Characterization of a cholesterol response element (CRE) in the promoter of the cholesteryl ester transfer protein gene: functional role of the transcription factors SREBP-1a, -2, and YY1.

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Abstract Cholesteryl ester transfer protein (CETP) is expressed in human adipocytes, where it acts to promote selective uptake of HDL-CE (Benoist, F., M. McDonnell, P. Lau, R. Milne, and R. McPherson. **1997.** ***J. Biol. Chem.*** 272: **23572–23577). In contrast to other major sterol-responsive genes such as 3-hydroxy-3-methylglutaryl coenzyme A reductase CETP expression is up-regulated rather than down-regulated in response to cholesterol. To define elements involved in cholesterol-mediated up-regulation of CETP gene expression, deletion derivatives of the CETP promoter were cloned into a luciferase reporter construct and transfected into the human liposarcoma cell line SW872, cultured in the presence or absence of lipoproteins. A fragment associated with a positive cholesterol response was identified between nucleotides −361 and −138 (relative to the initiation site of transcription) of the promoter. This region contains a tandem repeat of a sequence known to mediate sterol dependent regulation of the hamster HMG-CoA reductase gene. We have putatively denoted this region, the cholesterol response element (CRE). Using gel mobility shift assays we demonstrate that both YY1 and SREBP-1 interact with the CRE of CETP. Furthermore, in transient co-transfection experiments, both YY1 and SREBP-1a were found to *trans*-activate, in a dose-dependent manner, the luciferase activity of constructs harboring the CRE. We also demonstrate that SREBP-2, is able to *trans*-activate a luciferase construct harboring the CRE although much less effectively as compared to SREBP-1. Finally, functional analysis of the CRE confirms its regulatory role in modulating CETP gene expression through its interaction with YY1 and SREBP-1a.**—Gauthier, B., M. Robb, F. Gaudet, G. S. Ginsburg, and R. McPherson. **Characterization of a cholesterol response element (CRE) in the promoter of the cholesteryl ester transfer protein gene: functional role of the transcription factors SREBP-1a, -2, and YY1.** ***J. Lipid Res.*** **1999.** 40: **1284–1293.**

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Cholesteryl ester transfer protein (CETP) is a 74 kDa hydrophobic plasma glycoprotein that mediates the hetero- and homo-exchange of neutral lipid between apoA-I and apoB-containing lipoproteins (1–3). In humans, adipose tissue is the largest organ expressing CETP (4, 5). We have demonstrated, in both humans and hamsters, that there is a strong correlation between adipose tissue CETP mRNA abundance and plasma CETP concentrations (5, 6). Thus, adipocytes may contribute significantly to the plasma pool of this protein. Furthermore, we have recently defined a novel and important role for CETP in mediating the selective uptake of HDL-derived CE by human adipocytes (7). These findings suggest that adipocyte-derived CETP plays an important local role in cholesterol homeostasis. Therefore, molecular mechanisms that govern CETP gene expression in human adipocytes are of major interest.

The objective of the present study was to identify and characterize *cis*-acting elements(s) and their cognate transcription factors within the human CETP promoter that are involved in cholesterol-mediated modulation of gene expression in human liposarcoma cells, an adipocytic cell line. While CETP expression has been previously reported in cell culture, the very low activities and lack of published data demonstrating cholesterol regulation have precluded use of cells such as HepG2 to study the regulation of CETP gene expression by cholesterol. We have successfully used the human liposarcoma cell line, SW872, as a

Abbreviations: CETP, cholesteryl ester transfer protein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CRE, cholesterol response element; YY1, Ying Yang 1; SREBP, sterol regulatory element binding protein; 25-OH cholesterol, 25-hydroxy-cholesterol; LPDS, lipoprotein-depleted serum; CS, complete serum.

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model to study the regulation of CETP by cholesterol. This cell line was previously demonstrated to express CETP at levels 50-fold greater than those of HepG2 cells. Furthermore, CETP mRNA levels and protein secretion were shown to increase, in a dose-dependent manner, in the presence of either 25-OH cholesterol or LDL, demonstrating that CETP expression responds to cellular cholesterol loading (8).

MATERIALS AND METHODS

CETP reporter gene constructs

Chloramphenicol acetyl transferase reporter constructs harboring serial deletions of the CETP gene 5′ flanking region were described previously. Fragments of 125 bp, 600 bp, and 361 bp were excised from plasmids −836CETP.CAT, −636CETP.CAT and −300CETP.CAT, respectively, by double digestion with *Xba*I/*Ava*I, *Acc*I/*Ava*I, and *Hind*III/*Ava*I. These fragments were subsequently gel purified using the QIAEX II kit (QIAGEN Inc, Chatsworth, CA), made blunt ended using the Klenow fragment of DNA polymerase I and ligated into the pGL3-basic reporter vector (Promega Corp., Madison, WI) which had been cut with *Sma*I. The resulting clones were designated CETP125LUC (125 bp fragment), CETP361LUC (361 bp fragment) and CETP636LUC (600 bp fragment).

Mutagenesis of the cholesterol response element was performed by PCR according to the protocol of Higuchi. Two overlapping PCR products were generated using the following sets of primers: *1*) +GL3B, 5′-CCC TTT GAC GTT GGA GTG CAC G-3′ (pGL3-basic vector, position 4347 to 4368) and CETPmut2, 5′-CCC CTC **A**AC CA**G** CTG **A**AC CA**G** TTT TGC C-3′ [cholesterol response element (CRE) of the CETP promoter, position −124 to −151; mutated nucleotides are underlined] and *2*) −GL3B, 5′-CCA CGG TAG GCT AGC GAA ATG C-3′ (pGL3-basic vector, position 453 to 474) and CETPmut1, GGC AAA A**C**T GGT T**C**A G**C**T GGT **T**GA GGG G-3′ CRE of the CETP promoter, position −124 to −151; mutated nucleotides are underlined). The two overlapping PCR products were annealed and the gaps were filled in at 72°C using the Vent polymerase. Two nested primers, 5′-**GGT TAC GCG T**AA GCT TAT TCC TAG ATA TAT G-3′ (CETP promoter, position −300 to −279; underlined nucleotides depict *Mlu*I site) and 5′-**GGA ACT CGA G**CC CGA GCC GTT CAG CCT GGA GC-3′ (CETP promoter, position −22 to +1; underlined nucleotides depict *Xho*I site) were used for the second round of PCR. The amplified fragment was digested with *Mlu*I and *Xho*I and ligated into the *Mlu*I/*Xho*I sites of the pGL3-promoter vector (Promega Corp., Madison, WI). The resulting clone was designated mutCETP300LUC. The same protocol was followed using non-mutated CRE oligonucleotides to produce a wild type clone (wtCETP300LUC).

Multiple copies of the CRE were annealed together using T4 DNA ligase, made blunt ended using the Klenow fragment of DNA polymerase I, and ligated into the pGL3-promoter reporter vector (Promega Corp., Madison, WI) which had been cut with *Sma*I. Clones were sequenced to determine the orientation of the insert and the number of CRE copies. One clone, pGL3-PCRE$_3$, contained three copies of the CRE in the proper orientation.

Cell culture

The human liposarcoma cell line SW872 (American Type Culture Collection, Rockville, MD) was cultured in Dulbecco's modified Eagle's/HAM F12 (3:1) (Life Technologies, Burlington, ON) supplemented with 5% fetal bovine serum (Life Technologies, Burlington, ON), 10 mm HEPES, and 50 μg/ml gentamycin (NovoPharm, Toronto, ON) at 37°C in the presence of 5% CO_2. The effect of lipids and 25-hydroxy cholesterol (25-OH cholesterol) on levels of CETP mRNA was determined by incubating cells for 24 h in either lipoprotein-depleted serum (LPDS) or complete serum (CS) in the presence or absence of 5 μm 25-OH cholesterol (Sigma, Chemical Co., St. Louis, MO). Delipidated serum was prepared by separating lipoproteins from remaining plasma proteins by differential ultracentrifugation at density of 1.21 adjusted with KBr (11) and dialyzed against PBS for 24 h. For preparation of HDL, plasma was collected from healthy normolipemic donors and HDL (d 1.063–1.21g/ml), isolated by sequential ultracentrifugation (Beckman 55.2 Ti rotor, 40,000 rpm, 20 h, 8°C).

RNA extraction and Northern blot analysis

Total cellular RNA was isolated from SW872 cells with TRIzol™ reagent (Life Technologies, Burlington, ON) according to the manufacturer's recommendations. Subsequently, poly(A)$^+$ RNA was extracted using the PolyATtract mRNA isolation system as outlined by the supplier (Promega Corp.). For Northern blotting, 4 μg of poly(A)$^+$ RNA was size-fractionated on agarose/formaldehyde gels and transferred to MSI nylon membranes (MSI, Westborough, MA). Blots were prehybridized for 2 h in 5× SSPE (0.9 m NaCl, 50 mm NaH_2PO_4, and 5 mm EDTA [pH 7.7]) containing formamide (50%), 4× Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured herring testes DNA. CETP mRNA was detected by overnight hybridization at 42°C against a [α^{32}P]dCTP (Amersham Life Science Inc, Oakville, ON) random-primed cDNA corresponding to the coding region of this gene (specific activity of 5–8 × 10^8 cpm/μg). To correct for differences in RNA loading, blots were also hybridized with a β-actin cDNA (American Type Culture Collection, Rockville, MD) probe. Blots were exposed at −80°C to Kodak XAR films with an intensifying screen. The exposed X-ray films were analyzed densitometrically using a Sharp JX-325 and bands were quantified using the one-Dscan software.

Transient transfection assays

Confluent SW872 cells were trypsinized and seeded at a density of 2.5 × 10^5 cells/well in 6-well plates 48 h prior to transfection. In some experiments, fetal bovine serum was replaced by LPDS in the media 12 h preceding transfections. A total of 5 μg of DNA, comprised of 3 μg of either CETP600LUC, CETP361LUC, CETP125LUC, or pGL3-basic and 2 μg of herring testis DNA, was transfected into cultured cells using the calcium phosphate–DNA precipitate method (12). For co-transfection experiments with recombinant transcription factors YY1, SREBP-1a (mature form), and SREBP-2 (mature form), increasing amounts of either expression vectors pCMV-YY1 (13) or pCMV-CSA10 or −CS2 (14) were added to the DNA mixture. In some instances, the *Renilla* luciferase-bearing vector pRL-TK (Promega Corp.) was included in the transfection experiments as an internal control. The final amount of DNA in each transfection was maintained a 5 μg/plate. Four hours after transfection, cells were shocked with 15% glycerol for 2 min and washed twice with PBS before the addition of media. Cells were harvested 24 h post-transfection and disrupted in 200 μl of reporter lysis buffer (Promega Corp.) for 10 min.

Luciferase activities derived from both *Firefly* (CETP constructs) and *Renilla* (pRL-TK) proteins were measured using the Dual-Luciferase™ reporter assay system (Promega Corp.) and recorded using a Monolight 2010c luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). *Renilla* luciferase activity was then used to standardize for transfection efficiency. In instances where co-transfection experiments were performed in the presence of either SREBP-1a or −2, the *Firefly* luciferase activity of each construct was corrected for the total cellular protein content of each extract as previously described by others (15–18). This alternative method

was used because the *Renilla* luciferase activity of two independent internal controls (pRL-SV40 and pRL-TK) was shown to be modulated by these transcription factors (unpublished data, B. Gauthier, and R. McPherson). Protein concentrations were determined using the bicinchoninic acid (BCA) method as described by the supplier (Pierce, Rockford, IL). Protein content was highly constant from experiment to experiment and was not significantly altered by any of the interventions performed.

Preparation of nuclear protein extracts

SW872 cells were grown as described above in media supplemented with either complete serum or lipoprotein-depleted serum. Nuclear protein extracts were prepared from 3–6 $\times$ 10^8 cells according to the protocol of Zhang et al. (19) with the exception that the isolated proteins were resuspended in a nuclear buffer containing 25 mm HEPES (pH 7.6), 0.1 mm EDTA, 40 mm KCl, 10% glycerol, and 1 mm DTT. Resuspended protein extracts were then dialyzed overnight at 4°C against 100 volumes of nuclear buffer. Precipitates that formed after dialysis were removed by centrifugation and supernatants were frozen at −70°C in small aliquots. Protein concentrations were determined as described above.

DNA gel mobility shift assay

Double stranded oligonucleotides corresponding to the CRE of CETP (5′-GGCAAAAATG GTGCAGATGG TGGAGGGG-3′) were radioactively end-labeled with [γ^{32}P]-dATP using the polynucleotide kinase T4 and purified from unincorporated nucleotides by gel filtration over a G-50 column. For DNA binding assays performed with nuclear protein extracts, reactions were carried out on ice in 20 μl binding buffer (25 mm HEPES [pH 7.6], 5 mm $MgCl_2$ and 34 mm KCl) supplemented with 50 μg of BSA and 0.05 μg of poly dI:dC. Nuclear protein extracts (2–6 μg) isolated from SW872 were mixed with 60 fmol of ^{32}P-labeled oligonucleotides (specific activity of 333 cpm/fmol). These reactions were left on ice for 20 min. For gel mobility supershift assays, antibodies to transcription factor, YY1, were added to protein extracts 1 h prior to the binding assay. In experiments where recombinant SREBP-1a was used instead of nuclear protein extracts, the binding reaction was performed in a buffer containing 12.5 mm HEPES-KOH (pH 7.5), 6 mm $MgCl_2$, 5.5 mm EDTA, 50 mm KCl, 5 mm DTT, 0.25 mg/ml of low fat milk, 50 ng/μl dI:dC, and 10% glycerol (v/v). DNA/protein complexes were then resolved by electrophoresis through 5% polyacrylamide gels in 0.25× Tris-borate running buffer (20 mm Tris-acetate, pH 7.2, 0.5 mm EDTA).

Statistical analysis

Results are expressed as mean ± SE. Where indicated, the statistical significance of the differences between groups was estimated by Student's *t*-test.

RESULTS

CETP mRNA levels in confluent SW872 cells are modulated by lipids found in serum and by 25-OH cholesterol

To confirm an effect of cholesterol on CETP gene expression in the human liposarcoma cell line SW872, we carried out experiments in which cells were grown in media containing either complete or delipidated serum and 25-OH cholesterol. Northern blot analysis of the samples revealed that CETP mRNA increased by 110% in the presence of complete serum as compared to cells grown in the absence of lipoproteins (**Fig. 1**). The addition of 25-OH cholesterol to the complete serum resulted in a further 90% increase in levels of CETP mRNA (Fig. 1). Similar increases in endogenous levels of hepatic CETP mRNA have been previously reported in response to cholesterol feeding in mice bearing the human CETP transgene with its natural flanking sequences (20).

Deletion of the region spanning nucleotides −360 to −138 of the human CETP promoter results in the loss of lipid-mediated regulation of the gene

In order to delineate the region of the CETP promoter involved in cholesterol-mediated regulation, deletion derivatives containing 600, 361, and 125 bp of the 5′ flanking region were subcloned into the luciferase reporter vector pGL3-Basic. The relative luciferase activity of each construct was determined by short term transfection into confluent SW872 cells and was found to increase with gradual deletion of the promoter region (8008 ± 700, 3118 ± 410, and 450 ± 43 RLU/μg protein for CETP125LUC, CETP361LUC, and CETP600LUC, respectively) consis-

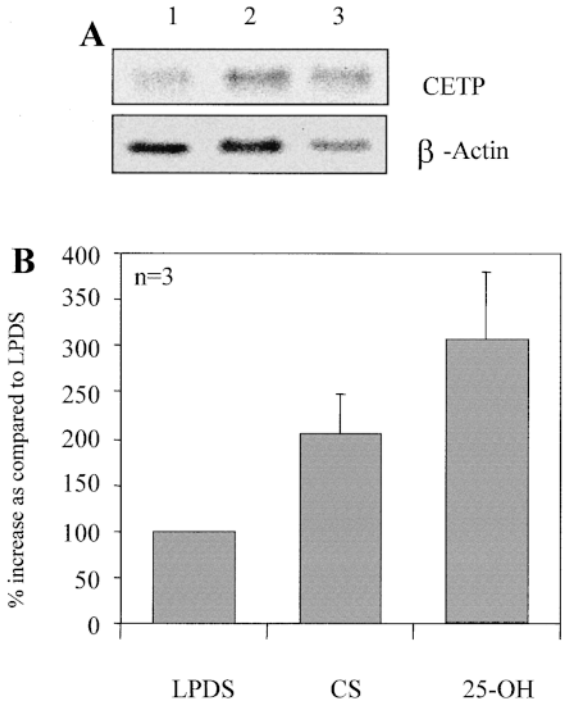

Fig. 1. CETP mRNA levels in confluent SW872 cells are modulated by lipids. The effect of lipids and 25-OH cholesterol on levels of CETP mRNA was determined by incubating SW872 cells for 24 h in DMEM/HAM F12 (3:1) supplemented with either lipoprotein-depleted serum (LPDS) or complete serum (CS) in the presence or absence of 5 μm 25-OH cholesterol. Cells were harvested and poly(A)$^+$ RNA was extracted using the polyATtract mRNA isolation system. (A) Four μg of poly(A)$^+$ RNA was size-fractionated on a agarose/formaldehyde gel and transferred to a nylon membrane. CETP mRNA was detected by overnight hybridization at 42°C against an [α^{32}P]dCTP random-primed cDNA corresponding to the coding region of this gene (specific activity of 5–8 $\times$ 10^8 cpm/μg). To correct for differences in RNA loading, blots were also hybridized with a β-actin cDNA probe. 1; LPDS, 2; CS, 3; 25-hydroxy-cholesterol. (B) Graphic representation of three Northern blot analyses. Results are expressed as the percentage of treated samples as compared to the LPDS sample; $P < 0.05$ for CS and 25-OH as compared to LPDS.

tent with previous reports (9). The ability of each construct to respond to cholesterol loading was then assessed by incubating transfected cells in media supplemented with delipidated or complete serum or complete serum plus 25-OH cholesterol (**Fig. 2**). Both luciferase reporter constructs CETP600LUC and CETP361LUC responded to lipoprotein supplementation with similar fold induction as compared to control cells cultivated in LPDS (2.5 and 2.8, respectively). Interestingly, the luciferase activity of CETP600LUC but not of CETP361LUC in the presence of 25-OH cholesterol increased 4.5-fold as compared to control levels (Fig. 2A). As opposed to CETP600LUC and

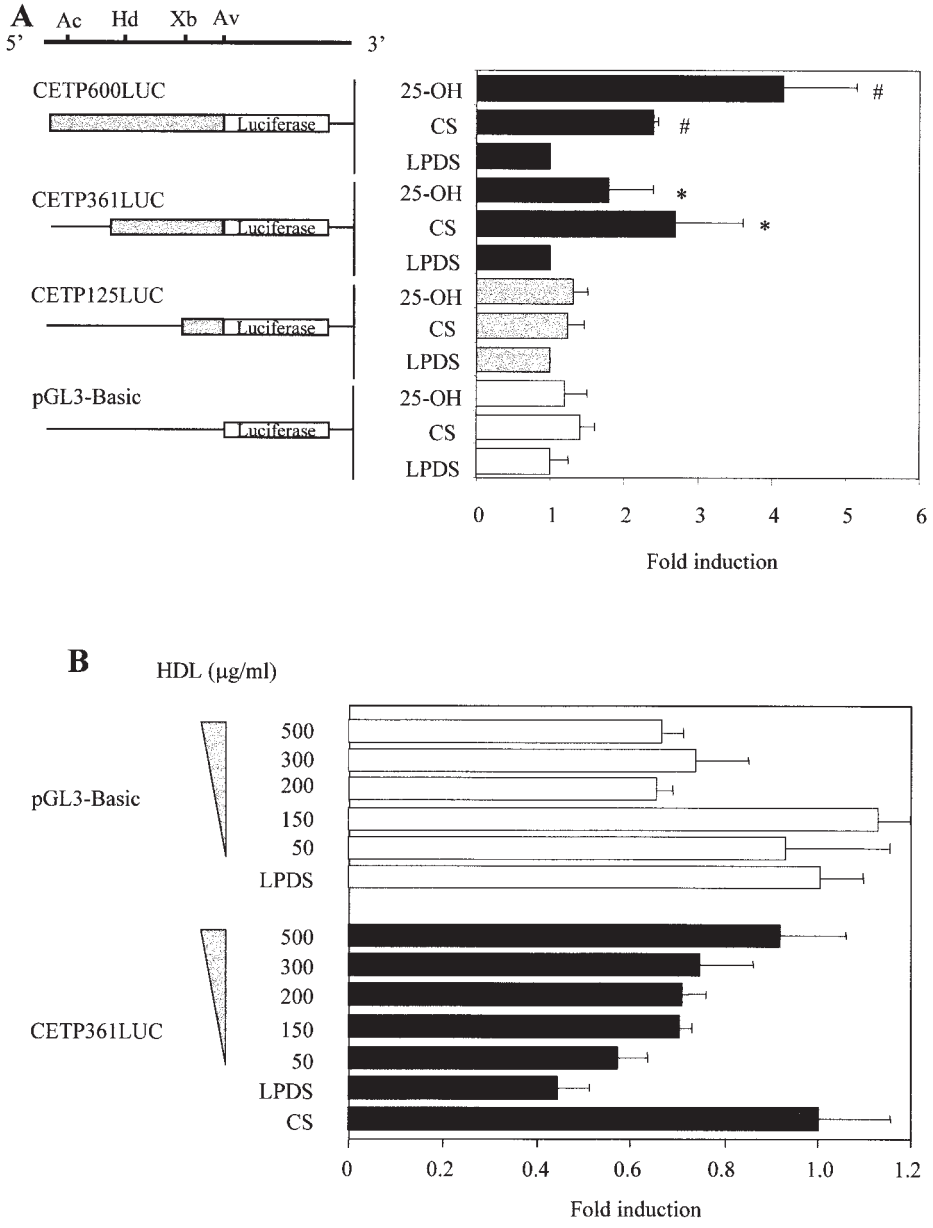

Fig. 2. Deletion of the region spanning nucleotides −361 to −138 of the human CETP promoter results in the lost of lipid-mediated regulation of the gene. (A) Serial deletion derivatives of the CETP gene 5′ flanking region were subcloned into the pGL3-Basic luciferase reporter vector. The effect of lipids on the expression of each construct was then assessed by transfecting SW872 cells with 3 μg of either CETP600LUC, CETP361LUC, CETP125LUC, or pGL3-Basic and 0.5 μg pRL-TK using the calcium phosphate–DNA precipitate method formed in BES. Cellular protein extracts were isolated 24 h after transfection and luciferase activities for both the *Renilla* and *firefly* gene were determined using the Dual-Luciferase™ reporter assay system. The luciferase activity of each construct was then normalized to the *Renilla* activity. Results are expressed as fold induction as compared to the LPDS-treated sample of each construct. All experiments were performed in triplicate on two separate occasions; * CETP361LUC, $P < 0.05$ for CS and 25-OH as compared to LPDS; # CETP600LUC $P < 0.05$ for CS and 25-OH as compared to LPDS. (B) Five μg of the luciferase construct CETP361LUC or pGL3-Basic were transfected into SW872 cells which were subsequently maintained in LPDS media with increasing concentrations of HDL particle.

CETP361LUC, the luciferase activity in CETP125LUC did not change in the presence of either lipoproteins or 25-OH cholesterol suggesting that the region between nucleotides −361 and −125 of the human CETP promoter may contain critical elements involved in lipid-mediated regulation of the gene (Fig. 2A). Lipoprotein cholesterol availability or the addition of 25-OH cholesterol to the media of cells transfected with the control vector did alter its luciferase activity, indicating that the effect observed on CETP600LUC and CETP361LUC is mediated by CETP promoter sequences. We have demonstrated that incubation with HDL results in net delivery of cholesterol to SW872 cells via a selective uptake process, which is mediated by CETP (7). The addition of HDL as a cholesterol source to LPDS resulted in the recovery of CETP361LUC luciferase activity as compared to complete serum (Fig. 2B). No increase in pGL3-basic luciferase activity was observed in the presence of HDL (Fig. 2B) indicating that lipids found in complete serum were conferring transcriptional regulation through potential interaction with CETP promoter sequences harbored between −361 and −125. As depicted in **Fig. 3A**, this region contains a tandem repeat (5′-**ATGGTG**-cag-**ATGGTG**-3′) which forms part of an element known to up-regulate the HMG-CoA reductase gene in response to cellular sterol depletion (21). Osborne and co-workers (21) have demonstrated that all residues contained within the equivalent half site of the sterol regulatory element of the HMG-CoA reductase gene are critical for binding of the transcription factors SREBP-1 (14) and YY1 (personal communication from T. Osborne) with the exception of the 3′ thymidine. This raises the possibility that YY1 and SREBP-1 may be involved in the lipid-mediated regulation of the human adipocyte CETP gene through their interaction with the tandem repeat which we have denoted the cholesterol response element (CRE). As each repeat can potentially interact independently with these factors, we have further divided the CRE into two sub-elements, cre-1 and cre-2 (Fig. 3A).

Transcription factors SREBP-1a and YY1 interact with the CRE of CETP

To determine whether or not the CRE could interact with factors such as SREBP-1a and YY1, we synthesized an oligonucleotide corresponding to this sequence (position −213 to −186) and carried out gel mobility shift assays in the presence of nuclear protein extracts derived from SW872 cells (Fig. 3B). The extracts were prepared from cells grown in media supplemented with either delipidated or complete serum. A retarded complex of similar mobility was produced using either LPDS or CS extracts indicating that the CRE may be occupied by nuclear factors regardless of lipid availability (Fig. 3B). The specificity of the lower mobility shift complex was demonstrated by showing that 4.5 pmol (200-fold molar excess) of unlabeled CRE competed effectively for the factors binding to the labeled element while similar molar excess of dIdC, a nonspecific competitor, had no effect (**Fig. 4A**). To determine whether or not YY1 could potentially be one of the nuclear factors interacting with the CRE, gel mobility supershift assays were performed using YY1 polyclonal antibodies. The results demonstrate that in the presence of YY1 antibody two additional retarded complexes are formed while preimmune serum exhibits no supplementary bands (Fig. 4B). These results indicate that the transcription factor, YY1, is a component of the CRE/protein complex. Gel mobility shift assay was performed with recombinant SREBP-1a (rSREBP-1a) (gift from Dr. T. Osborne) to determine whether or not this factor could also interact with the CRE. The addition of rSREBP-1a resulted in the appearance of a retarded complex demonstrating that this factor can interact with the CRE (Fig. 4C, lane 2). Therefore, our data indicate that both YY1 and SREBP-1a bind to the CRE.

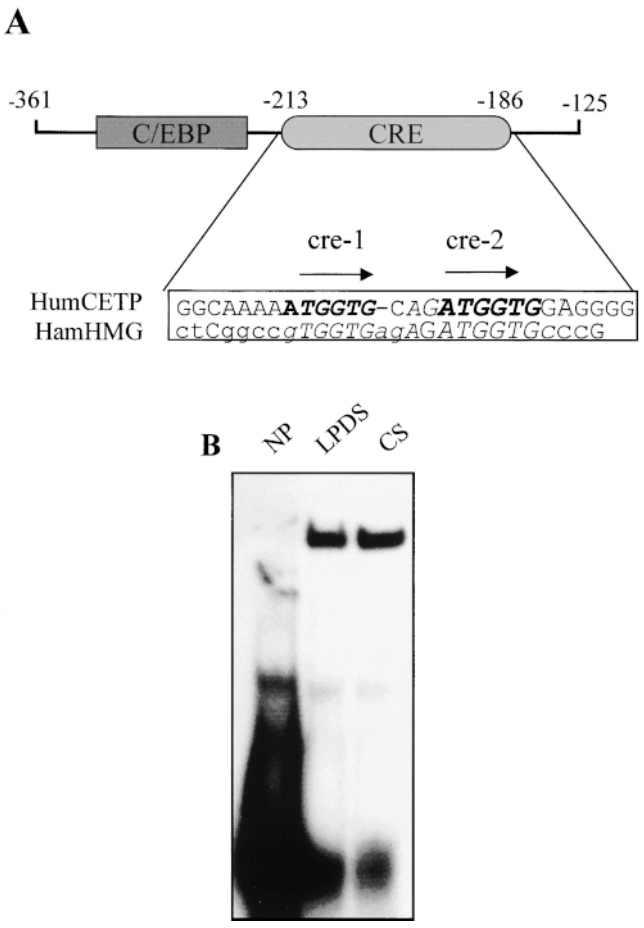

Fig. 3. (A) Schematic representation of the −361 to −138 region of the CETP promoter depicting the architecture of the cholesterol response element (CRE) and surrounding sequences. The sequence of the human CETP CRE (HumCETP) is shown and compared to the SRE of the hamster HMG-CoA reductase gene (HamHMG). The element contains two direct repeats (indicated in bold) which have been denoted as cre-1 and cre-2 (arrows). Similar sequences in the SRE of the hamster HMG-CoA reductase gene (depicted in italics) were shown to interact with the transcription factors YY1 and SREBP-1a. This region also contains a C/EBP site, which is important for hepatic expression of the CETP gene. (B) Gel mobility shift assays were performed using a labeled oligonucleotide (10,000 cpm/reaction) corresponding to the CRE (−213 to −186) of the CETP gene as shown in Fig. 3A and nuclear protein extracts (4 μg) isolated from SW872 cells grown in CS or LPDS. NP depicts no protein control.

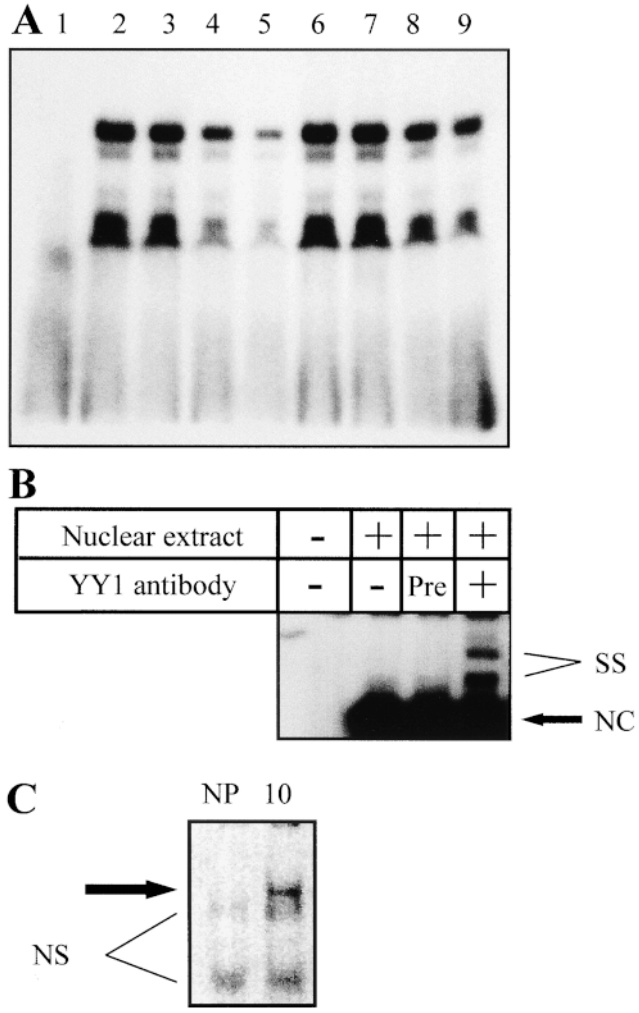

Fig. 4. Two of the factors interacting with the CRE of CETP are YY1 and SREBP-1a. (A) Competition assay demonstrating self-competition using CRE (lane 1: no protein; lane 2: 0 pmol CRE; lane 3: 0.15 pmol CRE; lane 4: 1.5 pmol CRE; lane 5: 4.5 pmol CRE) and non-specific competition using dIdC (lane 6: 0 pmol dIdC; lane 7: 0.075 pmol dIdC; lane 8: 0.75 pmol dIdC; and lane 9: 2.3 pmol didC). (B) Polyclonal antibodies raised against YY1 or pre-immune serum were added to 4 μg of nuclear protein extracts from cells cultured in complete (CS) or delipidated (LPDS) serum for 1 h prior to performing binding assays using a labeled CRE. SS depicts the supershift resulting from the interaction of the YY1 antibody to the CRE/protein complex. The native CRE/protein complex is depicted as NC. (C) Ten ng of recSREBP-1a was incubated with labeled CRE and the mixture was resolved on a 5% polyacrylamide gel. The arrow indicates the retarded complex formed with recSREBP-1a. Two non-specific retarded complexes, which are observed with skim milk, are depicted as NS.

To determine whether or not SREBP-1 and YY1 could modulate expression of the CETP gene through their interaction with the CRE, we performed transient transfection studies using fixed amounts of either CETP361LUC or CETP125LUC along with increasing amounts of expression vectors for each transcription factor (**Fig. 5**). Results of co-transfection experiments demonstrated that both YY1 and SREBP-1a increased the luciferase activity of CETP361LUC but not of CETP125LUC in a dose-dependent manner reaching maximum activity which was 4-fold (YY1) and 6-fold (SREBP-1a) higher than control samples (Figs. 5A and 5B). We also tested the ability of SREBP-2 to *trans*-activate CETP125LUC and CETP361LUC (Fig. 5C). Co-transfection experiments with increasing amounts of SREBP-2 resulted in the relatively mild activation of CETP361LUC luciferase activity (2.5-fold) but not of CETP125LUC. Neither YY1 nor SREBP-2 affected the luciferase activity derived from pGL3-basic while SREBP-1a caused a mild activation (1.8-fold). Members of the SREBP family of transcription factors are known to interact with ubiquitous factors such as Sp1 to promote transcription of target genes. As YY1 is a ubiquitous factor that binds to the CRE, we wished to determine whether or not it could interact with SREBP-1a and affect levels of CETP gene expression. SW872 cells were co-transfected with fixed amounts of CETP361LUC and YY1 expression vector along with increasing amounts of SREBP-1a expression vector. To reduce the possibility of quenching at higher doses, the amount of YY1 added to cells was less than that previously determined to be optimal for the activation of the luciferase gene under the control of the CETP promoter. As shown in **Fig. 6**, transfection of 500 ng of YY1 or 800 ng of SREBP-1a independently caused approximately a 4-fold increase in luciferase activity as compared to the control sample. Although an initial decrease in luciferase activity was observed with the addition of 200–400 ng of SREBP-1a to transfectants containing 500 ng of YY1, higher amounts of this factor resulted in a further increase in activity (7- to 8-fold). The effect of SREBP-1a and YY1 on the luciferase activity appear to be additive indicating that these factors may bind independently at non-interactive sites such as cre-1 and cre-2. However, it remains to be determined whether or not SREBP-1a and YY1 physically interact together once bound to their site.

The CRE is the functional *cis*-acting element involved in the lipid-mediated up-regulation of CETP gene expression through its interaction with SREBP-1a

To assess the function of the CRE in the regulation of inducible CETP promoter activity by SREBP-1a, the activity of a mutant reporter gene construct was measured in transient transfection experiments. Two nucleotides within each half sub-element of the CRE were mutated to residues which have been shown to effect binding of SREBP-1a to the SRE of HMG-CoA reductase (21). The basal activity of the new construct, mutCETP300LUC, was slightly lower as compared to a similar construct, CETP300LUC, harboring the wild-type sequence of the promoter (1943 ± 264 versus 2802 ± 106 RLU/(g protein). Co-transfection experiments were carried out using mutCETP300LUC, wtCETP300LUC, and SREBP-1a (**Fig. 7A**). Compared to wtCETP300LUC (4-fold increase in luciferase activity in the presence of 800 ng of SREBP-1a), the mutated plasmid, mutCETP300LUC, demonstrated a significant decrease in the ability of SREBP-1a to induce luciferase activity (2-fold versus 4-fold increase) (Fig. 7A). In order to further demonstrate that the increase in luciferase activity derived from CETP361LUC or wtCETP300LUC was mediated by the in-

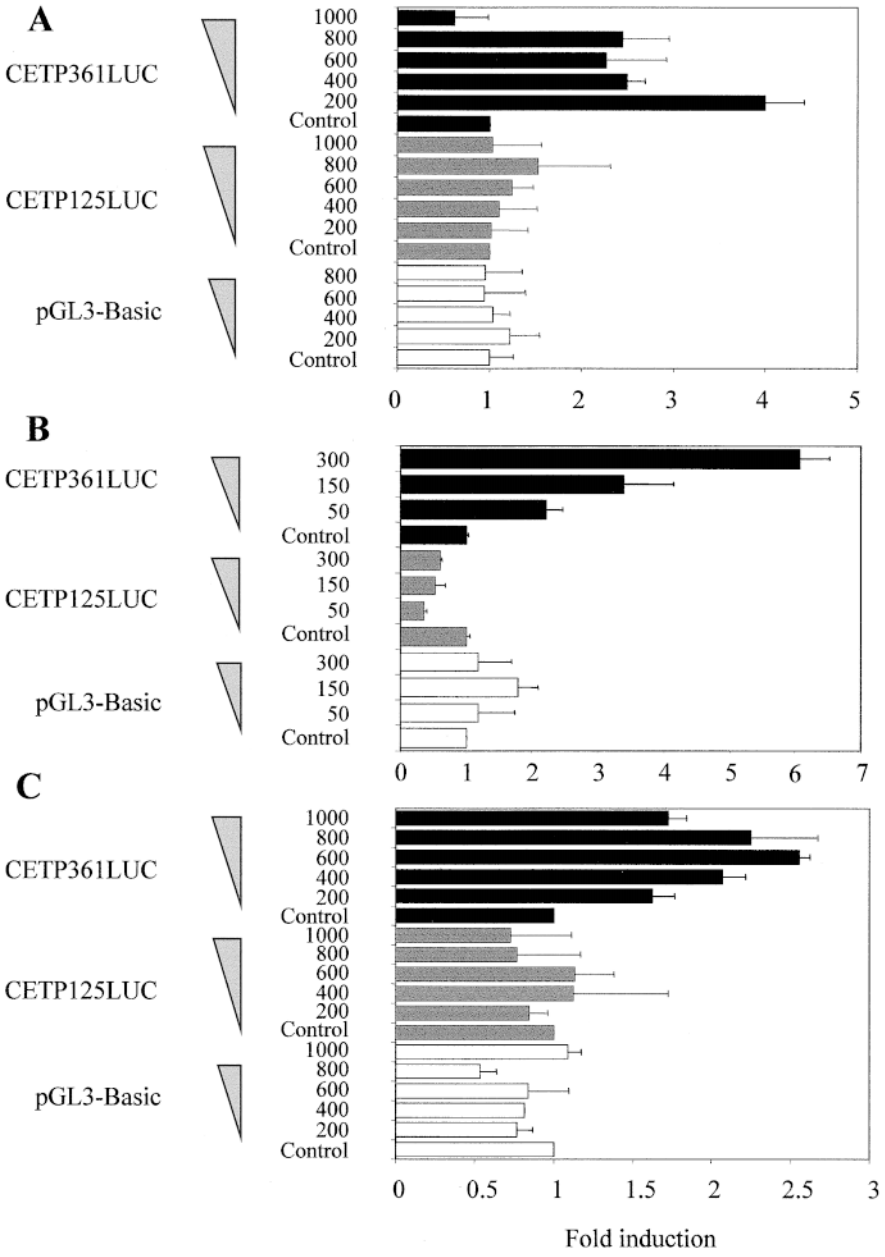

Fig. 5. SREBP-1a but not YY1 *trans*-activates only the luciferase activity of the construct harboring the CRE of CETP. Transient co-transfection studies using SW872 cells were performed with 3 μg of either luciferase reporter construct CETP361LUC or CETP125LUC and increasing amounts of expression vectors for: (A) YY1 or (B) SREBP-1a or (C) SREBP-2. As control, 3 μg of pGL3-basic was also transfected with increasing amounts of YY1, SREBP-1a, and -2. The total amount of DNA transfected was maintained at 5 μg/transfection using herring testis DNA. *Firefly* luciferase activity was assayed as described in Fig. 2 and normalized to *Renilla* luciferase activity (YY1) or total cellular protein content (SREBP-1a and -2). Results are expressed as fold induction compared to control sample. Experiments were performed in triplicate.

teraction of the CRE with SREBP-1a, three copies of the CRE were cloned into the pGL3-P vector upstream of the SV40 promoter. The luciferase activity of this new construct, pGL3-PCRE$_3$, was tested for inducibility by SREBP-1a in transient co-transfection assays (Fig. 7B). Although 800 ng of SREBP-1a resulted in a 2.7-fold increase in the luciferase activity of pGL3-promoter, the insertion of three copies of the CRE into the vector resulted in a further 10-fold increase in activity. To establish the functional role of the CRE in conveying lipid-mediated up-regulation of CETP gene expression, mutCETP300LUC or wtCETP300LUC were transfected into SW872 cells and cultured in the presence or absence of lipids. Lipids found in serum did not modulate the luciferase activity of the mutant construct, while the wild-type construct activity was increased by 2-fold (Fig. 7C). Taken together, these results demonstrate that this protein-binding region is critically involved in CETP expression and functions as a positive lipid regu-

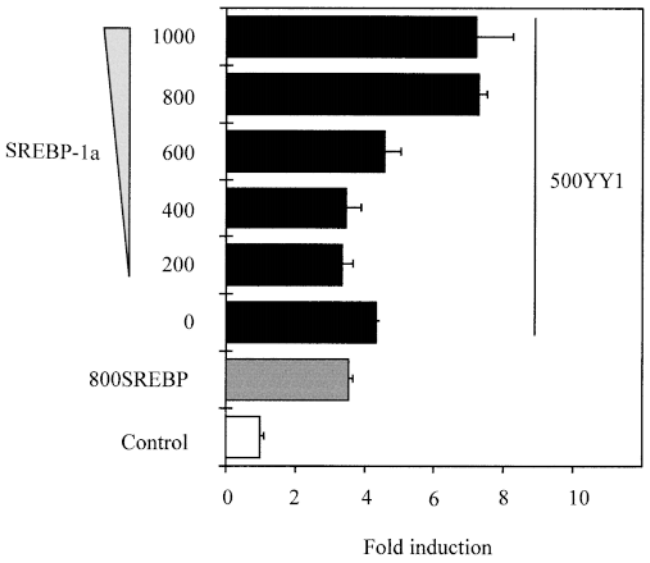

Fig. 6. Transcription factors YY1 and SREBP-1a act together to promote CETP300LUC luciferase activity. Transient co-transfection studies using SW872 cells were performed with 3 μg of the luciferase reporter construct CETP361LUC, 500 ng of YY1 expression vector, and increasing amounts of the expression vector for SREBP-1a. The total amount of DNA transfected was maintained at 5 μg/transfection using herring testis DNA. *Firefly* luciferase activity was assayed as described in Fig. 2 and normalized to total cellular protein content. Results are expressed as fold induction compared to control sample.

lator of promoter activity through its interaction with SREBP-1a.

DISCUSSION

Our results indicate that the transcription factors YY1, SREBP-1a, and -2, through their interaction with a cholesterol response element in the promoter region of CETP, are involved in the lipid-mediated up-regulation of this gene in the liposarcoma cell line, SW872. We suggest that the region of the human CETP promoter associated with lipid regulation of CETP in human liposarcoma cells resides between nucleotides −361 to −125. This region has previously been shown to be important for the sterol up-regulation of CETP gene expression in liver derived from transgenic mice bearing the human CETP gene (20) and contains an element similar to the sterol regulatory element (SRE) found in the promoter of the hamster HMG-CoA reductase gene. This element, 5′-ATGGTG-3′, was shown to be critical for the binding of the transcription factors YY1 (Red25) and/or SREBP-1. It was proposed that both factors bind to overlapping sequences within the SRE and that their relative levels determine the overall activity of the HMG CoA reductase gene (14, 21). The CRE of CETP contains two tandem repeats of the SRE sequence, which are capable of interacting with YY1 and SREBP-1a. It is presently unknown which of the subelements of the CRE interacts with YY1 or SREBP-1a. However, as cre-2 contains a classical E-box consensus sequence (CANNTG), we anticipate that this site is the SREBP binding element while cre-1 may interact with YY1 due to the presence of the inverted sequence CCATNTT which is known to bind this factor. (22, 23).

The functional importance of SREBPs in controlling transcription of sterol-regulated genes is well established. However, the physiological role that the ubiquitous nuclear factor YY1 plays in the sterol-dependent regulation of CETP gene expression remains to be determined. YY1 activity does not appear to be modulated by lipoprotein availability and therefore may not be directly involved in sterol regulation of this gene. However, in all SREBP-regulated promoters studied to date, additional co-regulatory transcription factors are required. SREBPs are inherently weak activators by themselves and function synergistically with ubiquitous factors such as NF-Y and Sp1 that bind in proximity to achieve a high level of promoter activation (24–28). In the present study, we demonstrate an additive effect of YY1 and SREBP-1a on the luciferase activity of CETP361LUC indicating a possible interaction between the two factors. These results are therefore consistent with the concept that SREBPs require co-factors to promote gene transcription and, in accord with previous studies on the HMG-CoA reductase promoter by Vallett et al. (14) and Osborne and colleagues (21), would define YY1 as a third class of ubiquitous factors that can cooperate with SREBPs. However, it remains to be determined whether or not the sterol-mediated regulation established by SREBP-1a on CETP gene expression is conditional on the binding of YY1 to the CRE.

To date, members of the SREBP family have been shown in cell culture to increase gene expression under conditions of cellular sterol depletion (25, 29–31). Limited data are available on the regulation of SREBPs in adipocytes or adipocytic cell lines such as human liposarcoma cells. Recently, Kim and Spiegelman (32) and Kim et al. (33) have demonstrated that the nuclear form of SREBP-1 (ADD1/SREBP-1c) plays a role in adipocyte differentiation and gene expression via production of endogenous ligand for PPARγ. In adipocytic cell lines such as 3T3-L1 cells, SREBP-2 mRNA exceeds that of SREBP-1a by 10-fold and SREBP-1c is virtually absent (34). In contrast, in mouse and human adipose tissue, SREBP-2 is very low and SREBP-1c is the predominant SREBP-1 transcript, exceeding that of SREBP-1a by 2- to 3-fold (34) and SREBP-1c may play a specific role in maintaining fatty acid homeostasis. Adipocytes synthesize very little cholesterol and rely on lipoproteins for cholesterol accumulation (7). Interestingly, targeted disruption of mouse SREBP-1a and 1c resulted in a marked increase in hepatic SREBP-2 and in hepatic cholesterol synthesis but no change in adipocyte expression of SREBP-2 (35). Overexpression of SREBP-1a caused overproduction of fatty acids and cholesterol in the liver but a decrease in adipose triglyceride stores (36). These findings suggest that adipocyte and hepatocyte SREBPs may not be regulated in an analogous fashion.

We have also demonstrated a much greater effect of SREBP-1a, as compared to SREBP-2, on the transcriptional activity of the CETP promoter. Previous in vivo data

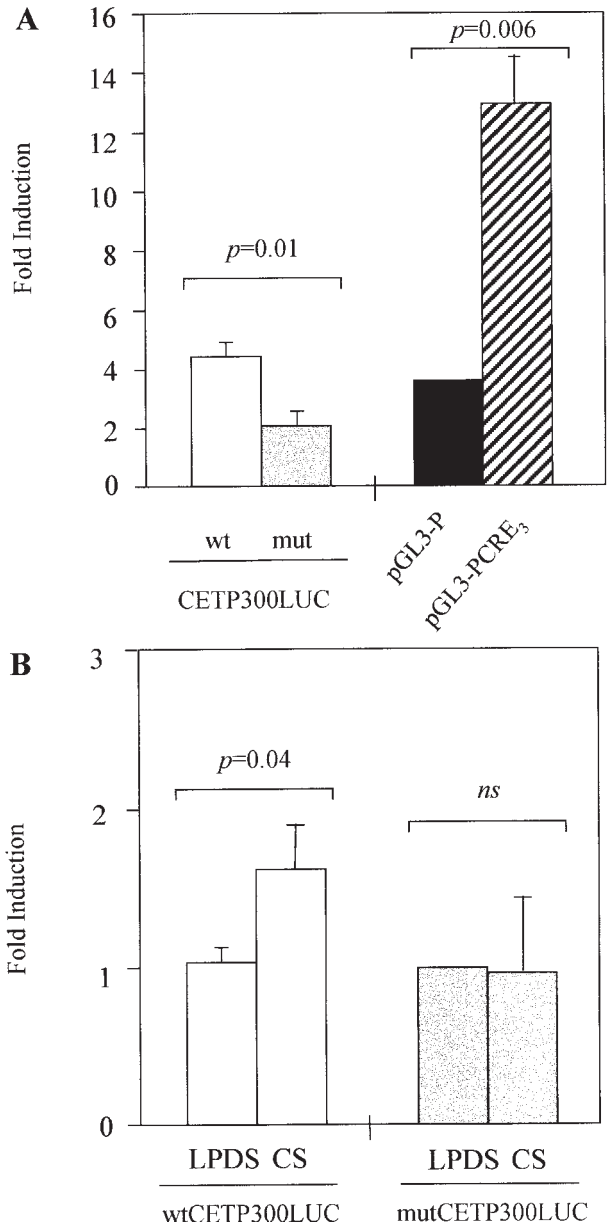

Fig. 7. The CRE is the functional *cis*-acting element involved in lipid-mediated up-regulation of CETP gene expression through its interaction with SREBP-1a. (A) Mutagenesis of the CRE was performed by PCR-mediated site directed mutagenesis. The mutated nucleotides were selected due to the critical role that each plays in binding SREBP-1a and YY1 to the SRE of the HMG-CoA reductase gene. Compared to a wild-type plasmid wtCETP300LUC, the mutated plasmid, mutCETP300LUC, demonstrated a significant decrease in the ability of SREBP-1a to induce luciferase activity (2- fold vs. 4-fold increase). Three copies of the CRE were also concatemerized and cloned into the pGL3-promoter vector to produce pGL3-PCRE$_3$. The ability of these constructs to respond to SREBP-1a (800 ng) was compared to parental vectors (wtCETP300LUC and pGL3-Promoter). Three μg of either wtCETP300LUC (Wt), mutCETP300LUC (Mut), pGL3-Promoter (pGL3-P), or pGL3-PCRE$_3$ were co-transfected along with 800 ng of SREBP-1a into SW872 cells. *Firefly* luciferase activity was measured as described in Fig. 2 and normalized to total cellular protein content. Results are expressed as fold induction of basal luciferase activity obtained in the absence of SREBP-1a. Although 800 ng of SREBP-1a resulted in a 2.7-fold increase in the luciferase activity of pGL3-promoter, the insertion of three copies of the CRE into the vector resulted in a further 10-fold increase in activity. (B) The ability of mutCETP300LUC and wtCETP300LUC to respond to the lipid content of serum was measured by transfecting 3 μg of either construct into SW872 cells along with 500 ng of pRL-TK. Cells were maintained in media supplemented with either LPDS or CS. *Firefly* and *Renilla* luciferase activities were measured and values were normalized to *Renilla* activity. Results are expressed as fold induction compared to the control sample (LPDS); ns, non-significant. The data demonstrate that lipids found in serum did not modulate the luciferase activity of the mutant construct, while the wild-type construct activity was increased by two-fold.

demonstrated that SREBP-1 and SREBP-2 are independently regulated by sterols (37). Studies using hamster liver showed that in cholesterol-fed animals, hepatic levels of mature SREBP-1 (later identified as SREBP-1c) were elevated and mainly localized to the nucleus while levels of mature SREBP-2 were low. In contrast, in animals treated with a statin and a bile acid sequestrant to deplete hepatic sterol concentrations, hepatic SREBP-2 expression and activity were induced with no change in SREBP-1a mRNA, a decrease in SREBP-1a proteolytic processing, and a decrease in SREBP-1c mRNA (34). Within the normal physiologic range, SREBP-1a has been shown to be 10 times as potent as SREBP-1c in increasing promoter activity of HMG-CoA reductase but only 2-fold more effective in increasing activity of the fatty acid synthase promoter (38).

We have not tested the effect of SREBP-1c on CETP promoter activity but our data strongly indicate that SREBP-1a is involved in up-regulation of human liposarcoma CETP gene expression, under conditions where cells are cholesterol replete. Under these conditions, increased production of CETP may facilitate reverse cholesterol transport. Cholesterol-depleted cells express relatively higher levels of SREBP-2, which we have shown to *trans*-activate the CETP promoter much less effectively than SREBP-1a. Thus it is becoming clear that SREBP-responsive genes may not all respond equally to different members of this family of transcription factors.

In summary, we have identified and characterized a lipid response element in the promoter region of CETP, which mediates cholesterol-dependent up-regulation of CETP gene expression in adipocytes. This element, denoted CRE, was shown to interact with transcription factors YY1 and SREBP-1a. We propose that, in circumstances where cells are cholesterol-replete and nuclear SREBP-2 is low, SREBP-1a binds to one of the CRE repeats and increases CETP expression. This interaction is likely stabilized by the presence of YY1 on the second repeat. When cholesterol levels are low, release of SREBP-2 to the nucleus exceeds that of SREBP-1a. We propose that the newly vacant repeat of the CRE interacts with either a second YY1 molecule or SREBP-2 which maintains a basal transcriptional activity of the gene.

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