

Human Cholesteryl Ester Transfer Protein Gene Proximal Promoter Contains Dietary Cholesterol Positive Responsive Elements and Mediates Expression in Small Intestine and Periphery While Predominant Liver and Spleen Expression Is Controlled by 5'-distal Sequences

CIS-ACTING SEQUENCES MAPPED IN TRANSGENIC MICE*

(Received for publication, July 9, 1996, and in revised form, September 20, 1996)

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The plasma cholesteryl ester transfer protein (CETP) facilitates the transfer of high density lipoprotein cholesteryl esters to other lipoproteins and appears to be a key regulated component of reverse cholesterol transport. Earlier studies showed that a CETP transgene containing natural flanking sequences (–3.4 kilobase pairs (kbp) upstream, +2.2 kbp downstream) was expressed in an authentic tissue distribution and induced in liver and other tissues in response to dietary or endogenous hypercholesterolemia. In order to localize the DNA elements responsible for these effects, we prepared transgenic mice expressing six new DNA constructs containing different amounts of natural flanking sequence of the CETP gene. Tissue-specific expression and dietary cholesterol response of CETP mRNA were determined. The native pattern of predominant expression in liver and spleen with cholesterol induction was shown by a –3.4 (5'), +0.2 (3') kbp transgene, indicating no major contribution of distal 3'-sequences. Serial 5'-deletions showed that a –570 base pairs (bp) transgene gave predominant expression in small intestine with cholesterol induction of CETP mRNA in that organ, and a –370 bp transgene gave highest expression in adrenal gland with partial dietary cholesterol induction of CETP mRNA and plasma activity. Further deletion to –138 bp 5'-flanking sequence resulted in a transgene that was not expressed *in vivo*. Both the –3.4 kbp and –138 bp transgenes were expressed when transfected into a cultured murine hepatocyte cell line, but only the former was induced by treating the cells with LDL. When linked to a human apoA-I transgene, the –570 to –138 segment

of the CETP gene promoter gave rise to a relative positive response of hepatic apoA-I mRNA to the high cholesterol diet in two out of three transgenic lines. Thus, 5'-elements between –3,400 and –570 bp in the CETP promoter endow predominant expression in liver and spleen. Elements between –570 and –370 are required for expression in small intestine and some other tissues, and elements between –370 and –138 contribute to adrenal expression. The minimal CETP promoter element associated with a positive sterol response *in vivo* was found in the proximal CETP gene promoter between –370 and –138 bp. This region contains a tandem repeat of a sequence known to mediate sterol down-regulation of the HMG-CoA reductase gene, suggesting either the presence of separate positive and negative sterol response elements in this region or the use of a common DNA element for both positive and negative sterol responses.

The cholesteryl ester transfer protein (CETP)¹ is a hydrophobic M_r 70,000 plasma glycoprotein that is synthesized by liver and small intestine as well as a variety of peripheral tissues (1). CETP mediates the net transfer of cholesteryl ester (CE) from HDL to triglyceride-rich lipoproteins (2) and appears to be a key component of reverse cholesterol transport, *i.e.* the transfer of cholesterol from the periphery back to the liver (3). By transferring CE to rapidly cleared triglyceride-rich lipoproteins, CETP stimulates the net movement of CE from HDL to the liver (4, 5). Acting in concert with hepatic lipase, CETP remodels HDL into smaller particles, which appear to be optimal substrates for the lecithin:cholesterol acyltransferase reaction (6) as well as the initial mediators of cellular cholesterol efflux (7, 8). In human genetic CETP deficiency, coronary heart disease is increased despite higher HDL levels, presumably reflecting the role of CETP in reverse cholesterol transport (9).

CETP gene expression is regulated by hormonal, inflammatory, and nutritional stimuli (10). Dietary cholesterol feeding induces increases in plasma CETP activity and CETP mRNA in a number of different species, including rabbits (11), hamsters

* This work was supported by National Institutes of Health Grant HL 54591. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U71187 and U71188.

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¹ The abbreviations used are: CETP, cholesteryl ester transfer protein; apo, apolipoprotein; CE, cholesteryl ester; HDL, high density lipoprotein(s); HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDL, low density lipoprotein(s); MT, metallothionein; SREBP, sterol response element binding protein; Tg, transgene; bp, base pair(s); kbp, kilobase pair(s); HFHC, high fat and high cholesterol.

(12), monkeys (13), and humans (14). The major sites of CETP mRNA expression are liver, spleen, adipose tissue, small intestine, kidney, and adrenal (1). Transgenic mice expressing a human CETP minigene linked to its natural flanking sequences show a tissue-specific pattern of CETP mRNA expression similar to that in humans (except that adipose tissue expression is relatively higher in humans) as well as an increase in CETP mRNA in liver and other tissues in response to a high cholesterol diet (15). The dietary induction of plasma CETP activity and hepatic CETP mRNA is entirely due to increased CETP gene transcription (15). Hepatic CETP gene expression is also up-regulated in response to endogenous hypercholesterolemia, as shown by breeding the CETP transgene into apoE and LDL receptor knockout backgrounds (16). The increase in CETP gene expression is proportional to plasma cholesterol levels, with 2–8-fold induction of hepatic CETP mRNA depending on the degree of hypercholesterolemia (16). CETP gene up-regulation in response to hypercholesterolemia may represent a homeostatic mechanism to enhance reverse cholesterol transport.

A number of genes are regulated in response to changes in plasma or cellular cholesterol levels. In particular, the LDL receptor (17, 18) and several genes of the cholesterol biosynthetic pathway, such as HMG-CoA reductase and synthase (19, 20) and farnesyl pyrophosphate synthase (21) are subject to sterol-mediated feedback repression. In the case of the LDL receptor, this response is mediated by a 10-bp sterol response element, which binds transcription factors termed SREBP-1 and SREBP-2 (22–24). The SREBPs reside in the endoplasmic reticulum in an inactive form. In response to sterol depletion conditions, an N-terminal proteolytic fragment enters the nucleus and promotes transcription of the LDL receptor gene (25). Although the transcription of a number of genes is induced by cholesterol (26–29), the DNA sequences and mechanisms responsible are poorly understood.

In this study we have used a transgenesis approach to define cis-acting sequences mediating cholesterol up-regulation and tissue-specific expression of the CETP gene. The transgenesis approach has provided unique information on cis-acting elements responsible for tissue-specific gene expression (30–32). In addition, we were not able to obtain reproducible sterol responses of the CETP gene in HepG2 and CHO cells.² Recently, a broad region mediating sterol up-regulation of cholesterol 7 α -hydroxylase gene expression has been mapped using both transgenesis and cell culture approaches (28). The latter involved the use of a highly differentiated murine hepatocyte line, termed H2.35 cells (33). Thus, this cell line was used as a secondary approach to confirm sterol-responsive CETP gene promoter regions.

EXPERIMENTAL PROCEDURES

CETP DNA Constructs and Development of Transgenic Mice—The construction of Tg A (–3400 bp) was described previously (15). Serial deletions in the CETP flanking regions were performed to create the following transgenes (Fig. 1). The *HindIII-HindIII* fragment in the 3' genomic region (34) was ligated to the *EcoRV-EcoRV* cDNA fragment and *BamHI-EcoRV* fragment in the 5' genomic region to generate Tg B (–3400) with the short 3'-flank. Digestion of Tg B with *KpnI* generated Tg C (–570). Digestion of Tg A with *KpnI* generated Tg D (–570). Tg E (–370) was prepared by digestion of Tg A with *EcoRI* and religation of the 109-bp fragment to the 5'-fragment of the remaining Tg A. Tg F (–138) was created by digestion of Tg A with *XbaI*. A CETP promoter-driven apoA-I transgene was obtained by blunt ligation of a 432-bp *KpnI-XbaI* fragment from the 5'-region of the CETP gene to the 2.2-kbp *SmaI* fragment of the apoA-I gene. All transgenes were subcloned into the pBluescript KS+ vector (Stratagene, La Jolla, CA). The transgenes

were injected into the pronuclei of fertilized mouse eggs taken from superovulated (C57BL/6J \times CBA/J) F1 females as described by Walsh *et al.* (35). Screening of CETP transgenic mice and subsequent breeding was performed according to Agellon *et al.* (36). Screening of CETP/apoA-I founders was performed by standard Southern blotting analysis of mice genomic DNA digested with *FokI* and probed with a 0.7-kbp *SacI-PstI* human apoA-I genomic probe (37). Subsequent generations of the CETP/apoA-I founders were screened by immunodetection of human apoA-I in dot blots of 0.5 μ l of mouse plasma, using the ECL detection kit (Amersham Corp.). Control nontransgenic plasma produced no apoA-I signal.

The relative copy number of CETP transgenes in Tg A to Tg F was determined by Southern blotting analysis of the founder genomic DNA. Briefly, 10 μ g of transgenic mouse genomic DNA was digested with *EcoRV*. Hybridization under high stringency conditions with a 0.98-kbp *EcoRV* fragment of human CETP cDNA probe produced the expected 0.98-kbp band. Copy number was estimated by comparing the signals by densitometric scanning of the autoradiograms and expressed relative to the lowest expressing line. Control nontransgenic mouse DNA did not produce any signal.

Dietary Studies—Heterozygous transgenic mice of both sexes from F1 and F2 generations were used in all experiments. Three to six-month-old animals were fed a chow (Purina Chow 5001, Ralston Purina Co., St. Louis, MO) or a high fat and high cholesterol (HFHC) diet containing 1% cholesterol and 20% coconut oil (Formulation C11037, Research Diets Inc., New Brunswick, NJ) for 1 week ($n = 6$ –12 animals/group). Blood was collected from the retro-orbital plexus under methoxyflurane anesthesia, and transgenic mice were sacrificed between 9:00 a.m. and 2:00 p.m. Twelve organs were excised, immediately frozen in liquid nitrogen, and stored at -70°C until RNA analysis. The tissues examined were small intestine, spleen, liver, heart, lung, kidney, adrenal, gonads, adipose, skeletal muscle, and brain.

RNA Analysis—Total RNA was isolated by the guanidinium thiocyanate method (38) using RNeasy B (Cinna/Biotech, Friendswood, TX). CETP mRNA was determined by a solution hybridization-ribonuclease protection assay as described previously (11). Briefly, a human CETP antisense cRNA probe was prepared from the human cDNA fragment, which included part of exon 16 and the 3'-untranslated region. A second riboprobe prepared from a mouse β -actin cDNA fragment (Ambion, Austin, TX) was included in the assay to normalize the amount of RNA per lane.

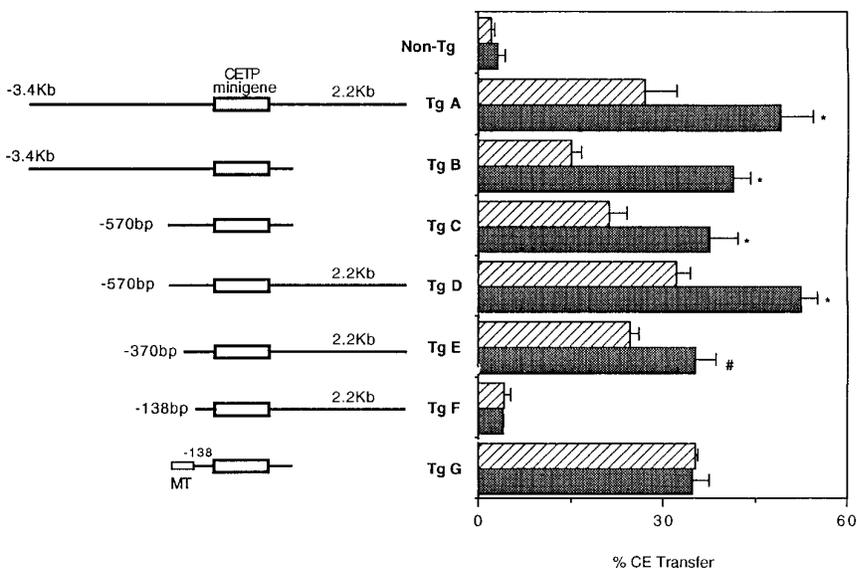
Hepatic human and mouse apoA-I mRNA levels were also determined by the solution hybridization ribonuclease assay according to Azrolan and Breslow (39). Specific riboprobes were prepared from a 188- and 230-bp fragment of mouse and human apoA-I cDNA, respectively, kindly provided by Dr. Neal Azrolan (Wyeth Ayerst Co.) (37). Five to ten μ g of total RNA were hybridized in solution with both mouse and human [³²P]UTP-labeled riboprobes for 16 h at 65 $^{\circ}\text{C}$. Samples were digested with RNase T1 and analyzed by electrophoresis on an 8 M urea, 5% polyacrylamide gel and autoradiography. This gave rise to two different sized protected fragments, representing endogenous mouse apoA-I mRNA and human apoA-I mRNA derived from the transgene. To normalize for recovery and nonspecific variation, the human apoA-I mRNA amount (optical density units from densitometry) was divided by the mouse apoA-I mRNA amount in each sample. For each line of mice the normalized human apoA-I mRNA amount was determined on chow or high cholesterol diet, and the high cholesterol diet's response was determined as a percentage of the chow response (Fig. 7).

Plasma CETP Activity and Cholesterol Levels—CETP activity was determined in diluted plasma using ³H-labeled CE-HDL as CE donor and a mixture of very low density lipoprotein and LDL as CE acceptor as described previously (40). Plasma total cholesterol levels were determined enzymatically (Wako Bioproducts, Richmond, VA).

Cell Culture and Transfection Experiments—The temperature-sensitive, SV40-transformed mouse hepatocyte cell line H2.35 (Ref. 33; from ATCC) was maintained in Dulbecco's modified Eagle's medium supplemented with 4% fetal bovine serum at 37 $^{\circ}\text{C}$ in a 5% CO₂ humidified atmosphere. Cells were cotransfected with 15 μ g of Tg A or Tg F and 1 μ g of pSV3-neo (ATCC) by the calcium phosphate method using the Stratagene mammalian transfection kit (Stratagene, La Jolla, CA). Selection with G418 (0.5 mg/ml) was maintained for 2–4 weeks. Resistant colonies were pooled and expanded in mass culture in the presence of 0.5 mg/ml of G418 (Life Technologies, Inc.). To induce differentiation, cells (approximately 5×10^6 cells) were plated in 100-mm diameter dishes containing rat tail type I collagen prepared as described by Zaret *et al.* (33) and cultured in 8 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and G418 at 39 $^{\circ}\text{C}$ for 3–5 h. On day 0, the medium was replaced by serum-free medium (33) con-

² H. C. F. Oliveira, R. A. Chouinard, L. B. Agellon, and A. R. Tall, unpublished results.

FIG. 1. Effect of HFHC diet on the plasma CETP activity of mice expressing human CETP transgenes containing variable lengths of flanking sequences (Tg A to Tg G). The boxes represent a CETP minigene containing all exons and 5 of the 15 introns (36). CETP activity was determined by an isotopic assay in diluted plasma of mice fed a chow (cross-hatched bars) or HFHC (dark bars) diet for 1 week. Results represent mean \pm S.E., $n = 6$ –12 animals/diet/group. Comparison between chow and HFHC diets is shown (*, $p < 0.0005$; #, $p < 0.002$).



taining G418, and the culture was incubated at 39 °C. Cells were refed every day with serum-free medium. On day 2, medium on replicate plates was supplemented with either 150 μ g/ml of LDL-cholesterol or saline solution (control). On day 3, cells were harvested by a brief, 1 mg/ml collagenase digestion (Sigma) and washed in phosphate-buffered saline, and their RNA was extracted with RNazol B.

Statistical Analysis—Results are expressed as mean \pm S.E. The statistical significance of the differences between the groups was estimated by Student's *t* test (two-tailed).

RESULTS

Plasma CETP Responses to HFHC Diet in CETP Transgenic Mice with Different Amounts of Flanking DNA Sequence—We used a transgenesis approach to localize cis-acting DNA sequences mediating dietary cholesterol regulation and tissue-specific expression of the CETP gene. Seven different constructs containing a CETP minigene and variable lengths of flanking sequence were used to generate transgenic mice (Fig. 1 and Table I). Two of these, transgenes A and G, were partly characterized previously (15). For each of the new transgenes, two to six different lines of mice were characterized.

Fig. 1 shows plasma CETP activity on the chow or HFHC diet for one representative line containing each transgene. Plasma CETP activity was measured on individual plasma samples ($n = 6$ –12 per line), using an isotopic assay indicative of CETP mass (41). The HFHC diet induced significant increases in plasma CETP levels for transgenes A–E. The -fold induction was 1.2 ± 0.2 (non-Tg), 1.9 ± 0.2 (Tg A), 2.9 ± 0.2 (Tg B), 1.9 ± 0.3 (Tg C), 1.7 ± 0.1 (Tg D), 1.3 ± 0.1 (Tg E), and 1.0 ± 0.2 (Tg F). For transgene F (–138), plasma CETP levels on either diet were not significantly different from the low background found in nontransgenic control animals. Transgene G (MT) was previously shown to be nonresponsive to the HFHC diet in the absence of Zn^{2+} induction (15). To see if Zn^{2+} induction of transgene G led to cholesterol responsiveness, this line was studied with zinc supplementation of drinking water on chow or HFHC diet. Zn^{2+} induced plasma CETP to levels similar to those observed for transgenes A–E, but did not endow cholesterol responsiveness.

A comparison of the -fold responses of transgene A versus B, and transgene C versus D indicates no contribution, or even a dampening effect, of 3'-flanking sequences on the cholesterol induction of plasma CETP activity. The positive response of transgenes A–E and the lack of response of transgenes F and G suggest a minimal region of upstream sequence between –138 and –370 that is required for dietary cholesterol induction of

CETP expression. The diminished response of transgene E relative to transgenes A–D could indicate a partial contribution of sequences between –370 and –570. In order to confirm these findings and to gain insights into potential regulatory regions driving tissue-specific expression of the CETP transgene, we next determined CETP mRNA levels in 12 different tissues for each of the lines. These results are summarized in Figs. 2–5.

Expression of CETP mRNA in Different Tissues and Response to High Cholesterol Diet in CETP Transgenic Mice with Different Amounts of Flanking DNA Sequences—Previous studies showed that the major sites of expression of transgene A (–3400) are liver and spleen, with lower levels of expression in small intestine, kidney, and adipose tissue (15). Similarly, in three independent lines of transgene B, liver and spleen were the predominant tissues expressing the CETP mRNA (shown for line B1 in Fig. 2). Although expression of CETP mRNA was not detected in secondary sites such as small intestine and kidney in lines B1, B2, and B3, this may be because of relatively low transgene copy number and levels of CETP mRNA near the limits of detection (10 pg of CETP mRNA/mg of total RNA) (Table I, Fig. 2). In addition to liver and spleen expression, transgenic line B4 showed low levels of CETP mRNA in small intestine and adrenal and higher levels in testis and kidney (Table I). In all lines of Tg B, hepatic CETP mRNA was significantly increased after 1 week of HFHC diet, as illustrated for line B1 in Fig. 2. These results suggest that the 3.4 kbp of 5'-flanking sequence contain the major elements providing liver and spleen expression as well as dietary cholesterol induction of CETP mRNA.

A markedly different pattern of expression was found for transgene C (–570, Fig. 3). The dominant tissue expressing this transgene was small intestine. Note that line C1 had a high transgene copy number and, accordingly, high expression levels of CETP mRNA (Table I, Fig. 3). Predominant small intestinal expression was confirmed in a second line of this transgene (Table I). These findings indicate that the deleted region upstream of –570 bp contains positive elements determining predominant liver and spleen expression for the CETP transgene. On the HFHC diet, there were significant increases in CETP mRNA in small intestine and muscle and apparent increases in pooled samples of some other peripheral tissues (Fig. 3).

Transgene D gave rise to a similar pattern of CETP mRNA in tissues as did transgene C (Table I). Both transgenes have 570

TABLE I
Expression of CETP in different transgenic mouse lines

Transgene copy number was determined by Southern blotting analysis and expressed relative to the lowest expressing line (B3). Sterol regulation of plasma protein activity and CETP mRNA is shown. +, yes; -, no; ND, not done.

Transgenes	Lines	Copy number	Plasma CETP expression	Major sites of expression	Sterol regulation
A (-3400)	5203	10	+	Liver, spleen	+
B (-3400)	1	2	+	Liver, spleen	+
	2	ND	+	Liver, spleen	+
	3	1	+	Liver, spleen	+
	4	ND	+	Testis, kidney, liver	+
C (-570)	1	15	+	Small intestine, brain, muscle	+
	2	ND	+	Small intestine	ND
D (-570)	1	7	+	Widespread	+
	2	5	+	Widespread	ND
	3	4	+	Widespread	ND
E (-370)	1	3	+	Adrenal, liver, spleen	+
	2	1	-	ND	ND
	3	2	-	ND	ND
	4	1	-	ND	ND
	5	2	-	ND	ND
	6	2	-	ND	ND
F (-138)	1	1	-	-	-
	2	3	-	-	-
E (MT)	MT	ND	+	Small intestine, heart, liver	-

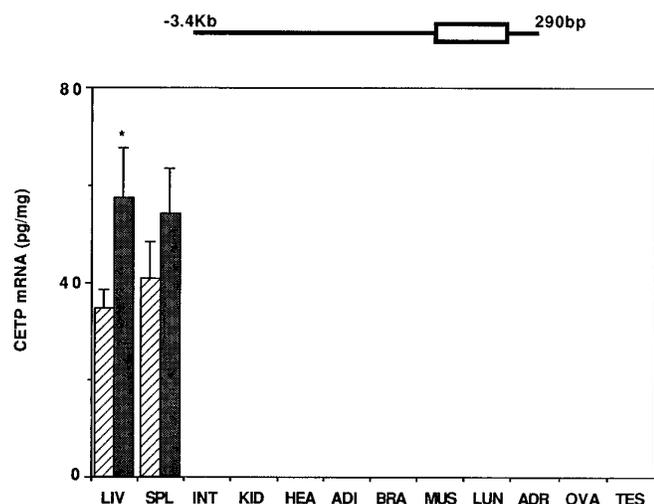


FIG. 2. Abundance of the CETP mRNA in the tissues of Tg B (-3400), line B1, fed rodent chow diet (cross-hatched bars) or HFHC diet (dark bars) for 1 week. Values shown are mean \pm S.E. ($n = 11$ animals/group). *, differences on the two diets were significant, with $p < 0.05$ (Student's t test). LIV, liver; SPL, spleen; INT, small intestine; KID, kidney; HEA, heart; ADI, adipose; BRA, brain; MUS, skeletal muscle; LUN, lung; ADR, adrenals; OVA, ovary; TES, testis.

bp of upstream sequence but 2.2 and 0.29 kbp of downstream sequence, respectively. Transgene D showed loss of predominant expression in liver and spleen but widespread expression in secondary sites (small intestine, kidney, lung). The intestinal expression was not as prominent for transgene D as for transgene C. CETP mRNA in adipose tissue, skeletal muscle, and adrenal was increased in response to the HFHC diet (not shown). Thus, the additional 3'-flanking sequences in transgene D did not appear to make a major contribution to the pattern of CETP mRNA expression nor to the induction by the HFHC diet.

Only one line of transgene E (-370) gave rise to detectable

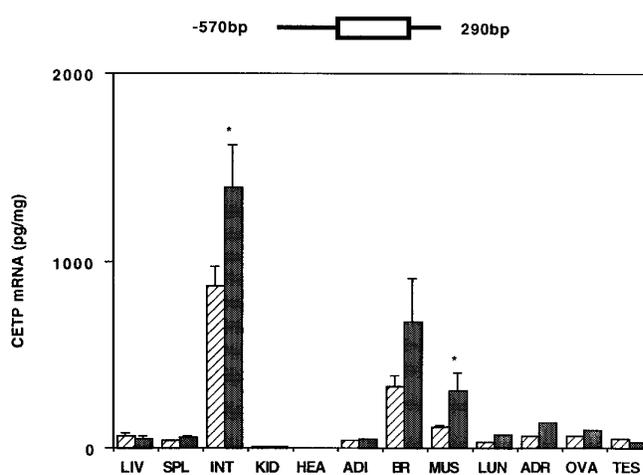


FIG. 3. Abundance of the CETP mRNA in the tissues of Tg C (-570), line C1, fed rodent chow diet (cross-hatched bars) or HFHC diet (dark bars) for 1 week. Values shown are mean \pm S.E. ($n = 6-8$ animals/group), and results for kidney, adipose, lung, adrenals, ovary, and testis represent a pool of RNA samples from at least 4 animals/group. *, $p < 0.05$. Abbreviations are as in Fig. 2.

CETP mRNA expression (Fig. 4, Table I). In this line there was loss of small intestinal expression and low level expression in liver, spleen, kidney, and adrenal, with adrenal containing the highest levels of CETP mRNA. The levels of expression in these tissues were comparable with that of transgene C (compare Figs. 3 and 4; note difference in y axis scale). Thus, elements mediating expression in intestine, brain, muscle, and possibly gonads, adipose, and lung, appear to have been lost with deletion of the -370 to -570 bp region. In parallel with changes in plasma CETP levels (Fig. 1), the HFHC diet induced a small but significant ($p < 0.05$) increase in hepatic CETP mRNA. A similar induction was also observed in pooled adrenals (data in Fig. 4 shown for six animals on each diet). Assays of tissue from two lines of transgene F (-138) failed to show any CETP

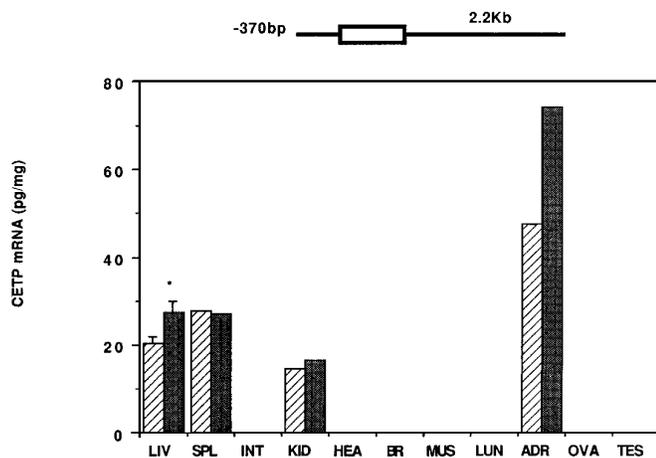


FIG. 4. Abundance of the CETP mRNA in the tissues of Tg E (-370) fed rodent chow diet (cross-hatched bars) or HFHC diet (dark bars) for 1 week. Values for liver are mean \pm S.E. ($n = 12$ animals/group), and results for other tissues represent a pool of RNA from 6 animals/group. *, $p < 0.05$. Abbreviations are as in Fig. 2.

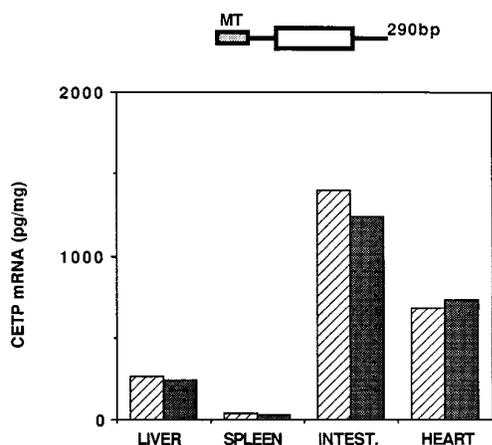


FIG. 5. Abundance of the CETP mRNA in the tissues of Tg G (MT) fed rodent chow diet (cross-hatched bars) or HFHC diet (dark bars) for 1 week. Results are average of two pools of 2 animals/group.

mRNA, just as there was no plasma CETP activity above the low level of nontransgenic mice (Fig. 1, Table I). Fig. 5 shows that Zn^{2+} induction of transgene G (*MT*) gave rise to high level expression of CETP mRNA in heart and small intestine (the latter comparable with that of transgene C in Fig. 3). However, there was no dietary cholesterol response.

Response of Transgenes to Cholesterol Loading in H2.35 Cells—These results suggest that the proximal 138 bp of the CETP promoter and the CETP minigene are not sufficient to mediate the dietary cholesterol response. However, since transgene F was not expressed and transgene G involved a foreign promoter, this conclusion was tentative. To further address this issue, we transfected mouse H2.35 hepatocytes with transgenes A and F. The stably transfected cells were induced to differentiate, and CETP mRNA was assayed in control (cholesterol-deprived) and cholesterol-loaded (LDL-treated) cells. The CETP mRNA was readily detected in H2.35 cells expressing either transgene (Fig. 6). With cholesterol loading, there was a significant increase in CETP mRNA ($p < 0.005$) in cells expressing transgene A (-3400), whereas there was a small decrease in CETP mRNA in cells expressing Tg F (-138). The cholesterol induction of transgene A was not detected in undifferentiated cells (not shown), as reported previously for cholesterol 7α -hydroxylase (28). This result confirms that elements

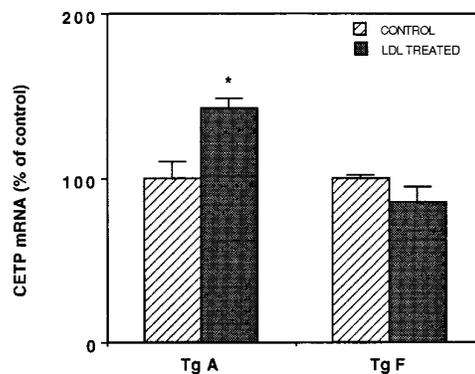


FIG. 6. Abundance of CETP mRNA in differentiated H2.35 cells transfected with Tg A (-3400) or Tg F (-138). Cells were differentiated by growth on rat type I collagen matrix, in serum-free media at 39 °C for 3 days. Cells were treated with 150 μ g/ml of LDL-cholesterol (dark bars) or saline (cross-hatched bars) for the last 24 h before harvesting for RNA analysis. Results for Tg A-transfected cells ($n = 8$ plates/treatment) and Tg F-transfected cells ($n = 5$ plates/treatment) are the average of three independent experiments. *, $p < 0.005$.

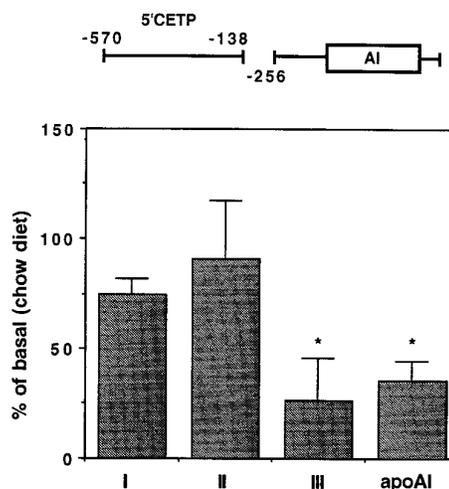


FIG. 7. Human/mouse apoA-I mRNA ratio in the liver of mice expressing a fusion of CETP -570 to -138 bp promoter region and apoA-I gene (lines I, II, and III) and the apoA-I gene alone. Results shown for animals fed a HFHC diet are expressed as the percentage of values of animals fed chow diet ($n = 6$ animals/line/diet treatment). *, $p < 0.003$ for HFHC versus chow values; $p < 0.006$ for line I versus apoA-I on HFHC diet; $p = 0.076$ for line II versus apoA-I on HFHC diet. See "Experimental Procedures" for additional details.

required for cholesterol up-regulation of the CETP gene lie upstream of the -138 site.

Cholesterol Response of a Fusion Transgene Containing CETP Promoter and Human ApoA-I Sequences—The transgenesis and cell culture studies together suggest that sequences between -138 and -570 are necessary for the full sterol response. We next attempted to determine if this region could act in an enhancer-like fashion to endow a cholesterol response on a reporter gene. In experiments where upstream segments (-3.4 kbp) of the CETP promoter were linked to the bacterial chloramphenicol acyltransferase reporter gene, we were unable to demonstrate cholesterol induction of chloramphenicol acyltransferase activity in differentiated H2.35 cells (not shown). In a further attempt to demonstrate enhancer-like activity of the CETP gene promoter, we used the human apoA-I gene as a reporter in transgenic mice. The relevant region of the CETP gene promoter was placed upstream of a human apoA-I gene fragment that is known to be well expressed in the liver in transgenic mice (35, 37). A fragment containing the entire apoA-I gene and 256 bp of upstream sequence was placed

Mapping of Positive Sterol Response Elements

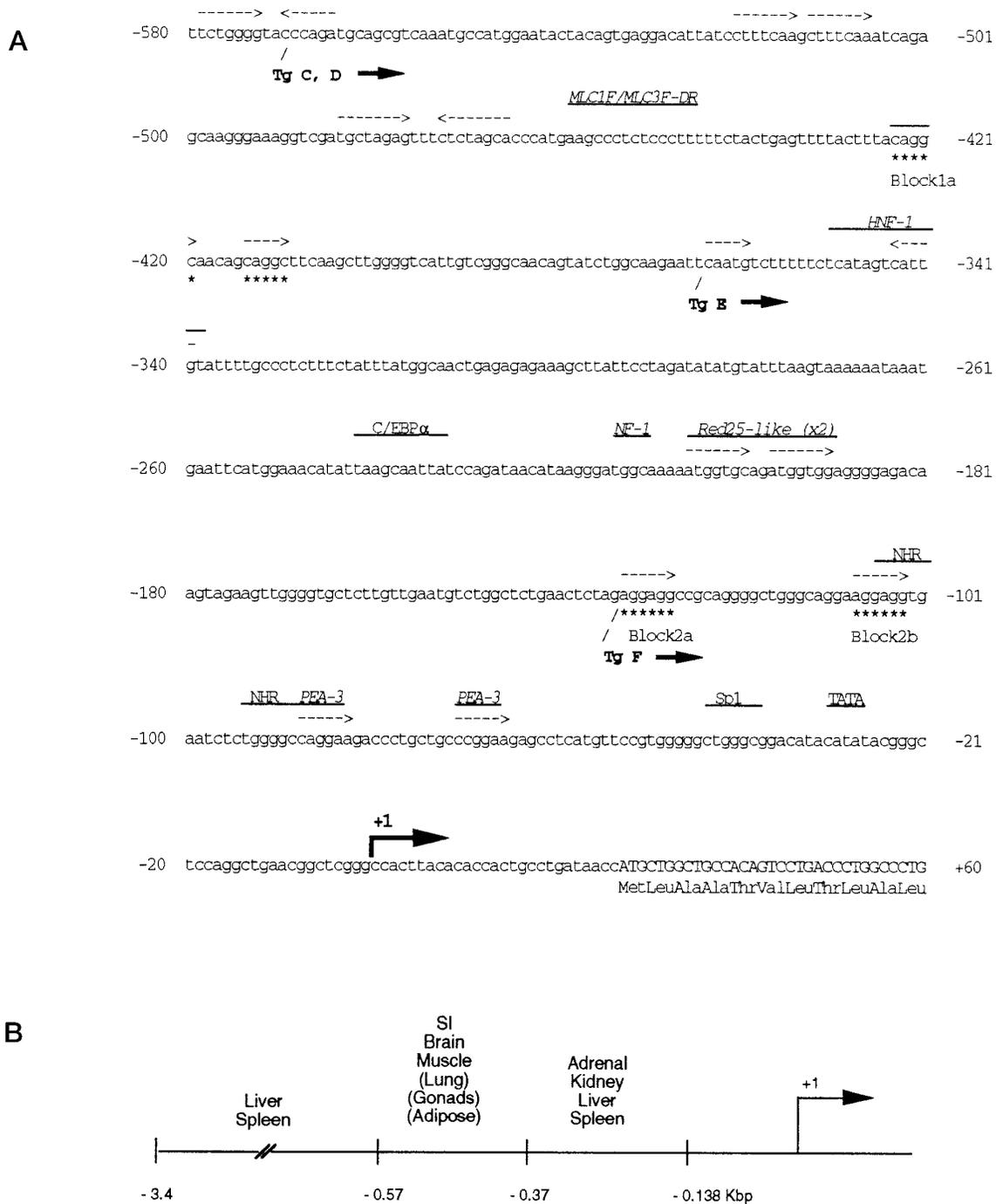


FIG. 8. A, complete nucleotide sequence between +60 and -580 of the human CETP promoter region. Consensus elements for known transcription factors are shown: a tandem repeat of the sterol regulatory element of the HMG-CoA reductase gene (Red 25), the ubiquitous nuclear factor-1 (*NF-1*), *C/EBP α* , hepatocyte nuclear factor-1 (*HNF-1*), a nuclear hormone receptor (*NHR*), *PEA-3*, *Sp1*, and the muscle-specific element *MLC1F*. Arrows indicate direct and inverted repeats. Asterisks indicate sequences found using the MACAW program (51) in the proximal promoter of rat, mouse, hamster, and human cholesterol 7 α -hydroxylase, human PLTP, and human, rat, and mouse apoE (GenBankTM accession numbers Z18860, L23754, L04690, L13460, U38950, M10065, J02582, and D00466, respectively). The 5'-ends of Tg C, D, E, and F are also indicated. B, distribution of tissue-specific elements in the 3.4 kbp of the human CETP promoter. Data in Figs. 2-5 are summarized by indicating tissues and the regions in the promoter that contain positive expression elements for them. Tissue names *in parenthesis* indicate the presence of regulatory elements with minor effects.

downstream of the -138 to -570 bp segment of the CETP gene promoter (Fig. 7). Three lines of transgenic mice expressing this fusion transgene were obtained and compared with an existing line of human apoA-I transgenic mice, expressing the same human apoA-I gene fragment (line 427, Ref. 37). All of these lines were found to express human apoA-I mRNA in the liver but not in the small intestine. Hepatic human apoA-I mRNA abundance was determined in mice fed chow or HFHC

diets. Compared with the human apoA-I transgene, two of the lines of mice expressing the fusion transgene had a positive response to the HFHC diet, and one of the lines had a similar response (Fig. 7). We considered the possibility that CETP promoter sequences may have been deleted during the integration of the transgene in the nonresponsive line III mice. However, polymerase chain reaction analysis using human CETP promoter-specific primers showed this was not the case. The

data suggest that the CETP gene promoter fragment endows a relative positive cholesterol response to the human apoA-I transgene, with the different behavior of line III mice attributed to positional effects.

DISCUSSION

In earlier studies the up-regulation of CETP gene expression in response to dietary cholesterol or endogenous hypercholesterolemia was shown to be dependent on the natural flanking sequences of the CETP gene (15, 16). In this study we have used a combination of transgenesis and cell expression approaches to define a minimal 232-bp region within the CETP proximal promoter that appears to contain one or more cholesterol-responsive elements. This region lies between -138 to -370 bp upstream of the transcription start site. The response of transgene E (-370) was less pronounced than transgene D (-570) (Figs. 1 and 3 *versus* Fig. 4), suggesting that there may be additional sequences contributing to the sterol response between -370 and -570 bp. However, the combined data of plasma activities and mRNA responses provided no evidence for a contribution of further upstream sequences or any downstream sequence to the positive sterol response.

A comparison of the minimal region of the CETP promoter (between -138 and -370) required for cholesterol regulation with promoter sequences of other sterol-regulated genes revealed a number of interesting homologies. In particular, there was a sequence with a striking similarity to the sterol regulatory element of the HMG-CoA reductase promoter (42, 43) (Fig. 8). Thus, the sequence between -217 and -192 bp in the CETP promoter consisted of a direct tandem repeat of the Red 25 binding site found at a similar position in the reductase promoter and contained all nucleotides shown by point mutagenesis studies to be required for sterol down-regulation of the latter gene (42, 43). Moreover, an NF-1 binding site required for sterol regulation of the reductase gene (42) is also found at a similar position upstream of the Red 25 binding sequence within the CETP gene promoter (Fig. 8). If this site is functional within the CETP gene promoter, there are two possibilities: 1) the Red 25 site acts independently as a negative sterol response element as in the HMG-CoA reductase promoter, and in this case there would be both positive and negative sterol response elements within this region of the CETP gene promoter; or 2) this region is directly or indirectly involved in the sterol induction of the CETP gene, acting as a positive element in the context of the CETP promoter. Recently, this site in the HMG-CoA reductase promoter has been shown to bind SREBP-1 (44), as well as Red 25 (42), suggesting the possibility of a complex interaction of different transcription factors mediating the positive sterol response.

A comparison of the CETP promoter with that of other genes up-regulated by sterols indicates some other regions of sequence conservation (Fig. 8). The proximal 342 bases of the rat cholesterol 7 α -hydroxylase promoter have been shown to be sufficient for cholesterol induction (28). Two six-base sequence blocks, CAGGC(A/C) and AGGAG(G/T), indicated in Fig. 8A as *Block 1* and *Block 2*, are present in the proximal 570 bases of the human CETP promoter as well as at conserved positions in the rat, human, hamster, and mouse cholesterol 7 α -hydroxylase. These sequences are also present in the proximal 500 bases of two other genes that appear to be cholesterol-inducible, human phospholipid transfer protein and human, rat, and mouse apoE (29, 45). The occurrence of these two sequences at similar positions in the promoters of these four genes suggests that they may be functionally important. There is also a cluster of potential regulatory sequences at about -350 bp (Fig. 8). This region contains a putative hepatic nuclear factor-1 binding site that overlaps an inverted repeat. Recently, it has been

shown that SREBP-1 is involved in the regulation of genes of fatty acid metabolism (46) and recognizes several different, very weakly related DNA consensus sequences (21). Determination of which if any of the implicated CETP promoter sequences is involved in cholesterol regulation of the CETP gene will require more detailed analysis by mutagenesis.

A secondary objective of our study was to define elements mediating tissue-specific expression of the CETP gene. Fig. 8B provides a tentative assignment of tissue-specific elements based on CETP mRNA expression in 12 tissues in different lines of mice. The results suggest that the region between -570 and -3400 bp confers predominant expression of the CETP gene in liver and spleen. Recently, it was shown that sequences between -138 and -33 bp in the CETP gene promoter are both necessary and sufficient to promote high levels of CETP gene transcription in HepG2 and CaCo-2 cells (47). This region binds the nuclear hormone receptor, apoAI regulatory protein-1, which can be either a repressor or inducer of CETP gene transcription, depending on the amount of 5'-flanking DNA. By contrast, our study indicates that additional elements between -570 bp and -3.4 kbp and between -370 and -570 bp are required for hepatic and intestinal expression, respectively. Thus, while the sequences defined *in vitro* may play a role in tissue-specific expression, they are not sufficient *in vivo*. These findings are reminiscent of studies of the apoE and apoA-I genes, where distant elements required for specific expression in liver and small intestine were only detected by studying expression in transgenic animals (31, 32, 37). Recent evidence shows that such enhancer-like elements bind distinctive sets of transcription factors and interact synergistically with transcription factors binding to sequences within the proximal promoter of their cognate genes (48, 49).

Deletion of sequences from -570 to -370 bp abolished muscle expression of CETP, indicating the presence of a muscle-specific element in this region. At position -459 lies a sequence found to be responsible for enhanced expression from the myosin alkali light chain 1 promoter (MLC/F) in differentiated muscle cells (50). The length of this functional element is 14 bp, making its presence in the CETP promoter highly unlikely to be a spurious occurrence.

Although the changes in CETP mRNA in response to the high cholesterol diet were modest in magnitude (1.4–2.5-fold), they are proportional to the relatively small (1.8–2.8-fold) change in plasma cholesterol induced by the HFHC diet in mice. By contrast, more marked hypercholesterolemia produces much larger changes in CETP mRNA (up to 8-fold), also involving a response of the natural flanking sequences (16). Similar moderate (1.7–2.5-fold) mRNA induction in response to cholesterol has been shown for several other genes. These include cholesterol 7 α -hydroxylase (28), the microsomal triglyceride transfer protein (26), apoE (29), and acyl-CoA:cholesterol acyltransferase (27). Through various pathways these different molecules all help to prevent the cellular accumulation of free cholesterol. The present study raises the possibility that common molecular mechanisms may underlie both up-regulation and down-regulation of gene expression by cholesterol.

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