures and incubated for 40 h in serum-free medium containing either 0.5 μM dexamethasone in ethanol or ethanol alone. Normalized promoter activities are expressed as percentage of control and represent the mean ± SD of three independent determinations; (*) indicates difference at $P < 0.0005$; (**) indicates difference at $P < 0.0005$.

are about 50% of pGL2-Promoter activity (Figs. 2 and 4). This implies that repressor sequences are located upstream of nt -416 and activator sequences downstream of nt -416.

Regulation of the CYP7 promoter activity by glucocorticoid hormones

Previous studies have shown that glucocorticoid hormones can stimulate cholesterol 7α-hydroxylase activity (39), mRNA level and transcription rate in rat primary hepatocytes (6, 40) and HepG2 cells (26). When HepG2 cells were transfected with p-3644Luc and treated with increasing concentrations of dexamethasone, a synthetic glucocorticoid hormone, the maximum effect (about 2.5-fold increase) was observed at 0.1–1 μM dexamethasone (**Fig. 3A**). In order to amplify the magnitude of glucocorticoid stimulation, HepG2 cells were cotransfected with CYP7 promoter/luciferase chimeric genes and a mammalian expression vector carrying the cDNA for the glucocorticoid receptor (6RGR) (34). The plasmid p-3644Luc responded with a 5- to 10-fold induction of luciferase expression in the presence of 0.5 μM dexamethasone when cotransfected with 6RGR. Without the ligand, the overexpression of the glucocorticoid receptor did not affect the promoter activity of p-3644Luc. The data in Fig. 3B show that the deletion of the promoter to nt -2803 drastically decreased the dex-

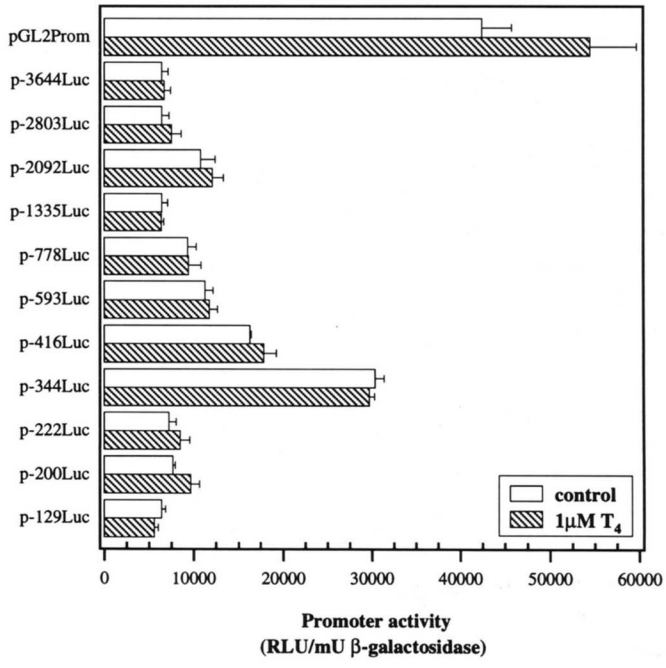

Fig. 4. Effect of L-thyroxine on the promoter activity of the CYP7 gene in HepG2 cells. Confluent cultures of HepG2 cells were transfected with the indicated rat CYP7/luciferase chimeric genes and incubated for 40 h in serum-free medium in the presence of 1 μM L-thyroxine or vehicle alone. The results are expressed as the ratio of luciferase and β-galactosidase activity and represent the mean ± SD of three independent determinations

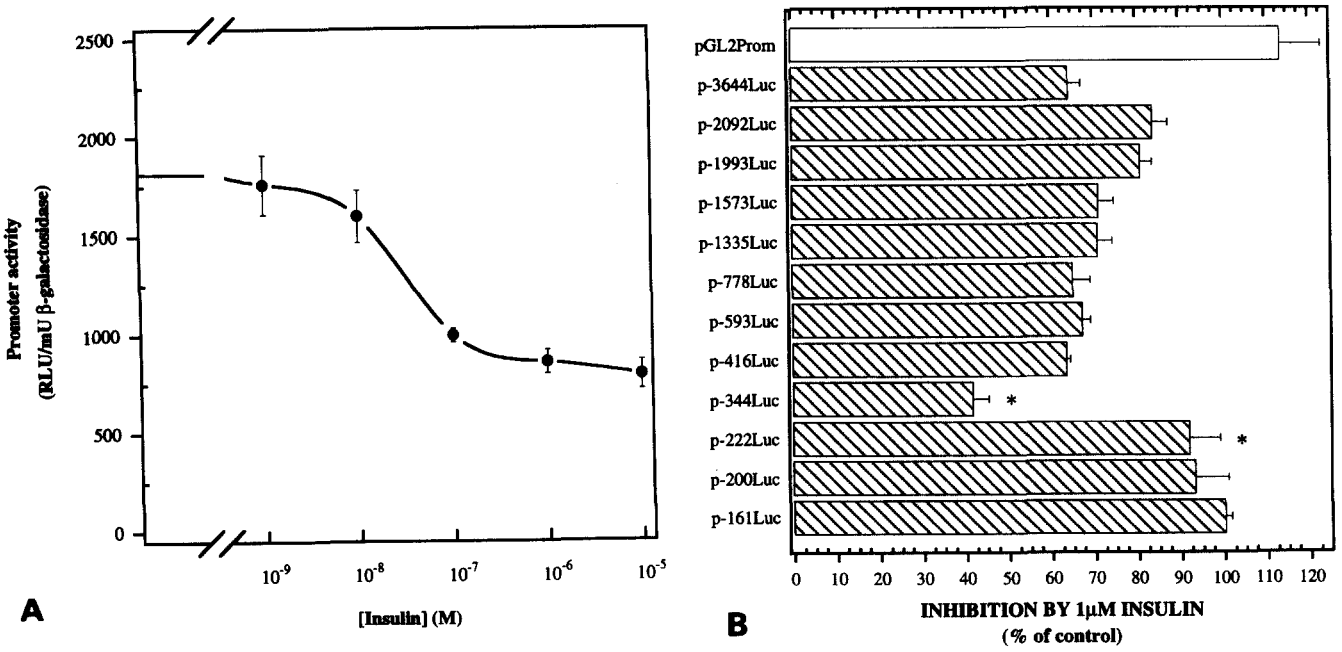

Fig. 5. Effect of insulin on the promoter activity of the CYP7 gene in HepG2 cells. Panel A, confluent HepG2 cultures were transfected with p-3644Luc and incubated 40 h in serum-free medium containing increasing concentrations of insulin. The results are expressed as the ratio of luciferase and β-galactosidase activity and represent the mean ± SD of three independent determinations. Panel B, confluent cultures of HepG2 cells were transfected with the indicated rat CYP7/luciferase chimeric genes and incubated for 40 h in serum-free medium in the presence or absence of 1 μM insulin. Normalized promoter activities are expressed as percentage of control (mean ± SD). Representative graph shown is from two independent experiments; (*) indicates difference at $P < 0.0001$.

amethasone induction to about 4-fold and deleting down to nt -222 further dropped the induction to less than 2-fold. Apparently, two regions may mediate the glucocorticoid effect, one located between nt -3262 and -2803, and the other between nt -344 and -222.

Thyroid hormones do not affect the transcription rate of the CYP7/luciferase fusion genes

Thyroid hormones have been shown to stimulate the cholesterol 7α-hydroxylase mRNA levels in hypophysectomized rats (5, 41) and the transcription rate in cultured rat hepatocytes (6). In preliminary experiments carried out in our laboratory, T_4 increased the CYP7 mRNA levels in HepG2 cultures (W. G. Karam, and J. Y. L. Chiang, unpublished results). Therefore, we tested the effect of L-thyroxine on the transcriptional activity of the CYP7/luciferase deletion mutants. As shown in **Fig. 4,** none of the clones tested responded to T_4. Cotransfection with thyroid hormone receptor expression plasmid also did not affect the CYP7 promoter activity (data not shown). We conclude that no thyroid hormone response element is present in the region 3644 nt upstream of the transcription start site in the rat CYP7 gene.

Effect of insulin on the promoter activity of the CYP7 upstream region

Insulin is known to exert a negative effect on bile acid synthesis (42–44), thus we tested the effect of this hormone on the transcriptional activity of the CYP7 promoter. Insulin repressed the promoter activity of p-3644Luc in a dose-dependent fashion (**Fig. 5A**). The inhibitory effect of insulin disappeared when the sequence from nt -344 to -222 was removed (Fig. 5B). Therefore, a negative insulin response sequence (IRS) must be located between nt -344 and -222.

Effect of protein kinase A on the CYP7 promoter activity

In preliminary experiments, we observed that cyclic AMP increased cholesterol 7α-hydroxylase mRNA levels (W. G. Karam, and J. Y. L. Chiang, unpublished results). Also, the transcriptional activity of p-3644Luc increased in the presence of cAMP (Fig. 7). As cAMP can activate protein kinase A (PKA), we carried out cotransfection experiments in HepG2 cells with the plasmid pCEV, a mammalian expression vector containing the cDNA for the catalytic subunit of the cAMP-dependent protein kinase (PKA) (35). A titration curve with increasing amounts of the catalytic subunit of PKA was performed and a maximum of 10-fold enhancement was seen when 1 μg of pCEV was cotransfected (data not shown). To define the region(s) mediating the induction by PKA, we cotransfected the 5′ deletion mutants of the CYP7/luciferase gene with 1 μg of pCEV. The induction of the promoter activity by PKA was dramatically re-

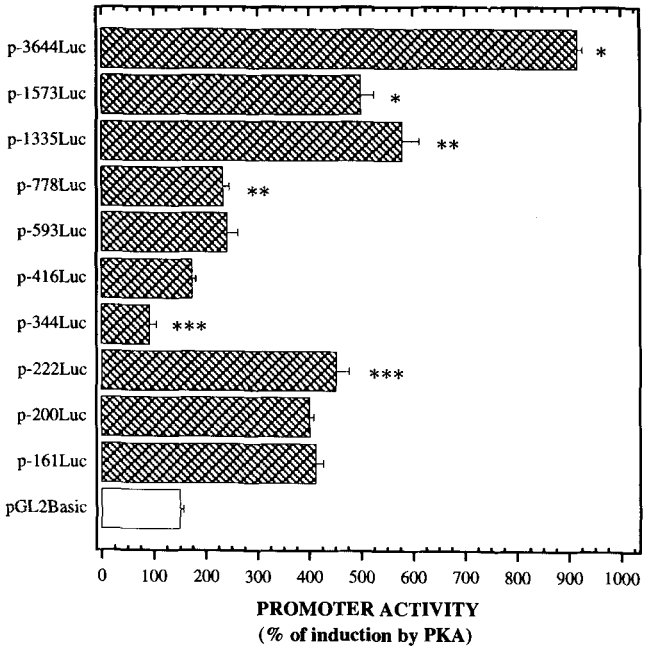

Fig. 6. Cotransfection of protein kinase A with CYP7/luciferase chimeric genes into HepG2 cells. Rat CYP7/luciferase chimeric genes were cotransfected with 1 ug of pCEV into confluent cultures of HepG2 cells. Transfected cells were incubated for 24 h in serum-free medium. Data shown are expressed as percentage of control of normalized promoter activities (mean ± SD). Representative graph from two independent experiments; (*) indicates difference at $P <$ 0.0001; (**) indicates difference at $P \pm$ 0.0001; (***) indicates difference at $P <$ 0.0001.

duced when the upstream region from nt -3644 to -344 was deleted (**Fig. 6**). Surprisingly, the stimulation was partially restored when the promoter sequence was further deleted to nt -222 (Fig. 6). The pattern of the activation of the CYP7/luciferase deletion clones seems to be quite complex and the effect of PKA may be mediated through several regions in the CYP7 promoter, i.e., positive effects in the regions -3644 to -1573, -1335 to -778, -593 to -344, and -161 to +32; negative effect in the region -344 to -222.

Effect of insulin on glucocorticoid- and cyclic AMP-stimulated CYP7 gene expression

To study the interplay of different hormones and second messengers on the promoter activity of the CYP7 gene, we treated transfected HepG2 cells with insulin, dexamethasone, cAMP alone, and with combinations of these factors. Dexamethasone and cAMP stimulated the promoter activity by 2.5-fold, as opposed to 50% repression of activity with insulin (**Fig. 7**). Surprisingly, insulin completely abolished the stimulatory effect of dexamethasone (e.g., insulin effect is greater in the presence of dexamethasone) (Fig. 7). Insulin also decreased the effect of cAMP, however, the promoter activity of p-3644Luc in the presence of both cAMP and insulin was higher than in the presence of insulin alone (Fig. 7). Thus, the effect of insulin seems to be dominant over the effect of dexamethasone but does not completely antagonize that of cAMP.

Phorbol esters inhibit the promoter activity of the CYP7 gene

To determine whether a protein kinase C (PKC)-mediated signal transduction pathway is involved in the regulation of the CYP7 gene transcription, we performed transfection experiments with phorbol 12-myristate 13-acetate (PMA), a PKC activator. As long exposures to phorbol esters are known to deplete the cell of PKC activity (45), we carried out a time course experiment to find the optimal incubation time. The promoter activity of p-3644Luc was inhibited up to 40% in the first 4–6 h of incubation with 1 μM PMA (**Fig. 8A**). Then, the promoter activity started to recover to the control level after 8 h and reached 125% of the control at 16 h (Fig. 8A). Further exposure to PMA reduced the promoter activity. This biphasic pattern was specific for the CYP7 promoter as the expression of the luciferase gene driven by the herpes simplex virus thymidine kinase (hsv-TK) promoter was rapidly enhanced 3-fold by PMA. The maximum inhibition was achieved at 0.1–1 μM PMA after incubation for 4 h (data not shown). To define the

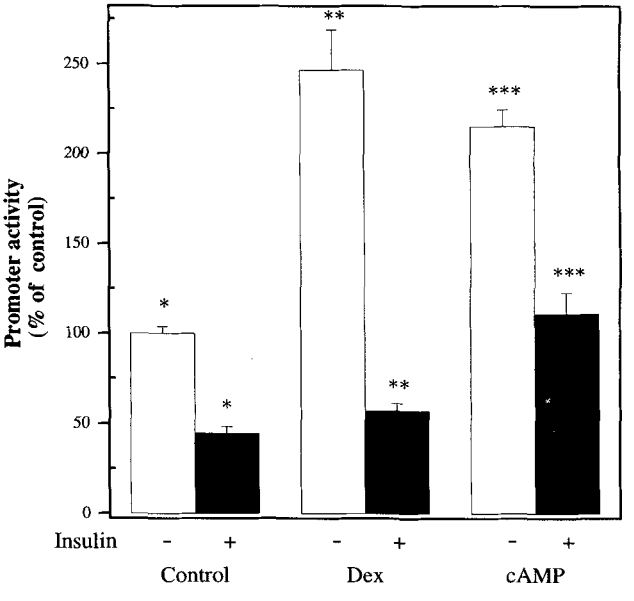

Fig. 7. Effect of insulin on glucocorticoid- and cAMP-stimulated CYP7 promoter activity. Confluent HepG2 cultures were transfected with p-3644Luc as described and incubated for 40 h in serum-free medium (control) and medium containing 0.1 μM dexamethasone or 0.1 mM dibutyryl cAMP in the presence (+) or absence (-) of 1 μM insulin. Dibutyryl cAMP was added during the last 12 h of incubation. Luciferase activities are expressed as percentage of control (mean ± SD). The graph shown is representative of two independent experiments; (*) indicates difference at $P <$ 0.0005; (**) and (***) indicate difference at $P <$ 0.005.

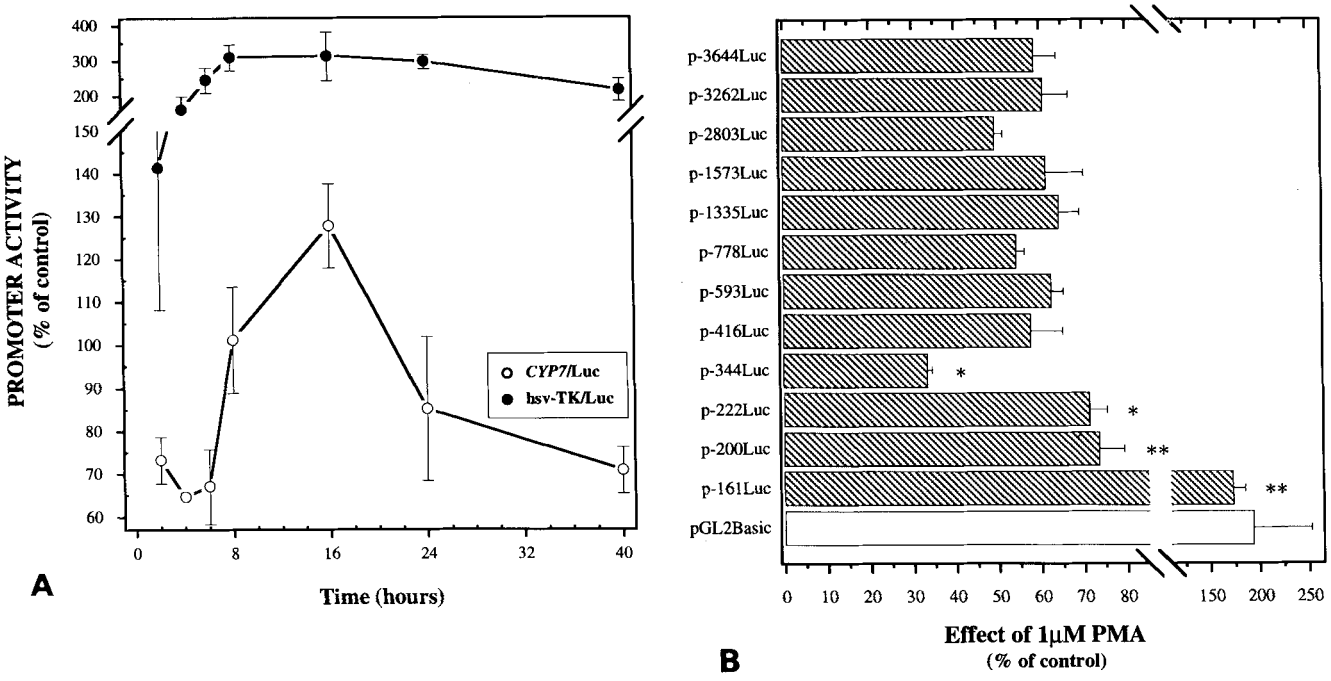

Fig. 8. Effect of phorbol esters on the promoter activity of the CYP7 gene in HepG2 cells. Panel A, confluent HepG2 cultures were transfected with either p-3644Luc or pFlashII (luciferase gene driven by hsv-TK promoter) and refed with serum-free medium containing 1 μM PMA in ethanol or ethanol alone. Cells were harvested at the times indicated and reporter enzyme activities expressed were determined. Data are expressed as percentage of control of normalized luciferase activity and represent mean ± SD of three separate observations. Panel B, confluent HepG2 cells were transfected with the rat CYP7/luciferase gene constructs. Cells were incubated in serum-free medium containing 1 μM PMA in ethanol or ethanol alone and harvested after 4 h. The normalized promoter activities are expressed as percentage of control and represent mean ± SD of three separate observations. The graph shown is representative of two independent experiments; (*) indicates difference at $P < 0.0005$; (**) indicates difference at $P < 0.0005$

region containing a phorbol ester response sequence (PRS), HepG2 cultures were transfected with several CYP7 promoter/luciferase chimeric genes and treated with 1 μM PMA for 4 h. As can be seen in Fig. 8B, the promoter activity of the deletions down to nt -200 was inhibited by PMA, whereas the activity of p-161Luc was 1.7-fold enhanced by PMA. Thus, it appears that strong negative PRSs are located in region from -344 to -222, and from -200 to -161, and positive PRSs are located from -416 to -344, and from -161 to +32.

All-*trans*-retinoic acid is a potent stimulator of the CYP7 gene transcription

Because retinoids play an important role in the regulation of genes involved in lipid and cholesterol metabolism (46–48), we tested the effect of all-*trans*-retinoic acid on the promoter activity of the CYP7 gene. The compound stimulated the promoter activity of p-3644Luc at a concentration as low as 10 nM and the maximum effect (4.5-fold) was observed at 1–10 μM (**Fig. 9A**). As shown in Fig. 9B, the promoter activity of all the deletion mutants was induced by all-*trans*-retinoic acid except p-129Luc, which was not responsive to all-*trans*-retinoic acid. The promoter activity of p-161Luc was still induced by retinoic acid but to a lesser extent (1.5-fold). Thus, it appears that a major retinoic acid response element (RARE) is located in the region from nt -200 to -129, although the further upstream region may contain other RARE/RXRE-like sequences that can enhance the retinoic acid mediated activation of the CYP7 gene.

DISCUSSION

The aim of this study was to identify and characterize upstream regions of the promoter involved in the transcriptional regulation of the CYP7 gene, in basal conditions and under the effect of physiological stimuli. **Figure 10** illustrates the nucleotide sequence and location of regulatory regions mapped in this study and also those elements identified previously. The proximal promoter region contains a repressor element in footprint A (FpA, Fig. 10) (D. Stroup, M. Crestani, and J. Y. L. Chiang, unpublished results), which is recognized by a previously identified protein named Direct Repeat Binding Protein (DRBP) (31). Two strong activator regions are located between -344 and -130 relative to the transcription start site and are linked to the repressor by a TC-rich hinge. Another repressor region is located upstream of the proximal promoter.

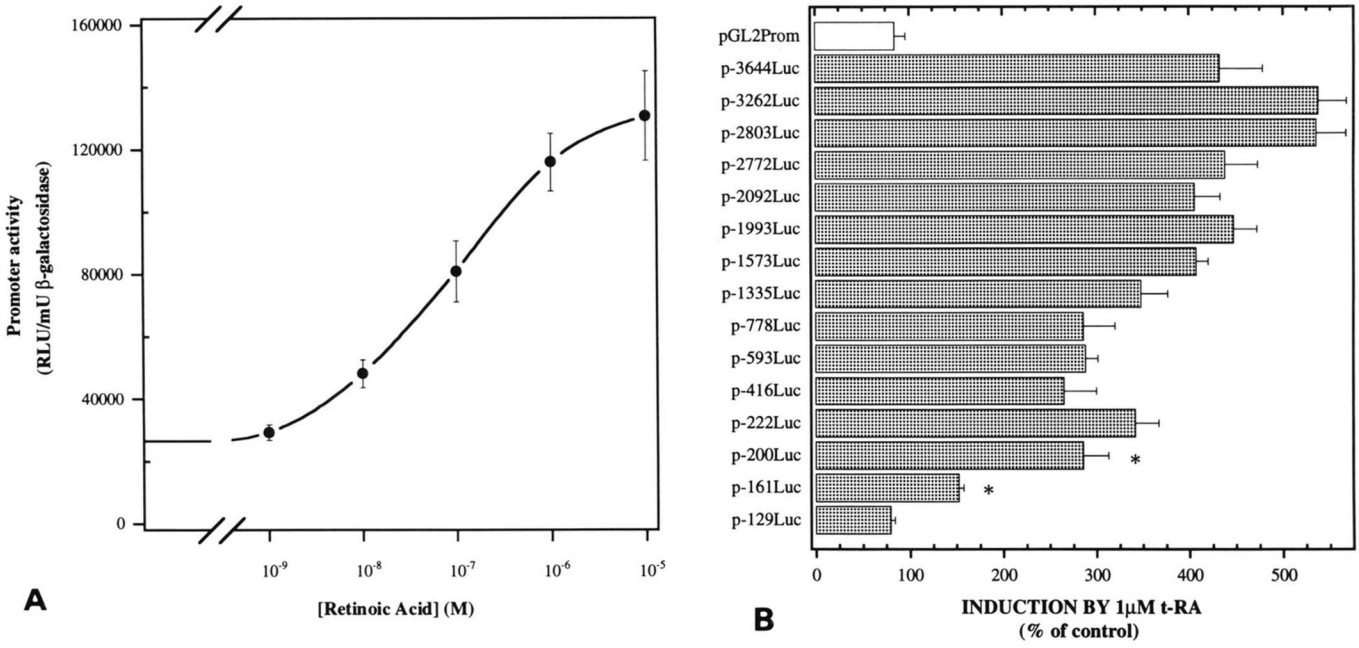

Fig. 9. Effect of all-*trans*-retinoic acid on the promoter activity of the CYP7 gene in HepG2 cells. Panel A, confluent HepG2 cultures were transfected with p-3644Luc and incubated 40 h in serum-free medium containing increasing concentrations of all-*trans*-retinoic acid. The results are expressed as the ratio of luciferase and β-galactosidase activity and represent the mean ± SD of three independent determinations. Panel B, rat CYP7/luciferase chimeric genes were transfected into confluent cultures of HepG2 cells. Transfected cells were incubated for 40 h in serum-free medium containing 1 μM all-*trans*-retinoic acid in ethanol or vehicle alone. Data illustrated are expressed as percentage induction relative to the control and are mean ± SD of six independent observations from two separate experiments; (*) indicates difference at $P < 0.0005$.

As in the case of other liver-specific genes (49, 50), it has been shown that the CYP7 expression is related to the stage of hepatocyte differentiation (32). Our data show that the expression of the CYP7 gene is increased in confluent HepG2 cultures. Previous investigations have demonstrated that this cell line exhibits a fetal-like phenotype when cultured at low density, whereas confluent HepG2 cultures display an adult-like phenotype (23, 24). Moreover, cholesterol 7α-hydroxylase mRNA level is developmentally regulated and rises dramatically at weaning in rats (51, 52). In this regard, it is interesting that the region downstream of nt -416 contains the information required for the developmental regulation and the maintenance of the expression of the CYP7 gene. Perhaps C/EBP and DBP, two liver-enriched transcription factors that are highly expressed in adult liver (53), may mediate, at least in part, this phenomenon. DBP and C/EBP can up-regulate the rat CYP7 promoter and the strongest binding sites are located in this region (8, 33) (Fig. 10). It is noteworthy that some authors have recently proposed an "establishment versus maintenance" model for C/EBP and DBP in the developmental regulation of other liver genes such as albumin (53) and phosphoenolpyruvate carboxykinase (PEPCK) (54). According to this model, C/EBP, which is expressed earlier in ontogeny, would trigger the expression of liver-specific genes and DBP, which is expressed later, would then maintain their expression in differentiated hepatocytes. Ramirez et al. (32) have identified an enhancer located 7 kb upstream the cap site that seems to be necessary for the tissue specific expression of the CYP7 gene in transgenic mice. Also, without this enhancer, the CYP7 promoter activity was very low and was not responsive to bile acids and cholesterol. It should be mentioned that these investigators used a mouse cell line and the CAT reporter gene, which is far less sensitive than the luciferase reporter gene used in this study. In our transient transfection system, the promoter constructs were quite active and could be regulated by bile acids (27) and hormones without the upstream enhancer. It is possible, however, that this enhancer cooperates with the proximal promoter region to direct the tissue-specific and developmental expression of the CYP7 gene.

Multiple hormones and second messengers regulate the CYP7 gene

Our data indicate that even though glucocorticoid hormones and PKA affect the transcription of the rat CYP7 gene through proximal and distal 5′-flanking sequences, the regulation of the CYP7 gene by hormones and other stimuli is mediated mainly through the proximal promoter region, downstream of nt -416 (Fig. 10). A striking result was that the effect of insulin was dominant over that of dexamethasone (Fig. 7). Twisk et al. (7)

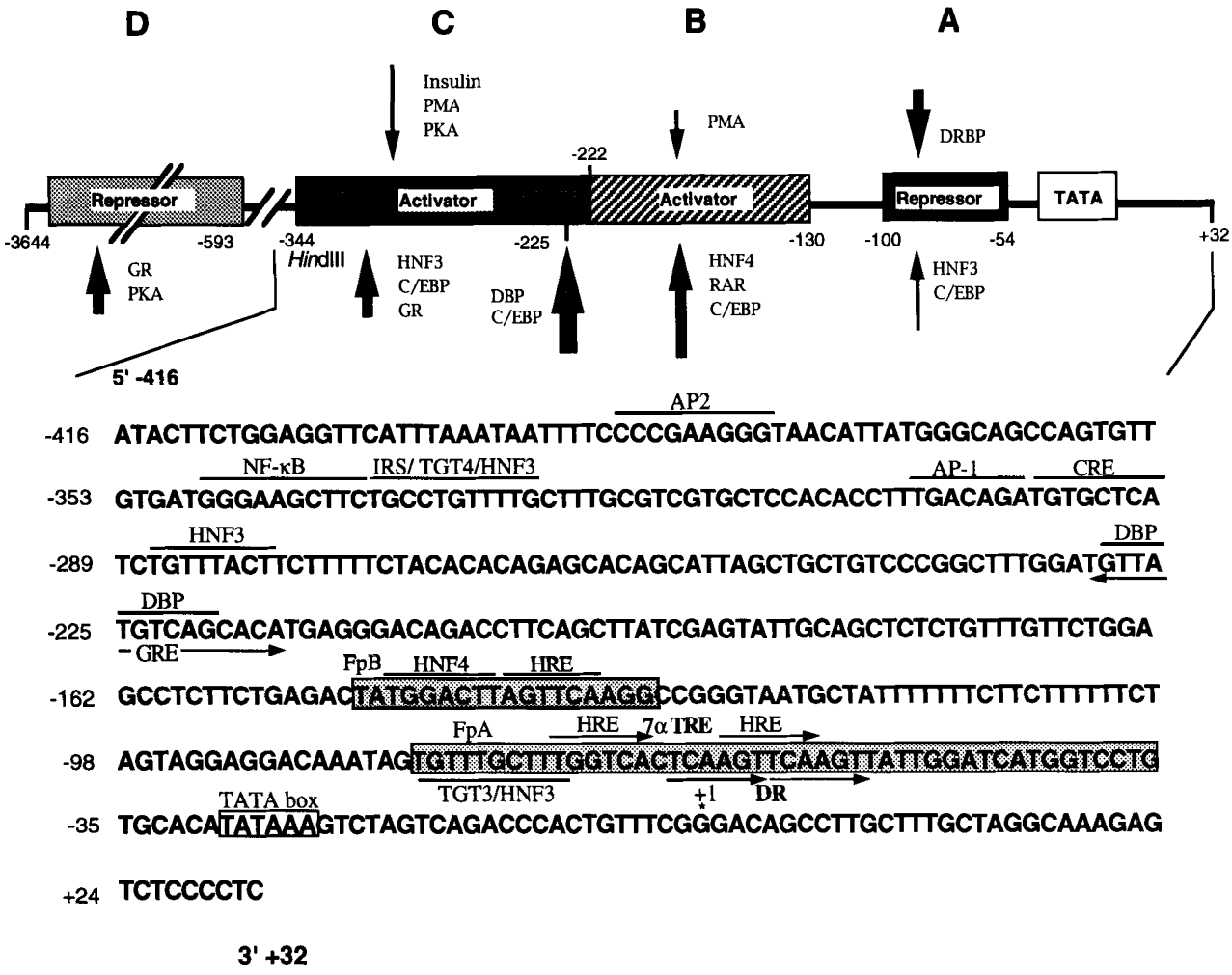

Fig. 10. The structure of the rat CYP7 promoter. Upper panel, a map of the 5'-flanking sequence has been divided in four domains (A, B, C, D). Within each domain the transcription factors and stimuli that affected the promoter activity are indicated. The direction of arrows indicates stimulation or inhibition of the promoter activity. Lower panel, sequence of the rat -416/+32 CYP7 promoter. Footprint A (FpA) and footprint B (FpB) are shaded. Putative consensus sequences for transcription factors and nuclear hormone receptors are marked. HRE, hormone response element; CRE, cAMP response element; IRS, insulin responsive sequence; DR, direct repeat; AP-1, activator protein-1; AP-2, activator protein-2; HNF3, hepatocyte nuclear factor 3; HNF4, hepatocyte nuclear factor 4; NF-κB, nuclear factor κB.

have also shown that the induction of cholesterol 7α-hydroxylase activity by dexamethasone was prevented by the simultaneous addition of insulin to rat hepatocyte cultures. A similar phenomenon has been described with another liver gene, PEPCK (55), which has a complex glucocorticoid response unit (GRU) containing two glucocorticoid receptor (GR) binding sites and two sequences bound by accessory factors (AF1 and AF2) (56). The AF2 element of the PEPCK gene is also an insulin responsive sequence (IRS) thus, explaining how it opposes dexamethasone-mediated stimulation (57). The responsive sequences for insulin repression and one of the putative GREs of the rat CYP7 promoter were mapped in the same region, between -344 and -222. Interestingly, we found an imperfect palindrome TGTTATgtaAGCACA located between nt -230 and -216 (Fig. 10), which resembles a typical glucocorticoid response element (GRE), and the sequence TGCCTGTTTTG located between nt -338 and -328 (Fig. 10), which is very similar to TGGTGTTTTG, the IRS of the PEPCK gene (57). It is possible that the CYP7 gene also contains a complex GRU composed of a GRE, IRS, and other sequences mediating the insulin effect.

We also tested the effect of thyroid hormones on the promoter activity of the CYP7 gene but, surprisingly, we did not detect any effect. Hoekman et al. (30) reported that a putative thyroid hormone response element may reside in the region upstream of nt -344, as the transcriptional activity of 3.6kb 5′-flanking sequence linked to the CAT reporter gene was higher in the presence of 1 μM T_4. To date, the reason for the discrepancy is not known and it might be explained with the different cell context (cultured rat hepatocytes vs. HepG2 cells). However, it has been shown that the expression of liver specific

genes is abolished in primary hepatocytes cultures unless hormones are added to serum-free medium (49, 58). Moreover, the expression of liver specific genes is decreased in hepatocytes cultured in the presence of serum (59) and in particular the mRNA levels and the transcription rate of CYP7 in HepG2 cells in complete medium is lower than in serum-free medium (26). We also observed that the promoter activity of CYP7/luciferase chimeric genes was higher in serum-free medium (data not shown). Therefore, the stimulation observed by these investigators could be explained by the absence of serum in the medium of cells transfected and then treated with T_4. As T_4 can stimulate CYP7 mRNA levels in HepG2 cells, it is possible that a thyroid hormone response (TRE) element may be located further upstream. In fact, this is the case of the chicken malic enzyme gene, which contains a region conferring thyroid hormone response between 3888 and 3688 bp upstream of the transcription start site (60).

Phorbol 12-myristate 13-acetate (PMA), a known activator of PKC, repressed the transcriptional activity of the CYP7 gene. PMA is known to affect the transcription of several genes through phosphorylation of transcription factors such as NF-κB, c-Jun, and c-Fos. The biphasic time course observed was probably caused by the well-known proteolytic inactivation of PKC after long-term exposures to PMA (45). We have mapped two positive PRS between nt -416 and -345 and downstream of nt -161, and two negative PRS between nt -344 and -222, and nt -200 to -161, respectively. AP-1- and AP-2-like sequences have been located in the proximal promoter, downstream of nt -416 (Fig. 10). As discussed above, the region between -344 and -222 includes the sequence TGCCTGTTTTG that resembles the IRS/PRS of the PEPCK gene (61). In addition, a putative NF-κB binding sequence GGGAAGCTTC is present between -348 and -339. It is noteworthy that the region between -344 and -222 was also responsive to bile acids (M. Crestani, D. Stroup, and J. Y. L. Chiang, unpublished results). It has been reported that bile acids can activate PKC in HepG2 and other cell lines (62–65) and hydrophobic bile acids are more potent PKC activators than hydrophilic bile acids (62, 63, 65). Therefore, it is plausible that bile acids may down-regulate the CYP7 gene transcription, at least in part, through the PKC signal transduction pathway as suggested by Stravitz et al. (65).

The sequence between nt -200 and -129 is strongly responsive to all-*trans*-retinoic acid, the hormonal form of vitamin A. There are evidences that retinoic acid plays an important role in lipid metabolism (46) and cholesterol homeostasis (47, 48). Retinoic acid had a stimulatory effect and, along with other signals, may be involved in the developmentally timed expression of the CYP7 gene. This is the first time retinoic acid has been shown to affect CYP7 expression. This implies that vitamin A derivatives may be important in the regulation of bile acid synthesis. The analysis of the DNA sequence between nt -200 and -129 disclosed only a hormone response element half site (AGTTCA) between -139 and -134, preceded by a HNF-4 binding site in a previously identified footprint B region (Fig. 10) (31). HNF-4 is a member of the steroid hormone receptor superfamily (66). Interestingly, in the same region we have also mapped a PRS. Moreover, a reverse AP-1-like site TGAATCA, located between -143 and -137, overlaps with the hormone response element mentioned above. As in the case of insulin and dexamethasone, it is significant that two signals, characterized by opposite effects, mapped in the same region of the CYP7 gene. This suggests that distinct classes of transcription factors can converge to the same regulatory sequences, a phenomenon commonly referred to as cross-coupling (67).

The effect of cAMP on the transcription of the CYP7 gene seems to be quite complicated. Insulin effect did not oppose that of cAMP, as the promoter activity of p-3644Luc in the presence of both agents was higher than that of insulin alone. This would indicate that insulin and cAMP effects are independent and mediated through distinct sequences. Both positive and negative elements responsive to PKA were identified in the CYP7 promoter (Fig. 10). A cAMP response element (CRE)-like sequence is located between -297 and -290 (TGTGCTCA, Fig. 10). It should be mentioned, however, that other authors have shown that cAMP rapidly decreased cholesterol 7α-hydroxylase mRNA levels in primary rat hepatocyte cultures (6). The reason for this discrepancy may be also due to the different cellular environment discussed above. Further studies will be necessary to understand the mechanisms underlying cAMP effect on CYP7 expression in rats and humans.

In this paper we have described the regulation of the CYP7 gene transcription by several physiological stimuli. Our data seem to correlate well with previous observations from other laboratories. However, the discrepancy in the response to thyroid hormones and cAMP underscores the importance to carefully evaluate the cellular environment (e.g., HepG2 cells vs. hepatocytes) and the effect of the species (e.g., man vs. rat) before extrapolating data to in vivo condition. Perhaps, the most intriguing finding of this study is that most effects of physiological stimuli are mediated through response elements located in the region downstream of nt -416. Especially, between -344 and -222, there are response elements for, insulin, phorbol esters, cAMP, and glucocorticoid hormones. Competition for binding to overlapping consensus sequences and interactions between these transcription factors may determine the level of CYP7 gene expression under various physiological con-

ditions. The clustering of the sequences that mediate the physiological stimuli in a relatively short region of the CYP7 promoter must be significant in the cross-talk of different signal transduction pathways. It is possible that this represents a means to finely tune the expression of the gene in response to changes of external conditions. This study paves the way for future experiments to identify the response elements and the transcription factors involved in the CYP7 gene regulation.

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