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Research Article

To investigate the regulation of expression of the human cholesteryl ester transfer protein (CETP) gene, transgenic mice were prepared using a CETP minigene linked to the natural flanking sequences of the human CETP gene. By using a transgene containing 3.2 kb of upstream and 2.0 kb of downstream flanking sequence, five different lines of transgenic mice were generated. The abundance of CETP mRNA in various tissues was determined on standard laboratory diet or high fat, high cholesterol diets. In three lines of transgenic mice the tissues expressing the human CETP mRNA were similar to those in humans (liver, spleen, small intestine, kidney, and adipose tissue); in two lines expression was more restricted. There was a marked (4-10-fold) induction of liver CETP mRNA in response to a high fat, high cholesterol diet. The increase in hepatic CETP mRNA was accompanied by a fivefold increase in transcription rate of the CETP transgene, and a 2.5-fold increase in plasma CETP mass and activity. In contrast, CETP transgenic mice, in which the CETP minigene was linked to a metallothionein promoter rather than to its own flanking sequences, showed no change in liver CETP mRNA in response to a high cholesterol diet. Thus (a) the CETP minigene or natural flanking sequences contain elements directing authentic tissue-specific expression; (b) a high cholesterol diet induces CETP transgene […]

Dietary Cholesterol Increases Transcription of the Human Cholesteryl Ester Transfer Protein Gene in Transgenic Mice

Dependence on Natural Flanking Sequences

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Abstract

To investigate the regulation of expression of the human cholesteryl ester transfer protein (CETP) gene, transgenic mice were prepared using a CETP minigene linked to the natural flanking sequences of the human CETP gene. By using a transgene containing 3.2 kb of upstream and 2.0 kb of downstream flanking sequence, five different lines of transgenic mice were generated. The abundance of CETP mRNA in various tissues was determined on standard laboratory diet or high fat, high cholesterol diets. In three lines of transgenic mice the tissues expressing the human CETP mRNA were similar to those in humans (liver, spleen, small intestine, kidney, and adipose tissue); in two lines expression was more restricted. There was a marked (4–10-fold) induction of liver CETP mRNA in response to a high fat, high cholesterol diet. The increase in hepatic CETP mRNA was accompanied by a fivefold increase in transcription rate of the CETP transgene, and a 2.5-fold increase in plasma CETP mass and activity. In contrast, CETP transgenic mice, in which the CETP minigene was linked to a metallothionein promoter rather than to its own flanking sequences, showed no change in liver CETP mRNA in response to a high cholesterol diet. Thus (*a*) the CETP minigene or natural flanking sequences contain elements directing authentic tissue-specific expression; (*b*) a high cholesterol diet induces CETP transgene transcription, causing increased hepatic CETP mRNA and plasma CETP; (*c*) this cholesterol response requires DNA sequences contained in the natural flanking regions of the human CETP gene. (*J. Clin. Invest.* 1992. 90:1290–1295.) Key words: atherosclerosis • cholesteryl ester transfer protein • cholesterol • gene expression • lipoprotein

Introduction

Cholesteryl ester transfer protein (CETP)[1] is a hydrophobic plasma glycoprotein that mediates transfer and exchange of neutral lipids and phospholipids between the plasma lipoproteins (1). Plasma CETP activity is variable in different species and tends to be correlated with species susceptibility to dietary atherosclerosis (2–4). Mice and rats lack plasma CE transfer activity. To study the effects of CETP expression on mouse lipoprotein metabolism, CETP transgenic mice were recently prepared by using a human transgene driven by a mouse metallothionein promoter (MT). When the transgene is induced by dietary zinc, these animals show reduced levels of plasma HDL cholesterol (5).

The CETP mRNA shows a fairly widespread tissue distribution and is inducible by dietary cholesterol. The major tissues expressing the human CETP mRNA are liver, small intestine, spleen (6), and adipose (4). In monkeys variation in plasma CETP concentration is highly correlated with the abundance of liver CETP mRNA and with the output of CETP in liver perfusates, suggesting that liver synthesis is the main determinant of plasma CETP in primates (7). In response to a high cholesterol, high fat diet, several species show an increase in plasma CETP activity and mass (2, 4, 7, 8) associated with an increased abundance of the CETP mRNA in liver (2, 7, 9) and in adipose tissue (4, 9).

Whereas several genes are down-regulated by dietary cholesterol and the mechanisms are well understood (10), only a few genes are known to be up-regulated by dietary cholesterol and the mechanism is largely unknown. A transgenesis approach has been successfully employed to study tissue-specific and developmental regulation of a variety of genes (11, 12), including apolipoprotein genes (13, 14). In the current studies we have used a transgenesis approach to investigate the regulation of expression of the human CETP gene in different tissues and its induction by dietary cholesterol. We show that a version of the human gene containing 5 of the 15 native introns (Fig. 1) and relatively limited amounts of natural flanking region (NFR) DNA sequences shows a tissue pattern of expression similar to that in humans, as well as marked induction in response to a high fat/high cholesterol diet. These results are contrasted with the response obtained in MT-CETP transgenic animals, where the transgene is not induced by dietary cholesterol.

Methods

Development of CETP transgenic mice

An 11-kb pair synthetic CETP structural gene was assembled by combining genomic and cDNA fragments. The 5′-genomic region (including a 3.2-kb flanking sequence, exon 1, intron 1, and part of exon 2; a BamHI-EcoRV genomic fragment) and the 3′-genomic region (including part of exon 12, exons 13–16, introns 12–15, and 2.0 kb of 3′ flanking sequence; an EcoRV-EcoRI genomic fragment) were linked together through a fragment taken from the human CETP cDNA (EcoRV-EcoRV fragment). This fusion resulted in the complete re-

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1. *Abbreviations used in this paper:* CETP, cholesteryl ester transfer protein; MT, mouse metallothionein promoter; NFR, natural flanking region.

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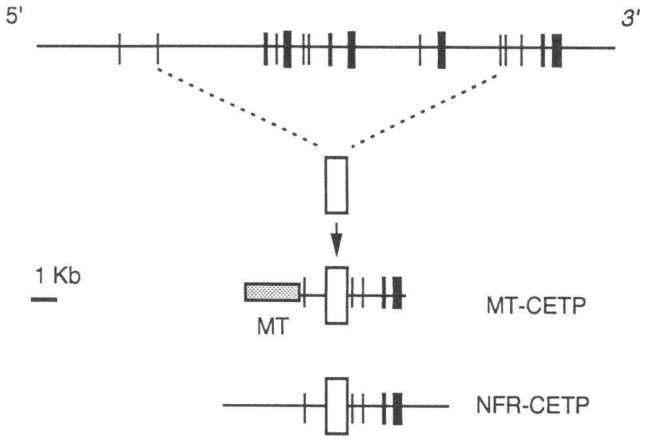

Figure 1. The organization of the human CETP gene is shown (*top*). The vertical bars represent exons. A minigene derivative was constructed by combining genomic fragments with a portion cDNA, representing exons 2–12, and placed under control of the mouse metallothionein-I promoter (*MT-CETP*), or assembled with intact natural flanking regions of the CETP gene (*NFR-CETP*).

moval of introns 2–11 and the generation of one synthetic exon. No alterations were made to the remaining CETP sequence. To generate transgenic mice, the transgene (designated NFR-CETP) was microinjected into the male pronuclei of fertilized mouse eggs taken from superovulated (C57BL/6J × CBA/J) F1 females. Screening and subsequent breeding was performed as described previously (5).

Animals used in dietary studies

Heterozygote transgenic mice were used in all experiments. The mice (both transgenic and nontransgenic littermates) were housed in metabolic cages and given free access to food and water.

Long-term high cholesterol and fat feeding study. NFR-CETP or MT-CETP were mice (n = 5 per group) were fed either the high cholesterol, high fat diet (15% fat, 1.25% cholesterol, 0.5% sodium cholate [formulation TD 90221, Teklad Premier Laboratory Diets, Madison, WI), or a standard laboratory diet diet (Purina Laboratory rodent Chow 5001) for 1 mo before being killed.

Short-term high cholesterol and high saturated fat feeding study. NFR-CETP mice (n = 5 per group) were fed a Chow diet, a high cholesterol diet (Chow +1% cholesterol), a high fat diet (Chow + 20% coconut oil), or a combined diet (Chow + 1% cholesterol + 20% coconut oil) for 1 wk. These four diets were purchased from Research Diets, Inc., New Brunswick, NJ (formulations C11000, C11035, C11036 and C11037, respectively).

Time-course study. To evaluate the time course of changes in liver CETP mRNA in NFR-CETP transgenic mice, three mice were killed at each time point (0, 2, 4, 8, 16, and 32 d after beginning the high cholesterol, high fat diet).

RNase protection analysis

RNA from various organs was isolated immediately after sacrifice (4). Total RNA (30 μg) from tissues was analyzed for CETP mRNA by a solution hybridization-RNase protection assay using a riboprobe specific for human CETP mRNA (5).

Nuclear run-on analysis

Liver nuclei from three mice were isolated and pooled (15). Approximately 4×10^7 nuclei were stored at −70°C in 200 μl nuclei storage buffer (50 mM Tris, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 5 mM EDTA). The in vitro elongation reaction was carried out as described (16). The DNA probes were denatured in 0.1 N NaOH for 30 min at room temperature, neutralized in 6× SSC, and applied to Hybond-N membrane (10 μg per slot) using a slot-blot apparatus. ^{32}P-labeled RNA ($1–4 \times 10^6$ cpm/ml) was hybridized to the membranes in a buffer containing 10 mM Hepes, pH 7.5, 10 mM EDTA, 0.3 M NaCl, 1% SDS, 1 × Denhardt's (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% BSA), and 250 μg/ml tRNA at 45°C for 3 d. The membranes were washed four times for 5 min each in 2 × SSC at room temperature, incubated in 2 × SSC containing 10 μg/ml RNase A for 30 min at 37°C, then washed twice for 30 min each in 0.5 × SSC, 0.1% SDS at 65°C. The signal was detected by autoradiography for 1–3 d, and quantitated by laser densitometry.

Measurement of plasma CETP concentration and activity

The mass and activity of CETP in mouse plasma were determined as described previously (17, 18).

Results

In earlier studies (5) of the function of plasma CETP a minigene was constructed by fusing the second exon of the CETP gene to the twelfth exon, using a portion of the CETP cDNA. This CETP minigene was placed under the control of the mouse metallothionein promoter (*MT-CETP,* Fig. 1). In the current studies a CETP transgene, consisting of the structural CETP minigene linked to the natural flanking sequences of the human CETP gene (*NFR-CETP,* Fig. 1), was used to generate several lines of transgenic mice. All of these lines gave rise to authentic human CETP in plasma, with activities ranging from 80% to 350% of human plasma CETP activity. These results are similar to those obtained previously with the MT-CETP transgene (5).

In five lines of NFR-CETP transgenic mice the tissue distribution of the human CETP mRNA was evaluated by RNase protection using an assay specific for human CETP mRNA (5). The results from four of the lines are shown in Fig. 2, *A–D,* and are contrasted with the results for the MT-CETP transgenic line shown in Fig. 2 *E.* The tissues examined were liver, spleen, small intestine, kidney, adipose, heart, skeletal muscle, brain, brain, skin, and lung. Only the tissues which gave a detectable signal in the RNase protection assay are shown (sensitivity ~ 10 pg/mg total RNA).

In two lines of mice (5171 and 5203) the pattern of expression of the CETP mRNA in various tissues was similar to that observed in human with readily detectable CETP mRNA in liver, spleen, small intestine, kidney, and adipose tissue; expression was detected in heart and brain in line 5171 (Fig. 2 A) but not in line 5203 (Fig. 2 *B*). There was no signal detected in skeletal muscle, lung, or skin. In a third line (5177, not shown) the pattern of expression in the founder was similar to that of line 5171, but the line was lost before dietary studies were completed. Two other lines of NFR-CETP transgenic mice displayed a more restricted tissue distribution, with expression only in liver and small intestine (5180, Fig. 2 *C*) or liver and spleen (5216, Fig. 2 *D*). In the basal state (i.e., non–Zn-induced), the MT-CETP transgene was expressed in liver and a variety of other tissues, particularly heart (Fig. 2 *E*); with Zn induction, liver becomes the major site of expression (5).

When placed on a high fat, high cholesterol diet for more than one week, all four lines of NFR-CETP transgenic mice showed a dramatic increase in CETP mRNA abundance in the liver. In the different lines there was a 4–10-fold increase over the baseline (chow diet) values (Fig. 2, *A–D*). In addition, the high fat, high cholesterol diet produced smaller increases in CETP mRNA abundance in spleen, small intestine, and adi-

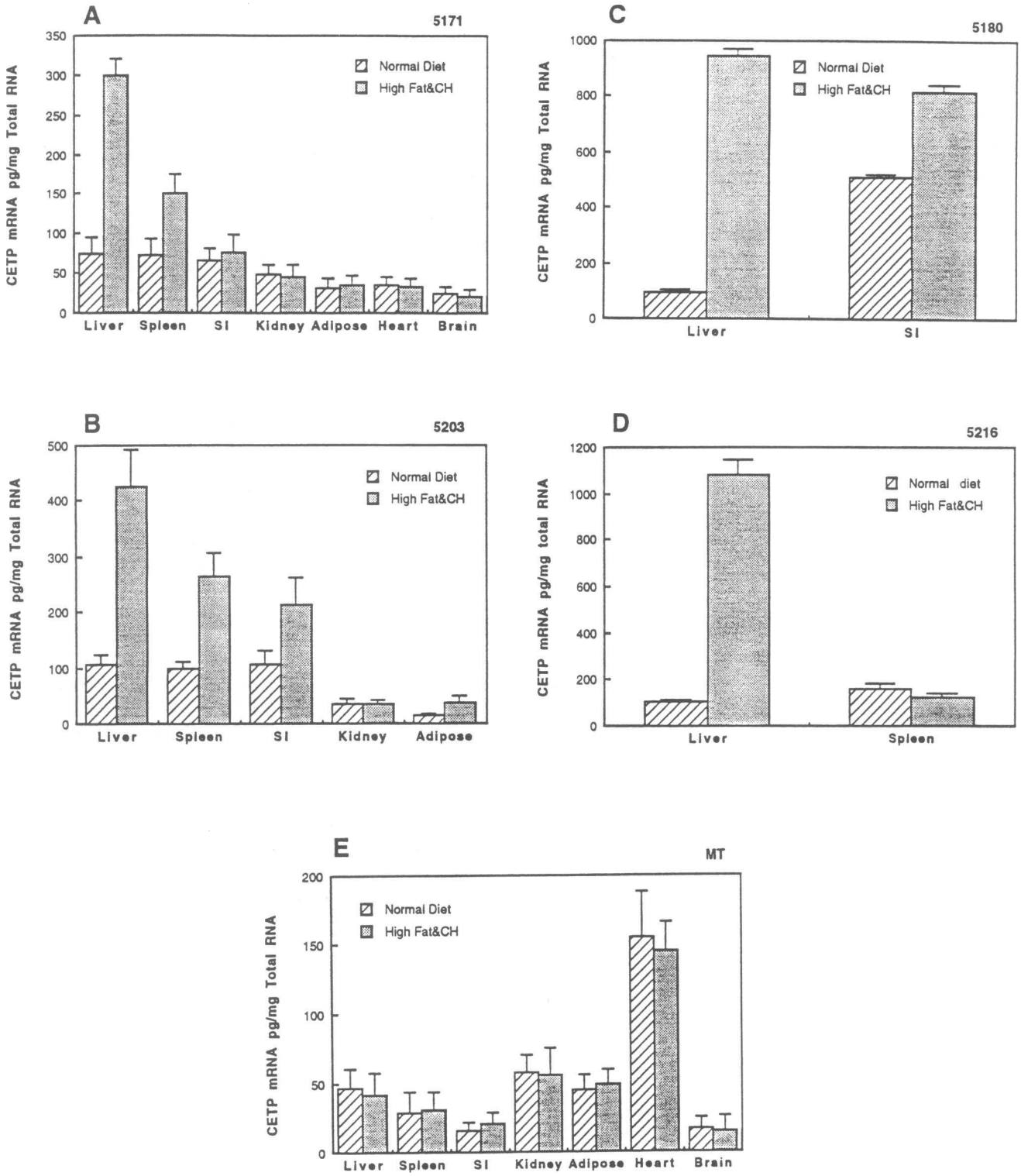

Figure 2. Abundance of the CETP mRNA in the tissues of various lines of NFR-CETP transgenic mice (*A–D*) or mT-CETP transgenic mice (*E*) fed standard laboratory or high fat, high cholesterol (CH) diets. Values shown are means±SE (n = 5 mice per group). Differences on the two diets were evaluated using Student's t test: (*A*) liver, $P < 0.001$, spleen, $P < 0.01$; (*B*) liver, $P < 0.001$, spleen, $P < 0.01$, small intestine, $P < 0.05$, adipose tissue, $P < 0.05$; (*C*) liver, $P < 0.001$, small intestine, $P < 0.05$; (*D*) liver, $P < 0.001$.

pose tissue; however, these changes were not uniformly observed in the different lines of mice (Fig. 2). In marked contrast to the induction of liver CETP mRNA in the NFR-CETP transgenic mice, there was no change in CETP mRNA abundance in any tissue when the MT-CETP transgenic animals were placed on the high fat, high cholesterol diet (Fig. 2 *E*). In parallel with these findings, in the NFR-CETP transgenic mouse line 5171, there was an ~ 2.5-fold increase in plasma

CETP mass and activity in response to the high fat, high cholesterol diet, but no change in plasma CETP mass or activity in the MT-CETP transgenic mice when placed on the same diet (Table I).

The NFR-CETP transgenic mouse line 5171, in which the tissue-specific pattern of expression of the CETP mRNA most closely resembles that of humans, was chosen for more detailed studies. RNA blot analysis showed that the CETP mRNA was the same size as in human tissues (2.1 kb) (6) and confirmed the increase in abundance of CETP mRNA in liver and spleen in response to the high cholesterol, high fat diet (Fig. 3). There was no evidence for the appearance of transcripts of different size on the high cholesterol diet. A time course study revealed an eightfold increase in CETP mRNA abundance 48 h after beginning the high fat, high cholesterol diet, with a four- to sixfold increase at later time-points (Fig. 4). In order to analyze which component of the atherogenic diet was responsible for the increase in CETP mRNA, animals were studied on diets supplemented with cholesterol, or saturated fat, or both simultaneously (Fig. 5). There was a fourfold increase in response to cholesterol alone, a slight but statistically significant ($P < 0.05$) increase in response to fat alone, and a synergistic sixfold increase when both components were present. The three diets (Fig. 5) lacked the bile salts which form a part of the atherogenic diet (19) given in the earlier studies (Fig. 2). Thus, dietary cholesterol is largely responsible for the increase in CETP mRNA, but this component acts synergistically with dietary fat to increase CETP mRNA abundance.

The increase in CETP mRNA in NFR-CETP transgenic mice but not in MT-CETP transgenic mice suggests that the increase is not mediated by changes in mRNA stability. To assess the possibility that the increase is mediated by increased gene transcription, a nuclear run-on assay was performed (Fig. 6). The labeled RNA from the liver of nontransgenic mice showed only a trace amount of hybridization with the human cDNA probe (Fig. 6, *inset*), indicating specificity of the signal in the transgenic mice. In the transgenic animals there was a fivefold increase in elongation of initiated transcripts, comparing the high fat, high cholesterol diet with the chow diet. There was no significant change in the control (*GAPD*). Thus, the increase in CETP mRNA abundance in response to the high fat, high cholesterol diet can be entirely accounted for by an increase in transcriptional initiation.

Table I. Plasma CETP Concentration and Activity in Transgenic Mice Fed Standard Laboratory Diet or High Cholesterol and Fat Diets, without Zn Induction (Metal-free Water)

	CETP concentration	CETP activity
	ng/μl	*cpm/μl*
Mt-CETP transgenic mice		
Normal diet	1.82±0.41*	720±64*
High CH and fat	1.86±0.65*	672±53*
NFR-CETP transgenic mice		
Normal diet	1.91±0.21*	398±51‡
High CH and fat	4.32±0.93*	992±142‡

Plasma was obtained from mice fed chow or high fat/high cholesterol (CH) diets and metal-free water for 7 d. CETP mass was determined by RIA (17) and CETP activity by isotopic assay in diluted plasma (18). Values within columns with different superscript symbols are significantly different, $P < 0.05$. Values are mean±SE of five animals/group.

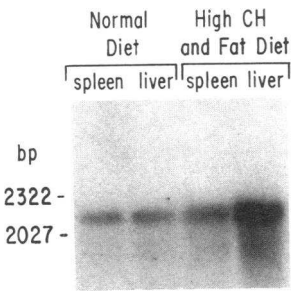

Figure 3. RNA blot analysis of tissue RNA in NFR-CETP transgenic mice (line 5171), on standard laboratory or high cholesterol (CH) diets. Each lane contains 5 μg of polyA+ RNA, prepared from a pool of tissues from five mice fed either standard laboratory or high fat, high cholesterol diets. A full-length ^{32}P-labeled CETP cDNA was used as a probe.

Discussion

Transgenic mice have been prepared using a version of the human CETP gene containing several of its native introns, as well as relatively limited amounts of 5′ and 3′ NFR DNA sequences. In several lines of mice the pattern of expression of the CETP mRNA in different tissues is similar to that in humans, suggesting that the transgene contains the major regulatory elements determining expression in human tissues. In response to increased dietary cholesterol, there is a pronounced increase in hepatic CETP transgene transcription, leading to increased CETP mRNA abundance, and increased plasma CETP mass and activity. The increased plasma CETP concentration and activity are similar to those observed previously in several mammalian species in response to a high cholesterol, high fat diet, and the results suggest that altered transcription of the CETP gene in liver may be the major mechanism underlying diet-induced changes in plasma CETP concentration and activity in humans (20) and some other species (2, 4–7). A high cholesterol diet produced no change in CETP mRNA in MT-CETP transgenic animals, suggesting that the natural flanking

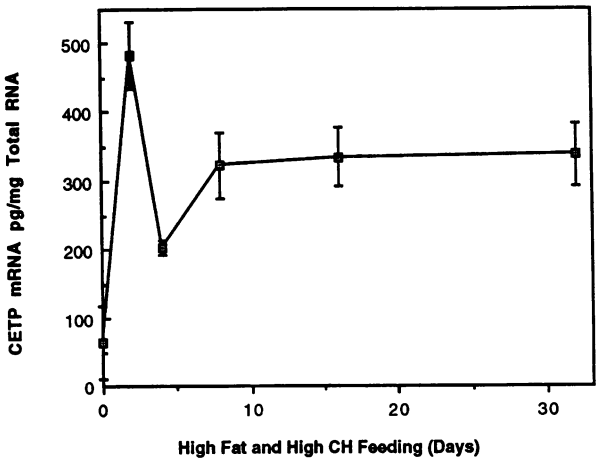

Figure 4. Time course of changes in hepatic CETP mRNA in NFR-CETP transgenic mouse line 5171 in response to a high fat, high cholesterol diet. Transgenic mice were fed a standard laboratory diet ($t = 0$) or a high fat, high cholesterol (CH) diet. At each time point three mice were killed and CETP mRNA was determined by RNase protection assay. Each time point represents the mean±SD for three mice.

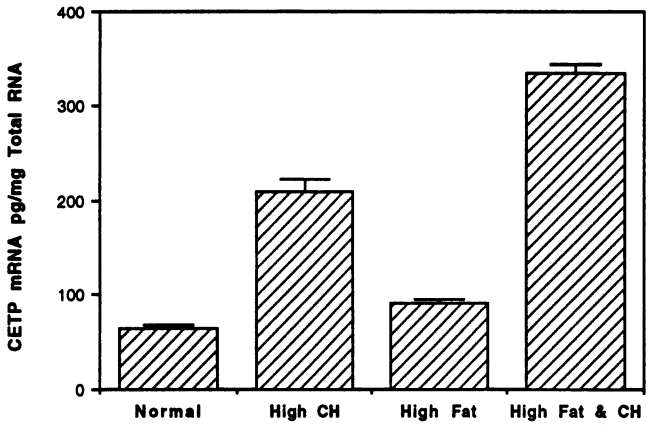

Figure 5. Effects of different diets on hepatic CETP mRNA abundance in NFR-CETP transgenic mice (line 5171). Mean±SE differences (n = 5 per group) are shown. Differences in CETP mRNA levels were evaluated using Student's t test. Compared to standard laboratory diet, high cholesterol (CH) ($P < 0.001$), high fat ($P < 0.05$), and CH/fat ($P < 0.001$); compared to high CH, high fat ($P < 0.01$), high CH/fat ($P < 0.01$); compared to high fat, high CH/fat ($P < 0.001$).

sequences of the human CETP gene contain elements that are necessary to produce this effect.

Overall, the NFR-CETP transgenic mice displayed a pattern of expression of the CETP mRNA in tissues similar to that in primates, i.e., with expression in the liver and small intestine, but also in several peripheral tissues, such as spleen and adipose tissue. Although the CETP mRNA shows a widespread distribution, it is not found in all tissues (2, 4, 6), and was not detected in lung, skin, and skeletal muscle in the present studies. Despite the general resemblance to the tissue-specific pattern of expression of the human CETP mRNA, there was considerable variation in the results in different lines of transgenic mice. All NFR-CETP transgenic lines displayed significant expression of CETP mRNA in the liver, suggesting that the regulatory elements mediating liver-specific expression are present in the construction and are insensitive to position of integration of the transgene in the mouse chromosome. However, in two lines of mice there was no detectable CETP mRNA in several peripheral tissues, such as adipose. The reason for the more variable expression in nonhepatic tissues is uncertain. One possibility is that the elements driving peripheral expression are more dependent on positional effects. This could arise, for example, if peripheral tissue-specific enhancers require looping of intervening DNA sequences to gain access to the proximal promoter (21), a mechanism which could be sensitive to local chromatin structure.

A highly consistent finding was that all lines of NFR-CETP transgenic mice displayed a marked increase in hepatic CETP mRNA abundance in response to a high fat, high cholesterol diet. Two lines of evidence indicated that this effect was mediated by increased gene transcription. First, there was no increase in hepatic CETP mRNA in MT-CETP transgenic mice placed on a high cholesterol diet, excluding a role for post-transcriptional processes, such as increased mRNA transport or mRNA stabilization. Second, nuclear run-on assays showed an increase in transcriptional rate of the CETP transgene of similar magnitude to the increase in CETP mRNA. The increased transcription of the NFR-CETP transgene in response to increased dietary cholesterol indicates that the CETP minigene or its flanking sequences contain elements which respond to factors induced by the high cholesterol diet. The lack of a similar response of the MT-CETP transgene suggests that there may be one or more cholesterol response elements within the natural flanking sequences of the CETP gene, i.e., within the 3 kb of upstream, or 2 kb of downstream flanking sequence (Fig. 1).

In the NFR-CETP transgenic mice, the high cholesterol diet caused an increase in plasma CETP activity and mass, resembling that observed in monkeys or rabbits fed similar diets (2, 7–9). Quig and Zilversmit (22) have hypothesized that, because CETP is found in plasma only in association with lipoproteins, its concentration may be regulated not only by a balance between its secretion and removal from plasma, but also by the availability of lipoprotein particles to which CETP can bind. Thus, its concentration might increase passively as plasma cholesterol concentration is increased in response to a high cholesterol diet. However, in the present study, MT-CETP transgenic animals fed the high cholesterol diet showed no change in plasma CETP mass or activity, even though these animals are known to become hypercholesterolemic when fed this diet (5). Thus, in transgenic mice the increase in plasma CETP mass and activity is due to increased gene transcription, and is unrelated to diet-induced changes in plasma lipoproteins that are independent of CETP expression.

A limited number of genes are known to be up-regulated by dietary cholesterol. Apolipoprotein E mRNA may be increased in macrophages as a result of cholesterol loading (23), but, in most tissues, including liver, has generally not found to be changed by alterations in dietary cholesterol (24). 7-α-Hydroxylase, the rate-limiting enzyme in the conversion of cholesterol to bile acids in the liver, is induced by increased dietary cholesterol in rodents (25–27); the increase in mRNA is also mediated by an increase in gene transcription (26). The cholesterol down-regulation of the transcription of several genes,

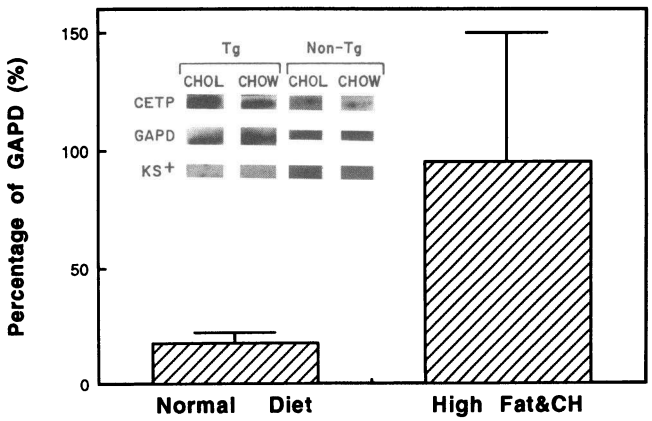

Figure 6. Effect of high cholesterol, high fat diet on CETP mRNA transcriptional rate in nontransgenic and NFR-CETP transgenic (line 5171) mice. The inset shows the hybridization signals for one run-on assay (probes, full-length CETP cDNA; glyceraldehyde 3-phosphate dehydrogenase (*GAPD*) cDNA, and Bluescript KS+ vector without insert). The bar graph shows the mean±SD signal for specific hybridization, relative the GAPD signal for three independent experiments.

such as the LDL receptor or the 3-hydroxy-3-methylglutaryl coenzyme A reductase, is mediated by a highly conserved proximal promoter element, called the sterol regulatory element (10). It is interesting to note that the proximal upstream sequences (to ~ 1 kb) of both 7-α-hydroxylase (28) and CETP genes (29, and unpublished observation) do not contain DNA sequences similar to the sterol regulatory element. This suggests that novel *cis*-acting elements are involved in the up-regulation of 7-α-hydroxylase and CETP genes by dietary cholesterol.

Both CETP and 7-α-hydroxylase are thought to be involved in a process of "reverse cholesterol transport," i.e., the movement of cholesterol from peripheral tissues to the liver via the plasma compartment with subsequent excretion in bile (30). The increased expression of CETP in liver and in peripheral tissues in response to increased dietary cholesterol probably helps to recycle excess cholesterol from peripheral tissues to the liver, where it may be reused or excreted in bile. The marked responsiveness of the human CETP gene to changes in dietary cholesterol suggests that its transcriptional regulation may play an important role in cholesterol homeostasis.

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