The CCAAT/Enhancer-binding Protein trans-Activates the Human Cholesteryl Ester Transfer Protein Gene Promoter*

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The plasma cholesteryl ester transfer protein (CETP), primarily synthesized in the liver of several species, is expressed at very low levels in a number of transformed human liver cell lines. The human CETP gene promoter contains a sequence that closely resembles the binding site for the transcription factor CCAAT/enhancer-binding protein (C/EBP). This site is capable of binding C/EBP, as shown by electrophoretic mobility shift and DNase I footprint analyses. Transient expression of the bacterial chloramphenicol acetyltransferase gene under the control of the human CETP gene promoter gave low activities in the human hepatoma cell line, HepG2. However, in the presence of C/EBP, CAT activity was markedly elevated indicating that CETP gene promoter activity was enhanced. In primary cultures of isolated hepatocytes, CETP mRNA was lost rapidly and in parallel with the C/EBP mRNA. C/EBP may play an important role in the proper maintenance of CETP gene promoter activity, and its low levels in proliferating or cultured cells may account for the low level of the CETP gene expression in immortalized human liver cell lines or cultured hepatocytes.

The transfer and exchange of neutral lipids between plasma lipoproteins is mediated by cholesteryl ester transfer protein (CETP), a 74,000 plasma glycoprotein. This protein facilitates the net transfer of cholesteryl esters from high density lipoproteins to lower density lipoproteins, and the net reciprocal transfer of triglycerides. In human genetic CETP deficiency, cholesteryl esters are greatly increased in high density lipoprotein particles, illustrating the key role that CETP plays in human high density lipoprotein metabolism. The liver represents a major site of plasma CETP synthesis in several species (e.g. humans, monkeys, rabbits) and in transgenic mice expressing human CETP under the control of its natural promoter. However, the cell types expressing the CETP gene in the liver are not clearly defined. A recent report showed that isolated monkey hepatocytes (parenchymal cells) did not contain CETP mRNA, and the expression of the CETP gene in liver was attributed to the peri-sinusoidal cells. On the other hand, isolated human hepatocytes have been shown to contain CETP mRNA and the human hepatocyte derived cell line HepG2 is also known to secrete low levels of CETP into the culture media.

Previously, we described the primary structure of the human CETP gene. Computer analysis of the promoter region revealed only minimal sequence similarities with known cis-regulatory elements. Notably however, the CETP gene promoter contains a sequence that bears a strong resemblance to the binding site of the transcription activator C/EBP (also known as C/EBPα). C/EBP is normally abundant in terminally differentiated and growth-arrested cells, but not in actively proliferating cells. In the liver, C/EBP is produced exclusively in hepatocytes. A role for C/EBP in the expression of the human CETP gene could explain the low levels of CETP mRNA in transformed human liver cell lines and in cultured human hepatocytes. In this report, we describe studies that assess the possible role of C/EBP in the expression of the human CETP gene.

MATERIALS AND METHODS

Probes and Nuclear Extracts—A double-stranded C/EBP binding site was prepared by annealing equimolar amounts of synthetic oligonucleotides bearing the sequences described by Landschultz et al. (12). For electrophoretic mobility shift assays, a HindIII/XbaI fragment (−133/−360; 168 bp) containing site A (Fig. 1) was isolated from the cloned human CETP gene promoter (11) or generated by polymerase chain reaction amplification using primers positioned upstream of the HindIII site and downstream of the XbaI site. After digestion with HindIII and XbaI, both strands were labeled by incorporation of [%32P]-labeled deoxynucleotides. For DNase I footprint analyses, the same DNA fragment was labeled only at the 5′ end of the sense strand. A rat liver nuclear extract was prepared as described previously (16). In some experiments, the extract was incubated at 95 °C for 5 min to enrich for C/EBP DNA binding activity (12).

Electrophoretic Mobility Shift and DNase I Footprint Analyses—Electrophoretic mobility shift assays and DNase I footprint analyses were done using standard procedures (17). DNA binding reactions contained 2 μg of rat liver nuclear extract, ~1 ng of labeled probe, and 2 μg of polyclonal to suppress nonspecific binding. For competitive DNA binding assays, extracts were incubated with an excess (see figures) of unlabeled synthetic C/EBP binding site for 10 min at 25 °C prior to the addition of labeled probe. Bound complexes were resolved on a 3% native polyacrylamide gel. Products of DNase I footprint analyses were resolved on an 8% polyacrylamide-urea sequencing gel. Markers were generated from the probe by chemical cleavage at the purine residues.

Cell Transfection and CAT Assays—HepG2 cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with glutamine and 10% fetal bovine serum. HeLa cells were maintained in Eagle’s minimum essential medium containing Earle’s salts and supplemented with glutamine, essential and nonessential amino acids, pyruvate, and 10% fetal bovine serum. For trans-activation studies, a 650-bp fragment of the human CETP gene promoter was linked to the promoterless bacterial CAT gene in pCAT-basic (Promega). The plasmid pHMSV-C/EBP
was used to direct the synthesis of C/EBPα in transfected cells (14). DNA was transfected into cells using the calcium phosphate method (18). Extracts of transfected cells were prepared 40 h after the introduction of DNA, and assayed for CAT activity as described previously (19). The pCAT-control plasmid (Promega) was used to assess the efficiency of transfection.

Isolation and Culture of Liver Parenchymal Cells—Parenchymal cells (hepatocytes) were isolated from NFR-CETP transgenic (line 5171) mouse (7) livers following the method of Hendriks et al. (20). Hepatocytes were harvested from the initial cell suspension by low speed centrifugation (20 × g for 2 min). Contaminating nonparenchymal cells were removed by repeated washing (4 times) and low speed centrifugation as described above. The extent of nonparenchymal cell contamination (<5%) at the end of the isolation procedure (time zero) was determined by a binding assay using fluorescently labeled acetylated human low density lipoproteins. Hepatocyte primary cultures were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (without hypoxanthine) supplemented with penicillin (100 units/ml), streptomycin (10 mg/ml), insulin (5 μg/ml), dexamethasone (0.1 mM), and 10% fetal bovine serum. RNA was extracted from cells immediately after the isolation procedure and after 3, 6, 12, and 24 h of culture. Cell viability was determined by trypan blue exclusion. Cell death that occurred over the time course of the experiment was less than 10%. CETP mRNA level was determined by solution hybridization-ribonuclease protection as described previously (21). C/EBPα and actin mRNA were detected by RNA blotting using the rat C/EBPα and mouse actin cDNA probes.

RESULTS

Previously, we showed that the human CETP gene promoter contains a “TATA” box and a potential SP1 binding site immediately upstream of the transcription start site (Fig. 1) (11). A further search for consensus regulatory sequences revealed the existence of an additional element similar to the binding site (Fig. 1, site A) for the transcription activator C/EBP (12).

To determine if C/EBP is involved in CETP gene expression, we first evaluated the ability of site A to bind C/EBP in vitro. A fragment (168 bp) of the CETP gene promoter containing site A was purified and analyzed for protein binding by electrophoretic mobility shift assay. Heated rat liver nuclear extract was used as a source of C/EBP (12). Avid protein binding to the probe was evident in heated rat liver nuclear extract (Fig. 2). To determine if the binding was sequence-specific, a synthetic double-stranded C/EBP binding site (Fig. 1) (12) was used as a competitor. Addition of increasing amounts of competitor to the assay mixture resulted in the reduction of probe binding (Fig. 2). Greater than 90% of the binding activity was blocked when a 10-fold molar excess of cold competitor, relative to the labeled probe, was added to the binding reaction (Fig. 2). These results indicate that the binding activity is sequence specific and imply C/EBP binding to the CETP gene promoter fragment.

Probe binding to nonheated rat liver nuclear extracts was

![Fig. 1. Structure of the human CETP gene promoter. The top diagram shows the location of the TATA box, the putative SP1 binding site, and the putative C/EBP binding site (site A). The sequence of site A, the core sequence of the synthetic C/EBP binding site (12), and the C/EBP binding site consensus (34) are also shown below. The common residues are represented in uppercase boldface letters.](http://www.jbc.org/content/257/34/22337/F1)

![Fig. 2. Protein binding to the CETP gene promoter. The CETP gene promoter fragment containing site A was tested by electrophoretic mobility shift assay as described under “Materials and Methods.” The arrow shows the position of the bound probe, in heated rat liver nuclear extract (left panel) and nonheated rat liver nuclear extract (right panel). An excess of unlabeled synthetic C/EBP binding site (10-, 100-, and 1000-fold molar excess relative to the probe concentration) was added to the binding reactions prior to the addition of the labeled probe. [comp], relative competitor concentration.](http://www.jbc.org/content/257/34/22337/F2)

![Fig. 3. DNase I footprinting analysis of the human CETP gene promoter fragment containing site A. The figure shows DNase I digestion products generated in reactions containing heated rat liver nuclear extracts (20 and 5 μg, left lanes), no extract added (middle lane), and purified recombinant C/EBP (5 and 20 ng, right lanes). The bracket shows the region (site A) of the CETP gene promoter protected from DNase I digestion.](http://www.jbc.org/content/257/34/22337/F3)

![Fig. 4. Transactivation of the human CETP gene promoter by C/EBP. Transient expression studies in HepG2 cells were conducted as described under “Materials and Methods.” The left panel shows a representative thin layer chromatogram of CAT reaction products catalyzed by extracts prepared from mock transfected (no DNA) cells (left lane), cells transfected with 650-CETP.CAT alone or together the C/EBP expression plasmid pMSV-C/EBP (middle lanes), and pCAT-control (right lane). The arrows show the position of the CAT reaction products. The right panel shows the relative CAT activity. Values shown are mean ± S.E. of four separate experiments done in triplicate.](http://www.jbc.org/content/257/34/22337/F4)
transgenic mouse liver parenchymal cells (hepatocytes) were analyzed.

22338

Fig. 5. Decay of CETP mRNA levels in cultured liver parenchymal cells. Equal amounts of RNA extracted from CETP transgenic mouse liver parenchymal cells (hepatocytes) were analyzed for CETP, C/EBPα, and actin mRNA levels. CETP mRNA was detected by solution hybridization-ribonuclease protection assay as described previously (21). C/EBPα and actin mRNA was detected by RNA blotting using rat C/EBPα and mouse actin cDNA probes. L, RNA extracted from whole liver; 0–24, time (h) in primary culture.

also tested. As with heated extracts, avid probe binding was observed (Fig. 2). Increasing amounts of cold competitor also caused a reduction of probe binding. However unlike heated extracts, probe binding to nonheated extracts was not completely blocked even by a large excess of the synthetic C/EBP binding site residual binding activity was still evident even when the extracts were pretreated with the cold synthetic C/EBP binding site at a 1000-fold molar excess relative to the labeled probe (Fig. 2), suggesting that it might arise from a factor other than C/EBP.

To evaluate further the binding of protein to site A, the same CETP gene promoter fragment used in electrophoretic mobility shift assays was analyzed by DNase I footprinting. Heated rat liver nuclear extract protected the probe from nuclease digestion at a single region centered at site A (Fig. 3). An identical pattern of nuclease protection was also obtained with purified recombinant C/EBP (Fig. 3). These results show that site A of the human CETP gene promoter interacts with C/EBP.

The role of C/EBP in controlling CETP gene promoter function was assessed by transfection studies in HepG2 cells. A chimeric gene (650-CETP.CAT) was constructed by linking a CETP gene promoter fragment containing the C/EBP binding site (site A) to the bacterial chloramphenicol acetyltransferase (CAT) gene. Transfection of the 650-CETP.CAT plasmid into HepG2 cells resulted in low but detectable CAT activity (Fig. 4). However when the 650-CETP.CAT plasmid was introduced into HepG2 cells together with a C/EBPα expression plasmid, an average of 6.3-fold enhancement of CAT activity (p < 0.02, n = 4) was observed (Fig. 4). By contrast the 650-CETP.CAT, by itself or together with the C/EBP expression plasmid, showed no detectable CAT activity above the background in transfected HeLa cells (not shown). The results indicate that C/EBPα is capable of activating the CETP gene promoter in HepG2 cells. Failure to detect CETP promoter activity in transfected HeLa cells suggests that additional transcription factors not present in HeLa cells may be required for proper CETP gene promoter function, reflecting the tissue-specific expression of the CETP gene.

It has been suggested that hepatocytes do not have the capacity to synthesize CETP (8). Recently, a new line of transgenic mice in which the expression of the human CETP minigene remains under the control of its natural promoter was established. CETP transgene expression in these mice closely resembles the human tissue-specific pattern of CETP gene expression (7). Expression of the CETP transgene was detected in the liver and also in freshly isolated hepatocytes (Fig. 5). To determine if CETP gene expression is related to C/EBP levels, the abundance of both CETP and C/EBP mRNA in isolated hepatocytes was determined as a function of time in primary culture. As shown in Fig. 5, the C/EBP mRNA disappeared rapidly and was undetectable after 24 hours of primary culture. CETP mRNA abundance was also markedly decreased, and the decline occurred in close parallel with that of the C/EBP mRNA. By contrast, the abundance of the actin mRNA remained unchanged over the same time period. These results show that hepatocytes have the capacity to express the CETP gene and that the mRNA levels of CETP and C/EBPα are tightly linked.

DISCUSSION

A number of genes containing a C/EBP binding site in their promoters have now been identified. These genes have also been shown to be responsive to C/EBP in transfection studies (14, 22–27). Since C/EBP levels become elevated after terminal differentiation, C/EBP may function to coordinately activate or enhance the expression of some genes when cells reach their fully differentiated state (13). Consequently, reduction of C/EBP levels may also cause the extinction of expression of responsive genes. For example, a mutation in the promoter of the factor IX gene, which disrupts the C/EBP binding site and simulates the loss of C/EBP function, causes drastically reduced plasma factor IX levels and is associated with the development of hemophilia B (28). In the present study, we show that the activity of the CETP gene promoter is strongly dependent on the presence of C/EBP.

The existence of a C/EBP binding site in the human CETP gene promoter raised the possibility that C/EBP may play a role in controlling CETP gene expression. The results presented in this paper show that C/EBP can bind to an element in the CETP gene promoter. However, in nonheated liver extracts, failure to completely compete probe binding with the synthetic C/EBP binding site suggested either the binding of additional factors to other sites within the probe, or competitive binding of different factors to site A. The second possibility seems more likely since only one probe-protein complex was detected in nonheated extracts. The significance of the additional binding activity is unknown. The second factor is heat-labile and appears to have a high affinity for site A. This may indicate that site A of the human CETP gene promoter, like element D of the albumin promoter, is a target for multiple regulatory factors (14, 29–31). From the present results, it is not possible to determine if site A interacts with multiple regulatory factors made by a single cell type or by different cell types, since the nuclear extract tested contained a complex mixture of DNA binding activities derived from the different cell types that make up the liver.

Biochemical data obtained by a recent study did not support the existence of CETP mRNA in isolated monkey hepatocytes (parenchymal cells), leading the authors to ascribe CETP gene expression to the nonparenchymal cells of the monkey liver (8). Our present findings showing the rapid loss of the CETP mRNA from cultured hepatocytes (Fig. 5) suggest that the absence of CETP mRNA in hepatocytes reported in the earlier study (8) may have resulted from the decay of CETP gene expression due to the loss of C/EBP (or C/EBP function) during cell isolation. In the same study, in situ detection of CETP mRNA in monkey liver sections was thought to show a clustering of signal around the hepatic sinusoids, in a pattern different to that of the monkey apolipoprotein A-I mRNA (8). However, these differences were not quantitated, and the cell type was not identified.

Studies done by our laboratory indicate that hepatocytes in the liver of transgenic mice bearing the human CETP gene under control of its natural promoter contain CETP mRNA.
These results are concordant with the fact that the human hepatocyte-derived HepG2 cells contain a low level of CETP mRNA (~10% of normal human liver content), and that CETP activity is detectable in the culture media (9, 10). Data from the transfection experiments carried out in the present study confirm that the CETP gene promoter is active in HepG2 cells. However, the more significant finding is the ability of C/EBP to trans-activate the CETP gene promoter activity in HepG2 cells.

CETP gene expression is most likely regulated (positively and negatively) by multiple trans-regulatory factors, some of which may be active only in specific cell types. This could account for the differences in cell type- and tissue-specific pattern of CETP gene expression. Other members of the C/EBP family (32, 33) may also be involved in regulating the CETP gene through site A. The present study evaluated the role of the transcription factor C/EBP (also known as C/EBPα) in the expression of the CETP gene in hepatocytes, and the results obtained indicate that the CETP gene promoter is responsive to C/EBP function. It therefore appears that CETP gene expression in hepatocytes is directly linked to the level of C/EBP. The low levels of C/EBP in HepG2 cells (14), and likely in other immortalized human hepatocyte-derived cell lines and cultured hepatocytes, may be responsible for the low level of CETP gene expression.

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