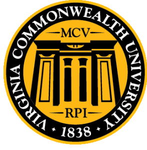

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This is to certify that the dissertation prepared by Ramesh Natarajan entitled "Molecular Cloning and Analysis of the Promoter for Rat Hepatic Neutral Cholesterol Ester Hydrolase" has been approved by his committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

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MOLECULAR CLONING AND ANALYSIS OF THE PROMOTER FOR RAT HEPATIC NEUTRAL CHOLESTEROL ESTER HYDROLASE

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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December, 1997

ACKNOWLEDGEMENT

I would like to thank Dr. Grogan, my advisor, for giving me the opportunity to work in his laboratory. I am grateful to him for keeping me focussed in my research and for guiding me through an important phase in my career. I would also like to thank my committee members Dr. Jim Collins, Dr. Richard Franson, Dr. Phil Hylemon and Dr. John Bigbee for their patience and helpful suggestions throughout my Ph.D. career.

I would always be indebted to Dr. Shobha Ghosh, my mentor and friend, for her help and support, both in the laboratory and outside of it. I would also like to acknowledge my lab-mates Britt Langston and T.J. Wallace for all the good times and for introducing me to the birdie, par and bogey world of golf.

I am extremely grateful to my parents who were always there in my time of need and for letting me choose my road in life. Without the love and support of both my parents and in-laws, this goal would have been a dream left unfulfilled. I would also like to thank my brother, Rajesh, for encouraging me every step of the way. Finally, this work would not have been possible without my wife, Seema, who was always a pillar of strength for me to lean on. I am grateful to her for being with me on this "roller-coaster ride".

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LIST OF ABBREVIATIONS

ACAT	acyl-coenzymeA:cholesterol acyltransferase
ADD1	adipocyte determination- and differentiation-dependent factor 1
AP-1	activator protein 1
C7H	cholesterol 7α-hydroxylase
C/EBP	CCAAT/enhancer binding protein
CEH	Cholesterol ester hydrolase
DMSO	dimethyl sulfoxide
GRE	glucocorticoid response element
GRU	glucocorticoid response unit
HMGCoAR	3-hydroxy-3-methyl-glutaryl coenzyme A reductase
HMGCoAS	3-hydroxy-3-methyl-glutaryl coenzyme A synthase
HNF	hepatocyte nuclear factor
HRE	hormone response element
HSL	hormone sensitive lipase
IRS	insulin responsive sequence
LDLR	low density lipoprotein receptor
Luc	luciferase gene

MMTV	murine mammary tumor virus
NF-Y	nuclear factor-Y
nt	nucleotide
PEPCK	phosphoenolpyruvate carboxykinase
PMA	phorbol-12-myristate-13-acetate
PRS	PMA responsive sequence
RLU	relative light units
SRE	sterol response element
SREBP	sterol response element binding protein
T4	L-thyroxine
TRE	thyroid hormone response element

ABSTRACT

MOLECULAR CLONING AND ANALYSIS OF THE PROMOTER FOR RAT HEPATIC NEUTRAL CHOLESTEROL ESTER HYDROLASE

By Ramesh Natarajan, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 1997.

Major Director: W. McLean Grogan, Professor, Department of Biochemistry and Molecular Biophysics

Neutral cholesterol ester hydrolase (CEH) is a key enzyme in regulating hepatic free cholesterol. Using the CEH specific cDNA sequence in the 5'-untranslated region as a primer, 1.3 kb of sequence upstream of the ATG initiation codon was amplified and cloned. Primer extension analysis with total RNA from rat primary hepatocytes identified a transcription initiation site, 60 bases upstream from the initiation codon. No typical TATA-box sequences were found upstream from the transcription start site. However, a consensus GC-box, which can bind the positive transcription factor SP1, was found 35 bases upstream from the transcription start site. In addition the promoter also contained several hormone responsive half elements, sterol response elements, ubiquitous transcription factor binding sites and liver specific elements.

To determine the promoter activity of the rat CEH gene, the 1.3 kb of 5'-flanking region was fused to a luciferase reporter gene. Smaller 5'-deletion constructs were obtained by generation of unidirectional nested deletion breakpoints in the full-length construct with Exonuclease III. Basal promoter activity, as well as transcriptional regulation by hormones, signal transduction pathways and agents perturbing cholesterol metabolism were studied in human hepatoblastoma HepG2 cells and cultured primary rat hepatocytes by transient transfection assays of the promoter activity of the deletion constructs. Functional glucocorticoid response elements, phorbol ester responsive sequences and sterol responsive sequences were mapped with both the culture systems. Results indicate that the first 599 base pairs upstream of the initiation codon and the region between nucleotides -1317 and –1190 regulate the effects of various physiological stimuli. The effects of various stimuli used in this study were similar in the two cell lines. The rat CEH gene appears to be finely regulated by distinct signals converging to consensus promoter regulatory sequences.

INTRODUCTION

Cholesterol ester hydrolases (CEH) are a family of ubiquitous enzymes that are found in many organisms. In mammals they occur in many tissues and are associated with different functions, depending on location. They have been found in liver (Deykin and Goodman, 1962), pancreas (Calame *et al*, 1975), adrenals (Trzeciak and Boyd, 1974), testis (Durham and Grogan, 1982), intestine (Gallo *et al*, 1980), mammary glands (Martinez and Botham, 1990), placenta (Chin and Morin, 1971), brain (Eto and Suzuki, 1972), adipose tissue (Pittman *et al*, 1975), macrophages (Small *et al*, 1989), artery (Hajjar *et al*, 1983) and corpus luteum (Cook *et al*, 1983), where they catalyze the release of free cholesterol and fatty acids from cholesteryl esters, an intracellular storage form of cholesterol. Multiple forms of CEH have been reported in testis (Durham and Grogan, 1984), brain (Eto and Suzuki, 1972), adrenals (Pittman and Steinberg, 1977) and liver (Deykin and Goodman, 1962; Nilsson, 1976). They have been identified in different sub-cellular organelles including cytosol (Deykin and Goodman, 1962), lysosomes (Lundberg *et al*, 1990), endoplasmic reticulum (Gandarias *et al*, 1987), mitochondria (Deykin and Goodman, 1962) and nuclei (Deykin and Goodman, 1962). While the pancreatic and intestinal CEH's have a digestive role, the CEH in adipose tissues and steroidogenic organs provide substrate for steroidogenesis. The lysosomal CEH on the other hand hydrolyses cholesteryl and glyceryl esters during lipoprotein degradation. Although rat

liver contains both lysosomal and microsomal CEH activities, the major hydrolytic activity is associated with a unique cytosolic cholesterol ester hydrolase that is appropriately located, rationally regulated and is capable of mobilizing free cholesterol from cholesteryl ester stores (Natarajan *et al*, 1996a). However, because most of the research to date has focussed on the bile salt-stimulated cholesterol esterase found initially in the pancreas and the hormone sensitive lipase / cholesterol esterase found in steroidogenic tissues, I will briefly review these enzymes prior to discussing the properties of the hepatic cytosolic CEH.

Bile salt-stimulated cholesterol esterase

The bile salt stimulated cholesterol esterase has been purified from many different sources including rat and human pancreatic juice (Gallo, 1981; Lombardo *et al*, 1978), pancreas from different species (Wang, 1988; Jacobson *et al*, 1990; Rudd *et al*, 1987), rat liver (Camulli *et al*, 1989) and human milk (Rudd and Brockman, 1984; Wang and Hartsuck, 1993). This enzyme has a broad substrate specificity. In addition to the hydrolysis of cholesteryl esters, the enzyme is capable of hydrolyzing mono-, di-, and triacylglycerol, phospholipids, and esters of fat-soluble vitamins. Enzyme activity against these substrates can be activated several fold by bile salt, with the trihydroxylated bile salts being more potent than their dihydroxylated counterparts (Hernell and Olivecrona, 1974; Blackberg and Hernell, 1993). This enzyme can also hydrolyze lysophospholipid (Han *et al*, 1987). However, this activity is bile salt-independent. The bile salt-stimulated cholesterol esterase has also been demonstrated to have lipoamidase activity (Hui *et al*, 1993). Han *et al* were the first to report the primary sequence of this

protein, based on isolation and cloning of a 2 kb full-length transcript from rat pancreas. Hui *et al* used an independent approach, based on immunoreactivity, partial protein sequencing, and cloning of the cDNA to confirm identity between the pancreatic lysophopholipase and cholesterol esterase. The sequence of bile salt-stimulated lipase found in milk was also shown to be identical to the pancreatic cholesterol esterase (Hui and Kissel, 1990; Nilsson *et al*, 1990), thereby establishing the identity between these proteins. The cholesterol esterase sequence displays a high degree of homology with other serine esterases, such as acetylcholinesterase and cholinesterase. These proteins contain the catalytic triad serine-histidine-acidic residue at similar locations of the protein.

The complete sequence of the bile salt-stimulated cholesterol esterase gene has been determined in the rat (Fontaine *et al*, 1991), and partial sequences with intron-exon junctions have been reported for both the human and the mouse cholesterol esterase gene (Lidmer *et al*, 1995; Lidberg *et al*, 1992). Both the rat and the mouse cholesterol esterase genes are single copy genes in their respective genomes, spanning approximately 7 kb. The human gene is approximately 9 kb in length, and is present in the human genome along with a cholesterol esterase-like gene. The 5' flanking region of the cholesterol esterase gene shares several homologous domains with that of the pancreatic lipase gene (Mickel *et al*, 1989). There are 5 distinct sequences each spanning at least 10 residues, that are similar between the cholesterol esterase and pancreatic lipase genes. Since the pancreatic lipase has a key role in dietary lipid absorption, it has been suggested that the cholesterol esterase synthesized by the pancreas may be involved in a similar process.

The 5' flanking region also contains consensus sequences for pancreas specific enhancer elements (Fontaine *et al*, 1991; Lidberg *et al*, 1992), a tissue-specific mammary gland factor recognition sequence (Lidberg *et al*, 1992) and an 11 bp conserved sequence present in a number of milk protein genes (Lidberg *et al*, 1992) such as lactalbumin and casein. This consensus sequence may be responsible for cholesterol esterase gene expression in lactating mammary glands.

In addition to transcriptional regulation, cholesterol esterase biosynthesis is also regulated post-transcriptionally in response to gastric hormones like cholecystokinin, secretin and bombesin. The hormone-induced cholesterol esterase biosynthesis is not dependent on *de novo* RNA synthesis and does not alter the level of cholesterol esterase mRNA (Huang and Hui, 1991). Instead hormonal treatment increases translational efficiency of the cholesterol esterase mRNA. The hormone-induced cholesterol esterase biosynthesis is mediated via calcium mobilization and protein kinase C-dependent mechanisms (Brodt-Eppley and Hui, 1995).

Disruption of the cholesterol esterase gene in knockout experiments abolished cholesterol esterase activity in the intestinal tract. However, while cholesterol absorption efficiency was unaffected, the absorption of cholesteryl esters was dramatically decreased in the cholesterol esterase knockout mice (Howles *et al*, 1996), suggesting that cholesterol esterase is not involved in the cholesterol absorption process but is only important for the absorption of substrates with carboxylester bonds.

Hormone sensitive lipase

Hormone sensitive lipase (HSL) is another enzyme with high cholesterol esterase

activity. Although this protein was originally isolated based on its ability to hydrolyze triglyceride, subsequent characterization of the protein revealed its ability to hydrolyze cholesteryl esters with equal efficiency. The HSL is most abundant in adipose tissue, heart, skeletal muscle and in steroidogenic tissues such as the adrenals, ovary and testes. In contrast to the bile salt-stimulated cholesterol esterase, which requires direct interaction of the bile salt with the enzyme for activation, bile salt is not a required cofactor for HSL.

The primary structure of HSL was deduced by cloning and sequencing of the cDNA, from both rat and human adipose tissue (Holm *et al*, 1988; Lombardo *et al*, 1993). The HSL contains within its sequence the GXSXG motif, a characteristic of other serine esterases and lipases. However, the histidine and acidic amino-acid residue in the catalytic triad have yet to be identified.

As the name implies, the HSL hydrolytic activity is stimulated by lipolytic hormones such as catecholamines. The mechanism of action is through cAMP-dependent protein kinase-mediated phosphorylation of a serine residue (Holm *et al*, 1988). In contrast, the inactivation of the HSL by anti-lipolytic hormones, such as insulin, is indirect through lowering of the cAMP level by a cAMP phosphodiesterase.

The HSL gene spans approximately 10 kb and contains 9 exons (Langin *et al*, 1993). The intron-exon organization of the HSL gene resembles that of the bile salt-stimulated cholesterol esterase, with each exon encoding a key functional domain of the protein. The 5' flanking region of the mouse HSL gene has adipose tissue specific elements (Rice *et al*, 1990) and an element responsible for protamine 1 gene expression

in the testes (Tamura *et al*, 1992). The presence of the consensus sequences in the regulatory region of the HSL gene is consistent with its expression in these tissues. Consensus sequences for Ets-1 and Pu.1 are also present, suggesting that these may represent *cis*-acting elements responsible for monocyte/macrophage expression of this gene (Klemsz *et al*, 1990). In addition, a preadipocyte repressor element (Swick and Lane, 1992) and sterol regulatory element-1 sequences (Goldstein and Brown, 1990) are also present.

Although HSL activity / immunoreactivity was originally found in the adipose tissue, it has been shown to be present in the heart, skeletal muscle, adrenals, ovaries, placenta and in some macrophage cell lines (Kraemer *et al*, 1993). It has been proposed that the HSL in adipose tissue, skeletal muscle and heart serves as a triacylglycerol lipase in supplying fatty acids to these tissues. On the other hand, its role in ovaries and adrenals may be related to its cholesteryl ester hydrolytic activity and it possibly mobilizes cholesteryl ester stores for steroid biosynthesis. HSL is also found in the testes where it is expressed in a stage-dependent manner, coinciding with the onset of spermatogenesis. Testicular expression of the gene was localized to Sertoli cells instead of the Leydig cells (Holst *et al*, 1994), suggesting that the role of this protein in the testis is most likely related to mobilization of lipid droplets in Sertoli cells rather than for steroid biosynthesis in Leydig cells. As mentioned before, the HSL is also found in several mouse macrophage cell lines. The functional significance for macrophage expression of the HSL is speculated to be the hydrolysis of cholesteryl esters liberating free cholesterol for efflux from the cells (Khoo *et al*, 1981; Small *et al*, 1989). Based on

these observations, the importance of this enzyme in dictating macrophage foam cell formation and in athergenesis has been postulated.

Cytosolic Cholesterol Ester Hydrolase : Isolation and cloning

The cytosolic CEH represents the major regulated cholesterol ester hydrolytic activity in rat liver. Conventional protein purification procedures have been used in this laboratory to purify this hepatic CEH from rat livers (Ghosh and Grogan, 1991). The enzyme was purified 12,600 - fold by ammonium sulfate precipitation, cation exchange chromatography and gel permeation high pressure liquid chromatography with an overall yield of 20%. The identity of this enzyme has been a controversy for over a decade. Gallo *et al* first suggested that hepatic CEH is different from the pancreatic CEH, by showing that antibodies to pancreatic CEH did not cross react with hepatic CEH. But Harrison *et al* reported that the anti-pancreatic CEH antibodies (supplied by Gallo) could inhibit 72% of hepatic CEH activity and therefore suggested identity between these enzymes. Camulli *et al* isolated a liver protein using anti-pancreatic CEH antibodies on an antibody affinity column. However this protein had properties different from the hepatic CEH. Kissel *et al* then cloned the cDNA for rat pancreatic CEH and demonstrated the presence of corresponding RNA in rat liver. However the CEH purified by Ghosh and Grogan (1991) differs from the pancreatic CEH in several ways. This hepatic CEH has orders of magnitude greater total activity than pancreatic CEH, is protein kinase A activated, is bile salt independent and has no cross reactivity with anti-pancreatic CEH antibodies. Also a specific neutralizing antibody to hepatic CEH generated in this laboratory cross reacted only weakly with pancreatic CEH on Western

blots (Ghosh and Grogan, 1992). More recently a 1923 bp cDNA for rat hepatic CEH was cloned by screening a rat liver lambda gt 11 cDNA library with an end-labelled synthetic oligonucleotide derived from the conserved active site sequence of rat and human pancreatic cholesterol esterase and human milk bile salt stimulated lipase (Ghosh *et al*, 1995). This cDNA hybridized to a single strong 2.3 kb band on Northern blots of rat liver mRNA and included an open reading frame coding for 565 amino acids, corresponding to a protein of molecular weight 62143 (unglycosylated), consistent with the 66 kDa mobility of rat liver CEH on SDS-PAGE. The cDNA sequence for hepatic CEH has only 44% homology with pancreatic CEH, whereas the predicted amino acid sequence has only 31% identity. The identity of the cDNA obtained with the purified CEH was demonstrated by immunoreactivity of antibodies to hepatic CEH with the fusion protein produced by the recombinant phage and with protein expressed in a prokaryotic expression system, and also by production of high levels of hepatic CEH activity and a 66 kDa immunoreactive protein in COS cells transfected with an expression vector containing the full-length cDNA (Ghosh *et al*, 1995).

Characterization of hepatic cytosolic CEH

The purified hepatic CEH exhibited activity over the pH range 5.0 - 9.0 with optimal activity at pH 7.0 to 7.5 (Ghosh and Grogan, 1991). The purified enzyme eluted as a single peak from a FPLC chromatofocussing column corresponding to a pI value of 5.5 (Ghosh and Grogan, 1991). As mentioned earlier, hepatic CEH is activated by cAMP-dependent protein kinase (PKA) and also by Ca^{2+}- phospholipid dependent protein kinase (PKC). Thus, CEH is regulated by reversible phosphorylation, with the

phosphorylated form being the more active form (Ghosh and Grogan, 1989). Whereas the cytosolic enzyme is bile salt independent, the purified enzyme is activated by increasing taurocholate concentrations up to 1 mM. The activation remained more or less constant up to 10 mM and then declined with further increases in concentration (Ghosh and Grogan, 1991). Purified hepatic CEH is strongly inhibited by 20 - 40 mM taurocholate in marked contrast to the pancreatic CEH which is known to be activated by bile salts in this concentration range. While the purified CEH requires a minimal level of taurocholate (0.25 mM) to prevent aggregation of the enzyme to an inactive state, bile salts can also modify the physical state of the substrate. The purified enzyme hydrolyzes oleoyl esters of both cholesterol and glycerol, although the activity was consistently higher with cholesteryl oleate than with triolein at all taurocholate concentrations (Natarajan, *et al*, 1996a). With cholesteryl oleate as a substrate, enzyme activity rapidly increased between 2.5 - 6.5 μM and gradually increased thereafter with further increases up to 300 μM. Thus, typical of enzymes with insoluble substrates, this CEH did not saturate with substrate within the range of feasible substrate concentrations. The activity of purified CEH is also independent of divalent metal ions like Zn^{+2}, Cu^{+2} and Cd^{+2}. However these cations were mildly inhibitory at one or more concentrations from 1 - 1000 μM (Natarajan *et al*, 1996a).

CEH belongs to the family of serine esterases which are characterized by the presence of a conserved catalytic triad (serine, histidine and an acidic residue). Similar to other cholesterol esterases, purified CEH was inhibited by phenylmethylsulfonylfluoride (PMSF), with 50% inhibition at 0.1 mM PMSF (Natarajan *et al*, 1996a). This is

consistent with the identification of the consensus serine esterase active site. The sulfhydryl specific agent mercury benzoate, inhibited the enzyme activity 30% at 3 mM (Natarajan *et al*, 1996a). Inasmuch as iodoacetamide and N-ethylmaleimide affected a modest activation, inhibition by Hg^{+2} probably reflects a general effect of divalent cations, rather than an interaction with an essential sulfhydryl group. Since CEH catalyzes the hydrolysis of uncharged esters and is inhibited by Cu^{+2} and Hg^{+2}, but not by iodoacetamide, it has the properties of a class A esterase, as described by Aldridge, 1953. In addition to its ability to hydrolyze physiologically relevant substrates like cholesteryl oleate and triolein, this cytosolic CEH can also hydrolyze paranitrophenyl (PNP)- esters of fatty acids. While esterases like lipoprotein lipase and pancreatic CEH can efficiently hydrolyze the water soluble substrate PNP-acetate, hepatic CEH demonstrated the lowest activity towards this substrate. Activity increased with chain length, peaked with the more lipophilic PNP-caprylate and then declined gradually with increasing chain length > C_8 (Natarajan *et al*, 1996a). However hydrolytic activity was consistently higher with the more lipophilic esters than with PNP-acetate.

Serine esterases such as lipoprotein lipases and hepatic lipase have highly variable trypsin-labile loop structures formed by disulfide bridges, which confer substrate binding specificity. Trypsin cleavage at a specific site in this loop abolishes lipoprotein lipase activity with triolein but not with more hydrophilic tributyrin. Mild trypsin digestion of the purified CEH results in progressive and selective loss of activity with cholesteryl oleate and triolein, but not with more hydrophilic PNP-caprylate. Analysis of CEH by SDS-PAGE after 24 hrs. of trypsin digestion revealed only a single band at 66 kDa,

indistinguishable from the mobility of the unmodified protein (Natarajan *et al*, 1996a). By analogy with lipoprotein lipase, this is consistent with the cleavage of a single peptide bond within a loop domain which confers substrate specificity on CEH. Analysis of secondary structure for CEH by the GCG program (PEPTIDESTRUCTURE), using algorithms for hydrophilicity (Kyte-Doolittle), surface probability (Emini), chain flexibility (Karplus-Schulz) and secondary structure (Chou-Fasman and Garnier-Osguthorpe-Robson), predicts such a loop between Cys^{87} and Cys^{116}, containing a highly exposed trypsin cleavage site at Arg^{104}. This site has a high hydrophilicity index (2.4), a high surface probability (5.8) and a predicted turn.

The hepatic cytosolic CEH is also developmentally regulated in the rat. In male rats, age-related differences in mRNA, protein mass and catalytic activity suggest transcriptional regulation and indicate an important role for CEH in cholesterol homeostasis in the developing rat (Natarajan *et al*, 1996). On the other hand, in female rats it appears that hepatic CEH is subject to gender-specific multivalent regulation by post-translational mechanisms (Natarajan *et al*, 1997).

Regulation of cholesterol homeostasis in the liver

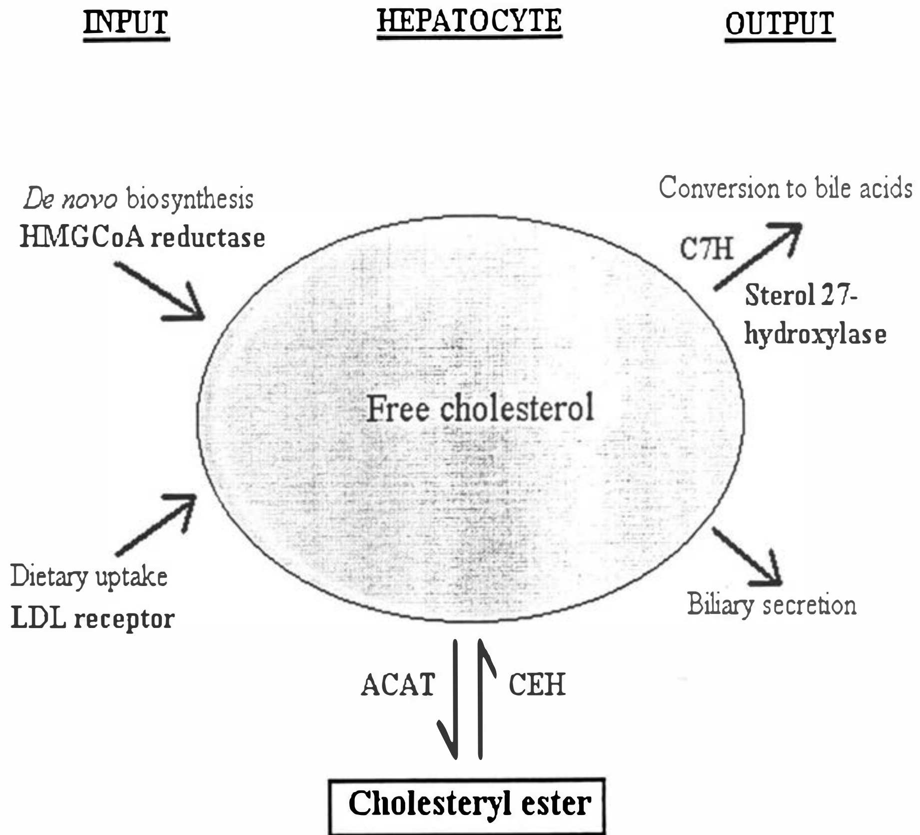
As mentioned before, CEH releases free cholesterol from cholesteryl esters. Free cholesterol is an important structural component of cellular membranes. It has profound effects on physical properties of membranes, as well as membrane associated activities including enzymes and receptors involved in cholesterol metabolism and homeostasis (Liscum and Underwood, 1995). This free cholesterol is not evenly distributed among the various membrane classes. While plasma membranes are relatively rich in

cholesterol, the endoplasmic reticulum has much lower cholesterol concentrations. The mechanisms required to maintain this gradient are not well understood as yet.

Hepatic free cholesterol pools play important roles in the regulation of receptor mediated uptake of cholesterol, in the *de novo* biosynthesis of cholesterol, in bile acid synthesis, in the esterification of free cholesterol and in the hydrolysis of stored cholesteryl esters. Intracellular levels of free cholesterol are maintained relatively constant over a broad range of metabolic states and cholesterol fluxes through the liver (Gould, 1977; Klauda *et al*, 1978; Erickson *et al*, 1980). While *de novo* cholesterol biosynthesis and uptake of dietary cholesterol as lipoproteins are the two input pathways for cholesterol, conversion of hepatic cholesterol to bile acids and biliary secretion of cholesterol are the only significant output pathways (Fig. 1). In response to cholesterol influx or efflux, sterol balance in the hepatocyte is maintained by altering the flux of cholesterol through 1) endogenous cholesterol biosynthesis regulated by 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCoAS) and the rate limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAR), 2) lipoprotein uptake, synthesis and secretion which is partly regulated by the low density lipoprotein (LDL) receptor, 3) conversion of cholesterol to bile acids which is regulated by sterol-27-hydroxylase and the rate limiting enzyme cholesterol-7α-hydroxylase (C7H) and 4) reversible conversion of excess cholesterol to cholesteryl esters by acylcoenzymeA:cholesterol acyltransferase (ACAT) and hydrolysis of stored cholesteryl esters to free cholesterol by CEH.

Regulation of the LDL receptor and the other enzymes HMGCoAS, HMGCoAR,

Figure 1. Regulation of cholesterol homeostasis in the rat liver. This figure shows the input and output pathways involved in the regulation of cholesterol homeostasis in the rat liver as described in the text.


INPUT
HEPATOCYTE
OUTPUT
De novo biosynthesis
HMGCoA reductase
Conversion to bile acids
C7H
Sterol 27-
hydroxylase
Free cholesterol
Dietary uptake
LDL receptor
Biliary secretion
ACAT
CEH
Cholesteryl ester

C7H, sterol-27-hydroxylase and ACAT have been studied in considerable detail. HMGCoAS, HMGCoAR and the LDL receptor are actively regulated by transcriptional, translational and post-translational modes (Goldstein and Brown, 1990). C7H is primarily regulated at the transcriptional level (Spady and Cuthbert, 1992). There is some controversy regarding the identity of ACAT and its regulatory mechanisms are as yet unclear. While a detailed explanation of the regulation of these enzymes is beyond the scope of this dissertation, it is useful to review their regulation at the transcriptional level.

The promoters for the LDL receptor (Osborne *et al*, 1988), HMGCoAS (Smith *et al*, 1988) and HMGCoAR (Dawson *et al*, 1988) have been identified and characterized. They are regulated through sterol regulatory elements (SRE-1) in their promoters that behave as conditional positive or conditional negative regulators of transcription when bound by the sterol regulatory element binding protein (SREBP). The details of this process are described in the following section. The C7H promoter has also been identified and characterized (Jelinek and Russell, 1990; Nishimoto *et al*, 1991; Chiang *et al*, 1992). No SRE's have yet been found in the C7H promoter. However the gene is regulated by different physiological stimuli including bile acids, hormones and signal transducing agents (Crestani *et al*, 1994; Chiang and Stroup, 1994; Crestani *et al*, 1995). While an ACAT cDNA has recently been identified, no information is available on its promoter (Chang *et al*, 1993).

Another common mode of regulation is the short-term regulation of HMGCoAR, C7H, ACAT and CEH by reversible phosphorylation. In contrast to HMGCoAR which

is inhibited by phosphorylation and activated by dephosphorylation, the activities of C7H and ACAT are increased by *in vitro* phosphorylation (Gavey *et al*, 1983; Goodwin *et al*, 1982; Beg *et al*, 1987). As mentioned before, CEH is also regulated by reversible phosphorylation and the phosphorylated form is the active form. Based on these observations there appears to be a coordinated control of the enzymes involved in regulation of cholesterol homeostasis in the liver by reversible phosphorylation. Under conditions of increased phosphorylation in the cell, HMGCoAR would be inhibited and the free cholesterol pools would be depleted due to increased activities of ACAT and C7H. CEH, which is more active in the phosphorylated state, would then be important in maintaining the free cholesterol level in the cell. However, a mechanism by which cellular cholesterol levels might affect the phosphorylation status of the various enzymes has not been demonstrated.

Unlike free cholesterol, the levels of intracellular cholesteryl esters vary greatly with rates of influx and efflux of cholesterol from the liver. They serve as an inert storage pool that is capable of absorbing considerable amounts of cholesterol. Since the physical properties of cholesteryl esters are not conducive to the formation of membrane bilayers, their concentration in membranes is very low. Therefore, most cholesteryl esters are incorporated into lipoproteins or triglyceride rich cytoplasmic droplets. These cytoplasmic droplets serve as substrates for CEH and provide a reservoir for mobilization of cholesteryl ester stores.

Promoters and control of gene expression

The DNA of a cell has information responsible for the maintenance of that cell

and ultimately the entire organism. Genetic information is first transcribed by RNA polymerase and then translated into the finished protein product. Cells have developed complex systems to regulate these processes at every step. Transcription initiation is a complex process involving both protein:DNA and protein:protein interactions. While the details of transcription initiation from mRNA encoding genes by RNA polymerase II and associated factors are beyond the scope of this dissertation, it should suffice to mention some of the essential features of this process. Eukaryotic promoters generally consist of *cis*-acting elements close to the transcription initiation site, which help to direct gene expression. Many promoters contain a TATA-box (consensus sequence TATANA; where N indicates a position that can be occupied by any nucleotide) approximately 30 bases upstream of the transcription initiation site, which is recognized by the multi-subunit complex TFIID. Interaction of TFIID with other initiation factors correctly positions RNA polymerase on the DNA and allows transcription to proceed.

Another common promoter element called the CAAT-box (consensus sequence CCAAT) is found between -80 and -60 nucleotides upstream of the transcription initiation site for RNA polymerase II. Although not palindromic, this sequence occurs naturally in both orientations and is bound by either the CTF/NF-1 or CP1 proteins (Pearson *et al*, 1991, Dutta *et al*, 1990).

Transcription initiation also occurs from promoters that lack a TATA-box and/or a CAAT-box. These promoters frequently contain another element whose consensus sequence is GGGCGG. This sequence called the GC-box binds the positive transcription

factor SP1. It is functional in either orientation and can be present in one or multiple copies.

In addition to these elements, eukaryotic promoters also contain other *cis*-acting elements that bind different transcription factors and thus finely regulate the expression of various genes. Some of the pertinent *cis*-acting elements and the transcription factors that bind them are discussed below.

Glucocorticoid response element

Comparison of available sequence information shows the consensus glucocorticoid response element (GRE) to be (T/G)GTACAnnnTGTTCT (Beato, 1989). The GRE is a transcription enhancer because it functions through heterologous promoters and in a relatively position- and orientation-independent manner (Chandler *et al*, 1983; Yamamoto, 1985). A single GRE works when located immediately upstream of the TATA-box, but multiple copies are required when the GRE's are located at a distance (Strahle *et al*, 1988). There is considerable variation in the number and location of GRE's. The long terminal repeat of the murine mammary tumor virus (MMTV) gene has multiple GRE's clustered near the transcription initiation site (Payvar *et al*, 1983). On the other hand, the clustered GRE's of the tyrosine aminotransferase gene are located 2.5 kb from the transcription start site (Jantzen *et al*, 1987). The rat phosphoenol pyruvate carboxykinase (PEPCK) gene has a complex glucocorticoid response unit (GRU) that spans 110 base pairs and includes two glucocorticoid receptor binding elements designated GR1 and GR2 (Imai *et al*, 1990), plus two accessory factor binding elements

(AF1 and AF2). The rat C7H gene also has a similar GRU located 220 base pairs upstream of the transcription start site (Crestani *et al*, 1995).

The classical mechanism for glucocorticoid hormone action involves binding of the hormone to a cytoplasmic form of the receptor, followed by a conformational change ("activation" or "transformation") that leads to its intranuclear translocation and binding to chromatin. However the steroid-free glucocorticoid receptor binds specifically to the GRE of MMTV *in vitro*. It is possible that upon binding of the hormone, the oligomeric complex of glucocorticoid receptor and a 90 kDa heat shock protein dissociates, leaving the DNA binding domain of the receptor free to bind to chromatin. One molecule of the receptor binds to the TGTTCT half-site of the GRE, and a second molecule then binds to the TGTACA half-site in a cooperative manner. The receptor contacts the DNA through two zinc fingers in the DNA binding domain. The amino-terminal finger interacts specifically with one half of the GRE, whereas the carboxyl-terminal finger interacts with the DNA helix flanking the GRE consensus sequence. Transcription is enhanced, presumably by bringing into close proximity the transactivation domain(s) of the receptor with one or more components of the initiation complex.

Thyroid hormone response element

Unlike the GRE, much less is known about the thyroid hormone response element (TRE). The proposed consensus sequence for the TRE is TCAGGTCA---TGACCTGA (Beato, 1989). The "---" refers to gaps of 1 to 6 base pairs between the two half sites. This consensus sequence contains half palindromes similar to the estrogen response element but with different spacing.

Based on amino acid and structural similarities, the thyroid hormone receptor belongs to the class of steroid hormone receptors that regulate gene expression in a ligand dependent manner. Similar to the glucocorticoid receptor, the thyroid hormone receptor-DNA binding is mediated by a conserved sequence motif that is thought to form a tertiary structure of loops or "fingers" coordinated by a metal cation like Zn^{+2}. While thyroid hormone can up regulate transcription through the TRE, it has also been demonstrated to down regulate transcription of several genes including the thyroid-stimulating hormone gene (Shupnik *et al*, 1985). The thyroid hormone receptor can bind both TRE as well as the closely related estrogen response element. However, this binding does not result in transcriptional activation. Instead it causes a decrease in the level of expression. Since the consensus sequence of the TRE has centrally located gaps and the estrogen response element is a variant of the TRE with a gap of 3 base pairs, the functional consequence of thyroid hormone receptor binding to the estrogen response element is to decrease the estrogen-dependent gene expression. Thus the presence or absence of a 3 base pair gap in the DNA recognition sequence dictates positive or negative transcriptional control by thyroid hormone.

Phorbol ester response element

Since phorbol esters are activators of protein kinase C (PKC), before elaborating on phorbol ester response sequences (PRS), I shall briefly discuss the role of phosphorylation in the regulation of transcription. Phosphorylation events are post-translational modifications that affect the activity of various DNA binding proteins. They

not only result in a conformational change in the protein but also affect electrostatic interactions. There are three main levels of regulation by phosphorylation:

Transcription factors may be sequestered in the cytoplasm, bound to inhibitory proteins. In response to various stimuli, the inhibitory protein is phosphorylated and this causes dissociation of the transcription factor, which now enters the nucleus and binds DNA to regulate transcription. An example of this type of regulation is the phosphorylation of the inhibitory protein IκB that causes it to dissociate from complexes with other transcription factors, thereby enabling these transcription factors to enter the nucleus and bind to chromatin (Hunter and Karin, 1992).

Secondly, phosphorylation can also affect the binding activity of transcription factors. It can increase binding, as is observed with phosphorylation of the serum response factor by casein kinase II, or decrease binding activity as seen by phosphorylation of c-Myb by the same casein kinase II (Hunter and Karin, 1992; Luscher *et al*, 1989).

Finally phosphorylation could affect transactivation events mediated by a transcription factor. For example, the cAMP responsive binding protein when phosphorylated by protein kinase A in the activation domain leads to an increase in gene expression driven by this element (Gonzalez and Montminy, 1989).

Phosphorylation events are controlled by kinases, which in turn are controlled by receptor initiated signals conveyed by multiple cell signalling pathways. While there are several signalling mechanisms, this discussion will be limited to the protein kinase C (PKC) pathway, since phorbol esters are PKC activators. Activation of the PKC pathway

is initiated by binding of ligand to receptor in the plasma membrane. Generally the receptor is thought to interact next with a specific heterotrimeric G protein (G_q) which requires bound GTP for activity. Association of the receptor with G_q causes the α subunit to dissociate and interact with the enzyme phosphoinositide-specific phospholipase C (PLCγ). PLC cleaves phosphoinositide 4,5-bisphosphate (PIP_2) which is located in the inner leaflet of the plasma membrane, to generate inositol triphosphate (IP_3) and diacylglycerol (DAG). Here, the pathway divides into two branches, each of which is critical to the signalling process. IP_3, which is water soluble, diffuses into the cytosol and causes the release of Ca^{+2} from the endoplasmic reticulum. This is mediated by the binding of IP_3 to special Ca^{+2} channels in the endoplasmic membrane. The initial rise in cytosolic calcium levels results in the movement of PKC from the cytosol to the cytoplasmic side of the plasma membrane. Secondly, DAG, along with phosphotidylserine which is associated with the plasma membrane, can bind to PKC, thereby increasing the affinity of the enzyme for calcium and resulting in enzyme activation. While calcium ionophores mimic the affects of IP_3, phorbol esters mimic the affect of DAG. DAG can also be cleaved to produce arachidonic acid, which can either be utilized for prostaglandin synthesis or other lipid signalling moieties. After activation PKC phosphorylates cellular proteins on serine or threonine residues. These proteins may themselves further phosphorylate other cellular proteins leading to potentiation of the signal.

The consensus PRS identified in many genes transcriptionally activated by phorbol esters is TGACTCA (Deutsch *et al*, 1988). It is believed that 4β-phorbol-12-

myristate-13-acetate (PMA), a phorbol ester, affects transcription through phosphorylation of transcription factors like NF-κB, c-Jun and c-Fos. AP-1 and AP-2 can also confer PMA responsiveness.

Sterol response element

Sterol response elements (SRE) are sequences found in the 5'-flanking region of genes that respond to the levels of sterols in the cell. The consensus sequence for the SRE is CACC(C/G)(C/T)AC (Smith *et al*, 1988). This element enhances transcription in sterol depleted cells and is inactivated when sterols accumulate in the cell. In tissue culture cells the most potent inactivators of SRE are oxysterols, which are derivatives of cholesterol with additional hydroxy- or keto- groups that enhance their solubility. SRE is also inactivated by cholesterol when it is delivered to cells in low density lipoproteins.

Two proteins designated sterol regulatory element binding proteins 1 and 2 (SREBP-1 and 2) bind to SRE-1 with a nucleotide specificity that precisely matches the requirement for sterol regulated transcription (Wang *et al*, 1994). SREBP's are members of the basic-helix-loop-helix-leucine zipper family of transcription factors. They bind to DNA either as homodimers or as heterodimers. DNA binding is mediated by the basic region and dimerization is mediated by the helix-loop-helix and leucine zipper structures. The basic helix-loop-helix region is preceded by an acidic amino-terminal region which is a putative transcription activation domain.

Three alternatively spliced forms of SREBP-1 have been identified (Kawabe *et al*, 1994). They are called SREBP-1a, SREB-1b and SREBP-1c. The three forms show no functional difference in SRE-1 binding or transcriptional activation in transfected cells.

No alternatively spliced forms of SREBP-2 have been identified as yet. SREBP-1a and SREBP-2 differ from other basic helix-loop-helix zipper proteins in two important aspects; firstly they are larger in size, and secondly they fail to recognize the palindromic hexanucleotide sequences that are targets for all other basic helix-loop-helix zipper proteins. Instead they recognize the SRE-1 sequence which contains a direct repeat of CAC.

SREBP-1 is synthesized as a 125 kDa precursor that is bound intrinsically to membranes of the nuclear envelope and the endoplasmic reticulum. In sterol depleted cells, the precursor is cleaved proteolytically to generate amino-terminal fragments of apparent molecular masses in the range of 66 kDa to 70 kDa that translocate to the nucleus and bind to SRE-1. When cells are exposed to regulatory sterols, proteolysis of the 125 kDa precursor form of SREBP-1 is interrupted and the amount of the mature nuclear form declines due to rapid turnover (Wang *et al*, 1994).

SRE-1 sequences have been found in the 5'-flanking sequences of genes for the LDL receptor (Osborne *et al*, 1988), HMGCoAS (Yokoyama *et al*, 1993), HMGCoAR (Dawson *et al*, 1988), farnesyl diphosphate synthase (Jackson *et al*, 1995), squalene synthase (Guan *et al*, 1995), fatty acid synthase (Bennett *et al*, 1991) and acetyl CoA carboxylase (Kawabe *et al*, 1996; Lopez *et al*, 1996). In addition to SRE-1 sequences, the promoters for the LDL receptor and HMGCoAR also have sequences that bind to positive transcription factors. In the LDL receptor gene the major positive element is the nuclear factor SP1 (Sudhof *et al*, 1987; Dawson *et al*, 1988). The HMGCoAR promoter contains multiple binding sites for proteins in the nuclear factor-1 (NF-1) family (Gil *et*

al, 1988). These positive elements are required for the functioning of the SRE-1 element. In the LDL receptor and HMGCoAS promoters, the SRE-1 acts as a conditional positive element, enhancing transcription in the absence of sterols. In the HMGCoAR promoter the SRE-1 also acts as a conditional negative element, repressing transcription in the presence of sterols.

Rationale for study

Cholesterol ester hydrolases are a group of ubiquitous enzymes that are present in many tissues and are associated with different functions, depending on location. They catalyze the release of free cholesterol and fatty acids from cholesteryl esters, an intracellular storage form of cholesterol. The major cholesteryl ester hydrolytic activity found in rat liver is associated with a unique cytosolic CEH that is appropriately located, rationally regulated and capable of mobilizing free cholesterol from cholesteryl ester stores.

Rat liver cytosolic CEH has been purified to homogeneity. The cDNA for this enzyme has been cloned and expressed. The purified enzyme has been extensively characterized biochemically and its physiological relevance has been well established. It plays a crucial role in the regulation of hepatic levels of free and esterified cholesterol along with HMGCoA reductase, cholesterol 7α-hydroxylase and acylcoenzyme A:cholesterol acyl transferase. While HMGCoA reductase is actively regulated at the transcriptional, translational and post-translational modes, cholesterol 7α-hydroxylase is primarily regulated at the transcriptional level. CEH has been demonstrated to be regulated at the post-translational level by reversible phosphorylation. Recently

regulation at the transcriptional level was also studied by measuring the steady-state mRNA levels under various conditions.

While most of the data reported to date suggest that CEH is regulated at the transcriptional level, there is no direct evidence to support this mode of regulation. Therefore, isolation and characterization of the 5'-flanking portion of the CEH gene would enable us to understand the regulation of this enzyme at the transcriptional level.

Specific objectives to be addressed are as follows:

1. Using the sequence of the CEH cDNA, isolate and clone the 5'-flanking portion of the CEH gene.
2. Identify the transcription start site of the CEH gene.
3. Identify putative *cis*-acting elements regulating the expression of the CEH gene.
4. Map regulatory regions in the 5'-flanking portion using reporter gene constructs and transient transfection assays in human hepatoblastoma HepG2 cells and cultured primary rat hepatocytes.
5. Determine if the two culture systems used in this study have different effects on the CEH promoter.

EXPERIMENTAL PROCEDURES

Isolation of the rat hepatic CEH promoter

The PromoterFinder DNA Walking Kit (Clontech) was used to isolate the rat hepatic CEH promoter. The kit contains five "libraries" of uncloned adaptor-ligated genomic DNA fragments. The "libraries were prepared by digesting rat genomic DNA with 5 different restriction enzymes that recognize a 6-base site and leave blunt ends, viz. EcoR V, Sca I, Dra I, Pvu II and Ssp I. These fragments were ligated to a PromoterFinder adaptor, the unique features of which eliminates non-specific amplification among the general population of DNA fragments. A combination of long distance PCR, suppression PCR and "touchdown" PCR were then used isolate the 5'-flanking portion of the CEH gene.

The gene specific primer SEQP8 5'- TACCCCCAAGCTGTGCACGCAGCAAG –3', corresponding to positions 56 to 31 of the rat hepatic CEH cDNA and the anchor primer AP1 5'-GTAATACGACTCACTATAGGGC-3' of the PromoterFinder kit were used in a primary PCR with the five different "libraries" of genomic DNA as template. Primary PCR was carried out in 1 X Tth PCR reaction buffer (Clontech), 10 mM each dNTP, 25 mM $Mg(OAc)_2$, 10 μM adaptor primer AP1, 10 μM gene specific primer SEQP8, 1 μl of Tth (2U/μl)/ Vent polymerase (2U/μl)/Tth Start^{TM} Antibody (2.2 mg/μl) (Clontech) mixture (20:1:5 ratio respectively) and 1 μl of each genomic "library".

Thermal cycling parameters were : 7 cycles of 94°C for 25 seconds and 72°C for 4 min, 32 cycles of 94°C for 25 seconds and 67°C for 4 minutes and then a 4 minute extension at 67°C after the final cycle.

Primary PCR products were diluted 1 to 50. One μl of the diluted products were used as template in a secondary PCR with nested gene specific primer PAS3 5'-GCGCATTGTGGAAGGAACAAATAGCCC-3', corresponding to CEH cDNA specific positions 6 to -21 in the 5' untranslated portion, and nested anchor primer AP2 5'-ACTATAGGGCACGCGTGGT-3'. The secondary PCR was carried out in a buffer of the same composition as the primary PCR. Thermal cycling conditions were also identical to the primary PCR except that the number of cycles were reduced from 7 to 5 for the first step and from 32 to 20 for the second step.

The PCR products (5 μl) were analyzed on a 0.6% agarose/TAE gel along with DNA size markers in the form of a 1 kb ladder. The secondary PCR products from "libraries" 3 and 4 were TA-cloned into pGEMT-Easy vector (Promega) and sequenced by the Dye Terminator Cycle Sequencing System.

Construction of rat CEH promoter/luciferase reporter genes

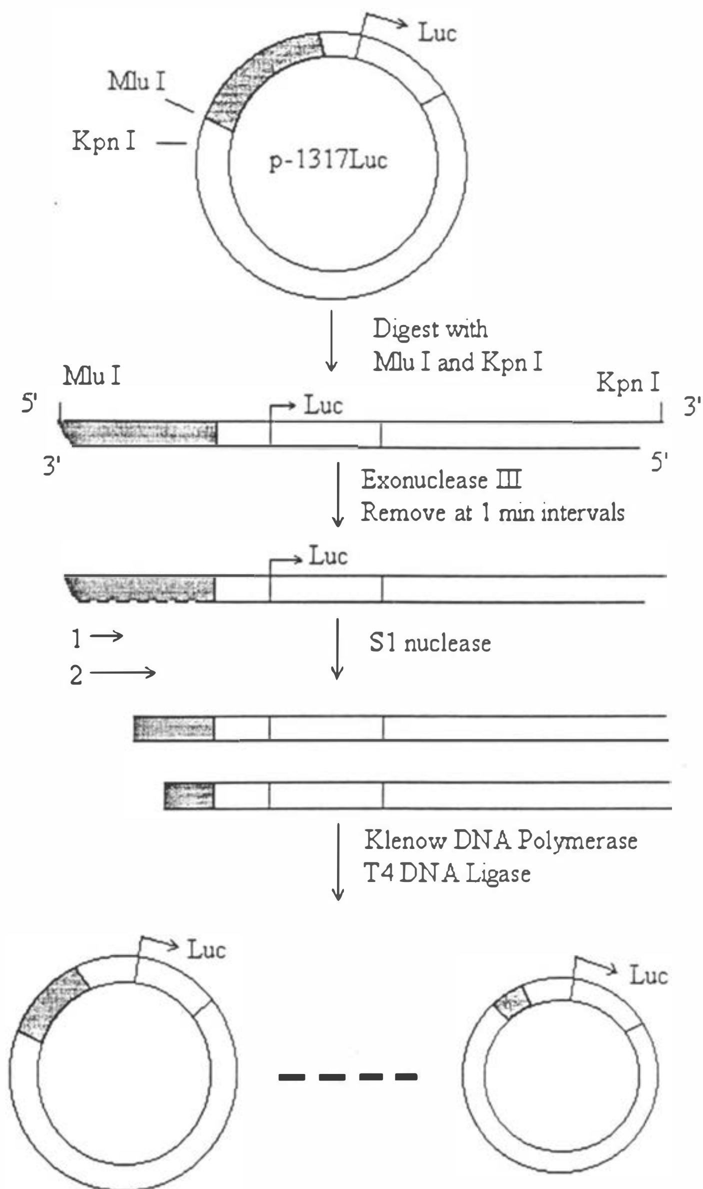
In order to clone the full length promoter into the luciferase reporter vector pGL3-Basic (Promega), PCR was performed with the primer PAS5BglII 5'-gcaagatctGATGACAGAAAGCTCTC -3' (upper case letters indicate gene sequence), bearing a Bgl II restriction site (underlined) and corresponding to position -37 upstream of the initiation ATG codon, and anchor primer AP2 which has a Mlu I restriction site. The 1317 bp PCR product was then cloned into the Bgl II-Mlu I sites of pGL3-Basic to

give p-1317Luc. The remaining constructs were obtained by generation of unidirectional nested deletion breakpoints in p-1317Luc with Exonuclease III (Exo III) which can digest DNA from one end at a uniform rate (Henikoff, 1987). Briefly, p-1317Luc was cut with Kpn I, to generate 3' overhangs resistant to Exo III digestion, and Mlu I, to generate 5' overhangs susceptible to digestion by Exo III (Fig.2). The Kpn I-Mlu I cut p-1317Luc was then digested with Exo III at 30°C. Aliquots were removed at 1 minute intervals , followed by digestion of single stranded ends with S1 nuclease. The ends were filled with Klenow fragment of DNA polymerase, religated with T4 DNA ligase and transformed into TOP10F' cells. All plasmids were verified by restriction digestion analysis and sequencing. They were purified by double banding in cesium chloride gradients or with Qiagen columns (Qiagen) as per manufacturers instructions.

Identification of transcription start site

Primer extension analysis was performed to identify the transcription start site. The CEH cDNA specific primer PAS3 was radiolabelled in 1X T4 polynucleotide kinase buffer with 3 µl of [γ-^{32}P] ATP (at 3000 Ci/mmol, 10 mCi/ml) and 1 µl of T4 polynucleotide kinase (8-10 U/µl) at 37°C for 10 min. The T4 polynucleotide kinase was then inactivated by heating to 90°C for 2 min. The radiolabelled primer (1 pmol) was annealed to rat liver total RNA (15 µg), prepared by cesium chloride density centrifugation (Chirgwin *et al*), in 1X AMV primer extension buffer (100 mM Tris-HCl, pH 8.3, 100 mM KCl, 20 mM $MgCl_2$, 20 mM DTT, 2 mM each dNTP, 1 mM spermidine) with 40 mM sodium pyrophosphate at 58°C for 20 min. The mixture was

Figure2. Strategy for generation of 5' deletion constructs of rat CEH promoter/luciferase reporter genes from p-1317Luc. The shaded region corresponds to the 1317 bp 5'-flanking portion of the rat CEH gene cloned into pGL3-Basic. The full-length construct p-1317Luc was digested with Kpn I and Mlu I to generate 3' and 5' overhangs respectively. The Kpn I-Mlu I cut p-1317Luc was digested with Exonuclease III at 30°C. Aliquots were removed at 1 min intervals followed by treatment with S1 nuclease, Klenow fragment of DNA polymerase and T4 DNA ligase as explained in Experimental Procedures to obtain the 5' deletion constructs.


Luc
Mlu I
Kpn I
p-1317Luc
Digest with
Mlu I and Kpn I
Mlu I
Kpn I
5'
3'
Luc
3'
5'
Exonuclease III
Remove at 1 min intervals
Luc
1
2
S1 nuclease
Klenow DNA Polymerase
T4 DNA Ligase
Luc
Luc

allowed to cool at room temperature for 10 min. The primer was then extended by AMV reverse transcriptase (1.3 U) for 30 min. at $42^{O}C$. Primer extension products were analyzed on an 8% denaturing polyacrylamide gel followed by autoradiography for 16 hrs. Radiolabelled standards were used to determine the size of the primer extension products.

Culture of human hepatoblastoma HepG2 cells

Human hepatoblastoma HepG2 cells were grown in 75 cm^2 tissue culture flasks in Minimal Essential Medium containing L-glutamine (0.292 mg/ml), non-essential 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 $37^{O}C$ 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 confluence. Medium was changed every two days until the cells were confluent. These cells were then used for transient transfection analysis.

Isolation and culture of primary rat hepatocytes

Primary rat hepatocytes used in this study were kindly provided by Dr. P. B. Hylemon (Dept. of Microbiology, MCV/VCU). Hepatocytes were isolated from male Sprague-Dawley rats (250-300 g) using the collagenase perfusion technique of Bissel and Guzelian (1980). Prior to plating, cells were judged to be > 90% viable using Trypan blue exclusion. Parenchymal cells ($8.5x10^5$) were plated in 1.5 ml of William's E medium containing L-thyroxine (1 μM), dexamethasone (50 nM), penicillin (100 U/ml) and 10% fetal calf serum in 35 mm Primaria culture dishes (Falcon) at $37^{O}C$ in a 5% CO_2

atmosphere. After a 6 hr. attachment period, the hepatocytes were used for transient transfection analysis.

Transient transfections

Both HepG2 cells and the primary rat hepatocytes were transiently transfected by the calcium-phosphate DNA coprecipitation technique (Graham and van der Eb, 1973) with the MBS mammalian transfection kit (Stratagene). Specifically, 2.0 μg of test plasmid and 0.5 μg of pCMVß, 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). Culture medium in the dishes were replaced with 2 ml of fresh medium containing 6% modified bovine serum instead of 10% fetal calf serum. Following addition of DNA suspension, cells were incubated for 3 hrs at $35^{O}C$ under 3% CO_2. They were then washed three times with phosphate-buffered saline (PBS) and refed with appropriate serum-free medium with the indicated concentration of agent or vehicle. Dexamethasone was reconstituted in 100% ethanol. L-thyroxine was dissolved in a solution containing 70% ethanol and 15% 6N NaOH. The solution was vortexed well and kept in a $37^{O}C$ water bath for 10 min.. Fetal calf serum (15%) was then added prior to use. PMA was reconstituted in 100% DMSO. Mevalonolactone and squalestatin were reconstituted in water. Squalestatin was kindly provided by Glaxo Research Group, Middlesex, United Kingdom, UB6 OHE. Media containing dexamethasone, L-thyroxine and PMA were replaced after 20 hrs.. Transfected cells were incubated at $37^{O}C$ under 5% CO_2 for different periods of time as indicated in the figure legends.

Reporter enzyme assays

After the incubation period, both HepG2 cells and primary rat hepatocytes were washed twice with PBS, lysed and harvested with 200-300 µl of reporter lysis buffer as per manufacturers instructions (Promega). Luciferase activity was assayed by mixing room temperature luciferase assay reagent (100 µl) to cell extracts (5-20 µl) also at room temperature in a luminometer (Lumat LB9501, Berthold) (Wood, 1991) and measuring total light emission during the initial 20 seconds of the reaction. Luciferase activity was calculated as relative light units (RLU) per mg of protein. ß-galactosidase activity was measured in 50-100 µl of lysate using o-nitrophenyl ß-D-galactopyranoside as substrate (Nielsen *et al*, 1983). The product was detected by measuring the absorbance at 420 nm in a spectrophotometer. ß-galactosidase activity was expressed as units (nmols of o-nitrophenol/min./mg protein). Protein concentrations were determined using the Pierce BCA reagent (Smith *et al*, 1985). Luciferase activity was normalized, for inter-assay variabilities due to transfection efficiency and extract collection, by dividing the luciferase activity by the ß-galactosidase activity.

Statistical analysis

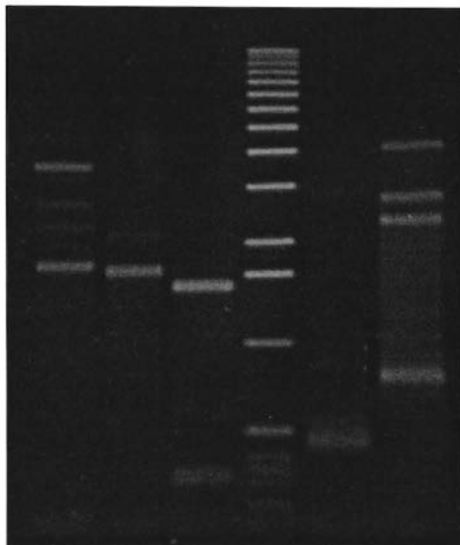
Data was analyzed for statistically significant differences by Student's t-test and $p<0.01$ was considered significant.

RESULTS

Cloning of the rat CEH promoter

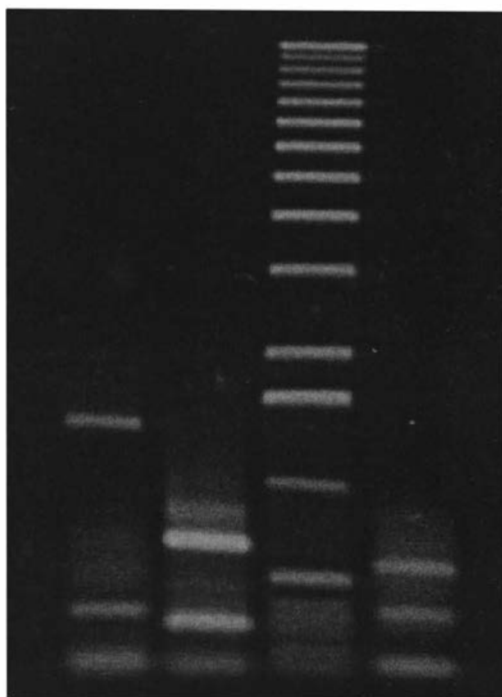
In order to isolate the promoter for the rat CEH gene, we used the PromoterFinder DNA Walking Kit (Clontech). This kit contains five "libraries" of uncloned adaptor-ligated rat genomic DNA fragments. A combination of long-distance PCR, suppression PCR and "touchdown" PCR were used to isolate the 5"-flanking portion of the CEH gene. The primary PCR with primers SEQP8 and AP1 (see experimental procedures) yielded products ranging from 250 bp to 4 kb in all five "libraries" (Fig.3). Primary PCR products were diluted and used as template for a secondary PCR with nested primers PAS3 and AP2 (see experimental procedures). Libraries 3 and 4 gave approximately 1.3 kb and 400 bp products respectively (Fig.4), in addition to other smaller size products. The 1.3 kb and 400 bp pieces were then TA-cloned into pGEMT-Easy vector and sequenced with the universal T7 and SP6 primers. The 1.3 kb product from library 3 overlapped with the 400 bp product from library 4 and was identical to it in sequence. Analysis of this sequence revealed the presence of several putative *cis*-acting elements in the 5'-flanking region upstream from the first ATG codon. These included hormone response elements (TRE, GRE, IRS), liver-specific elements (TGT3, HNF), ubiquitous transcription factor binding sites (NF-Y, AP1, SP1) and sterol responsive elements (SRE).

Figure 3. Agarose gel analysis of PCR products amplified from rat PromoterFinder libraries using anchor primer AP1 and cDNA specific primer SEQP8. Primary PCR was performed using the protocol for the PromoterFinder kit as explained in Experimental Procedures. Lanes 1-3, 5 and 6 correspond to PCR products amplified from libraries 1, 2, 3, 4 and 5 respectively. Lane 4 is a 1 kb DNA ladder (GIBCO BRL).



1 2 3 4 5 6

Figure 4. Agarose gel analysis of PCR products amplified from rat PromoterFinder libraries using anchor primer AP2 and nested cDNA specific primer PAS3. Secondary PCR was performed using the protocol for the PromoterFinder kit as explained in Experimental Procedures. Lanes 1, 2 and 4 correspond to secondary PCR products amplified from libraries 3, 4 and 5 respectively. Lane 3 is a 1 kb DNA ladder (GIBCO BRL).



1 2 3 4

Figure 5. Nucleotide sequence of the rat CEH promoter. The DNA sequence shown contains 1317 nucleotides of 5'-flanking DNA upstream of the ATG initiation codon. Bases in lower case are identical to the reported CEH cDNA sequence. The transcription start site identified by primer extension analysis of total liver RNA (shown in Fig. 6) is indicated by an asterisk (*). Putative transcription factor binding sites are either underlined or boxed. Sequence identity of the SRE-1 elements to the consensus SRE-1 sequence are indicated. AP1: activator protein 1; SRE-1: sterol response element-1; HRE: hormone response element ; NF-Y: nuclear factor-Y ; C/EBP: CCAAT/ enhancer binding protein ; PRS: phorbol ester response sequence ; IRS: insulin response sequence ; GRE: glucocorticoid response element ; HNF3: hepatocyte nuclear factor 3.

```
                                      HNF3                AP1
-1317 AAACAATAAAGGAAGTCTTAGCAAACATAAAGGATTGATTAACTAAATAT

-1267 AAAACGTACAATAATTTCTTTCATTGTGCTAGTTCTTAAGGGAATAAAAC
          GRE/IRS/HNF3
-1217 CAGAAAAACAAGAAAAATCTGTAGAAATGGAGACTGAACAATATAATTCA

-1167 CAATGAACAGCGATTCACTGAAGAAATAGGGGAAAGGGAGGGTTGTAAAA

-1117 TTCCTTAAGTGAAATAAGAACAGAAATACCGCATATAGGACTGAGATAAA

-1067 ACAAAAGTTGTTCTGGGGGAAATTCTCTATCATGACCATCTACAGTCTTA
                                                     AP1
-1017 TGTCTTGAAGAAGACATAATTATGCATCAAAGGGACTTAAATTCACAGAA

-967  TAGTACCTCCCTGGGCTGTAGAATACTTATTAGTTTATCAGCTCATGGAA

-917  ATTTTTCCAAAATAGATAAGTTGAAGAGATTTGATCTTAAAGGAACTAAA
                                   C/EBP
-867  GATTCCTAAAAATGAAGCAAATGATGTAGTAATGCATCCAGAGAACTTAA
                              GRE/IRS/PRS
-817  AAATAAGTGGTTGGGTAAAACAACCAAATACACAACAGGGGAATGAAAAG

-767  AGGTAGCCATACACAAAAGCATGAAGAGGCTACACTGTTGGAACAGGCTA

-717  GCCAGCAATTCCAGAGACACACCACTTTGCTGTACATTGTGCATTCCCAC

-667  GAAGAGAGAGGCCATGTACTTCAGTAAATGTTTGAAGAATCAGTGCACAA

-617  GTACATTCATGGATTATGAGGTGCCAGTAGAGTTTGTTTACCATATAAAC

-567  GTTATGTCCAAAGGTTCTTTCTCTGAGAGGTAAACTTCCTTGAGCCTGAG

-517  TATGCACCTTGCCTAGCCAAGTCACTTGACAGGAATACTGTGAAGATAAT

-467  TTAAGAATCGTCTGAAGGTCTGTAGGTGGTGAGGTTTCTTCCTAACTTGA

-417  GAATATGGATTCTAGGAAATGCCAGTCCCAGAGAGGCTGTGAGAAATGTG

-367  TTCTCACTTCCCATCTGAGCAGAGTGGGAATGAGAGGTATTCTAATATGT
        IRS/PRS                               SRE1 7/8
-317  TCATCTTTGTAAAAGGACCCCAGACCAACCCTTGCCACTAACTGCAGGCA
                                        PRS
-267  GCTGCCTGCTGCTGTCTGCTCTTGAGAGTTCAAGAGCATTGAATTGAGGT
                                  C/EBP
-217  GAGAGTGCTGGAGGGAAAACTGCTTATGTAAGAAGCTGTTGGATGAGTTT
       SRE1 6/8 HRE      AP1      NF-Y      AP1     HRE
-167  CTAGCCACTTGTAATCTGAGTATTGGGTACTGACTGTTCAGATAAAAGTG
                     SP1  SRE1 7/8
-117  GGTAAACTCTTGGTGGGCGTGGCTTGGAGATCCCAACTGGCACCCAGAGA
           *                   AP1
-67   GCTCTTTGGAAGGGAGAGCTTTTCTGTCATCTTAAATTACatctgtgggc
                          Met  Arg
-17   tatttgttccttccacaatgcgc
```

Fig.5 shows the nucleotide sequence of the cloned 1.3 kb of the promoter for the rat CEH gene as well as some of the putative *cis*-acting elements.

Primer Extension Analysis

Primer extension analysis was performed to identify the transcription start site. The CEH cDNA specific primer PAS3 was radiolabelled with [γ-^{32}P] ATP and annealed to rat liver total RNA (15 μg). The primer was extended with AMV reverse transcriptase (1.3 U) for 30 min. at 42°C. The primer extension products were analyzed on an 8% denaturing polyacrylamide gel followed by autoradiography for 16 hrs. (Fig.6, lane 2). Radiolabelled standards were used to determine the size of the primer extension product (lane 1). The primer by itself (lane 3) did not give any product. However the primer in the presence of RNA gave a single 66 bp product. Comparison of the length of this product to the DNA sequence of the 5'-flanking region allowed the approximate positioning of the 5' end of the CEH mRNA at nucleotide -60 upstream from the initiation ATG codon (Fig.5). Although no canonical TATA-box sequences were found around the transcription start site, a consensus GC-box which binds the positive transcription factor SP1, was found 35 bases upstream from the start site (Fig.5)

Generation of rat CEH promoter/luciferase chimeric genes

About 1.3 kb of the promoter was cloned into the luciferase reporter vector pGL3-Basic as detailed in Experimental Procedures, to generate p-1317Luc. The remaining constructs were obtained by generation of unidirectional nested deletion breakpoints in p-1317Luc with Exonuclease III. Fig.7 shows the products generated by this procedure.

Figure 6. Determination of transcription start site of rat CEH gene by primer extension analysis. Radiolabelled primer PAS3 corresponding to regions 6 to –21 of the published cDNA sequence was annealed to total liver RNA (15 μg) and extended with AMV reverse transcriptase (1.3 U) as explained in Experimental Procedures. DNA size markers are shown in lane 1. The primer extension product corresponding to 60 nucleotides upstream of the ATG initiation codon is shown in lane 2. Radiolabelled primer by itself is shown in lane 3.

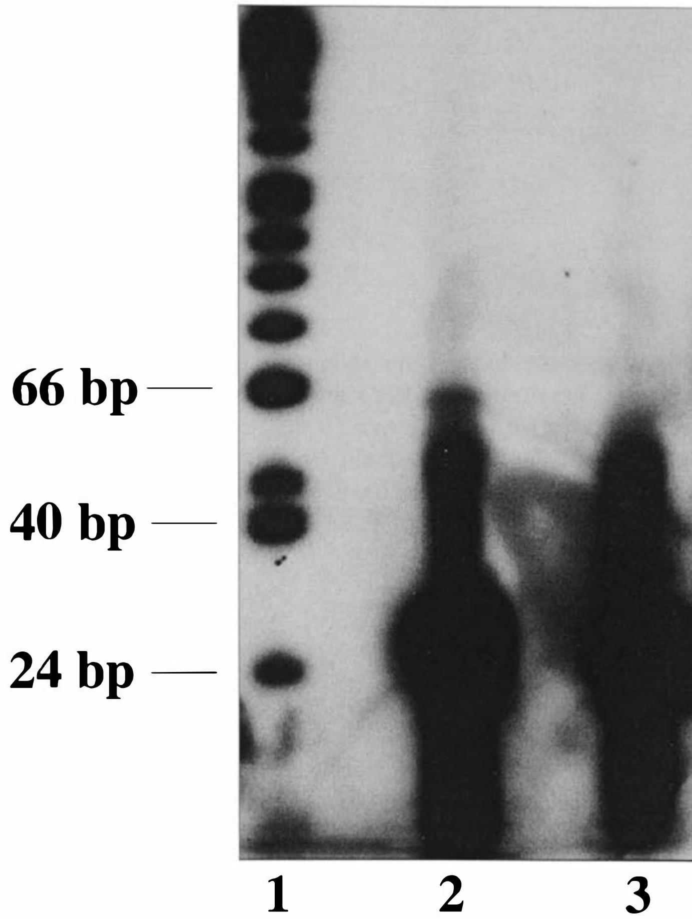
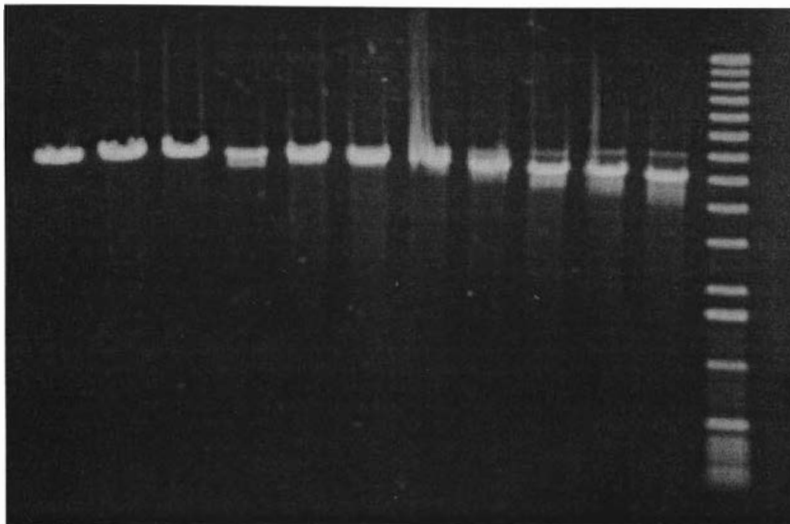

66 bp
40 bp
24 bp
1
2
3

Figure 7. Agarose gel analysis of deletion products obtained by digestion of p-1317Luc by Exonuclease III for generation of rat CEH promoter/luciferase chimeric genes. The full length construct p-1317Luc (lane 1) was digested with Exonuclease III as explained in Experimental Procedures. Aliquots removed at 1 min intervals were run on a 0.8% agarose gel (lanes 2-11). Size of the deleted products were estimated by comparison to a 1 kb DNA ladder (GIBCO BRL).



1 2 3 4 5 6 7 8 9 10 11 12

Aliquots removed at 1 min. intervals were run on a 0.8% agarose gel along with linearized p-1317Luc (Fig.7, lane 1). A 1 kb DNA ladder was run on the gel in order to estimate the size of the products obtained. These products were re-ligated and transformed into TOP10F' cells. The plasmids obtained were digested with restriction enzymes and sequenced with universal T7 and SP6 sequencing primers to determine the exact position of the breakpoint. They were then purified by double banding in cesium chloride or with Qiagen columns.

Promoter activity in HepG2 cells

Determination of basal CEH promoter activity

To determine the basal promoter activity of the rat CEH gene, confluent HepG2 cells were transfected with CEH promoter/luciferase chimeric genes shown in Fig.8. As shown in Fig.9 full-length and deletion clones were more active than the promoter-less pGL3-Basic by itself. CEH/luciferase chimeric genes downstream of, and including p-599Luc increased luciferase reporter activity 1.6 to 2 fold as compared to the longest construct p-1317Luc. This suggests that positive *cis*-acting elements are located downstream of nt-599 and repressor sequences upstream of nt-599.

Effect of dexamethasone on CEH promoter activity

Previous studies have shown that glucocorticoids can either stimulate or inhibit CEH activity when administered *in vivo* (Gandarias *et al*, 1984; Grogan *et al*, 1991). In cultured primary rat hepatocytes, dexamethasone was required to maintain steady-state CEH mRNA levels (Ghosh *et al*, 1997; manuscript submitted). When HepG2 cells were transfected with p-1317Luc and treated with increasing concentrations of dexamethasone,

Figure 8. Illustration of rat CEH/luciferase chimeric genes used in this study. Deletion clones were generated by PCR or by Exonuclease III digestion as described in Experimental Procedures. Numbers are relative to the ATG initiation codon of the CEH cDNA.

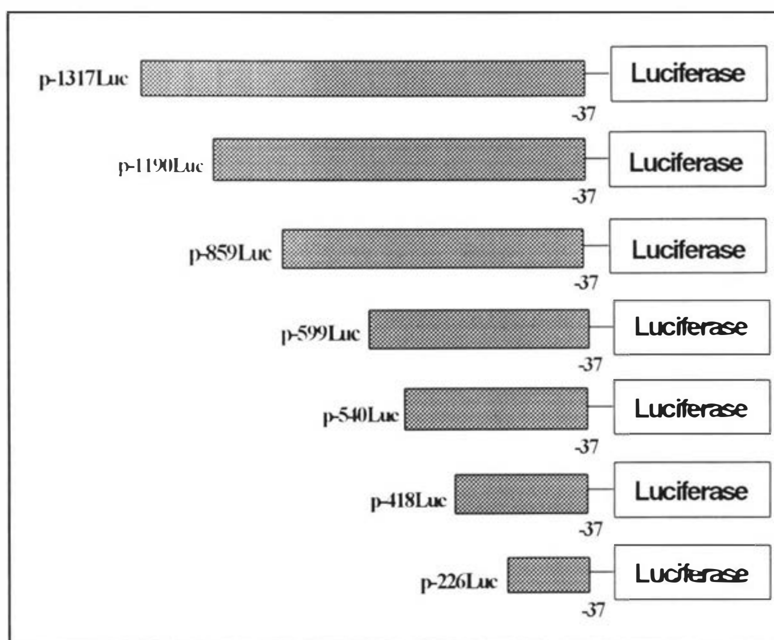

p-1317Luc
-37
Luciferase
p-1190Luc
-37
Luciferase
p-859Luc
-37
Luciferase
p-599Luc
-37
Luciferase
p-540Luc
-37
Luciferase
p-418Luc
-37
Luciferase
p-226Luc
-37
Luciferase

Figure 9. Basal promoter activity of the rat CEH/luciferase chimeric genes transfected into HepG2 cells. Confluent HepG2 cultures were transfected as described under Experimental Procedures and incubated for 40 hrs. in serum free medium. At the end of the incubation, cells were harvested and reporter enzyme activities were determined as described. Normalized promoter activities are expressed as percentage of control (pGL3-Basic) and represent the mean ± S. E. M. of 3 independent determinations; (*) indicates difference at $p<0.001$.

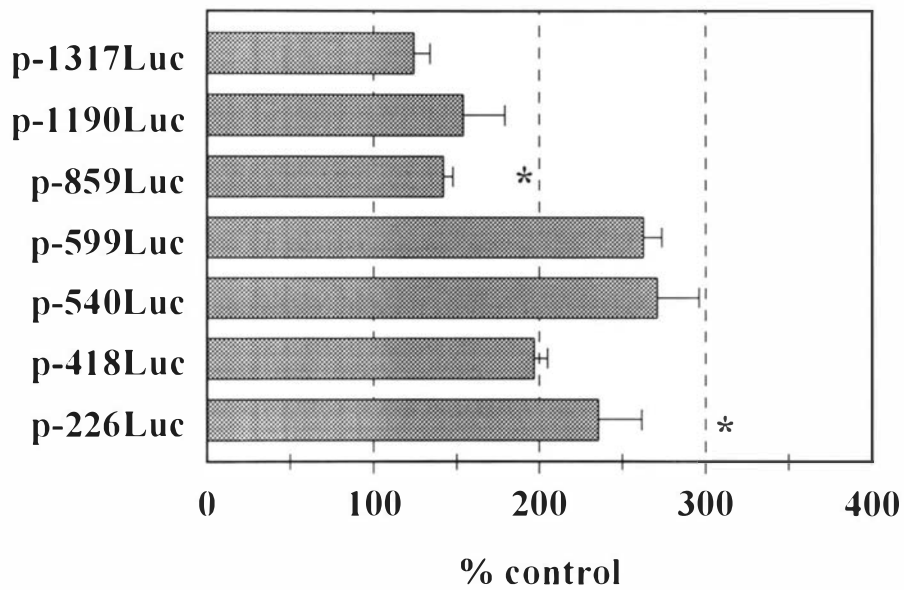
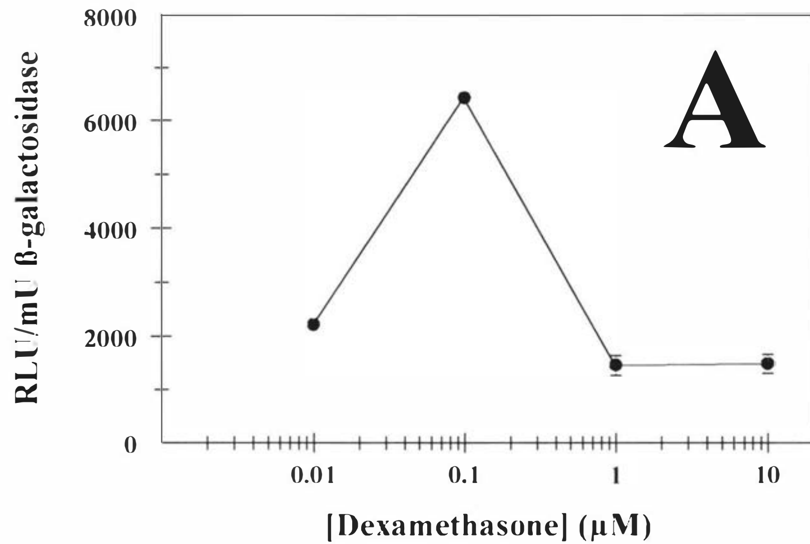
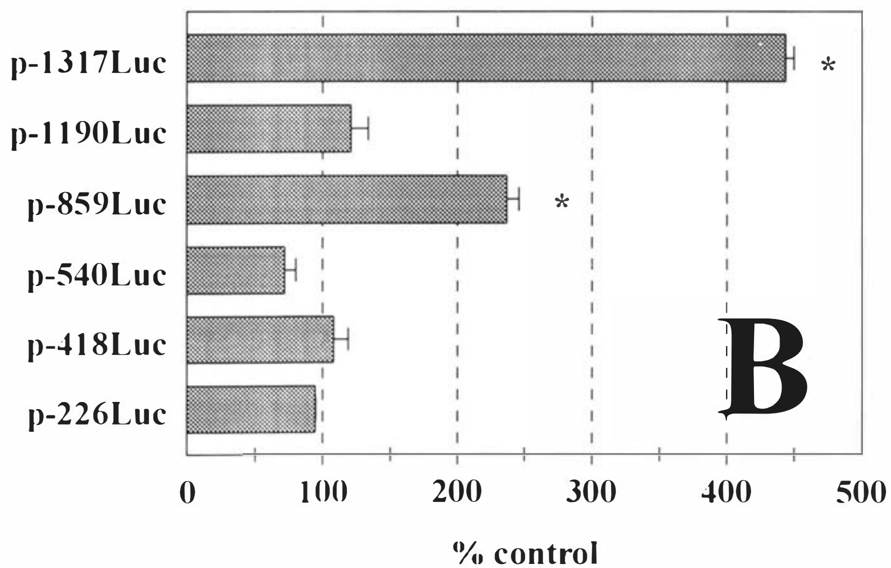

p-1317Luc
p-1190Luc
p-859Luc
p-599Luc
p-540Luc
p-418Luc
p-226Luc
*
*
0
100
200
300
400
% control

Figure 10. Effect of dexamethasone on the promoter activity of the CEH gene in HepG2 cells. **(A)** Confluent HepG2 cultures were transfected with p-1317Luc and incubated for 40 hrs. in serum free medium containing increasing concentrations of dexamethasone. Results are expressed as the ratio of luciferase and β-galactosidase activities and represent the mean ± S. E. M. of 3 independent determinations. Basal activity of p-1317Luc in the absence of dexamethasone was 1453 RLU/mU β-galactosidase. **(B)** Confluent HepG2 cultures were transfected with the indicated rat CEH/luciferase chimeric genes and incubated for 40 hrs. in serum free medium containing either 0.1 μM dexamethasone in ethanol or ethanol alone. Normalized promoter activities are expressed as percentage of control and represent the mean ± S. E. M. of 3 independent determinations; (*) indicates difference at $p<0.001$.



8000
6000
4000
2000
0
RLU/mU ß-galactosidase
A
0.01
0.1
1
10
[Dexamethasone] (µM)



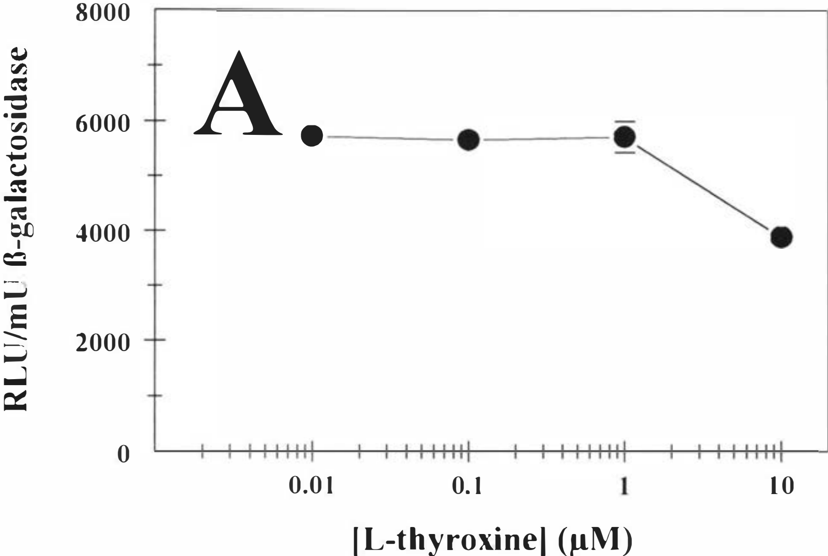
p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
*
*
B
0
100
200
300
400
500
% control

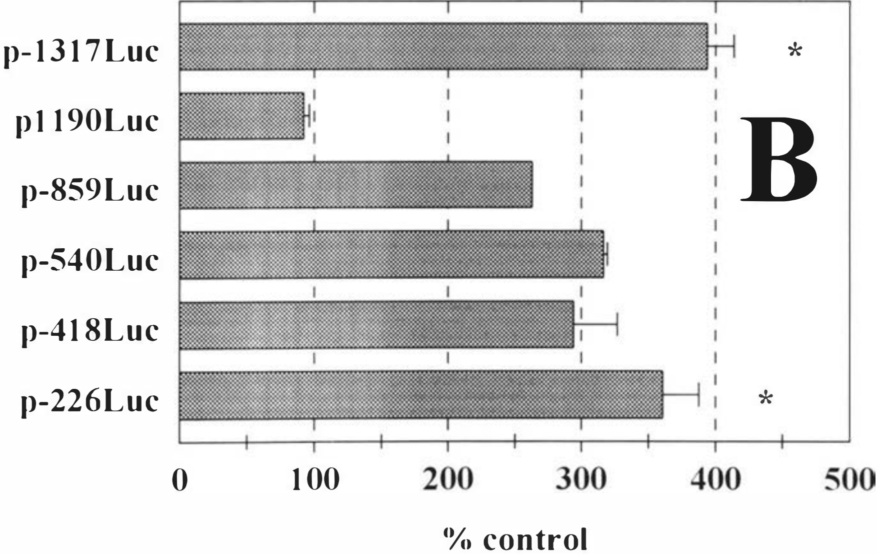
promoter activity increased approximately 4.5 fold at 0.1 μM dexamethasone (Fig.10A). In order to map the regions that respond to dexamethasone, reporter activity of each construct was measured in the presence of 0.1 μM dexamethasone (Fig.10B). Deletion of the promoter to nt-1190 eliminated the induction observed with the full-length construct. Further deletion down to nt-859 restored the activation to 2.4 fold. However the remaining constructs did not show any induction by dexamethasone suggesting that two regions mediate the glucocorticoid effect, one located between nt-1317 and nt-1190 and the other between nt-859 and nt-540.

Regulation of CEH promoter activity by thyroid hormones

Earlier work from this laboratory showed that L-thyroxine (T4) was required in conjunction with dexamethasone to maintain steady-state CEH mRNA levels in cultured rat primary hepatocytes (Ghosh *et al*, 1997; manuscript submitted). Hoekman *et al* (1993) reported that the C7αH gene is activated by T4. Karam and Chiang also observed that T4 increases C7αH mRNA levels in HepG2 cells. HMGCoAR mRNA also increased in the presence of T4 although this increase resulted from stabilization of the mRNA rather than increased transcription (Simonet and Ness, 1989). Therefore, we tested the effect of L-thyroxine on the transcriptional activity of the CEH promoter. As shown in Fig.11A, 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 of all the deletion clones tested except for p-1190Luc, for which the activity stayed at basal levels (Fig.11B). Therefore, two regions respond to T4, one between nt-1317 and nt-1190 and the other between nt-226 and nt-37.

Figure 11. Effect of L-thyroxine on the promoter activity of the rat CEH gene in HepG2 cells. **(A)** Confluent cultures of HepG2 cells were transfected with p-1317Luc and incubated for 40 hrs. in serum free media containing increasing concentrations of L-Thyroxine. The results are expressed as the ratio of luciferase and β-galactosidase activities and represent the mean ± S. E. M. of 3 independent determinations. Basal activity of p-1317Luc in the absence of L-thyroxine was 1453 RLU/mU β-galactosidase. **(B)** Confluent HepG2 cells were transfected with the indicated rat CEH/luciferase chimeric genes and incubated for 40 hrs. in serum free medium in the presence of 1.0 μM L-Thyroxine or vehicle alone. Results are expressed as the ratio of luciferase and β-galactosidase activities and represent the mean ± S. E. M. of 3 independent determinations; (*) indicates difference at $p<0.001$.


A.
RLU/mU ß-galactosidase
8000
6000
4000
2000
0
0.01
0.1
1
10
[L-thyroxine] (μM)

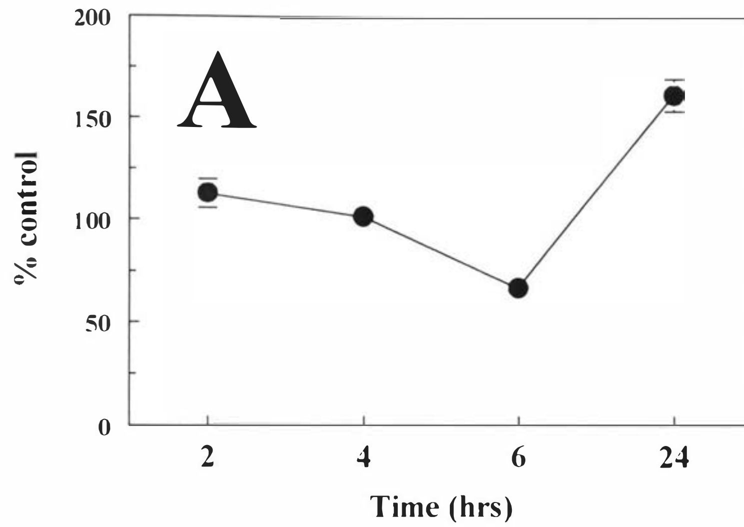

B
p-1317Luc
p1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
*
*
0
100
200
300
400
500
% control

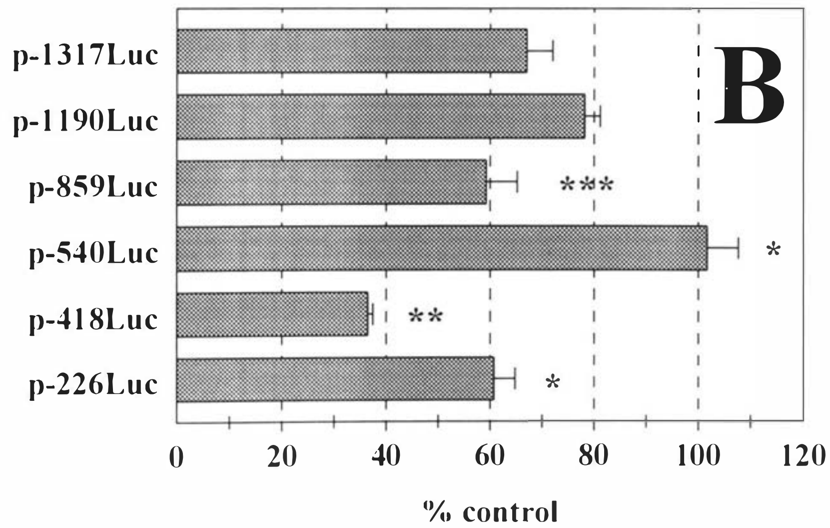
Effect of phorbol esters on the CEH promoter activity

Previous studies from this laboratory have shown that CEH is activated *in vitro* by reversible phosphorylation (Ghosh and Grogan, 1989). On the other hand, the protein kinase C (PKC) activator, 4β-phorbol-12-myristate-13-acetate (PMA), decreased CEH mRNA by 49% in cultured rat primary hepatocytes (Ghosh *et al*, 1997; manuscript submitted). This maximal decrease was observed after a 6 hr. incubation in PMA. In order to determine the involvement of a PKC-mediated signal transduction pathway in the regulation of CEH gene transcription, transfection experiments were performed in the presence of 1 μM PMA. Since long exposures to PMA depletes PKC activity in cells (Young *et al*, 1987), a time course experiment was performed to determine the optimal incubation time in PMA. When HepG2 cells were transfected with p-1317Luc and exposed to 1 μM PMA for different periods of time, the promoter activity was decreased by 34% after a 6 hr. incubation in PMA (Fig.12A). Further exposure to PMA for 24 hrs. caused the promoter activity to increase to 160% suggesting that the optimal incubation time in PMA was 6 hrs. In order to map the regions that respond to PMA, HepG2 cells were transfected with various CEH promoter/luciferase chimeric genes and exposed to 1μM PMA for 6 hrs. As seen in Fig.12B, the promoter activity of all the deletion clones except for p-540Luc were repressed. The maximal decrease was observed with p-418Luc, the activity of which was only about 36% of control, suggesting the presence of negative phorbol ester responsive sequences (PRS) in the region from nt-859 to nt-541, nt-418 to nt-227 and nt-226 to nt-37 and a positive PRS between nt-540 to nt-419.

Effect of agents that perturb cholesterol metabolism on CEH promoter activity

Figure 12. Effect of phorbol esters on the promoter activity of the rat CEH/luciferase chimeric genes in HepG2 cells. **(A)** Confluent HepG2 cells were transfected with p-1317Luc and fed with serum free medium containing 1 μM PMA in DMSO or DMSO alone. Cells were harvested at the indicated times and reporter enzyme activities were determined. Results are expressed as percentage of control of normalized luciferase activity and represent the mean ± S. E. M. of 3 independent observations. **(B)** Confluent HepG2 cells were transfected with the indicated rat CEH/luciferase gene constructs. They were incubated in serum free medium with 1 μM PMA in DMSO or DMSO alone for 6 hrs. and then harvested. Normalized promoter activities are expressed as percentage of control and represent the mean ± S. E. M. of 3 independent obsevations. (*) indicates difference at $p<0.001$; (**) indicates difference at $p<0.005$; (***) indicates difference at $p<0.01$.

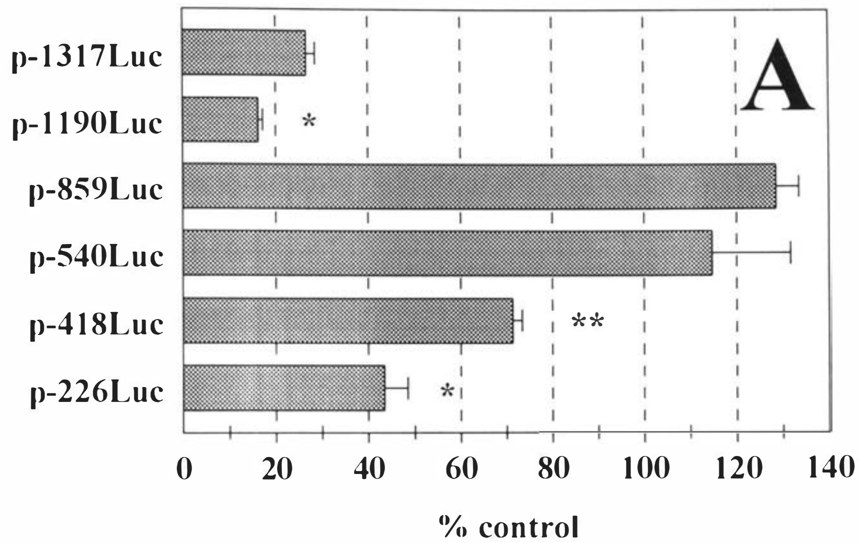

A
200
150
100
50
0
% control
2
4
6
24
Time (hrs)


B
p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
***
*
**
*
0
20
40
60
80
100
120
% control

Perturbations of cellular cholesterol metabolism by agents that increase or decrease levels of intracellular cholesterol have a marked effect on CEH mRNA, protein and activity. Intravenous infusion of mevalonate, an agent known to increase levels of intracellular cholesterol, caused a compensatory decrease in CEH activity and protein mass (Ghosh *et al*, 1997; manuscript submitted). Moreover, lovastatin, a potent competitive inhibitor of HMGCoAR, increased CEH mRNA 2 fold in cultured primary rat hepatocytes (Ghosh *et al*, 1997; manuscript submitted). Therefore, in order to determine the sterol responsiveness of the CEH gene, we tested the effect of mevalonate and squalestatin, an inhibitor of squalene synthase and cholesterol biosynthesis, on the transcriptional activity of the CEH promoter. Mevalonate (2 mM) treatment for 24 hrs. repressed reporter gene activity of p-1317Luc and p-1190Luc to 26% and 16% of control respectively (Fig.13A). Luciferase reporter activity was restored to basal levels in p-859Luc. However activity of p-226Luc was only 43% of control, suggesting that sterol response elements (SRE) are present between nt-1190 and nt-859, nt-418 and nt-227, and betwen nt-226 and nt-37. Simultaneous treatment for 24 hrs. with 2 mM mevalonate and 1 μM squalestatin restored the activity of p-226Luc (Fig.13B), but failed to bring the activity of p-1190Luc back to basal levels. It therefore appears that strong negative SRE's may be located between nt-226 and nt-37 and a positive SRE may be present between nt-418 and nt-227. Inasmuch as squalestatin did not reverse the inhibition associated with p-1190Luc, it is possible that the region between nt-1190 and nt-859 is uniquely responsive to non-sterols.

The positions of the variuos GRE's, TRE's, PRS and SRE's in the rat CEH promoter, that were identified by transient transfections in human hepatoblastoma HepG2

Figure 13. Effect of cholesterol perturbing agents on the promoter activity of rat CEH/luciferase chimeric genes in HepG2 cells. Confluent HepG2 cultures were transfected with the indicated rat CEH/luciferase promoter constructs and incubated for 24 hrs. in serum free medium in the presence or absence of 2 mM mevalonolactone alone, (A) or 2 mM mevalonolactone and 1 μM squalestatin, (B). Normalized promoter activities are expressed as percentage of control and represent the mean ± S. E. M. of 3 separate observations; (*) indicates difference at $p<0.001$; (**) indicates difference at $p<0.005$; (***) indicates difference at $p<0.01$.


p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
A
*
**
*
0
20
40
60
80
100
120
140
% control

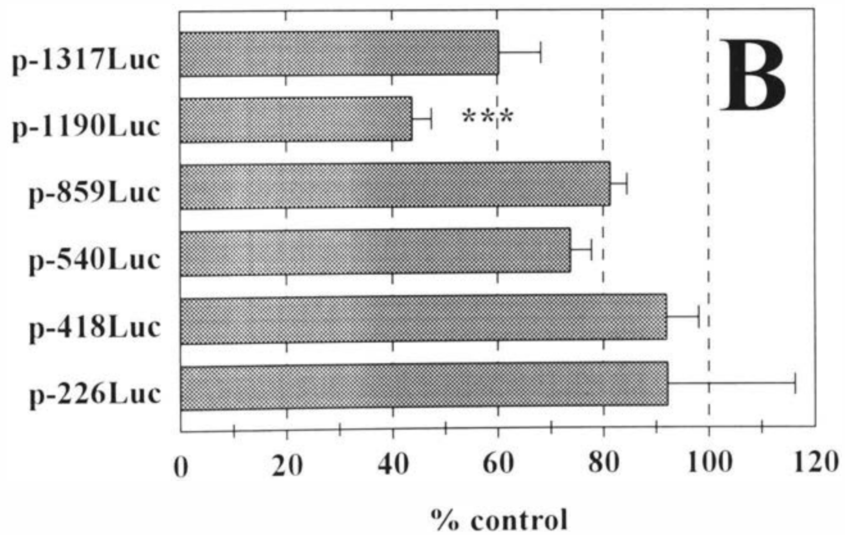
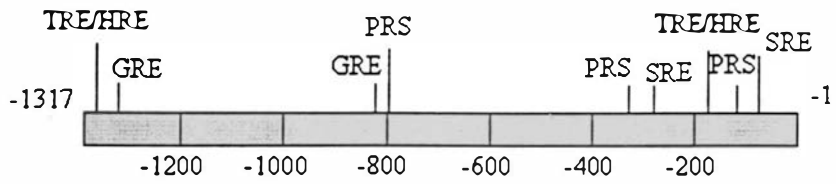

p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
B
***
0
20
40
60
80
100
120
% control

Figure 14. Position of *cis*-acting elements in the rat CEH promoter identified by transient transfections in HepG2 cells. This figure shows the approximate positions of the various response elements identified in the rat CEH promoter by transient transfections in HepG2 cells. The mapping of each individual response element is explained in the Results section. GRE: glucocorticoid response element ; HRE: hormone response element ; PRS: phorbol ester response element ; SRE: sterol response element ; TRE: thyroid hormone response element.


TRE/HRE
GRE
PRS
GRE
PRS
SRE
TRE/HRE
PRS
SRE
-1317
-1
-1200
-1000
-800
-600
-400
-200

cells, is shown in Fig. 14.

Promoter activity in primary rat hepatocytes

Determination of basal CEH promoter activity

The basal CEH promoter activity in primary rat hepatocytes was determined by transfection of cultured hepatocytes with the different CEH promoter/luciferase chimeric genes shown in Fig.8. As shown in Fig.15, full-length and deletion clones were more active than the promoter-less pGL3-Basic by itself. The CEH/luciferase chimeric genes downstream of, and including p-599Luc increased luciferase reporter activity 1.6 to 4 fold as compared to the longest construct p-1317Luc. This suggests that positive *cis*-acting elements may be located downstream of nt-599 and repressor sequences upstream of nt-599.

Effect of dexamethasone on CEH promoter activity

As shown earlier, dexamethasone at 0.1 μM increased CEH promoter activity in HepG2 cells. The same concentration of dexamethasone was also required, in conjunction with T4 to maintain the CEH steady state mRNA levels in cultured rat primary hepatocytes (Ghosh *et al*, 1997; manuscript submitted). Moreover, the C7αH promoter was maximally responsive to dexamethasone at 0.1 to 1.0 μM (Crestani *et al*, 1995). Therefore, in order to map the regions that respond to dexamethasone in primary rat hepatocytes, they were transfected with different CEH promoter/luciferase chimeric genes and incubated for 40 hrs in the presence or absence of 0.1 μM dexamethasone (Fig.16). The smallest construct p-226Luc increased luciferase reporter activity 4.5 fold. The activity of p-418Luc was only 1.8 fold. However p-540Luc also induced promoter

Figure 15. Basal promoter activity of the rat CEH/luciferase chimeric genes transfected into primary rat hepatocytes. Cultured primary rat hepatocytes were transfected as described under Experimental Procedures and incubated for 40 hrs. in serum free medium. At the end of the incubation, cells were harvested and reporter enzyme activities were determined as described. Normalized promoter activities are expressed as percentage of control (pGL3-Basic) and represent the mean ± S. E. M. of 3 independent determinations; (*) indicates difference at $p<0.001$.

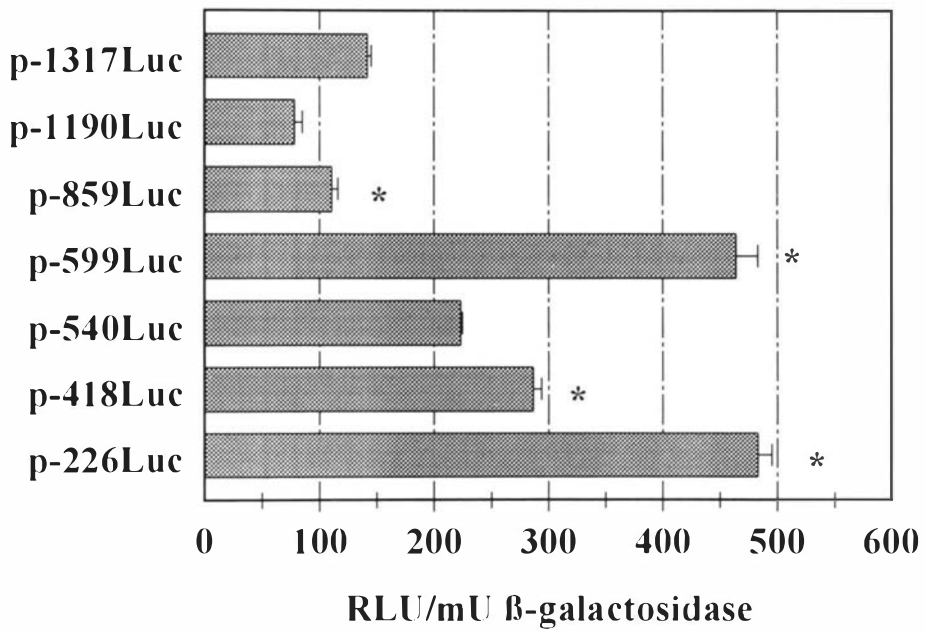
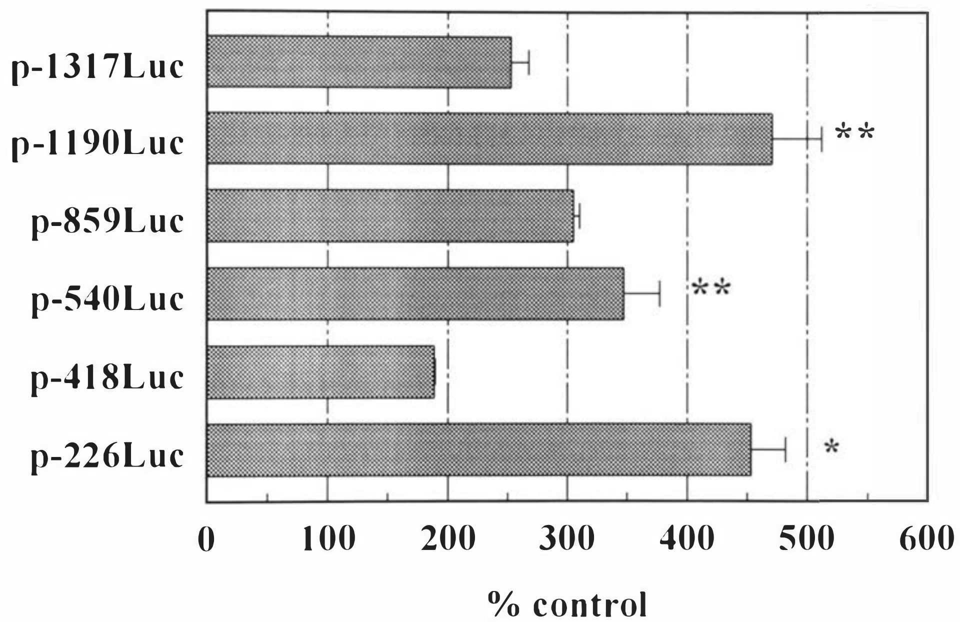

p-1317Luc
p-1190Luc
p-859Luc
p-599Luc
p-540Luc
p-418Luc
p-226Luc
*
*
*
*
0
100
200
300
400
500
600
RLU/mU ß-galactosidase

Figure 16. Effect of dexamethasone on the promoter activity of the CEH gene in primary rat hepatocytes. Cultured primary rat hepatocytes were transfected with the indicated rat CEH/luciferase chimeric genes and incubated for 40 hrs. in serum free medium containing either 0.1 μM dexamethasone in ethanol or ethanol alone. Normalized promoter activities are expressed as percentage of control and represent the mean ± S. E. M. of 3 independent determinations; (*) indicates difference at $p<0.001$; (**) indicates difference at $p<0.005$.


p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
**
**
*
0
100
200
300
400
500
600
% control

activity to 3.5 fold of control. Similarly p-1190Luc stimulated the promoter activity 4.7 fold. These results suggest that dexamethasone induces CEH promoter activity in primary rat hepatocytes through three regions, one located between nt-1190 and nt-859, one between nt-540 and nt-418 and the third between nt-226 and nt-37.

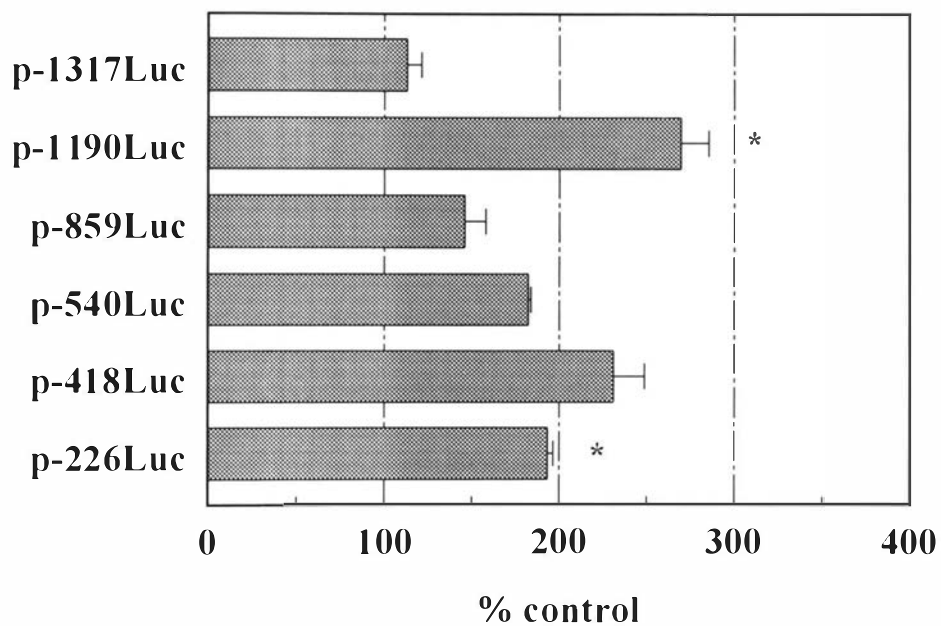
Regulation of CEH promoter activity by L-thyroxine

Previous work from this laboratory showed that T4 (1.0 μM) was required with dexamethasone to maintain CEH steady state mRNA levels (Ghosh *et al*, 1997; manuscript submitted). As shown earlier T4 stimulated CEH promoter activity in HepG2 cells at 0.01 - 1.0 μM. Therefore we tested the effect of T4 (1.0 μM) on the transcriptional activity of the CEH promoter in primary rat hepatocytes (Fig.17). T4 induced the promoter activity of p-1190 by 2.7 fold. Deleting down to p-859Luc decreased the induction to 1.5 fold. The remaining constructs demonstrated the same level of induction as p-859Luc suggesting that two regions mediate the T4 effect in hepatocytes, one located between nt-1190 and nt-859 and the other between nt-226 and nt-37.

Effect of phorbol esters on the CEH promoter activity

As mentioned before, the PKC activator, PMA, decreased CEH mRNA by 49% in rat primary hepatocytes (Ghosh *et al*, 1997; manuscript submitted). CEH promoter activity was also decreased by 64% in HepG2 cells after a 6 hr incubation in PMA. In order to determine the involvement of a PKC-mediated signal transduction pathway in the regulation of CEH gene transcription in primary rat hepatocytes, transfection

Figure 17. Effect of L-thyroxine on the promoter activity of the rat CEH gene in primary rat hepatocytes. Cultured primary rat hepatocytes were transfected with the indicated rat CEH/luciferase chimeric genes and incubated for 40 hrs. in serum free medium in the presence of 1.0 μM L-thyroxine or vehicle alone. Results are expressed as the ratio of luciferase and β-galactosidase activities and represent the mean ± S. E. M. of 3 independent determinations; (*) indicates difference at $p<0.001$.

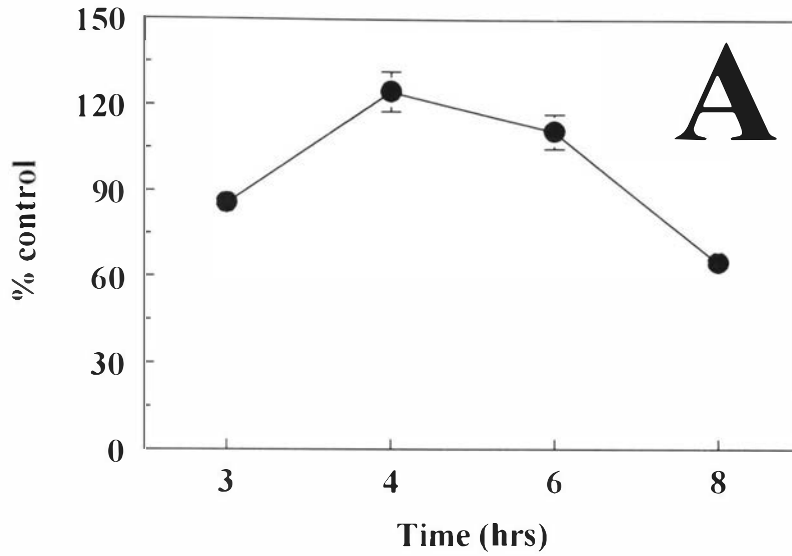

p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
*
*
0
100
200
300
400
% control

experiments were performed in the presence of PMA (1 μM). A time course experiment was first performed to determine the optimal incubation time in PMA. When primary rat hepatocytes were transfected with p-1317Luc and exposed to PMA for different periods of time, the promoter activity was decreased by 35% after an 8 hr. incubation in PMA (Fig.18A). To determine the PMA responsive regions, hepatocytes were transfected with various CEH promoter/luciferase chimeric genes and exposed to 1 μM PMA for 8 hrs. As seen in Fig.18B only the promoter activity of p-1317Luc was repressed. Therefore a negative PRS may be located in the region between nt-1317 and nt-1190.

Effect of agents that perturb cholesterol metabolism on CEH promoter activity

As mentioned before perturbations of cellular cholesterol metabolism have a marked affect on CEH mRNA, protein and activity. Therefore, in primary hepatocytes, as with HepG2 cells, we tested the effect of mevalonate and squalestatin on the transcriptional activity of the CEH promoter. Mevalonate (2 mM) treatment for 24 hrs. repressed the reporter activity of p-1317Luc to 49% of control (Fig.19A). Luciferase reporter activity was restored to basal levels in p-1190Luc. However activity of p-226Luc was only 74% of control, suggesting that sterol response elements (SRE) are present between nt-1317 and nt-1190 and between nt-226 and nt-37. Simultaneous treatment for 24 hrs. with 2 mM mevalonate and 1 μM squalestatin restored the activity of p-226Luc (Fig.19B), but failed to bring the activity of p-1317Luc back to basal levels. It therefore appears that a negative SRE may be located between nt-226 and nt-37. Inasmuch as squalestatin did not reverse the inhibition associated with p-1317Luc, it is

Figure 18. Effect of phorbol esters on the promoter activity of the rat CEH/luciferase chimeric genes in primary rat hepatocytes. (A) Cultured primary rat hepatocytes were transfected with p-1317Luc and fed with serum free medium containing 1 μM PMA in DMSO or DMSO alone. Cells were harvested at the indicated times and reporter enzyme activities were determined. Results are expressed as percentage of control of normalized luciferase activity and represent the mean ± S. E. M. of 3 independent observations. (B) Cultured primary rat hepatocytes were transfected with the indicated rat CEH/luciferase gene constructs. They were incubated in serum free medium with 1 μM PMA in DMSO or DMSO alone for 6 hrs. and then harvested. Normalized promoter activities are expressed as percentage of control and represent the mean ± S. E. M. of 3 independent obsevations. (**) indicates difference at $p<0.005$.


A
150
120
90
60
30
0
% control
3
4
6
8
Time (hrs)

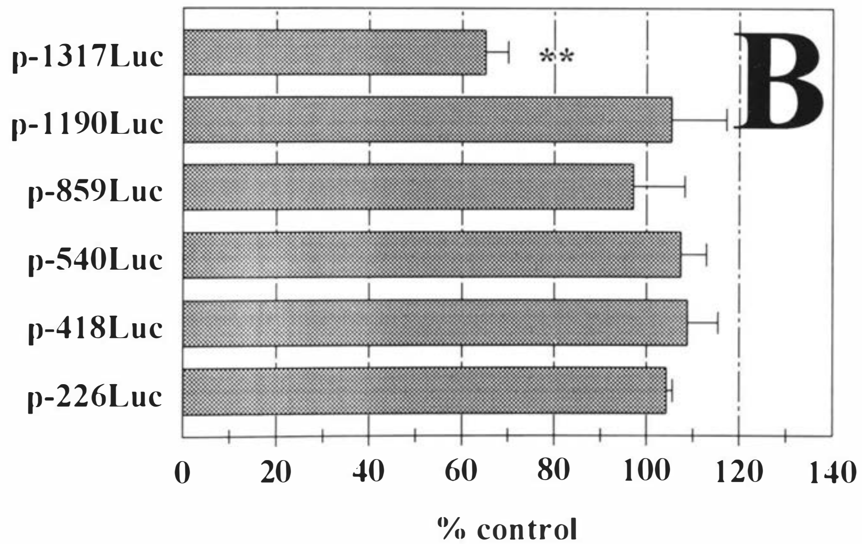
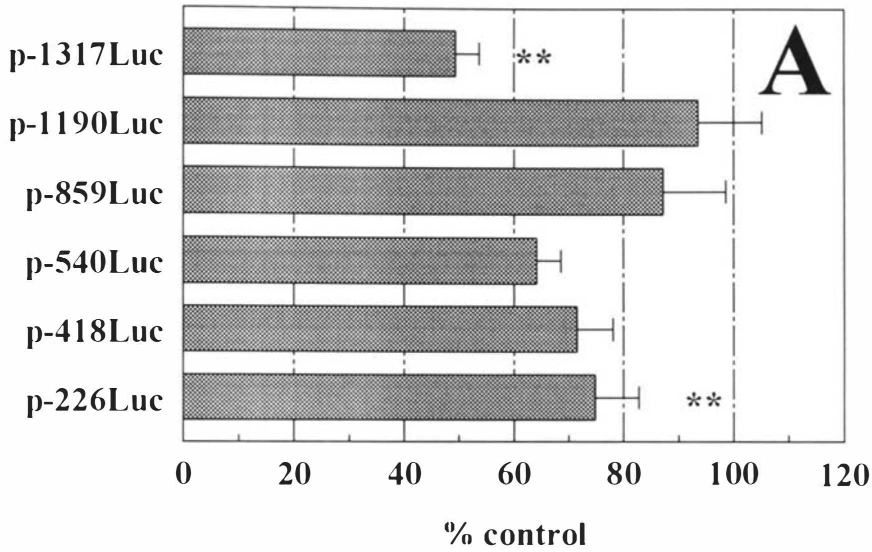

B
p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
**
0
20
40
60
80
100
120
140
% control

Figure 19. Effect of cholesterol perturbing agents on the promoter activity of rat CEH/luciferase chimeric genes in primary rat hepatocytes. Cultured primary rat hepatocytes were transfected with the indicated rat CEH/luciferase promoter constructs and incubated for 24 hrs. in serum free medium in the presence or absence of 2 mM mevalonolactone alone, **(A)** or 2 mM mevalonolactone and 1 μM squalestatin, **(B)**. Normalized promoter activities are expressed as percentage of control and represent the mean ± S. E. M. of 3 separate observations; (**) indicates difference at $p<0.005$.


A
p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
**
**
0
20
40
60
80
100
120
% control

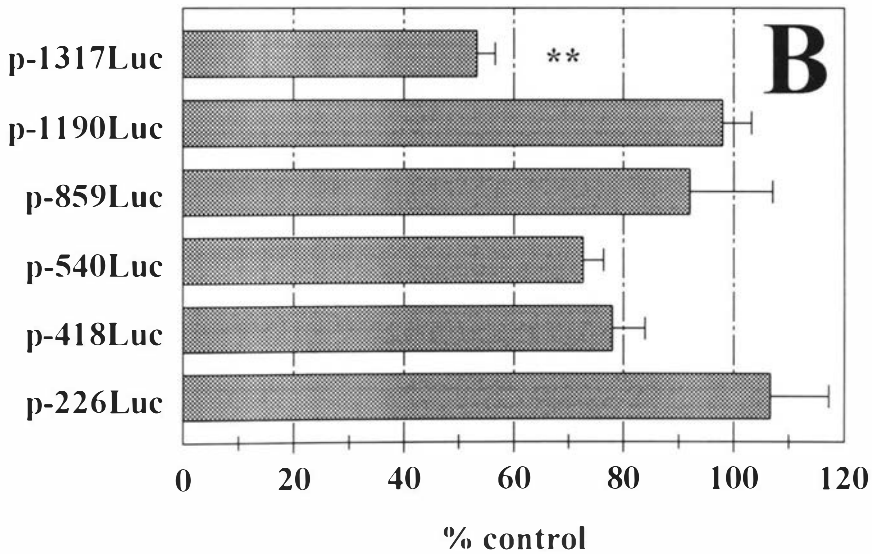
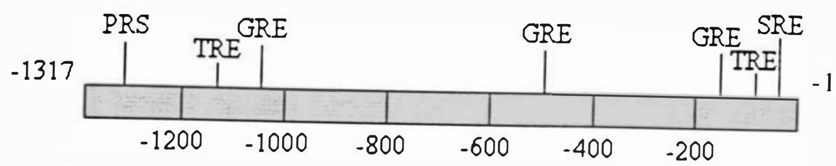

B
p-1317Luc
p-1190Luc
p-859Luc
p-540Luc
p-418Luc
p-226Luc
**
0
20
40
60
80
100
120
% control

Figure 20. Positions of *cis*-acting elements in the rat CEH promoter identified by transient transfections in primary rat hepatocytes. This figure shows the approximate positions of the various response elements identified in the rat CEH promoter by transient transfections in primary rat hepatocytes. The mapping of each response element is explained in the Results section. GRE: glucocorticoid response element ; PRS: phorbol ester response element ; SRE: sterol response element ; TRE: thyroid hormone response element.


PRS
TRE
GRE
GRE
GRE
TRE
SRE
-1317
-1
-1200
-1000
-800
-600
-400
-200

possible that the region between nt-1317 and nt-1190 is uniquely responsive to non-sterols. Figure 20 shows the positions of these *cis*-acting elements in the CEH promoter.

DISCUSSION

This dissertation describes the isolation and characterization of the rat neutral hepatic CEH promoter. The rat CEH promoter is unusual in that it has no consensus TATA-box sequences immediately upstream of the transcription initiation site. However the promoter does have a GC-box sequence, which can bind the positive transcription factor SP1 to drive transcription, and an inverted CCAAT box, which can bind another positive transcription factor NF-Y. In this respect the CEH promoter resembles the promoter for the HMGCoAR gene and the human squalene synthase gene, which also lack TATA-box like sequences but utilize several GC-box sequences to drive transcription. The upstream regions of the promoter involved in transcriptional regulation were also characterized. Two different cell lines were used for this purpose: a homologous system of rat primary hepatocytes and a heterologous system consisting of human hepatoblastoma HepG2 cells.

To gain insight into the molecular mechanisms involved in the regulation of CEH, an *in vitro* primary rat hepatocyte cell culture system was optimized in this laboratory by Ghosh *et al*. In this optimized system, insulin, dexamethasone (0.1 μM) and L-thyroxine (1.0 μM) were required to maintain CEH mRNA at steady-state levels. The HepG2 cell line is commonly used to study the regulation of cholesterol homeostasis. Most intermediates in the bile acid pathway have been detected in HepG2 cells, and the defects

previously reported were due to culture conditions. These defects have now been resolved. HepG2 cells exhibit an adult/terminal phenotype when grown to confluence and retain many cholesterol homeostatic functions (Kelly *et al*, 1989; Kosaki *et al*, 1993). They synthesize apolipoproteins B-100, C-II, C-III, E, A-I, A-II and A-IV (Gordon *et al*, 1983; Gordon *et al*, 1984; Rash *et al*, 1981; Tam *et al*, 1985; Zannis *et al*, 1981), express functional receptors for low density lipoproteins, high density lipoproteins, insulin, transferrin, estrogen and asialoglycoprotein (Wu *et al*, 1984; Hoeg *et al*, 1985), synthesize and secrete lipoproteins (Rash *et al*, 1981; Tam *et al*, 1985; Zannis *et al*, 1981), and express sterol-27-hydroxylase, HMGCoAR, lecithin:cholesterol acyltransferase and ACAT (Erickson and Fielding, 1986; Martin *et al*, 1993). Hence, this cell line is also a suitable model in which to study the regulation of CEH.

The hepatic CEH promoter was characterized both under basal conditions and in the presence of various physiological stimuli. Studies in both cell lines showed that the proximal 599 base sequences of the promoter contains activator elements that are linked to distal repressor regions by a TC-rich hinge located between nt-780 and nt-765, similar to the TC-rich hinge found in the C7H promoter. The promoter also contains putative *cis*-acting elements, including several hormone response half-elements, sterol response elements, ubiquitous transcription factor binding sites and liver specific elements, some of which are shown in Fig. 5. These putative elements were identified using the GCG program (SITEDATA). One liver-enriched transcription factor, C/EBP (CCAAT/enhancer binding protein) putatively binds to the promoter between nt-194 and nt-186, and between nt-843 and nt-835, and may be required for the developmental

regulation and maintenance of the expression of genes. CEH mRNA, protein and enzyme activity have been shown to be developmentally regulated both in male and female rats (Natarajan *et al*, 1996; Natarajan *et al*, 1997). Thus, the presence of putative binding sites for C/EBP and possibly DBP (albumin D-site binding protein) supports the "establishment v/s maintenance" model proposed in the developmental regulation of other liver-specific genes such as PEPCK (phosphoeneol pyruvate carboxykinase) and albumin (Crestani *et al*, 1995).

In HepG2 cells the response to glucocorticoids was seen primarily in the distal 5' flanking sequences. The activation, by dexamethasone, in the region between nt-1317 and nt-1190 was nearly twice that seen in the region between nt-859 and nt-540. This region between nt-1317 and nt-1190 has a sequence similar to the sequence within the glucocorticoid response unit (GRU) of the PEPCK gene. The complex GRU of the PEPCK gene has 2 glucocorticoid receptor (GR) binding sites and 2 sites recognized by accessory factors AF1 and AF2. The sequence in the CEH promoter, TTTTGGTCTTTTTGTTCTTTTTAGACATCT, is located between positions –1185 to –1156. It contains an imperfect palindrome TCTAGAgatTTTTCT which closely resembles the consensus glucocorticoid response element (GRE) (T/G)GTACAnnnTGTTCT, where n stands for any nucleotide. The consensus GRE directs the specific binding of the GR to DNA in the promoter region of target genes with one molecule of the GR binding to each of the two half-sites in a cooperative manner. The 5' portion of this sequence, TCTGGTTTT, located between nt-1177 and nt-1185 is virtually identical to the recognition sequence TGTGGTTTT, for accessory factor AF2 in

the PEPCK promoter. Imai *et al* 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 two 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 and C7H genes. Although we did not test the effect of insulin on the CEH promoter in this study, it has been shown that a minimum of 24 hrs. incubation of rat primary hepatocytes in insulin containing medium is essential to observe the synergistic increase in CEH mRNA by T4 and dexamethasone (Ghosh *et al*, manuscript submitted). 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 sequence TGTTGTGTATTTGGTTGTTTT at positions –781 to –801, which could also behave as a glucocorticoid response unit and account for the dexamethasone mediated stimulation in this region.

On the other hand, three entirely different glucocorticoid response regions were mapped by transient transfections in primary rat hepatocytes. These glucocorticoid response regions were located between nt-1190 and nt-859, between nt-540 and nt-418 and between nt-226 and nt-37. The region in the CEH promoter between nt-1190 and nt-859 has the sequence TCTTGT between nt-1101 and nt-1096. This sequence is identical to the human glucocorticoid response element 5 (HGRE5; von der Ahe *et al*, 1985). This

region also contains another sequence TCCTGA between nt-1081 and nt-1076 which is identical to the human glucocorticoid response element 7 (HGRE7; Cato *et al*, 1985). In addition the region between nt-540 and nt-418 has the sequence TGAAGA between nt-534 and nt-528 which closely resembles the core sequence of the GRE (TGTAGGA -5') reported by Langer and Ostrowski. Finally the region between nt-226 and nt-37 contains the sequence GGTACTGACTGTTCA between nt-142 and nt-128 which resembles the consensus sequence for the GRE (GGTACAnnnTGTTCT; Beato, 1989)

Thus, it is evident that while dexamethasone stimulated reporter gene activity in both HepG2 cells and primary rat hepatocytes, the glucocorticoid response regions mapped were different in each cell line. This variation could have arisen due to the different cellular context and underscores the importance of a careful evaluation of the cellular environment (HepG2 cells v/s cultured primary hepatocytes) and the effect of the species (man v/s rat) prior to extrapolating the data to an *in vivo* condition.

Thyroid hormones stimulated reporter gene activity in both HepG2 cells and primary rat hepatocytes. With HepG2 cells, activation regions were mapped between nt-1317 and nt-1190 and between nt-226 and nt-37. Both these regions have sequences that partially match the consensus type II steroid/thyroid hormone response element AG(G/T)TCA, although there were no sequences that exactly matched the consensus thyroid hormone response element (TRE) TCAGGTCA---TGACCTGA. Similarly in the case of primary rat hepatocytes, activation regions were mapped between nt-1190 and nt-859 and between nt-226 and nt-37. The region between nt-1190 and nt-859 contains a sequence that partially matched the consensus type II steroid/thyroid hormone response

element, but none that exactly matched the consensus TRE. As mentioned before, it has been shown that T4, along with insulin and dexamethasone, was essential to maintain CEH mRNA in cultured primary rat hepatocytes. It has also been shown that the expression of several liver specific genes in cultures of primary rat hepatocytes is abolished unless hormones are added to serum-free medium. Therefore it appears that further studies such as mutational analysis would be essential to identify and map the thyroid hormone response element in the CEH promoter.

The tumor promoter PMA repressed the transcriptional activity of the CEH gene, both in HepG2 cells and in primary rat hepatocytes. Whereas PMA is a known activator of PKC, long term exposures to PMA cause PKC to be proteolytically inactivated (Young *et al*, 1987). A time course experiment was therefore performed to determine the optimal incubation time of each cell line in PMA. In HepG2 cells maximal inactivation occurred after 6 hrs. exposure to PMA. On the other hand, in primary rat hepatocytes a time course experiment showed maximal inactivation after 8 hrs. exposure to PMA. With HepG2 cells, negative PRS were mapped between nt-859 and nt-541, nt-418 and nt-227, and nt-226 and nt-37. A positive PRS was also mapped in the region between nt-540 and nt-419. However in primary rat hepatocytes, only a single negative PRS was identified between nt-1317 and nt-1190. PMA affects transcription of several genes through phosphorylation of transcription factors like c-Jun, c-Fos and NFκB. Jun/Fos heterodimers mediate their effects by binding to AP-1 like elements. Several AP-1 like sequences with the invariant half-site TCA and the conserved T-nucleotide that are crucial for the binding of Jun/Fos are present between nt-226 and nt-37. The region

between nt-418 and nt-227 also has the sequence TCTTGT that resembles the IRS/PRS of both the rat and the hamster C7H gene. As discussed previously, the region between nt-859 and nt-541 includes the sequence TGTTGTGTATTTGGTTGTTTT that resembles the IRS/PRS of the PEPCK gene (Imai *et al*, 1990). These sites could therefore account for the effects observed with PMA in HepG2 cells.

Similarly the region between nt-1317 and nt-1190 has an AP-1 like sequence TGATTAA between nt-1282 and nt-1276 that could confer PMA responsiveness in primary rat hepatocytes. This region also includes the sequence, TTTTGGTCTTTTTGTTCTTTTTAGACATCT located between nt-1185 and nt-1156, that resembles the IRS/PRS of the PEPCK gene (O'Brien *et al*, 1991) as well as the IRS/TGT4/HNF3 of the C7H gene (Crestani *et al*, 1995). Thus it appears that signal transducing agents play a significant role in the transcriptional regulation of the CEH gene. Moreover, bile acids have been reported to down-regulate the C7H gene transcription, partly through the PKC signal transduction pathway (Stravitz *et al*, 1995). It would be reasonable to postulate that the CEH gene is regulated in a similar fashion by bile acids, especially since it has been shown that taurocholate suppresses CEH mRNA levels in primary rat hepatocytes (Ghosh *et al*, 1997; manuscript submitted)

In this study known cholesterol perturbing agents were used to alter intracellular cholesterol levels in HepG2 cells and in primary rat hepatocytes. The agents used were mevalanolactone and squalestatin. Cholesterol is the major product of the mevalonate pathway. In addition several biologically important non-sterol products such as isopentenyl adenine, dolichol, coenzyme Q, heme A and prenylated proteins are also

produced from mevalonate (Goldstein and Brown, 1990). Squalestatin, also called zaragozic acid A, is a competitive inhibitor of squalene synthase at picomolar concentrations and effectively lowers serum cholesterol in marmosets (Baxter *et al*, 1992). Squalene synthase catalyzes the committed step of cholesterol biosynthesis (Bruenger and Rilling, 1986). This step is the first on the pathway to cholesterol after it branches to the various non-sterol products mentioned above. Therefore inhibition of squalene synthase by squalestatin selectively lowers only sterol end products, but does not deprive the cell of important non-sterol products. Sterol and non-sterol products regulate the expression of proteins involved in cholesterol homeostasis at multiple levels including transcription, translation and protein degradation. In general, sterols regulate gene expression at the level of transcription, while non-sterols act at the level of translation.

Mevalonate has been shown to decrease HMGCoAR transcription rates (Goldstein and Brown, 1990) and both CEH (Ghosh *et al*, 1997; manuscript submitted) and HMGCoAR steady-state mRNA levels (Goldstein and Brown, 1990). In primary rat hepatocytes cultured in serum-free medium, squalestatin decreased C7H specific activity to undetectable levels and also decreased its steady-state mRNA levels and transcriptional activity (Kinchel *et al*, 1995). In the present study, mevalonate decreased CEH promoter activity by 57% for the smallest construct, p-226Luc and it also decreased the activity of p-1190Luc by 84% in HepG2 cells. In contrast, treatment with squalestatin along with mevalonate, restored the promoter activity of p-226Luc. Therefore functional sterol responsive sequences must be present between nt-226 and nt-

37. Two SRE-1 like sequences are present between nt-226 and nt-37, one being CACCGAAC which has a 7/8 base match and the other being CACCGATC which has a 6/8 base match with the consensus SRE-1 sequence CACC(C/G)(C/T)AC, found in the promoters of HMGCoAS, HMGCoAR and the LDLR genes (Smith *et al*, 1988). In primary rat hepatocytes, mevalonate decreased CEH promoter activity of p-1317Luc to 49% of control and that of p-226Luc to 74%. However, treatment with squalestatin, along with mevalonate, only restored the promoter activity of p-226Luc. Therefore the two SRE-1 elements identified with HepG2 cells, also appear functional in primary rat hepatocytes.

Thus, the CEH gene appears to have functional sterol responsive elements similar to those of LDLR, HMGCoAS, HMGCoAR, farnesyl diphosphate synthase, squalene syntahse, fatty acid synthase and acetyl CoA carboxylase. These genes not only have sterol responsive elements but also different positive promoter elements that are required for the SRE's to function. In the LDLR gene, the SRE-1 is a conditional positive element that enhances transcription in the absence of sterols, but not when they are present. The SRE-1 synergizes with two nearby sequences that are relatively weak binding sites for the positive transcription factor SP1 (Dawson *et al*, 1988). *In vivo*, The SRE-1 and the two SP1 sequences are all necessary for high level transcription in the absence of sterols. In the HMGCoAR promoter, the SRE-1 sequences actively repress transcription in the presence of sterols. The SRE-1 sequences are located in the midst of a cluster of eight protein binding sites, six of which bind proteins belonging to a family of transcription factors designated the CTF and the NF-1 group (NF-1, nuclear factor-1; CTF, CCAAT-

binding transcription factor) (Gil *et al*, 1988; Gil *et al*, 1988a; Santoro *et al*, 1988). The CEH promoter has potentially both SP1 and NF-Y binding sites at nt-104 and nt-146 respectively, that may be required for the functioning of the SRE.

The simplest interpretation of the data suggests the presence of a conditional negative element in the proximal 226 bases of the CEH promoter. In the presence of sterols, the elements may bind a negative transcription factor (SREBP) that competes for binding with the positive transcription factors and thus down-regulates transcription. Nevertheless, in order to determine if these octanucleotides are true sterol regulatory elements, it will be necessary to disrupt these elements by site-directed mutagenesis and then test transcription levels in the presence or absence of sterols.

The region between nt-1190 and nt-859 in the case of HepG2 cells, and the region between nt-1317 and nt-1190 in the case of primary rat hepatocytes, considerably reduced promoter activity in the presence of mevalonate and the activity was not restored to control levels by simultaneous treatment with squalestatin. It is possible that this region has a *cis*-acting element that responds to non-sterols in the cell. The existence of a non-sterol response element and its ability to down-regulate transcription is unique and contrary to the widely held view that non-sterols only act at the post-translational level. Identification of the nature of the non-sterol, the non-sterol response element and its putative DNA binding proteins will enable us to elucidate this novel mechanism of regulation.

In this study the rat CEH promoter was characterized by transient transfection assays in two different cell lines. One of the aims of this study was to determine if the

culture systems used had different effects on the CEH promoter. The basal CEH promoter activity in the human HepG2 cells was similar to that observed in primary rat hepatocytes. In the presence of dexamethasone, GRE's in the CEH promotor were mapped between nt-1317 and nt-1190 and between nt- 859 and nt-540 by transient transfection assays in human HepG2 cells. However the GRE's mapped by transient transfection assays in primary rat hepatocytes were located between nt-1190 to nt-859, nt-540 to nt-418, and between nt-226 to nt-37. The glucocorticoid response sequences mapped in these regions were the same, suggesting that the nature of the transcription factor involved, in this case the glucocorticoid receptor, could account for the differences observed in the location of the glucocorticoid response regions. Alternatively, the interaction between the transcription factor and the transcription initiation complex could also influence the rate of transcription of the gene. This, in turn, would depend on the nature of the transactivating domain of the transcription factor and its proximity to the transcription initiation complex. Phorbol esters are activators of PKC, which in turn phosphorylates other proteins. As mentioned earlier, phosphorylation events are post-translational modifications that affect the activity of DNA binding proteins in different ways. Thus depending on the nature of the phosphorylation event, transcription factors can be turned "on" or turned "off". The kinases involved could also be different in the two cell lines and this in turn could account for the differential effects observed in the two culture systems. Finally the SRE identified by transient transfection assays in the two cell lines were exactly identical, suggesting that the transcription factor/s that respond to the level of sterols in the two cell lines are similar. Thus it appears that a more

detailed and specific study would be required to detect differences between a homologous culture system and a heterologous culture system.

It is apparent that coordinate regulation of fatty acid and cholesterol metabolism is essential for balanced membrane biosynthesis and turnover, to accommodate metabolic fluctuations that occur during normal cellular growth. Moreover, these two important lipids are simultaneously required in the liver for regular, ordered assembly of very low density lipoprotein particles, which deliver their lipid load of cholesterol and fatty acids from the liver to other sites in the body to maintain lipid homeostasis (Brown and Goldstein, 1986). Therefore, not surprisingly, the genes in these pathways are also apparently co-regulated. They share common *cis*-acting elements and specific regulatory proteins that bind to these elements in order to coordinately regulate the expression of these genes. An example of such a regulatory sequence is the sterol response element (SRE-1). As mentioned before, SRE-1 sequences have been found in the promoters of genes that regulate cholesterol metabolism like LDLR, HMGCoAR, HMGCoAS, and squalene synthase. Moreover, these SRE-1 sequences have also been identified in the promoters of genes that regulate fatty acid metabolism like fatty acid synthase, an essential enzyme of fatty acid biosynthesis, acetyl coenzyme A carboxylase, the rate-controlling enzyme for fatty acid biosynthesis, and hormone sensitive lipase, an enzyme that plays a key role in adipocyte lipid metabolism. SREBP-1 is the regulatory protein that binds to the SRE-1 sequence in humans. The rat equivalent of SREBP-1 was cloned from an adipocyte cDNA expression library (Tontonoz *et al.* 1993). The rat SREBP mRNA was induced during adipocyte differentiation in cell culture, and so it was named

the adipocyte determination- and differentiation- dependent factor (ADD1). SREBP-1 binds to the SRE-1 sequence in the promoters of genes involved in cholesterol metabolism and regulates cellular sterol levels. Moreover, expression of the mRNA's for fatty acid synthase and acetyl coenzyme A carboxylase have been demonstrated to be regulated by sterols in a manner similar to genes that encode proteins of cholesterol metabolism and ADD1 is the regulatory protein implicated in the sterol responsiveness of these genes. Since ADD1 is the rat equivalent of SREBP-1, it is evident that SREBP-1/ADD1 serves as a direct molecular connection between the regulation of two different classes of cellular lipids that are both required for cellular growth and normal lipoprotein metabolism.

As mentioned before, CEH hydrolyzes cholesteryl esters to cholesterol and free fatty acids. This enzyme is therefore uniquely capable of regulating this aspect of both cholesterol and fatty acid metabolism. In this study we have identified and mapped functional SRE-1 sequences in the 5' flanking portion of the rat CEH gene, to which SREBP-1 or ADD1 could bind. Presence of these functional SRE-1 sequences in the CEH promoters indicates the importance of this enzyme in regulation of cholesterol metabolism. Previously, CEH has been shown to hydrolyze a broad range of substrates (Natarajan *et al*, 1996a) including cholesteryl esters and triglycerides. In the current study we also demonstrated the presence of functional hormone response elements. These hormone response elements have been previously identified in the promoters of genes that regulate fatty acid metabolism. Therefore, considering the ability of this enzyme to release free fatty acids from different substrates, and the presence of various

hormone response elements in the CEH promoter, similar to those present in the promoters of genes that regulate fatty acid metabolism, it is apparent that this enzyme could also play a significant role in fatty acid metabolism.

Thus, while coordinate regulation can be mediated by the involvement of SREBP-1/ADD1 in the transcriptional control of fatty acid synthesis and cholesterol homeostasis, the ability of CEH to release both cholesterol and fatty acids, the broad substrate specificity of this enzyme, and the presence of functional sterol response elements and hormone response elements in its promoter makes this enzyme a candidate for an additional level of coordinate regulation, which in turn could link the two pathways together.

In this dissertation the isolation and regulation of the rat hepatic neutral CEH promoter by various physiological stimuli has been described. It appears that the rat CEH gene is regulated by multiple physiological stimuli that fine tune the expression of the gene. Moreover, due to overlapping consensus sequences in the proximal 226 bases, competition for binding by transcription factors and their cross talk would eventually determine the level of CEH expression. Finally, determination of exact *cis*-acting sequences and the transcription factors that bind to them would enable us to have a greater understanding of the transcriptional regulation of the CEH gene.

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LITERATURE CITED

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