# Regulation of the rat neutral cytosolic cholesteryl ester hydrolase promoter by hormones and sterols: a role for nuclear factor-Y in the sterol-mediated response

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**Abstract** **Expression of the rat liver neutral cytosolic cholesteryl ester hydrolase (CEH) gene is regulated by glucocorticoids, thyroxine, and agents that perturb cholesterol metabolism. The present studies identify the putative hormone response elements in the CEH promoter. They also define the roles of two previously identified sterol regulatory elements (SRE-92 and SRE-160) and a putative nuclear factor-Y (NF-Y) binding site with a consensus ATTGG (inverted CCAAT) motif (Natarajan, R., S. Ghosh, and W. M. Grogan. 1998. *Biochem. Biophys. Res. Commun.* 243: 349–355). CEH promoter-reporter gene constructs were transiently transfected into HepG2 cells to evaluate promoter activity. Results indicated that the CEH gene has two complex glucocorticoid response units in distal portions of the promoter corresponding to consensus glucocorticoid regulatory sequences as well as putative thyroid hormone response elements. CEH promoter-reporter constructs with the proximal 189 bp of the wild-type or mutated sequences were also transfected into HepG2 cells. Activity of the wild-type construct increased when incubated in sterol depleted media or when co-expressed with a mature sterol regulatory element binding protein (SREBP-2). These responses were suppressed by mutations in SRE-92, SRE-160, or NF-Y, indicating that these *cis* elements are sufficient for sterol-mediated regulation of the CEH promoter. Gel mobility shift assays further demonstrated that NF-Y binds to the inverted CCAAT box motif and is required for the sterol-mediated regulation. These results indicate that multiple *cis*-elements regulate transcription of the cholesteryl ester hydrolase (CEH) gene, consistent with the reported regulation of CEH expression.—Natarajan, R., S. Ghosh, and W. M. Grogan. Regulation of the rat neutral cytosolic cholesteryl ester hydrolase promoter by hormones and sterols: a role for nuclear factor-Y in the sterol-mediated response. *J. Lipid Res.* 1999. 40: 2091–2098.**

**Supplementary key words** carboxylesterase • cholesteryl ester hydrolase • glucocorticoid response element • HepG2 cells • nuclear factor-Y • promoter • sterol response element

The neutral cytosolic cholesteryl ester hydrolase (CEH) characterized by this laboratory (1–3) is the principal enzyme catalyzing release of free cholesterol from hepatic cholesteryl ester stores in the rat. As such, this enzyme plays a key role in a tight regulation of intracellular free cholesterol, a necessary constituent of membranes and lipoproteins and the precursor to bile acids and biliary cholesterol. The importance of this role is indicated by patterns of expression and multivalent regulation similar to those reported for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, and in opposition to those reported for acyl-CoA:cholesterol acyltransferase (ACAT), which catalyzes cholesteryl ester synthesis. It has long been known that hepatic CEH activity is altered by various hormones and changes in cholesterol or bile acid flux (4–7), although the mechanisms involved have remained obscure until recently. CEH exhibits a pattern of regulation consistent with its proposed physiological function in response to perturbations of cholesterol metabolism (4, 6). Cholesterol feeding suppresses both CEH activity and messenger RNA (mRNA) (4, 6). Whereas stimulated synthesis of cholesterol by intravenous infusion of the cholesterol precursor, mevalonate, decreases CEH activity, protein, and mRNA, inhibition of synthesis by infusion of an HMG-CoA reductase inhibitor increases both CEH activity and mRNA (4). Moreover, stimulation of bile acid synthesis, the major cholesterol output pathway, with cholestyramine, a bile acid sequesterant, or by chronic biliary diversion produces 2-fold increases in hepatic CEH activity and mRNA (4).

We have recently provided additional evidence for transcriptional regulation of CEH by sterols (8). Promoter ac-

Abbreviations: CEH, cholesterol ester hydrolase; FPP, farnesyl diphosphate; GRE, glucocorticoid response element; GRU, glucocorticoid response unit; HMGCoAR, 3-hydroxy-3-methyl-glutaryl CoA reductase; HRE, hormone response element; IRS, insulin responsive sequence; LDL, low density lipoprotein; Luc, luciferase gene; NF-Y, nuclear factor-Y; nt, nucleotide; RLU, relative light units; SRE, sterol responsive element; SREBP-2, sterol response element binding protein-2; Sp1, stimulatory protein-1; T4, L-thyroxine; TRE, thyroid hormone response element.

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tivity of deletion constructs linked to the luciferase reporter gene identified three putative sterol response elements (SRE) at positions −92, −160, and −280 of the CEH promoter. Gel shift assays demonstrated binding of a sterol response element binding protein (SREBP-2) to the SRE at position −92 of the CEH promoter. In these studies, mevalonate was used to stimulate sterol synthesis in liver-derived HepG2 cells transiently transfected with the deletion constructs, while squalestatin, an inhibitor of squalene synthase, was used to selectively inhibit synthesis of sterol end products. However, the effect of sterols on CEH promoter activity was not directly determined.

Sterol response elements are often located in close proximity to other positive transcription factor binding elements, which are apparently essential for optimal regulation of sterol-sensitive genes. For example, SREs of low density lipoprotein (LDL) receptor (9, 10), acetyl CoA carboxylase (11), and fatty acid synthase (12, 13) promoters all require Sp1 sequences for expression of sterol responsiveness. Binding of SREBP to the SRE site stimulates the binding of Sp1 to an adjacent Sp1 binding motif. In contrast, the HMG-CoA synthase and farnesyl diphosphate (FPP) synthase promoters have nuclear factor-Y (NF-Y) sites, which are necessary for SRE activity (14, 15). Sp1 has two distinct domains (9), required for transcriptional activation and SREBP-dependent DNA binding to the LDL receptor promoter. In contrast, NF-Y is a heterotrimeric protein and all three subunits (A,B,C) are essential for DNA binding (16), which is accompanied by distortion of the DNA (17). However, the mechanisms for the synergistic activation of genes by SREBP and NF-Y or Sp1 are unknown. Although computer analysis of the CEH promoter identified potential Sp1 and NF-Y sites near the SRE sites, the role of these putative *cis*-acting elements was not addressed in previous studies.

The purpose of the present studies was to identify active hormone response elements that might mediate the previously reported regulation of CEH by hormones (4, 5, 7) and to further define the roles of *cis*-acting DNA elements involved in sterol regulation of CEH promoter activity.

## EXPERIMENTAL PROCEDURES

### Materials and general methods

l-Thyroxine, dexamethasone, cholesterol, 25-hydroxycholesterol, lipoprotein-deficient calf serum (LPDS) and o-nitrophenyl-β-d-galactopyranoside were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture media and other media supplies were purchased from GIBCO-BRL (Grand Island, NY). Reporter Lysis Buffer and luciferase assay system were purchased from Promega (Madison, WI). Reporter vector pCMVβ, the QuikChange Site-Directed Mutagenesis Kit and the MBS Mammalian Transfection Kit were purchased from Stratagene Cloning Systems (La Jolla, CA). Plasticware for tissue culture was obtained from Sarstedt (Newton, NC). The human hepatoblastoma cell line, HepG2, was supplied by American Type Culture Collection (Rockville, MD). Qiagen Plasmid Kit was purchased from Qiagen Inc. (Chatsworth, CA). [$\gamma$-$^{32}$P]ATP was purchased from NEN Research Products (Boston, MA). Oligonucleotides for site-directed mutagenesis and electrophoretic mobility shift assays were synthesized by Genosys Biotechnologies Inc. (The Woodlands, TX). Anti NF-YA (antibody against the A-subunit of human NF-Y protein) was purchased from Biodesign (Kennebunk, ME).

The rat CEH promoter-luciferase reporter gene, p1317-Luc, was constructed as described previously (8). Other smaller constructs were obtained by generation of unidirectional nested deletion breakpoints in p1317-Luc with Exonuclease III (8). The construct pCMV-CS2 is an expression plasmid that produces amino acids 1–481 of the human SREBP-2 protein.

### Cell culture and transfection

HepG2 cells were grown in culture as described previously (18). Briefly, cells were grown in 75 cm$^2$ tissue culture flasks in MEM containing l-glutamine (0.292 mg/ml), nonessential amino acids (1X), sodium pyruvate (0.11 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum at 37°C in a humidified incubator in the presence of 5% $CO_2$. Cells were seeded in 35-mm dishes with 2 ml of medium and grown to 70% confluency. Cells were then transiently transfected by the calcium–phosphate DNA coprecipitation technique (19) with the MBS mammalian transfection kit (Stratagene). Specifically, 2.0 μg of test plasmid and 0.5 μg of pCMVβ, an internal standard for the normalization of transfection efficiency, were incubated at room temperature for 10–20 min with $CaCl_2$ (0.125 mm) and BBS (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid and buffered saline, pH 6.95). Where indicated in the figure legend, 0.1 μg of the SREBP-2 expression vector, pCMV-CS2, was also included in the transfection mixture. Culture medium in the dishes was replaced with 2 ml of fresh medium containing 6% modified bovine serum instead of 10% fetal bovine serum. After addition of the DNA suspension, cells were incubated for 3 h at 35°C under 3% $CO_2$. They were then washed three times with phosphate-buffered saline and re-fed with serum-free medium at the indicated concentrations of agent or vehicle. Alternately, after transfection, the cells were incubated in media supplemented with either 10% LPDS in the absence (−) or presence (+) of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) or 10% fetal bovine serum as indicated in the figure legends. Transfected cells were incubated at 37°C under 5% $CO_2$ for different periods of time as indicated. Cells were lysed and luciferase and β-galactosidase activities were measured as described previously (8). Luciferase activity was normalized by dividing relative light units (RLU) by the β-galactosidase activity. All plasmids used for transfection were purified by double banding in CsCl gradients or with Qiagen columns according to manufacturers' instructions.

### Oligonucleotide directed mutagenesis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to introduce three separate mutations in the rat CEH promoter. The construct used for the generation of the replacement mutations was p226-Luc, which contains the proximal 189 bases of the CEH promoter (−37 to −226). The putative SRE at position −160 (mut1), the putative SRE at position −92 (mut2) and the inverted CCAAT box motif at position −142 (mut3) were mutated by this procedure. The primers used for these mutations are shown in **Table 1**. These mutagenic oligonucleotide primers have a high melting point ($T_m$) and anneal to the same sequence on opposite strands of the construct. Temperature cycling was performed according to manufacturer's instructions using *Pfu* DNA polymerase, which replicates both strands with high fidelity and without displacing the mutagenic primers. This generates a mutated plasmid containing staggered nicks. The product was treated with *Dpn*I endonuclease which specifically digests methylated and hemi-methylated parent DNA template and selects for mutation containing synthesized DNA. The nicked vector DNAs

TABLE 1. Oligonucleotides used for mutagenesis of rat CEH promoter

| Mutation | Oligonucleotide Sequence |
|---|---|
| SRE-160 (mut1) | **TAAGA**<br>$^{-177}$**GC . . . TTTCTA**GCCAC**TTGTAA . . . GG**$^{-141}$<br>**CG . . . AAAGAC**CGGTG**AACATT . . . CC**<br>**ATTCA** |
| SRE-92 (mut2) | **TCTTA**<br>$^{-118}$**GG . . . GTGGGC**GTGGC**TTGGAG . . . GC**$^{-77}$<br>**CC . . . CACCCG**CACCG**AACCTC . . . CG**<br>**AGAAT** |
| NFY-142 (mut3) | **GCCTT**<br>$^{-164}$**GC . . . CTGAGT**ATTGG**GTACTG . . . AG**$^{-120}$<br>**CG . . . GACTCA**TAACC**CATGAC . . . TC**<br>**CGGAA** |

The oligonucleotides used for the mutagenesis are displayed. Mutant primer sequences are in bold. Mutated bases are underlined. The numbers denote their position in the promoter.

containing the desired mutations were then transformed into *E. coli* XL-1 Blue supercompetent cells. The mutated vectors were sequenced to ensure presence of only the desired mutation.

### Preparation of nuclear extracts

Nuclear extracts for electrophoretic mobility shift assays and supershift assays were prepared from cultured primary rat hepatocytes. Isolation and culture of primary rat hepatocytes were performed by the collagenase perfusion technique of Bissel and Guzelian (20) as described elsewhere (4). Nuclear extracts were prepared essentially as described by Schreiber et al. (21). Briefly, $1 \times 10^6$ hepatocytes were grown in 60-mm dishes for 24 h. Plates were washed with cold PBS and cells were scraped and transferred to 1.5-ml Eppendorf tubes. They were then allowed to swell on ice for 15 min in cold buffer A (10 mm HEPES, pH 7.4, 10 mm KCl, 0.1 mm EDTA, 0.1 mm EGTA, 2 mm NaF). A 10% solution of Nonidet NP-40 was added and cells were vortexed at maximum speed for 10 sec. They were placed on ice for 3 min and centrifuged at maximum speed for 1 min at 4°C to separate the nuclear pellet. The nuclear pellet was then resuspended in buffer B (20 mm HEPES, pH 7.4, 0.4 m NaCl, 1 mm EDTA, 1 mm EGTA, 2 mm NaF) and rocked vigorously at 4°C for 20 min on a shaking platform. Protease and phosphatase inhibitors (2 mm $Na_3VO_4$, 25 μg/ml leupeptin, 10 μg/ml pepstatin, 0.1 mm PMSF) were added to buffers A and B just prior to use. The nuclear extract was centrifuged at 4°C for 5 min and the supernatants were used for the assays.

### Electrophoretic mobility shift assays

Complementary single-stranded oligonucleotides corresponding to positions −152 to −134 of the CEH promoter (8) and containing the inverted CCAAT box were annealed and used in these assays. A 10-μl reaction volume containing 20% glycerol, 5 mm $MgCl_2$, 2.5 mm EDTA, 2.5 mm DTT, 250 mm NaCl, 50 mm Tris-HCl, pH 7.5, and 0.25 mg/ml poly (dI-dC)and the indicated concentration of nuclear extract were incubated at room temperature for 10 min. The $^{32}$P-labeled double-stranded probe (30 fmol, $1 \times 10^5$ cpm) was added and incubated at room temperature for 20 min. Immuno-supershift assays were carried out under identical conditions except the nuclear extracts were pre-incubated with anti-NF-YA antibody for 60 min at 4°C prior to addition of the probe. After the binding reactions, the mixture was electrophoresed on a 4% nondenaturing polyacrylamide gel and the signal was detected by autoradiography.

### Statistical analysis

Where treated groups were compared to their own individual control groups, statistical significance of differences was determined by the unpaired 2-tailed *t*-test with and without a Welch correction for unequal variances. Where several treated groups were compared to a single control group, statistical significance was determined by one-way ANOVA and the Tukey-Kramer Multiple Comparisons Test. Each control and treated group consisted of data from at least three and sometimes four individual transfections. Differences with $P$ values $<0.0001$ were considered highly significant. Differences with $P$ values $<0.001$ were considered very significant.

## RESULTS

We have previously reported the isolation and characterization of the rat hepatic CEH promoter (8). We determined the CEH basal promoter activity and showed that positive *cis*-acting elements were located downstream of nt-599 and repressor sequences were located upstream of nt-599 of the CEH promoter. We also studied the effects of agents that perturb cholesterol metabolism on the CEH promoter activity. However, extensive characterization of the rat CEH promoter has not yet been reported.

### Effect of dexamethasone on CEH promoter activity

As an earlier study from this laboratory showed that dexamethasone was required to maintain steady-state levels of CEH mRNA in primary cultures of rat hepatocytes (4), we examined the effects of this glucocorticoid on promoter activity. When HepG2 cells were transfected with a full-length construct (p-1317Luc) and treated with increasing concentrations of dexamethasone, promoter activity increased 4.5-fold ($P < 0.0001$) at 0.1 μm dexamethasone (**Fig. 1A**). In order to map the regions that respond to dexamethasone, reporter activity of deletion constructs was measured in the presence of the optimal 0.1 μm dexamethasone (Fig. 1B). Deletion of the promoter to nt-1190 eliminated the induction observed with the full-length construct. Further deletion down to nt-859 restored the activation to 2.4-fold ($P < 0.002$). However, the remaining constructs showed no induction by dexamethasone, suggesting that two regions mediate the glucocorticoid effect, one located between nt-1317 and nt-1191 and the other between nt-859 and nt-540. Moreover, the region between nt-1190 and nt-860 has elements that repress the glucocorticoid-mediated activation by the region between nt-859 and nt-540. Similar studies with primary cultures of rat hepatocytes confirmed stimulation of promoter activity by dexamethasone, although regulatory elements could not be mapped with the same precision (data not shown).

### Regulation of CEH promoter activity by thyroid hormones

Earlier work from this laboratory showed that l-thyroxine was also required to maintain optimal CEH mRNA levels in primary cultures of rat hepatocytes (4). Therefore, we also tested the effect of l-thyroxine on activity of the

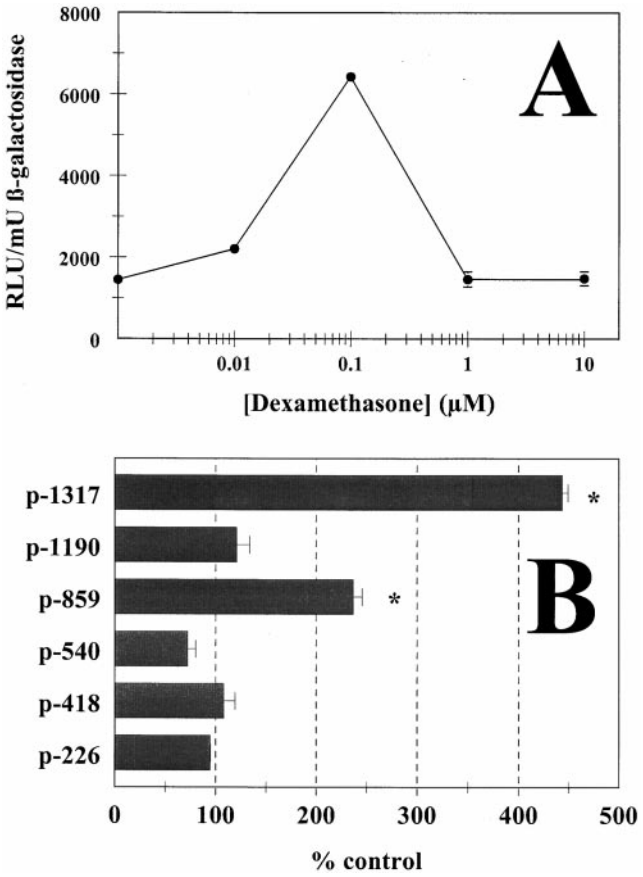


**Fig. 1.** Effect of dexamethasone on activity of CEH promoter and deletion constructs in HepG2 cells. A: Confluent HepG2 cultures were cotransfected with pCMV-β-gal and p-1317Luc and incubated 40 h in serum-free medium with indicated concentrations of dexamethasone. Results are expressed as the mean ratio of luciferase to β-galactosidase activities ± SEM of 3 or 4 (no addition) independent transfections. Initial data point is no additions. B: Confluent HepG2 cultures were transfected with the indicated rat CEH/luciferase chimeric genes and incubated 40 h in serum-free medium with 0.1 μm dexamethasone in ethanol or ethanol alone (controls). Luciferase activities are normalized to internal standard β-galactosidase and expressed as mean % control ± SEM for 3–4 independent transfections; (*) indicates difference between control and treated values is highly significant ($P < 0.0001$).

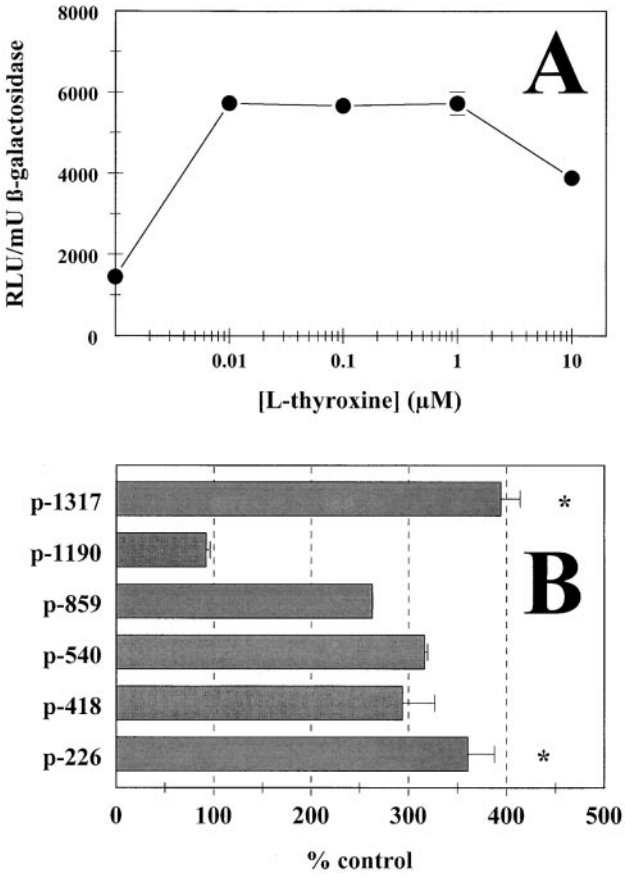


**Fig. 2.** Effect of l-thyroxine on the activity of CEH promoter and deletion constructs in HepG2 cells. A: Confluent cultures of HepG2 cells were cotransfected with pCMV-β-gal and p-1317Luc and incubated 40 h in serum-free medium with indicated concentrations of l-thyroxine. Results are expressed as the mean ratio of luciferase to β-galactosidase activities ± SEM for 3 independent transfections. Initial data point is no addition. B: Confluent HepG2 cells were transfected with the indicated rat CEH/luciferase chimeric genes and incubated 40 h in serum-free medium with 1.0 μm l-thyroxine or vehicle alone (controls). Luciferase activities are normalized to internal standard β-galactosidase and expressed as mean % control ± SEM for 3 independent transfections; (*) indicates difference between control and treated values is highly significant ($P < 0.0001$).

CEH promoter constructs. As shown in **Fig. 2A**, l-thyroxine (T4) stimulated the promoter activity of p-1317Luc at a concentration as low as 0.01 μm with maximal effect (4-fold) at 0.01–1.0 μm. T4 stimulated the activity ($P < 0.006$) of all the deletion constructs tested except for p-1190Luc, for which the activity stayed at basal levels (Fig. 2B). Therefore, two regions respond to T4, one between nt-1317 and nt-1191 and the other between nt-226 and nt-37. As with the glucocorticoid response, the region between nt-1190 and nt-860 repressed activation by the proximal but not the distal region. Similar studies with primary cultures of rat hepatocytes confirmed stimulation of promoter activity by l-thyroxine, although regulatory elements could not be mapped with the same precision (data not shown).

### Identification of *cis*-elements on CEH promoter that are required for sterol-regulated expression

We have previously reported that sterol-mediated regulation of CEH promoter activity resides primarily in the proximal 226 bases of the CEH promoter and that two putative sterol responsive sequences are located at nt-92 and nt-160 of the CEH promoter. This sterol responsiveness was confirmed in primary cultures of rat hepatocytes, although regulatory elements could not be mapped with the same precision (data not shown). An inverted CCAAT box that putatively binds the heterotrimeric NF-Y protein was also identified between these SREs at nt-142. In the present study, these *cis*-elements were individually mutated to confirm that these sequences were responsible for the sterol-regulated expression of the CEH promoter. Wild-type and mutated sequences for nt-90 to nt-170 are shown in **Fig. 3**.

After transient transfection with the 189 base wild-type

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          SRE160                    NF-Y142                    SRE92
5' TTTCTAGCCACTTGTAATCTGAGTATTGGGTACTG . . . . GGCGTGGCTTGGA 3'
     TAAGA (mut1)               GCCTT (mut3)              TCTTA (mut2)
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**Fig. 3.** Native and mutated sequences of elements critical for sterol-mediated regulation of rat CEH promoter. The three putative *cis*-elements required for sterol-regulated transcription of the rat CEH promoter between nucleotides −170 and −90 are shown. SRE160, SRE92, and NF-Y142 are underlined. Substituted bases in the three mutant constructs mut1, mut2, and mut3 are shown on the lower line.

(p-226Luc) and mutated (mut1, mut2, mut3) reporter gene constructs, HepG2 cells were incubated for 24 h in media (lipid-free fetal calf serum) either devoid of sterols (−) or supplemented with cholesterol and 25-hydroxycholesterol (+). Each of the three mutations suppressed promoter activity in the sterol-deprived cells, 59%, 69%, and 40%, respectively ($P < 0.001$), indicating that all of these elements are necessary for optimal promoter activity (**Fig. 4**). As expected, the wild-type CEH promoter activity was suppressed 68% ($P < 0.001$) by addition of sterols. In contrast, the reporter gene containing the 5 base pair mutation in the SRE sequence at nt-160 (mut1) was suppressed only 18% ($P < 0.05$) by sterols. Similarly, the reporter gene containing the 5 base pair mutation in the SRE sequence at position nt-92 (mut2) was suppressed only 19% ($P < 0.05$) by sterols. Moreover, mutation of the CCAAT box construct (mut3) also impaired suppression by sterols (27%, $P < 0.05$). Thus, it appears that each of the three *cis*-elements (SRE-160, SRE-92, and ATTGG) is essential for optimal sterol-mediated suppression of the rat hepatic CEH promoter.

To further demonstrate that these elements are essential for sterol-mediated regulation, mutated and wild-type constructs were coexpressed with a plasmid encoding low levels of mature SREBP-2. In a preliminary dose–response experiment to confirm activation of p-226Luc by the plasmid encoding SREBP-2, maximal activation was observed at 0.1 μg of DNA/plate (data not shown). As shown in **Fig. 5**, co-expression of SREBP-2 with wild-type CEH promoter increased luciferase activity 2.4-fold ($P < 0.001$), indicating the presence of active sterol regulatory elements. Activities of the constructs mutated in the SREs (mut1, mut2) were reduced by 73% and 72%, respectively ($P < 0.001$) in the absence of co-expressed SREBP-2, suggesting that these sequences contribute to the basal promoter activity under these culture conditions, whereas promoter activity of the inverted CCAAT box mutant construct (mut3) was not significantly impaired. In contrast to wild-type, constructs with mutant SREs (mut1 and mut2) showed no significant increase in luciferase activity when coexpressed with SREBP-2, suggesting that both SREs are necessary for activation by SREBP. Activity of the inverted CCAAT box mutant (mut3) increased 2-fold ($P < 0.001$), indicating that absence of an active inverted CCAAT box motif may be partially compensated under these conditions. Nevertheless, the activation was significantly lower than that seen with unmutated wild-type construct ($P < 0.001$).

### NF-Y binding to the inverted CCAAT box motif

Whereas deletion constructs and mutation analysis provide strong evidence that the three *cis*-acting elements are involved in sterol-mediated regulation, it is important to show that the putative regulatory proteins actually bind to the corresponding regulatory elements. In previous studies, we have shown that SREBP-2 actually binds to the SRE-92 sequence (8). Although others have demonstrated that the CCAAT box motif is absolutely essential for the binding of the heterotrimeric NF-Y to DNA, additional flanking nucleotides are also involved (22, 23). Therefore, we have used gel electrophoretic mobility shift assays and

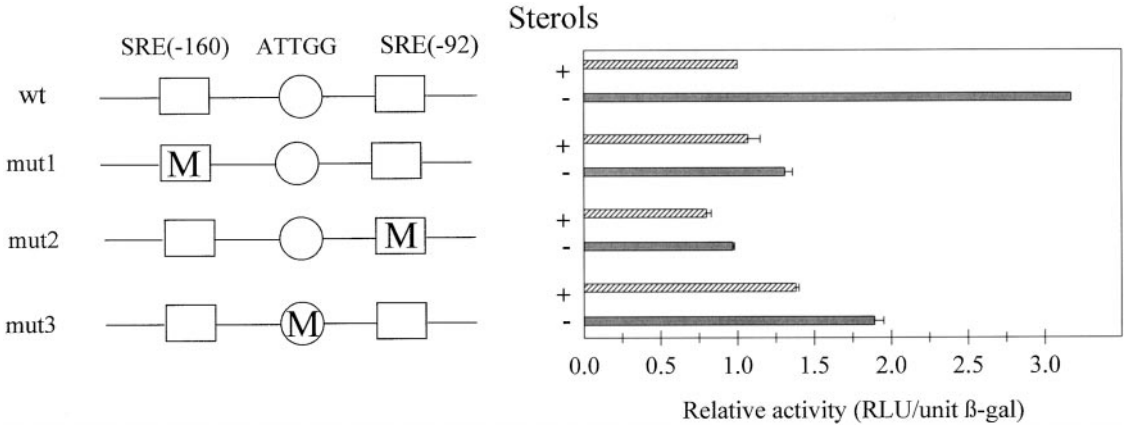


**Fig. 4.** Effects of sterols on promoter activity of p-226Luc and mutant constructs. HepG2 cells were cotransfected with pCMV-β-gal and either wild type p-226Luc or p-226Luc mutated in SRE-160 (mut1), SRE-92 (mut2), or NFY-142 (mut3). Mutated motifs are indicated by "M". Cells were incubated 24 h in 10% lipoprotein-deficient fetal calf serum with (+) or without (−) sterols (10 μg/mL cholesterol and 1 μg/mL 25-hydroxycholesterol). Results represent the mean ratios of luciferase to β-galactosidase activities ± SEM for 3 independent transfections, normalized to activity of the wild-type construct with addition of sterols. See Results for statistical signficance.

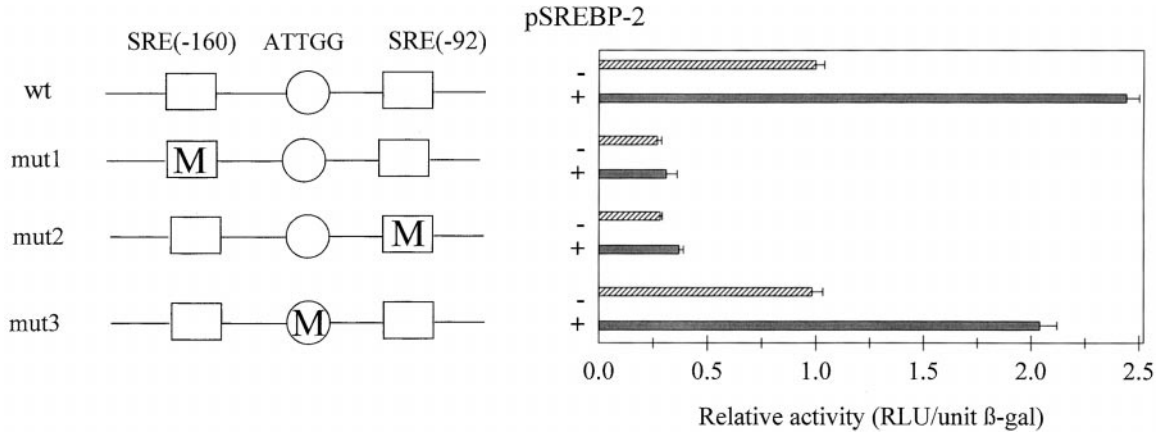


**Fig. 5.** Effects of mature SREBP-2 on promoter activity of p-226Luc and mutant constructs. HepG2 cells were transfected with wild type or mutated p-226Luc constructs as in Fig. 4, with (+) and without (−) 0.1 μg of pCMV-CS2, which co-expresses SREBP-2. Cells were incubated 24 h in 10% fetal calf serum before measuring luciferase activity. Results represent the mean ratios of luciferase to β-galactosidase activities ± SEM for 3 independent transfections, normalized to activity of the wild type construct without SREBP-2. See Results for statistical significance.

immuno-supershift assays to confirm specific binding of the NF-Y protein to the putative binding site on the CEH promoter.

Nuclear extracts from rat primary hepatocyte cultures were incubated with the $^{32}$P-labeled probe (13mer), consisting of the CCAAT box motif and flanking sequences from the CEH promoter, to detect specific binding to corresponding sequences in the nuclear DNA. Upon electrophoresis, these extracts gave one major radiolabeled band (**Fig. 6A, lane 1, Fig. 6B, lane 2**), which could be competed out with unlabeled probe (lanes 2–5), confirming specific binding to DNA. To confirm the identity of the protein/s that bind to this motif, the incubation was repeated in the presence of antibody to NF-Y A subunit. Under these conditions a specific super-shifted complex was obtained (Fig. 6B, lane 3), confirming the binding of endogenous NF-Y to the putative regulatory element.

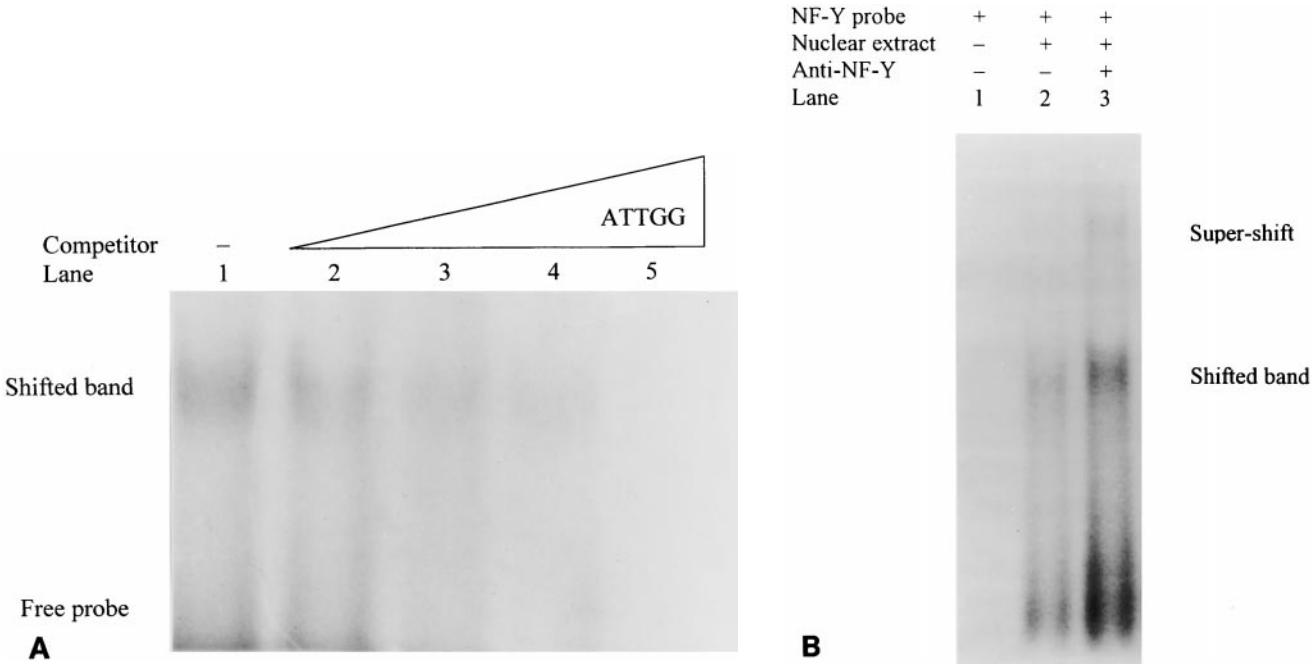


**Fig. 6.** Electrophoretic mobility shift assay showing binding of NF-Y to inverted CCAAT box motif. A $^{32}$P-labeled double-stranded oligonucleotide probe, corresponding to nt-152 to nt-134 of the rat CEH promoter and including the inverted CCAAT box, was used in the gel shift assay, as described in Experimental Procedures. A: Nuclear extract (5 μg protein) from primary rat hepatocytes was incubated with probe, with no addition (lane 1) or 50-, 100-, 500-, and 1000-fold molar excess of unlabeled wild-type competitor sequence (lanes 2–5, respectively). B: nuclear extract (5 μg protein) was preincubated with (lane 3) or without (lane 2) 2 μg anti-NF-YA antibody at 4°C for 60 min, prior to addition of probe as in Fig. 6A. Control incubation is without addition of antibodies or nuclear extract (lane 1). Bands correspond to free probe, the bound NF-Y/DNA complex (shifted band) and the super-shifted complex.

## DISCUSSION

Although previous studies from this laboratory have shown that the glucocorticoid dexamethasone stimulates CEH mRNA levels and CEH activity in rat hepatocytes (4), a mechanism has not been reported. The current study has localized dexamethasone responsive sequences in the distal 5′ flanking regions from nt-1317 to nt-1190 and from nt-859 to nt-540 of the CEH promoter. Consistent with these observations, the region between nt-1317 and nt-1190 has sequence similarities with the glucocorticoid response unit (GRU) of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene (24). The complex GRU of the PEPCK gene has 2 glucocorticoid receptor (GR) binding sites and 2 sites recognized by accessory factors AF1 and AF2. In the CEH promoter, this consensus GRU sequence, TTTTGGTCTTTTTGTTCTTTTTAGACATCT is located from nt-1185 to nt-1156. It contains an imperfect palindrome TCTACAgatTTTTCT which closely resembles the consensus glucocorticoid response element (GRE) T/GG TACAnnn-TGTTCT (25). The consensus GRE directs the specific binding of the GR to the promoter regions of target genes with one molecule of the GR binding to each of the two half-sites in a cooperative manner (26). The 5′ portion of this sequence TCTGGTTTT, located between nt-1177 and nt-1185 shows 8/9 identity to the recognition sequence TGTGGTTTT for accessory factor AF2 in the PEPCK promoter (24). Imai et al. (24) showed that proteins other than the GR are required for activation of glucocorticoid-inducible genes, and that accessory factors AF1 and AF2 do not act in the absence of the 2 GR binding regions. The AF2 element has also been shown to be an insulin response sequence (IRS) which opposes the dexamethasone-mediated stimulation, both in PEPCK (27) and cholesterol 7α-hydroxylase genes (28). Although we did not test the effect of insulin on the CEH promoter in this study, we have reported elsewhere that a minimum of 24 h incubation of rat primary hepatocytes in insulin-containing medium was essential for the synergistic increase in CEH mRNA by T4 and dexamethasone (4). Therefore, it appears that the CEH gene may also contain a complex glucocorticoid response unit with a glucocorticoid response element, an insulin responsive sequence and other sequences that mediate the insulin effect in the distal regions of the promoter.

Similarly, the region between nt-859 and nt-540 has the consensus sequence TGTTGTGTATTTGGTTGTTTT from nt-801 to nt-781 which could also behave as a glucocorticoid response unit and account for the dexamethasone-mediated stimulation in this region. Thus, we have mapped two regions that appear to have GRUs and seem to play an important part in the transcriptional regulation of the CEH gene.

In rat hepatocytes l-thyroxine was also required to maintain steady state CEH mRNA levels (4), consistent with the observation of others that addition of these hormones is necessary to maintain expression of liver specific genes in serum free media (29). In the current study, we demonstrated stimulation of CEH promoter activity by l-thyroxine and mapped activation sequences to regions from nt-1317 to nt-1190 and from nt-226 to nt-37. Although both these regions have sequences that partially match the consensus type II steroid/thyroid hormone response element (HRE) AGG/TTCA, none of these exactly matched the consensus thyroid responsive element (TRE) TCAGGTCA—TGACCTGA (25). Thus, further studies will be needed to identify and map the TRE in the CEH promoter.

In previous studies, we identified three consensus sterol regulatory sequences in the proximal portion of the rat CEH promoter and demonstrated binding of SREBP-2 to the SRE at nt-92 (8). In the current study, we demonstrate by mutation analysis that SRE-92, the SRE at nt-160, and an inverted CCAAT box motif at nt-142 contribute to the sterol-mediated regulation of the CEH promoter. Mutations of SRE-92 or SRE-160 almost abolish activation of the CEH promoter by sterol deprivation or SREBP-2 coexpression (Figs. 4 and 5), indicating that both elements are essential for sterol-mediated regulation of CEH transcription. SRE-92 is present in an inverted orientation and is similar to the Inv-SRE-3 element described for the farnesyl diphosphate synthase and human squalene synthase promoters (15, 30). While these two elements are a common feature of genes involved in the cholesterol biosynthetic pathway, this study establishes that they are functional in the CEH promoter.

Mutation of the inverted CCAAT box also diminishes activation of the CEH promoter by sterol deprivation in lipid-depleted media or by SREBP-2 coexpression in normal calf serum, although the effect was less pronounced in the latter case. Moreover, the heterotrimeric NF-Y protein was shown to bind to this inverted CCAAT box motif by electrophoretic mobility shift assays and immunosupershift assays. Others have reported that functional binding sites for SREBP and NF-Y are essential to induction of the promoters for FPP synthase (31), glycerol-3-phosphate acyltransferase (32), squalene synthase (30, 33), SREBP-2 (34), and HMG-CoA synthase (15) in sterol-deprived cells. Binding of heterotrimeric full-length and truncated NF-Y subunits to their binding sites is reported to induce a conformational change in the DNA (17). Moreover, the binding of the NF-Y core to its motif in the farnesyl diphosphate synthase promoter was necessary and sufficient to stimulate the binding of SREBP-1a to its cognate DNA binding site (31). In the CEH promoter, the NF-Y site is appropriately located between the two SRE sites, 13 base pairs 3′ to SRE-160 and 42 base pairs 5′ to SRE-92. We speculate that the NF-Y induced conformational change increases the SREBP:DNA complex formation by altering the conformation of the DNA bound protein.

These data indicate that the CEH promoter has functional response elements which interact with sterols, hormones, and corresponding transcription factors to regulate CEH enzyme levels, consistent with the role of this enzyme in cholesterol homeostasis.

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