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Down-regulation of the rat hepatic sterol 27-hydroxylase gene by bile acids in transfected primary hepatocytes: possible role of hepatic nuclear factor 1α

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Abstract

In vitro and in vivo studies have shown that the sterol 27-hydroxylase (CYP27) gene is transcriptionally repressed by hydrophobic bile acids. The molecular mechanism(s) of repression of CYP27 by bile acids is unknown. To identify the bile acid responsive element (BARE) and transcription factor(s) that mediate the repression of CYP27 by bile acids, constructs of the CYP27 5′-flanking DNA were linked to either the CAT or luciferase reporter gene and transiently transfected into primary rat hepatocytes. Taurocholate (TCA), taurodeoxycholate (TDCA) and taurochenodeoxycholate (TCDCA) significantly reduced CAT activities of the −840/+23, −329/+23, and −195/+23 mCAT constructs. A −76/+23 construct showed no regulation by bile acids. When a DNA fragment (−110/−86) from this region was cloned in front of an SV 40 promoter it showed down-regulation by TDCA. 'Super'-electrophoretic mobility shift assays (EMSA) indicated that both HNF1α and C/EBP bind to the −110 to −86 bp DNA fragment. Recombinant rat HNF1α and C/EBPα competitively bound to this DNA fragment. 'Super'-EMSA showed that TDCA addition to hepatocytes in culture decreased HNF1α, but not C/EBP, binding to the −110/−86 bp DNA fragment. A four base pair substitution mutation (−103 to −99) in this sequence eliminated TCA and TDCA regulation of the (−840/+23) construct. The substitution mutation also eliminated (>95%) HNF1α, but not C/EBP, binding to this DNA fragment. We conclude that bile acids repress CYP27 transcription through a putative BARE located between −110 and −86 bp of the CYP27 promoter. The data suggest that bile acids repress CYP27 transcriptional activity by decreasing HNF1α binding to the CYP27 promoter.

1. Introduction

The biosynthesis of bile acids represents a major pathway for cholesterol elimination from the body [1], accounting for approximately 50% of daily removal. Bile acid biosynthesis can be initiated in the liver by either microsomal cholesterol 7α-hydroxylase (CYP7) (E.C. 1.14.13.17) or mitochondrial sterol 27-hydroxylase (CYP27) (E.C. 1.14.13.15). The initiation of cholesterol degradation to bile acids by CYP7 and CYP27 are referred to as the 'neutral' and 'acidic' pathways of bile acid biosynthesis, respectively [1,2]. Recent evidence from both in vivo [3] and in vitro [4,5] models suggest that the 'acidic' pathway of bile acid biosynthesis may contribute as much as 50% of total bile acid output.

CYP7 is a liver specific enzyme and is rate limiting in the 'neutral' pathway of bile acid biosynthesis [6].

Abbreviations: CYP27: sterol 27-hydroxylase; CYP7: cholesterol 7α-hydroxylase; HNF1: hepatocyte nuclear factor 1; C/EBP: CAAT/enhancer binding protein; EMSA: electrophoretic mobility shift assay; CAT: chloramphenicol acetyl transferase; TCA: taurocholic acid; TDCA: taurodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; BARE: bile acid responsive element; LUC: Luciferase gene.

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The gene encoding cholesterol 7α-hydroxylase has been shown to be regulated by steroid and thyroid hormones [7,8], cholesterol [9,10], and hydrophobic bile acids [11]. Chiang and co-workers [12,13] have mapped two putative BAREs in the promoter of rat cholesterol 7α-hydroxylase. However, the transcription factor(s) interacting with these BAREs and how they are regulated have not been elucidated.

CYP27 is expressed in the liver as well as extra hepatic tissues [14]. This enzyme has been shown to utilize a number of sterols as substrates including cholesterol [15,16], bile acid intermediates [16] and vitamin D_3 [17]. The 'acidic' pathway may become the major route to chenodeoxycholic acids in patients with liver diseases, in whom the activity of CYP7 is low [3,16]. Moreover, recent data suggest that CYP27 may play an important role in the regulation of cellular cholesterol biosynthesis by generating 'oxysterols' which down-regulate HMG-CoA-reductase [18]. This enzyme may enhance cholesterol efflux from vascular endothelium and hence play an important role in reverse cholesterol transport [19]. Finally, genetic analysis of patients with cerebrotendinous xanthomatosis (CTX), have shown mutations in the CYP27 gene [20,21].

The c-DNA's encoding CYP27 have been cloned from rabbit [22], human [23] and rat [17]. In the rat, two mRNAs species of 2 and 2.3 kilobase (kb) have been observed [24]. In rat liver the 2 kb mRNA is the predominant species [16], while in the kidney the 2.3 kb species is the major form [24]. The rat gene has recently been cloned, sequenced, and the promoter responsible for generating the 2.0 kb mRNA has been characterized in regard to the transcription start site [24]. Hydrophobic, but not hydrophilic, bile acids have been shown to transcriptionally down regulate both hepatic CYP27 [25] and CYP7 [26], but the mechanism(s) of regulation is unknown.

In the present study, we mapped and characterized a putative bile acid responsive element (BARE) in the rat CYP27 proximal promoter. The data suggest that HNF1α plays an important role in bile acid regulation of the hepatic CYP27 gene.

2. Materials and methods

2.1. Materials

Bile acids were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant protein G Sepharose was from Gibco/BRL (Gaithersburg, MD). The (α-^{32}P)-dCTP and (γ-^{32}P)-ATP were supplied by Dupont NEN (Boston, MA). Anti-C/EBP (Δ198) polyclonal IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-HNF1 monoclonal antibody (RAD1) was a kind gift from Dr. GU Ryffel (Universitätsklinikum Essen, Essen, Germany) [27]. Restriction endonucleases were obtained from Gibco/BRL or New England Biolabs (Beverly, MA). All other reagents were of molecular biological grade and obtained from Sigma unless otherwise specified. The long CYP27 gene promoter −840/+23 mCAT and various 5′-deletion constructs with downstream ATG code mutated (mCAT) to allow proper translation were generated as described previously [24].

2.2. Animals

Sprague-Dawley rats weighing between 250–257 g were housed under controlled lighting as described previously [25]. Age- and weight-matched groups were fed regular powdered diet (Ralston Purina, St Louis, MO) or diet supplemented with 5% cholestyramine (CalBiochem, San Diego, CA), 1% sodium cholate and 0.25% deoxycholate acids (Sigma) for 14 days. Rats were housed under a reverse light cycle and sacrificed at 9.00 am. Equal portions of liver or other organs from three rats in each group were pooled for isolation of nuclear protein.

2.3. Primary culture of rat hepatocytes and cell viability examination

The isolation, plating and maintenance of hepatocytes from male Sprague-Dawley rats were performed essentially as described previously [5]. For transient transfection, hepatocytes (8×10^5) were plated in 1 ml 10% fetal calf serum in Williams' E medium containing dexamethasone (0.1 μM), insulin (0.25 u/ml) and penicillin (100 u/ml) on 35 mm Primaria plates (Falcon, Lincoln Park, NJ). For extracting nuclear protein, hepatocytes (3×10^6) were plated in 3 ml 10% fetal calf serum without insulin on 60 mm culture plates coated with rat tail collagen. After culture with various concentrations of TDCA, cell viability was assessed by measurement of 3(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) reduction. The assay was performed essentially as described by De Vries, et al. [28]. In short, cells on 35 mm plates (8×10^5 cells) in 1 ml of medium were incubated with bile acids. At the end of incubation, 25 μl of MTT solution(1 mg MT T/mL in PBS) was added to each plate for 30 min. The culture medium was aspirated, and 1.5 ml of 100% dimethyl sulphoxide was added to solubilize the formazan crystals. Absorbance at 545 nm was measured immediately.

2.4. Transient transfection and reporter gene construct assays

The various CAT plasmid constructs and PGL3-

LUC plasmid DNA was transfected into primary hepatocytes by lipofection [29]. Briefly, 4–6 h after plating, hepatocytes were washed once with 3 ml Williams' E medium. 10 μg of Lipofectin (Gibco/BRL, Gaithersburg, MD) in 100 μl William's E medium was mixed well in polypropylene microcentrifuge tubes with 3–4 μg of DNA, which had previously been diluted in 100 μl William's E medium. The mixture was incubated for 30 min at room temperature. Williams' E medium (800 μl) containing 0.1 μM dexamethasone was added to the DNA-lipid mixture, then dropped onto a culture plate. After 16 h in a 5% CO_2 incubator maintained at 37°C, the medium was aspirated and cells were rinsed twice with Williams' E medium. Williams' E medium containing 0.1 μM dexamethasone and various bile acids were added and incubated for indicated time periods. After incubation, cells were washed once with phosphate buffered saline (PBS) and 150 μl lysis buffer (Promega) was added to the plates and incubated for 15 min. Cells were then scraped from the plates. Protein concentration was determined using Bradford protein assay with reagent from Bio-Rad (Richmond, CA).

CAT assays were performed as described [30]. All test samples received equal amounts of protein, which varied from 25 to 30 μg. [^{14}C]-Chloramphenicol (0.5 μCi) (Dupont NEN) and 75 μg acetyl-CoA (Sigma) were added to reaction mixture tubes and incubated at 37°C for 30 min. Enzymatic products were extracted twice by ethyl-acetate and resolved on silica gel thin layer chromatography plates (TLC, JT Baker, Phillipsburg, NJ). CAT activity was quantitated by eluting spots from TLC plates followed by scintillation counting. CAT activities were normalized for transfection efficiency to β-galactosidase (β-Gal.). β-Gal. activity was quantitated by measuring the optical density of the reaction at 420 nm. The absorbance represents the relative β-Gal. activity of cleavage of o-nitrophenol β-D-galactopyranoside (ONPG). Luciferase activities were determined with the Luciferase assay kit (Promega) according to the manufacturer's instructions using a Berthold luminometer (LB9501, Berthold Systems Inc., Pittsburgh, PA).

2.5. Nuclear extracts

Nuclei were isolated using a modification of the method described by Cereghini, et al. [30]. Briefly, rat tissues were minced on ice and washed in buffer containing 25 mM Tris–HCl (pH 7.0), 137 mM NaCl and 5 mM KCl. Cells were disrupted using a Dounce homogenizer in buffer containing 10 mM Hepes (pH 7.6), 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose, 1 mM dithiothreitol (DTT), 25 μg/ml leupeptin and 0.3 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were layered over a cushion of the same buffer with 1.0 M sucrose and pelleted at 80,000 × g for 60 min. Nuclear protein was extracted by treatment with 10 mM Hepes (pH 7.6), 10 mM EDTA, 3 mM $MgCl_2$, 20 μg/ml leupeptin, 0.1 mM PMSF and lysed in 0.4 M ammonium sulfate. Chromatin was removed by centrifugation at 50,000 × g for 30 min. Protein was precipitated from the supernatant by addition of 0.33 g of solid ammonium sulfate per 1 ml buffer. Pellets were dissolved in 25 mM Hepes (pH 7.5), 0.1 mM EDTA, 0.5 mM DTT, 40 mM KCl, 5% glycerol and 20 μg/ml leupeptin and dialyzed against the same buffer overnight at 4°C.

Nuclear protein was isolated from primary cultures of rat hepatocytes according to the modified procedure described by Schreiber, et al. [31]. Briefly, 3×10^6 cells were washed twice with cold PBS (6 ml). Then, 400 μl cold buffer A (10 mM Hepes, (pH 7.9), 10 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate ($NaVO_4$), 0.5 mM PMSF, 10 mM NaF, 1 μM pepstatin A, 50 μg/ml each of leupeptin and aprotinin) was added to plates. Plates were kept on ice for 10 min. Cells were scraped from plates and allowed to swell on ice for another 10 min. Then, 20 μl of a 10% solution of Nonidet NP-40 (Sigma) was added and the tube was vortexed for 10 s. Homogenates were centrifuged for 30 s at 4°C. The nuclear pellet was resuspended in 50 μl ice cold buffer B (20 mM Hepes, (pH 7.4), 0.4 M NaCl, 1 mM $NaVO_4$, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 25 μg each of leupeptin and aprotinin, shaken vigorously at 4°C for 15–20 min and centrifuged for 5 min. The supernatants were frozen in aliquots at −70°C.

2.6. Electrophoretic Mobility Shift Assays (EMSA)

Promoter fragments from −329 to −195 and −195 to −76 were obtained by digesting the −840/+23 mCAT constructs with *Pst*I/*Sac*I and *Sac*I/*Xho*II, respectively. The DNA fragments were isolated by resolving on an 8% polyacrylamide gel. Gel pieces containing the probe were cut out and eluted with high salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M NaCl). The salt was removed by using a QIA quick nucleotide removal kit (QIAGEN Inc., Chatsworth, CA). Oligonucleotides corresponding to the −110 to −86 region (containing putative HNF1 and C/EBP elements) were synthesized by the Nucleic Acid Core Facility at the Medical College of Virginia Campus of Virginia Commonwealth University. DNA fragments were labeled with (γ-^{32}P)-ATP (3000 Ci/mmol) using T4 polynucleotide kinase (USB, Cleveland, OH) or labeled by filling with Klenow fragment of DNA polymerase. Oligonucleotides blunted with nonlabeled dNTPs were used as competitors in

EMSA. Binding reactions were carried out for 20 min at room temperature in 12 mM Hepes (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 3 μg of poly(dI-dC).poly(dI-dC), 3–5 μg of nuclear extracts, 15% glycerol, 20,000 cpm of DNA probe in a final volume of 25 μl. For 'super gel' shift assays, 1–2 μl of appropriate antibody was incubated with nuclear protein for 30 min before the addition of the labeled probe. The incubation period was extended for an additional 15 min at room temperature. Binding bands were separated on 4% nondenaturing polyacrylamide gel in 0.25 × TBE. Quantification of super gel shift bands was performed by using laser densitometry.

2.7. Mutagenesis

The four base pair substitution mutagenesis was performed by using the Quickchange Site-Directed Mutagenesis kit (Stratagene), according to the supplier's instructions using the −840/+23 CAT construct. The following oligonucleotide served as polymerase chain reaction (PCR) primers (mutated bases are shown in boldface: HEMY, −120/−80 GCATTTGTGTGTTCGA**TCGT**TCCTGATTGGAAGGGGTTATG and its complementary strand. After PCR, the resulting mutagenized plasmids were transformed into *E. coli* XL1-blue supercompetent cells and plasmid DNAs were sequenced. The plasmids containing the correct mutation were used for the transfection of primary hepatocytes.

Oligonucleotides corresponding to the wild type −110 to −86 region were synthesized with restriction sites at two ends and cloned to the upstream of SV40 promoter in the PGL3-Promoter plasmid (Promega, Madison, WI).

2.8. Immunoprecipitation and immunoblotting of nuclear protein

Nuclear lysate (0.25 ml) was precleaned by incubation with protein G Sepharose prior to the addition of 0.5 μl of anti-HNF1 monoclonal antibody (RAD1) to the resulting supernatant. The samples were incubated at room temperature overnight with continuous mixing. Protein G-Sepharose was added, and incubation continued for an additional 60 min with mixing. Immune complexes were washed three times with HNTG buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 5% Triton-X 100, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 100 μM $NaVO_4$, 10 μg/ml each of aprotinin and leupeptin, 1 mM PMSF). Immunoprecipitates were eluted by boiling in 25 μl SDS sample buffer containing 10% 2-mercaptoethanol. Samples were resolved by SDS-PAGE using a 12% gel. The resolved proteins were electrophoretically transferred to Immunolite membrane (Bio-Rad, Richmond, CA). Membranes were blocked by an 18 h incubation at room temperature with 5% nonfat dry milk in Tris-buffered saline (pH 7.4). Following extensive washing, the membranes were incubated for 3 h at 25°C with anti-HNF1 (1:1000) or anti-C/EBP (0.1 μg/ml) [27]. Goat anti-mouse or anti-rabbit Ig G, conjugated to alkaline phosphatase, were used as secondary antibodies (1:3000, Bio-Rad). Protein was detected using a chemiluminescent reaction, followed by exposure to X-ray film.

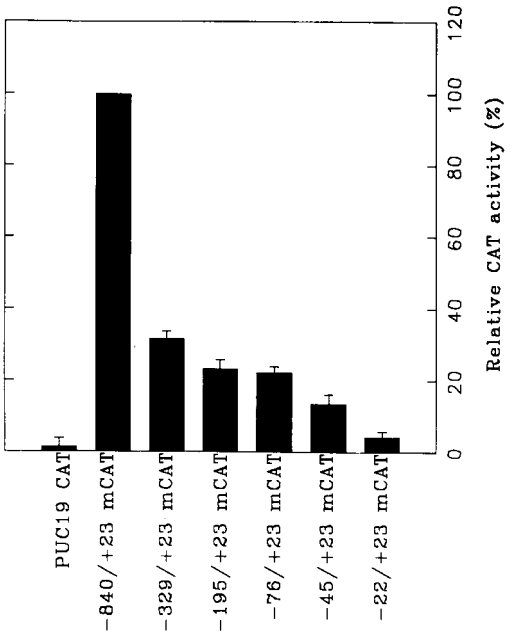

Fig. 1. Analysis of transcriptional activities of different CYP27 promoter constructs in primary cultures of rat hepatocytes. The intact −840/+23 mCAT and various 5′-deleted mCAT constructs were transfected into primary hepatocytes by lipofection and incubated for 18 h. CAT assays were performed as described in Section 2. The relative CAT activities (the CAT activity of −840/+23 mCAT was regarded as 100%) are from three independent experiments. The specific activity of the −840/+23 mCAT construct was approximately 80 nmol/h/mg protein.

2.9. In vitro coupled transcription/translation of HNF1 α and C/EBP α

One μg of Rc/CMV plasmid encoding c-DNA of rat HNF1 α (kindly provided by Dr. G.U. Ryffel) and PSP72 plasmid encoding c-DNA of rat C/EBP α (kindly provided by Dr. Steven L McKnight) were individually added into a rabbit reticulocyte lysate system (Promega, Madison, WI) using T7 polymerase to initiate transcription. When simultaneously expressing HNF1 α and C/EBP α, 0.5 μg of each plasmid DNA was added to reaction mixture. Reactions were incubated at 30°C for 60 min. To confirm the translation, [^{35}S]-methionine labeled protein was resolved in SDS-PAGE and autoradiography.

2.10. Statistical analysis

All experiments were replicated three times unless otherwise noted. Results are expressed as mean ± SD. When appropriate, statistically significant differences between treatment groups were tested by the means of student's *t*-tests using Sigma Plot Software (San Rafael, CA).

3. Results

3.1. Expression of CYP27/reporter chimeric gene constructs in primary cultured rat hepatocytes

In order to study CYP27 gene expression and regulation by bile acids, several CYP27 promoter/mCAT chimeric gene constructs containing proximal 5′-flanking regions (−840/+23, −329/+23, −195/+23, −76/+23, −45/+23, −22/+23) were transiently transfected into rat primary hepatocytes in culture. The −840/+23 mCAT construct yielded the highest activity, which was regarded as 100% (Fig. 1). The relative CAT activity of the −329/+23 mCAT construct was ~36%, indicating that *cis*-element(s) between −840 and −329 may be required to maintain high levels of CYP27 transcriptional activity in rat hepatocytes in culture. The mCAT constructs, −195/+23 and −76/+23, gave declining levels of CAT activities (24% and 22%, respectively) as compared to the −840/+23 mCAT construct. Together these results suggest that the activity of the −76/+23 mCAT may represent basal promoter activity, which may be up-regulated by various elements located upstream. The −45/+23 mCAT construct yielded only marginal CAT activity (~10%) suggesting that the TTTAAA motif alone may not be enough to maintain the basal CYP27 transcriptional activity in rat hepatocytes [24,33]. Moreover, the data also show that the −22/+23 mCAT construct, which deletes the TATA motif, yields little transcriptional activity (<5%, P=0.09 compared to background) indicating the importance of this motif for promoter activity. Additionally, the pCAT basic plasmid without any insert (pUC19 CAT) failed to obtain significant transcription activity (<1.5%) suggesting the specificity of the CAT reporter gene assays.

3.2. Effect of bile acids on the transcriptional activity of CYP27 gene promoter/CAT constructs

To assess the effect of bile acids on CYP27 transcription, the time course and concentration-dependence of TDCA treatments on CAT activity expressed by −840/+23 mCAT were determined. CAT activity declined 43 ± 7% after 24 h of incubation with 50 μM TDCA (data not shown). Repression of CAT activity

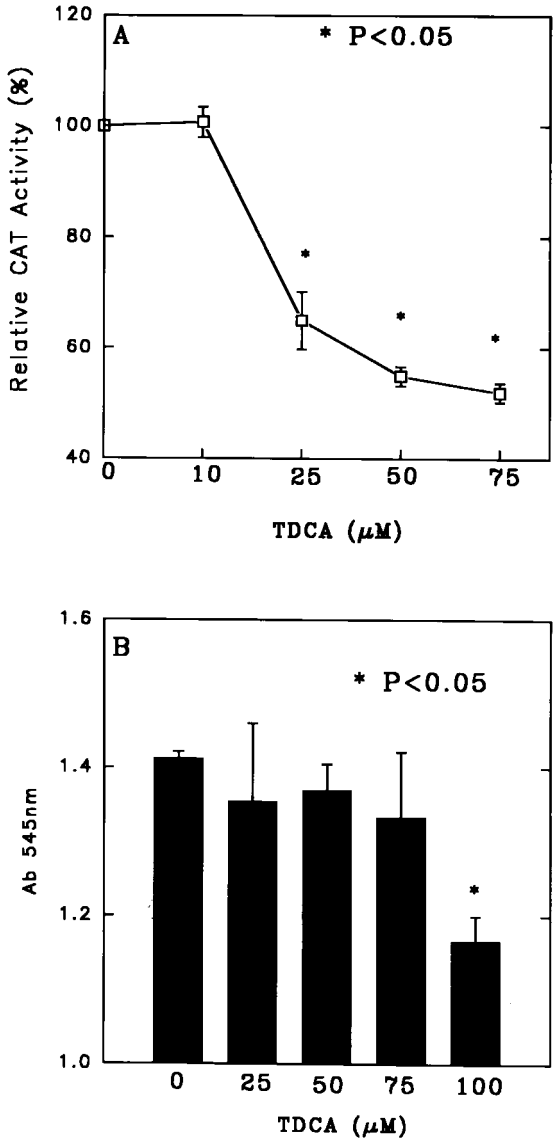

Fig. 2. Concentration-dependent repression of CAT activity of the CYP27 −842/+23 mCAT construct by TDCA. The intact −840/+23 mCAT construct was transfected into primary cultures of hepatocytes and CAT assays were performed as described in Section 2. The CAT activity values and standard deviation are based on three independent experiments. *Significant difference ($P < 0.05$) as compared with controls. (A) Rat hepatocytes were incubated with various concentrations of TDCA (0–75 μM) for 24 h, and cells assayed for CAT activity. (B) Hepatocyte viability was assessed by MTT assay. After 24 h exposure to TDCA, cells were harvested and assayed for MTT reduction by mitochondrial dehydrogenase as described in Section 2. Data are expressed as the means and standard deviations from three independent experiments. *Significant difference ($P < 0.05$) as compared with controls.

was a concentration-dependent process (Fig. 2A). Maximal reduction in CAT activity was reached at 50 μM TDCA (−44 ± 3%), a concentration of bile acid within the physiological range of bile acids in the portal blood of rats. In addition, MTT measurements

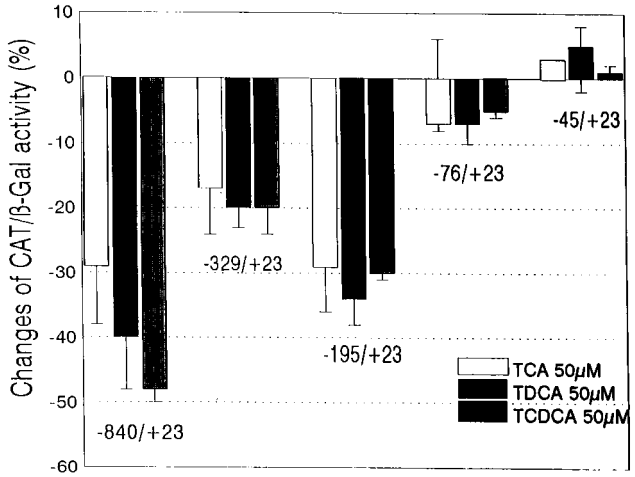

Fig. 3. Effects of different bile acids on transcriptional activities of different CYP27 gene promoter constructs in primary cultures of hepatocytes. CAT constructs were transiently transfected into rat primary hepatocyte cultures. Either 50 μM taurocholate (TCA), taurodeoxycholate (TDCA) or taurochenodeoxycholate (TCDCA) was added to culture medium and incubated for 24 h. CAT activities were determined in cell extracts and each construct was expressed as percentage of change of activity in transfected cells without bile acid treatment. Data are expressed as the means and standard deviations from three independent experiments. There was a significant difference ($P \leq 0.05$) between control and bile acid treated cells for the −840/+23, −329/+23, and −195/+23 constructs.

showed that TDCA did not have an adverse effect on cell viability at concentrations up to 50 μM (Fig. 2B).

To determine the location of a bile acid responsive element (BARE) in the proximal CYP27 promoter, five CYP27 promoter/mCAT constructs were transiently transfected into primary rat hepatocytes, and the effect of three bile acids (50 μM each of TCA, TDCA, TCDCA) on transcriptional activity determined. In this series of experiments, CAT activities were normalized for transfection efficiency using a plasmid encoding β-galactosidase. The data in Fig. 3 shows that TCA, TDCA and TCDCA reduce the CAT activity of −329/+23 and −195/+23 mCAT constructs from 16–35% ($P < 0.05$). In contrast, when these bile acids were added to the medium of hepatocytes transfected with −76/+23, and −45/+23 mCAT constructs, the CAT activity was not significantly changed. Therefore, these data suggest that the BARE is likely located in the region between −195 and −76 base pairs.

3.3. Effect of bile acid treatment on the DNA-protein interaction

In order to study the DNA-protein interaction in the *Sac*1/*Xho*II DNA fragment (−195 to −76), EMSA experiments were performed. A broad shifted band was observed when rat liver nuclear extract was mixed with labeled DNA probe (Fig. 4A). Nuclear extracts from kidney, spleen or brain (5 μg) showed no shifted band. Liver nuclear extract prepared from rats fed 0.25% deoxycholic acid (DCA) or 1% sodium cholate (CA) showed an increased shifted band intensity (Fig. 4A). In contrast, liver nuclear extracts from rats fed 5% cholestyramine (CSA) showed a decreased shifted band compared to the controls (CTRL). This shifted band could be competed out with a large excess (50×) of unlabeled oligonucleotide containing the binding site for HNF1-C/EBP (−110/−86). When nuclear extracts prepared from primary cultures of hepatocytes were used in EMSA, two shifted bands were observed using the Sac1/Xho II DNA fragment (Fig. 4B). Treatment of primary hepatocytes with TDCA increased the faster migrating shifted band (Fig. 4 B), but had little effect on the intensity of the slower migrating band. These shifted bands were lost or were markedly decreased when 10 or 25-fold unlabeled HNF1-C/EBP element oligonucleotide (−110/−86) was added to the binding assays (Fig. 4B). These results indicated that the oligonucleotide sequence containing the HNF1-C/EBP element might be a specific binding site for hepatic nuclear protein [27,32–36].

3.4. Substitution mutation alters bile acid regulation

A four base pair substitution mutation was synthesized and inserted (−103 to −99) into the −840/+23 promoter construct as described in Section 2. The mutated promoter construct was transformed into primary hepatocytes and the effects of TDCA and TCA on regulation determined. This mutation decreased transcriptional activity by ~50% and completely eliminated TDCA and TCA regulation (Fig. 5). These data suggest that the BARE lies within the −110/−86 gene fragment and indicates that no other BAREs are within the −840/+23 sequence.

3.5. Cloning of the putative BARE upstream of a heterologous promoter

Next, the −110/−86 DNA fragment was cloned upstream of the pGL3-SV40 promoter and transfected into primary hepatocytes. The insertion of this element increased overall transcriptional activity about 2.5-fold. There was approximately a 40% decrease in transcriptional activity of this promoter construct following the addition of 50 μM TDCA to the culture medium. The pGL3-SV40 promoter alone showed no down-regulation by TDCA under the same experimental conditions (Fig. 6).

3.6. Binding of HNF1α and C/EBP to putative BARE

To characterize the nature of nuclear protein bound to the putative HNF1-C/EBP element (−110/−86),

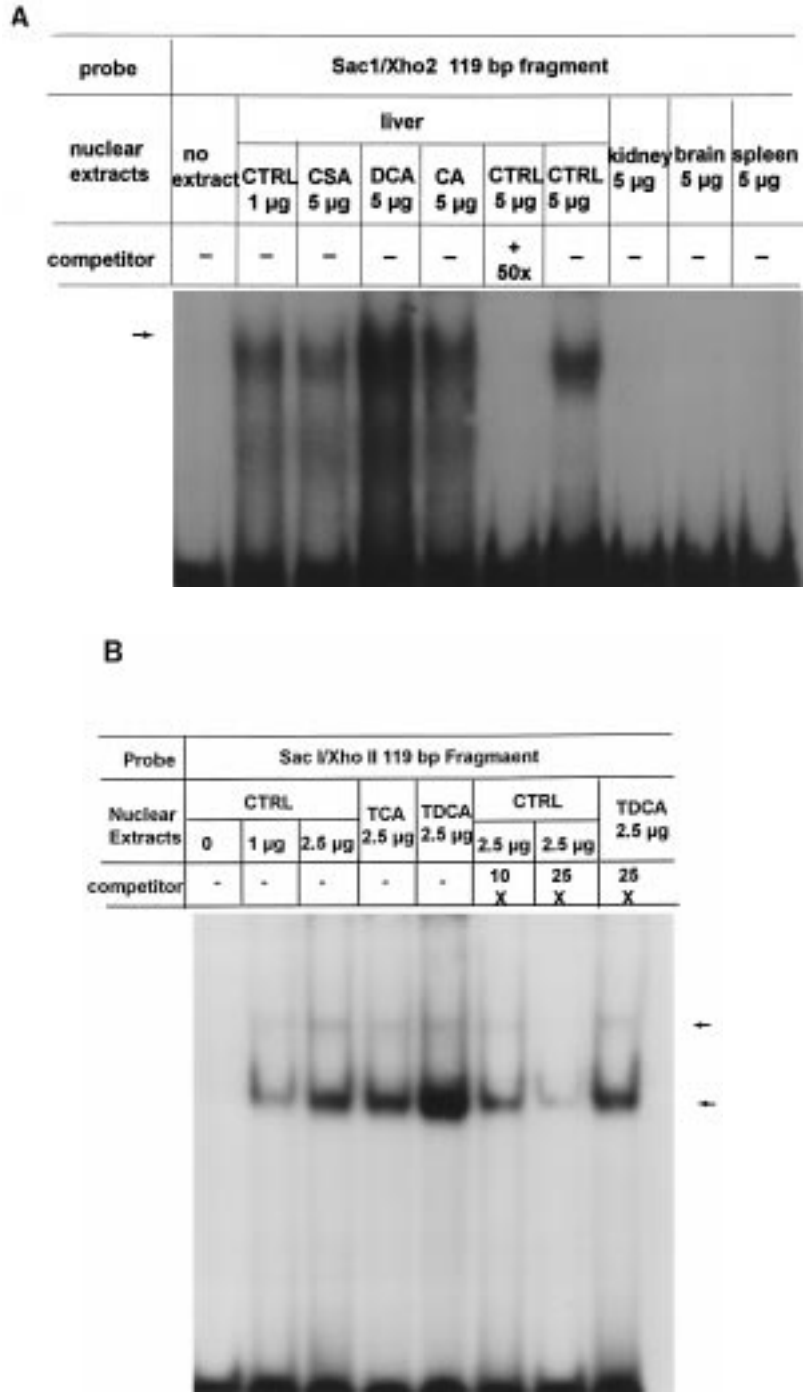

Fig. 4. Effect of bile acid treatment on hepatocyte nuclear protein binding to the *Sac*I/*Xho*II DNA fragment. (A) Liver nuclear extracts were isolated from rats fed a control diet (CTRL) or diets supplemented with 0.25% deoxycholic acid (DCA), 1% cholic acid (CA), or 5% cholestyramine (CSA). Nuclear extracts from other organs were isolated from rats fed with a control diet. Fifty-fold unlabeled oligonucleotide (−110/−86) containing the HNF1-C/EBP binding sites was used as competitor in the sixth lane from left (panel A). (B) Nuclear extracts were isolated from primary hepatocyte cultures incubated with 50 μM TCA or TDCA. Unlabeled oligonucleotide (10 or 25 fold excess) containing the HNF-C/EBP binding site was used as competitor where indicated.

'super' electrophoretic mobility shift assays were next performed. Specific antibodies against HNF1α (monoclonal) or C/EBP(polyclonal) were used. As shown in Fig. 7A, 'super'-EMSA demonstrated that HNF1α was bound to this oligonucleotide. Most notably, 'super'-EMSA with nuclear extract prepared from hepatocytes treated with TDCA or TCA revealed that the binding of HNF1α to DNA was consistently reduced despite

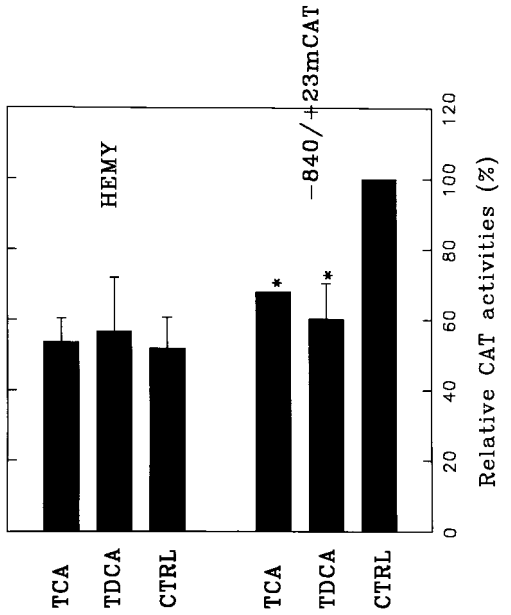

Fig. 5. Effect of a four base pair substitution mutation on regulation sterol 27-hydroxylase promoter activity by bile acids. The four base pair substitution mutation was generated and introduced into the homologous promoter (−840 to +23) as described in Section 2. The control and mutated promoter constructs were individually transfected into primary rat hepatocytes, incubated 24 h in the presence of either 50 μM TDCA or TCA, cells harvested, and assayed for CAT activity as described in Section 2. The mean ± SD from three experiments performed in triplicate and normalized to the wild-type (−840/+23) CAT activity are shown. $^{*}P < 0.05$ compared to untreated control.

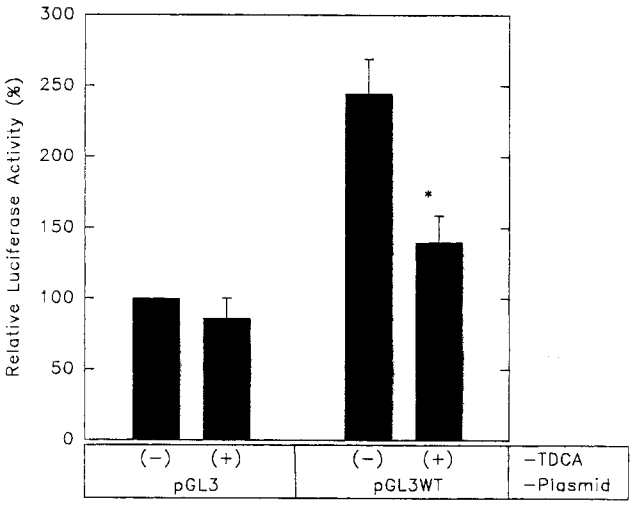

Fig. 6. Cloning of the putative bile acid responsive element upstream of a heterologous promoter. Primary hepatocytes were transfected with a pGL3 plasmid containing a SV40 promoter or pGL3-wt plasmid containing the putative bile acid responsive element (−110 to −86). An oligonucleotide containing the bile acid responsive element sequence was synthesized and cloned upstream of the SV-40 promoter in pGL3 as described in Section 2. After transfection, 50 μM TDCA (+) was added to the culture medium for 6 h before harvesting. Relative luciferase activities are shown as percent pGL3-promoter plasmid activity with (+) or without TDCA treatment (100%). The data are presented as the mean ± SD for three independent experiments performed in triplicate. $^{*}P < 0.05$ between the TDCA treatment and control.

no change in nuclear HNF1α concentration (Fig. 7B). A 'super'-EMSA using polyclonal antibody against rat C/EBP showed that this transcription factor was also bound to this site (Fig. 7C). However, in contrast to HNF1α binding, treatment of hepatocytes with TDCA (50 μM), did not change the apparent binding of C/EBP (Fig. 7C) or the relative nuclear content of C/EBP (Fig. 7D). These data suggest that HNF1α and C/EBP are normally able to bind to a common site in the proximal promoter of the rat CYP27 gene, and that treatment of primary hepatocytes with bile acids results in differential loss of HNF1α, but not C/EBP, binding. The loss of HNF1α is not due to its decreased nuclear expression in response to bile acid treatment (Fig. 7B).

To further investigate the DNA sequence required for nuclear protein binding, we analyzed the DNA protein interaction using the four base pair substitution mutation probe. The data in Fig. 8A show that the substitution mutation (mut) decreases (>95%) HNF1α binding, but not C/EBP, to this probe. These data strongly suggest that the slower migrating (upper) band is the HNF1α:DNA complex and the faster migrating band (lower) is the C/EBP:DNA complex. The upper and lower bands are shifted by HNF1α and C/EBP antibody, respectively (Fig. 8A). This is better illustrated in Fig. 8B where the gel was allowed to run longer in order to further separate the upper and lower migrating DNA:protein complexes. There was a marked decrease in HNF1α binding to the putative BARE using nuclear extracts prepared from hepatocytes treated with TDCA (compare wt and TDCA lanes). There was no significant effect of TDCA treatment on C/EBP binding and the C/EBP antibody shifted greater than 95% of the protein:DNA complex using the mutated DNA probe (See Fig. 8A lanes wt and TDCA mut).

3.7. Recombinant HNF1α and C/EBPα bind to the BARE in the CYP27 promoter

Finally, recombinant HNF1α and C/EBPα were synthesized in an in vitro transcription/translation system and used in EMSA. When both recombinant transcription factors were added to this assay, two shifted bands were observed using the wild type probe (−110/−86). Because only two, but not three shifted bands were seen, these data suggest that HNF1α and C/EBPα competes for the same binding site (Fig. 9). An oligonucleotide probe with a consensus HNF1 site bound recombinant HNF1α, yielding a protein:DNA complex with identical mobility as wild type −110/

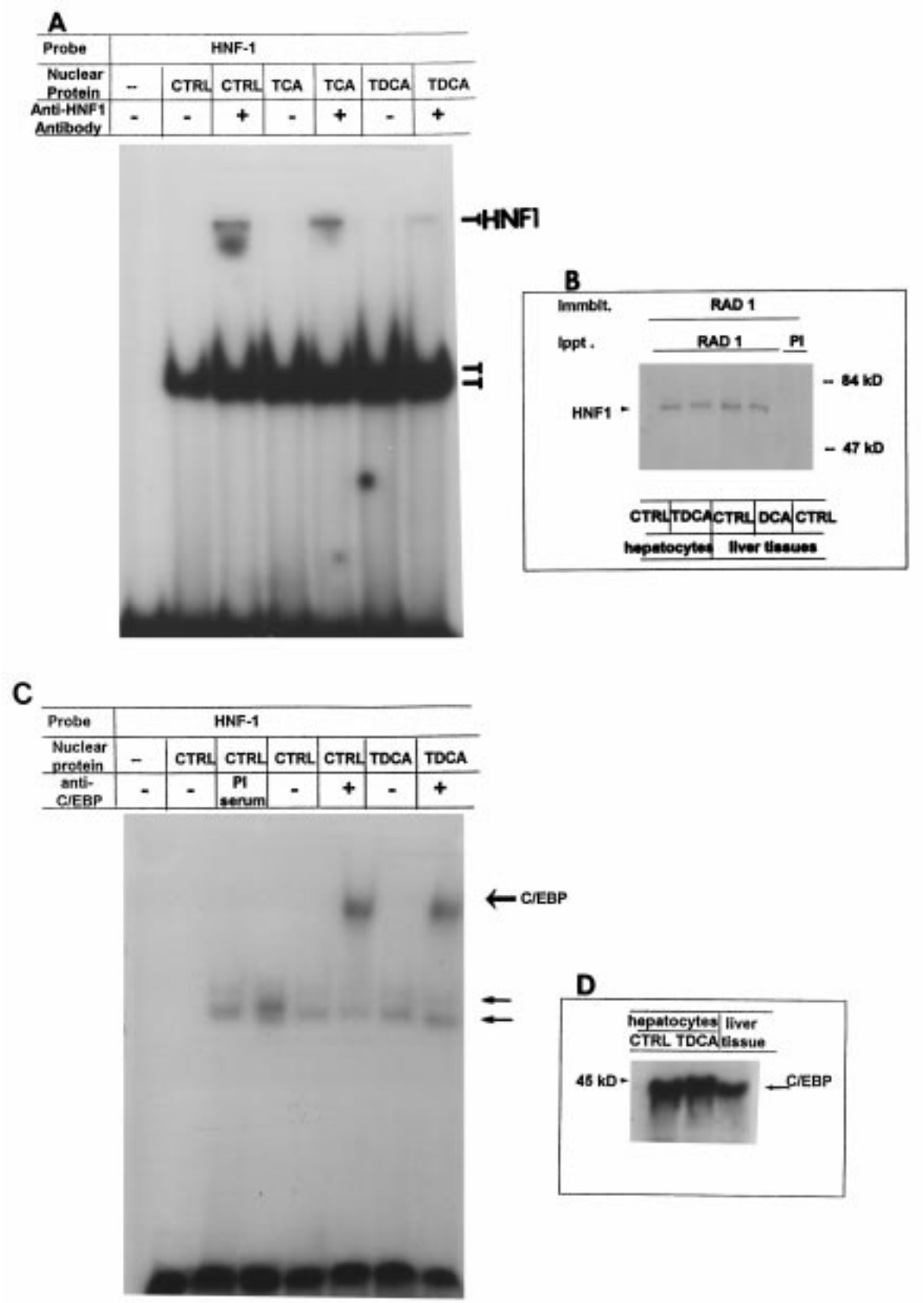

Fig. 7. HNF1α and C/EBP bind to the BARE of the rat CYP27 promoter. (A) 'Super'-EMSA was performed using 3 μl of monoclonal antibody (RAD1) (1:1000), raised against HNF1α [27] and nuclear extracts prepared from primary hepatocyte cultures. The gel position of DNA-HNF1α complex produced with RAD1 is indicated. (B) Immunoreactive HNF1α in crude rat liver nuclear extract prepared from normal diet (CTRL), deoxycholic acid (DCA) fed rats, and in nuclear extracts prepared from primary rat hepatocytes incubated with TDCA was detected by immunoprecipitation (Ippt.) and immunoblotting (Immblt.). (PI: preimmun serum). (C) 'Super'-EMSA with addition of 0.1 μg of polyclonal antibody (Δ198) against C/EBP. The gel position of DNA-C/EBP complex produced with Δ198 is indicated. (D) Immunoreactive C/EBP in crude rat liver nuclear extract and primary hepatocytes nuclear extracts was detected by immunoblotting.

− 86 probe. These data also show that the slow and fast migrating bands are HNF1α and C/EBP, respectively.

3. Discussion

Hepatic CYP27 and CYP7 are both transcriptionally down-regulated in vivo [9,25] and in vitro [5,15,26] by hydrophobic, but not hydrophilic, bile acids. Recent evidence reported by Stravitz, et al. [37,38] suggested that hydrophobic bile acids repress cholesterol CYP7 through a protein kinase C (PKC) dependent mechanism. In these studies, inhibitors of PKC blocked the ability of bile acids to repress CYP7 mRNA in primary rat hepatocyte cultures. In addition, phorbol esters, which are activators of Ca^{2+}-dependent and Ca^{2+}-independent PKC isoenzymes, down-regulated CYP7 m-RNA [38]. Activation of PKC activity in cell extracts was highly correlated with the hydrophobicity index of taurine conjugated bile acids [5]. Crestani, et al. [39] have shown that promoter constructs of the rat cholesterol CYP7 gene were repressed when transfected into HepG2 cells following the addition of hydro-

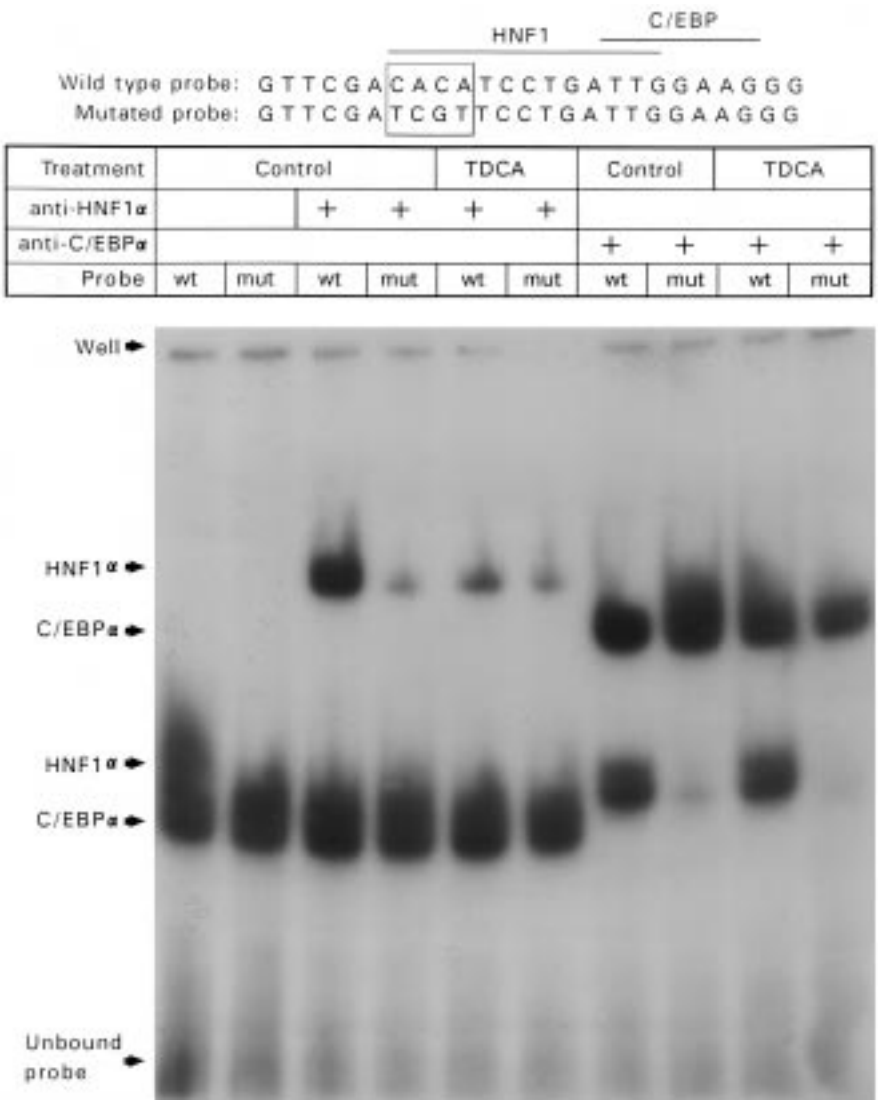

Fig. 8. Effect of four base pair substitution mutation on HNF1α and C/EBP binding to CYP27 BARE using nuclear extracts prepared from control and TDCA treated primary hepatocytes. (A) Radiolabeled oligonucleotides containing either the wild-type (wt) or mutated (mut) probe were used in 'super'-EMSA. Nuclear protein (3 μg) from TDCA or CTRL cells were preincubated 30 min with either monoclonal HNF1α antibody (1 μg) or polyclonal C/EBP polyclonal (1 μg) prior to addition of ^{32}P-labeled oligonucleotides. Nuclear protein:DNA complexes were separated on 4.5% polyacrylamide gels as described in Section 2. The upper and lower HNF1α and C/EBPα bands indicate 'super' shifts and shifts, respectively. (B) 'Super'-EMSA gels were allowed to run for an additional 30 min using wild type probe in order to further separate the HNF1α and C/EBPα DNA complexes.

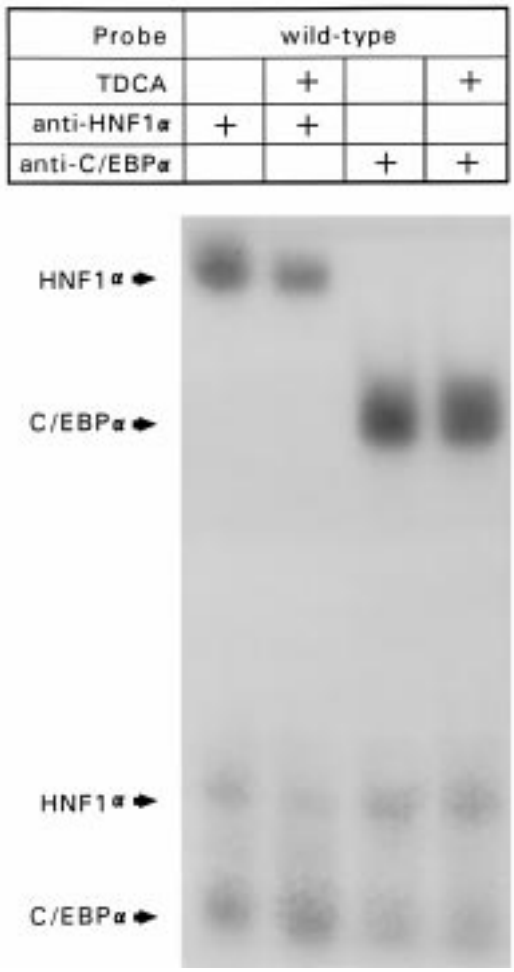

Fig. 8 (*continued*)

phobic bile acids or phorbol esters. Our laboratories have demonstrated that CYP27 transcriptional activity is down regulated by hydrophobic bile acids in the intact rat [25] and in primary rat hepatocytes [5].

Chiang and Stroup [12] have mapped a putative bile acid responsive element (BARE) in the rat CYP7 promoter to between −73 and −55 base pairs upstream from the transcription start site. More recent data indicate a second possible BARE/phorbol ester responsive element between −148 and −129 base pairs of the rat CYP7 promoter [13,40]. However, DNA sequence analysis (−840 to +23 bp) of the rat CYP27 promoter showed no sequences identical to the putative BARE sequences in the rat CYP7 promoter [13,24,41,42]. Moreover, phorbol esters failed to repress CYP27 promoter constructs in the current study (unpublished data). Therefore, it appears that the BARE in the CYP27 promoter is different from those in the CYP7 promoter.

We have mapped and characterized a putative BARE in the 5′-flanking region of the rat CYP27 gene using a combination of deletion analysis (Fig. 3), mutagenesis (Fig. 5), 'super' EMSA (Figs. 7 and 8), in vitro synthesis and binding studies of recombinant HNF1α and C/EBPα (Fig. 9), and cloning of the BARE upstream of a heterologous promoter (Fig. 6). The four base pair substitution mutation within the BARE of the homologous promoter (−840/+23) completely eliminated bile acid regulation indicating a single BARE in this promoter (Fig. 5). Our data show that the putative BARE is located between −110 and −86 bp of the proximal region of the gene. We observed that the BARE in the CYP27 promoter has an overlapping HNF1 and C/EBP DNA consensus sequence. Overlapping HNF1 and C/EBP binding sites have been reported in many gene promoters specifically expressed in liver, including: albumin, β-fibrinogen, α-fetoprotein, and factor VIII [32–35,43,44]. More importantly, these two overlapping elements are involved in gene transcriptional regulation by various regulators [34,43–45]. The proximal promoter (−840/+23) of CYP27 has several putative nuclear protein

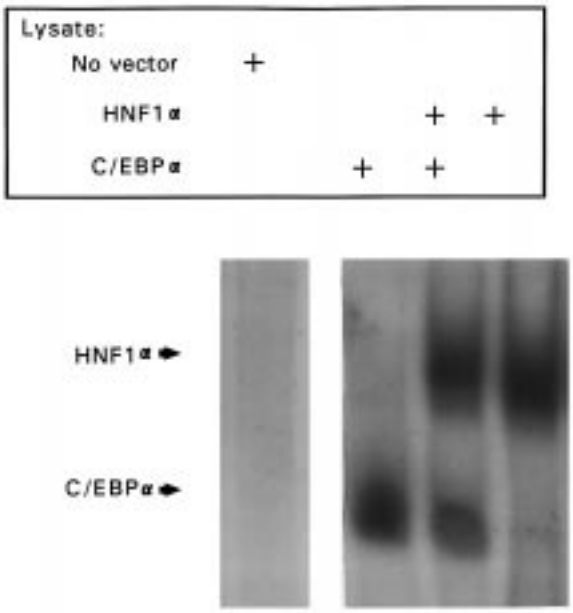

Fig. 9. Binding of recombinant HNF1α and C/EBPα to the putative BARE. EMSAs were performed as described in Section 2. Recombinant HNF1α and C/EBPα were synthesized using an in vitro transcription/translation system described in Section 2. Approximately 5 μl of the transcription/translation reaction mixture was added to the EMSA assays followed by separation of 5% polyacrylamide gels. The gel migration position of DNA:HNF1α and DNA:C/EBPα complex is indicated. The free probe was allowed to run off the gel.

binding sites, however, only those for HNF1 and C/EBP confer liver specific transcription or regulation [24]. Recent transfection studies with rat primary hepatocytes and Hep G2 cells also indicate that HNF1α directs the basal and tissue-restricted expression of the rat liver basolateral sodium-dependent bile acid cotransporter gene promoter [46].

'Super'-EMSA using specific antibody to HNF1α and C/EBP (Figs. 7 and 8) and in vitro synthesis of these transcription factors (Fig. 9) demonstrated that both HNF1α and C/EBPα bind to the BARE sequence. HNF1α and C/EBP are known as liver-enriched transcriptional factors [33,35,36,45] and function in unique combinations to positively or negatively regulate liver-specific gene transcription [24,32,43,44]. HNF1α is a POU (Pit-Oct-Unc) transcription factor containing a variant homeodomain while C/EBP belongs to the leucine zipper family [33,35]. The results suggest that these two transcription factors may compete for binding to the putative BARE. If true in vivo, this might allow for differential regulation of the CYP27 gene depending upon nutritional status and development stage of the animal.

Treatment of hepatocytes with hydrophobic bile acids resulted in an apparent loss of HNF1α, but not C/EBP, binding to the BARE sequence as assessed by 'super'-shift assays (Figs. 7 and 8). HNF1α is a strong transcriptional activator of many hepatic genes [33–35]. Therefore, it is likely that HNF1α also acts as a transcriptional activator of CYP 27 and the substitution mutation that eliminates HNF1α binding confirms this hypothesis (Fig. 5). If HNF1α binding is affected in vivo it may result in down-regulation of CYP27 transcriptional activity. Immunoprecipitation and immunoblotting revealed that HNF1α protein mass in nuclei isolated from hepatocytes treated with bile acids does not change. Therefore, the loss of HNF1α binding was not due to a decrease of HNF1α concentration in the nuclei. It is unknown how hydrophobic bile acids may induce the loss of HNF1α binding to the BARE. However, there are several possibilities. If the affinity of C/EBP were increased by bile acid treatment it might displace HNF1α. However, 'super' shift experiments did not indicate an increased amount of C/EBP binding using extracts prepared from bile acid treated cells (Figs. 7 and 8). Moreover, the four base pair substitution mutation which showed an almost complete loss of HNF1α binding (Fig. 8) but no change in C/EBP binding (Fig. 8) also argues against this mechanism. Bile acids might activate signaling pathways which result in the loss of HNF1α binding through post-transcriptional mechanisms. It is also possible that a bile acid induced 'repressor protein', with similar electrophoretic mobility properties as the HNF1α:DNA complex, is binding at or near the HNF1 site and decreasing HNF1α binding. This hypothesis is consistent with the apparent increase in protein binding to the BARE in nuclear extracts prepared from bile acid treated cells (Fig. 4). Moreover, the lack of a decrease in the putative HNF1α:DNA shifted complex using nuclear extracts prepared from TDCA treated cells and 'super' shifted with C/EBPα antibody is also consistent with a possible repressor protein that displaces HNF1α (Fig. 8A). However, additional studies will be required to determine the exact mechanism of bile acid induced down-regulation of the sterol 27-hydroxylase promoter.

In summary, we have mapped a putative BARE in the rat sterol 27-hydroxylase promoter between −110/−86 base pairs. HNF1α and C/EBPα was shown to bind to this DNA fragment. HNF1α binding, but not C/EBPα binding, to the BARE was decreased by an undefined mechanism following treatment of hepatocytes with bile acids.

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