Alternative Splicing of the Human Cholesteryl Ester Transfer Protein Gene in Transgenic Mice

EXON EXCLUSION MODULATES GENE EXPRESSION IN RESPONSE TO DIETARY OR DEVELOPMENTAL CHANGE*

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The plasma cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl ester from high density lipoprotein to other lipoproteins. The human CETP gene produces two forms of mRNA, with or without exon 9 (E9)-derived sequences. To study the function and regulation of alternative splicing of the CETP gene, transgenic mice were prepared 1) with the metallothionein (mT) promoter driving an E9-deleted construct (mT.CETP(−E9) transgene), and 2) with the natural flanking regions (NFR) controlling expression of genomic sequences which permit alternative splicing of E9 (NFR.CETP(±E9) transgene). With zinc induction, the mT.CETP(−E9) transgene gave rise to abundant E9-deleted CETP mRNA in liver and small intestine, but only relatively small amounts of E9-deleted protein were found in plasma. The E9-deleted form of CETP was inactive in lipid transfer and produced no changes in plasma lipoprotein profile. The NFR.CETP(±E9) transgene gave rise to full-length (FL) and E9-deleted forms of CETP mRNA in liver and spleen. In response to hypercholesterolemia induced by diet and breeding into an apoE gene knock-out background, the FL CETP mRNA was induced more than the E9-deleted mRNA, resulting in a 2-fold increase in ratio of FL/E9-deleted mRNA. The expression of CETP mRNA was found to be developmentally regulated. In NFR.CETP(±E9) transgenic mice CETP mRNA levels were markedly increased in the liver and small intestine in the perinatal period and decreased in adult mice, whereas CETP mRNA in the spleen was low in perinatal mice and increased in adults. The developmental increase in CETP mRNA in the liver and spleen was preceded by an increased ratio of FL/E9-deleted forms. Thus, the E9-deleted mRNA appears to be poorly translated and/or secreted, and the cognate protein is inactive in lipid transfer and lipoprotein metabolism. CETP gene expression was found to be highly regulated in a tissue-specific fashion during development. Increased CETP gene expression during development or in response to hypercholesterolemia is associated with preferential accumulation of the full-length CETP mRNA.

The plasma cholesteryl ester transfer protein (CETP)1 is a hydrophobic glycoprotein (476 amino acids, M, 74,000) that mediates the transfer and exchange of neutral lipids and phospholipids between the plasma lipoproteins (Tall, 1995). CETP mediates the transfer of cholesteryl ester from HDL and LDL to triglyceride-rich lipoproteins, as a result of an exchange of cholesteryl ester for triglyceride. The subsequent action of hepatic lipase on triglyceride-enriched HDL particles promotes the formation of smaller HDL species (Barter and Rye, 1994; Deckelbaum et al., 1986) that may be optimal mediators of cellular cholesterol efflux (Fielding et al., 1994) and optimal substrates for the lecithin:cholesterol acyltransferase reaction (Kunitake et al., 1992). Thus, the activity of CETP may lead to enhanced efflux of cell membrane cholesterol into HDL and facilitation of reverse cholesterol transport. Mice express very low levels of plasma cholesteryl ester transfer activity, and the introduction of a human CETP transgene results in decreased plasma HDL cholesteryl levels, formation of smaller HDL species, and stimulation of cholesteryl ester formation in the plasma lipoproteins (Agellon et al., 1991). In CETP transgenic mice with hypertriglyceridemia the HDL size changes are profound (Masucci-Magoulas et al., 1996), and the mice show decreased early atherosclerotic lesions in response to a high cholesterol diet (Hayek et al., 1995).

The human CETP gene consists of 16 exons encompassing 25 kilobase pairs of genomic DNA (Agellon et al., 1990). The CETP mRNA is primarily expressed in the liver, spleen, small intestine, adrenal, and adipose tissue (Jiang et al., 1991; Drayna et al., 1987). There is a similar pattern of mRNA expression in transgenic (Tg) mice expressing a human CETP minigene under the control of the natural flanking sequences of the CETP gene (Jiang et al., 1992). In response to a high cholesterol diet, CETP gene transcription is increased leading to increased mRNA levels in the liver and in peripheral tissues. Hepatic CETP mRNA is also markedly increased in response to endogenous hypercholesterolemia when the CETP transgene is crossed into apoE or LDL receptor gene knock-out backgrounds (Masucci-Magoulas et al., 1996). Thus, CETP appears to be a key regulated component of reverse cholesterol transport.

In all human tissues expressing the CETP gene, two species of mRNA are detected: a full-length form, giving rise to active M, 74,000 plasma CETP, and a shorter variant in which exon 9-derived sequences have been removed as a cassette (Inazu et al., 1992). The E9-deleted CETP mRNA is formed by alterna-

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1 The abbreviations used are: CETP, cholesteryl ester transfer protein; NFR, natural flanking regions; FL, full-length; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; Tg, transgenic; mT, metallothionein; E9, exon 9; apo, apolipoprotein.
ative splicing of the CETP gene transcript. E9 encodes 180 nucleotides or 60 amino acids in the central portion of the CETP sequence. Cell transfection of the E9-deleted cDNA gives rise to a shortened, poorly secreted protein which seems to be inactive. Co-transfection of full-length and E9-deleted cDNAs resulted in inhibition of the secretion of full-length, active CETP (Quinet et al., 1993). These studies have suggested that alternative splicing of the CETP gene may modulate the formation of active plasma CETP, and that the E9-deleted protein may have a dominant negative effect on expression of the full-length protein. Alternatively, the E9-deleted CETP variant might have an occult function in vivo, not detectable by expression in cultured cells. In this study we have used transgenic mice to study the function and possible regulation of alternative splicing of the CETP gene.

MATERIALS AND METHODS

Construction of Transgenes to Study Alternative Splicing of the CETP Gene—Two new CETP transgenes were constructed. In the first construct, E9 was removed from the previously described metallothionein-CETP transgene (Agellon et al., 1991), by swapping the central portion of the CETP gene, prepared from an EcoRV CETP cDNA fragment, with the cognate cDNA fragment lacking E9 sequences (see Fig. 1). In the second construct, designed to study the regulation of splicing, the NFR-CETP transgene (Jiang et al., 1992) was modified so that it contained exons and introns 6–10 as well as other exons and introns and natural flanking regions (NFR.CETP(±E9) transgene or transgene 12, Fig. 1). A BglII fragment of a genomic clone (lambda CG5) was cloned into BglII digested pGem3Z CETP cDNA; then an EcoRV fragment (corresponding to sequences of exon 2 to exon 12) was cloned into the EcoRV digested NFR.CETP minigene.

Transgenic mice were prepared by microinjection of DNA into C57Bl/6 × CBAJ mouse embryos. Screening was performed on tail-tip DNA by polymerase chain reaction and Southern blotting, using standard methods. Digestion of genomic DNA with EcoRV yielded two different sized fragments for mT.CETP(−E9) and NFR.CETP-transgenic mice, allowing these transgenes to be differentiated in crosses to develop compound mice. For phenotypic characterizations of control, transgenic and compound mice, littermates were compared. To study the effects of hypercholesterolemia, NFR.CETP(±E9) Tg mice were fed a high cholesterol, high saturated fat diet (1% cholesterol, 20% saturated fat) or crossed into the apoE0 background (Plump et al., 1992; Zhang et al., 1992).

Analysis of RNA—Total RNA was isolated by standard methods. RNazol B (Tel-Test Inc.) was added to the tissue to be homogenized. RNAase protection assay was performed using a CETP riboprobe which yields different sized fragments for full-length and E9-deleted forms of CETP mRNA (Quinet et al., 1993). A riboprobe for β-actin was also included as an internal standard. For the developmental studies 28 S ribosomal RNA was used as the internal standard. Protected fragments were quantitated by phosphorimaging, using a standard curve employing different amounts of a sense riboprobe standard. Data are expressed as absolute amount of RNA normalized to a constant amount of internal standard.

Quantitation of CETP in Liver Homogenates or Plasma—For preparation of liver homogenates, livers were perfused with oxygenated buffer via the portal vein to remove residual plasma. Liver (1 g) was homogenized in 1 ml of lysif buffer (0.75% deoxycholate, 0.6% Triton X-100, 0.1% SDS, 20 mM Tris, pH 7.5, 150 mM NaCl, 4 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM aprotonin) and subjected to ultracentrifugation at 60,000 rpm in a Beckman TL100 ultracentrifuge for 1 h. The supernatant was incubated with a CETP monoclonal antibody, TP2, immobilized on Sepharose beads (Hesler et al., 1988) in 500 μl of 1× TSE (Tris-saline-EDTA) overnight at 4°C. The beads were washed once with TSE and three times with 0.1 M ammonium bicarbonate, then analyzed by SDS-polycrylamide gel electrophoresis and Western blotting using 125I-TP2. Plasma was incubated in TSE buffer containing anti-proteases, then analyzed by SDS-polycrylamide gel electrophoresis and Western blotting using TP2. Plasma CETP mass was determined by radioimmunoassay (Brown et al., 1989). CETP activity was assayed in diluted plasma by addition of excess substrates, LDL, and HDL containing radiolabeled cholesteryl ester, as described by Tall et al. (1986).

Analysis of Plasma Lipoproteins—Plasma lipids were determined using kit assays (Waco, Osaka). Pooled plasma (200 μl, n = 4–6 mice per pool) was analyzed on a Superose 6HR column driven by a Pharmacia fast protein liquid chromatography system. Fractions were collected in microtiter plates and analyzed for total cholesterol content, using a reagent containing cholesterol esterase, cholesterol oxidase, 4-aminoantipyrine, and p-chlorophenol, followed by absorbance measurement at 490 nm. HDL cholesterol, cholesteryl ester, and phospholipid were determined after precipitation of apoB-lipoproteins with dextran sulfate. Native gel electrophoresis was carried out on 60 μl of plasma in native 4–20% polyacrylamide gradient gels. Samples were run in duplicate on different sides of the gel. Half of the gel was stained with Oil Red O to localize the HDL region, and the other half was subjected to Western blotting using 125I-TP2.

RESULTS

In this study we have developed two new lines of CETP Tg mice. The first line contained an E9-deleted CETP transgene under the control of the inducible metallothionein (mt) promoter (mt.CETP(−E9) Tg mice or transgene 8 (Fig. 1). This line was used to determine the effects of the E9-deleted CETP on the plasma lipoproteins. To investigate the regulation of alternative splicing, we also prepared Tg mice expressing a natural flanking region CETP transgene containing genomic sequences that provide the option to exclude E9 sequences (NFR.CETP(±E9) Tg mice or transgene 12). In Fig. 1, this line contrasts with the previously described CETP Tg mice (Jiang et al., 1992), in which cDNA was used to prepare the central portion of the minigene and thus only transcripts containing E9 are made (NFR.CETP(±E9) Tg mice or line 5203). To test a possible dominant negative effect of E9-deleted CETP on the expression of the full-length CETP (Quinet et al., 1993), we bred mice containing both mt.CETP(−E9) and NFR.CETP(−E9) transgenes. In these compound mice, we anticipated that zinc induction would increase the formation of E9-deleted CETP, but not the full-length CETP.

CETP mRNA and Protein Expression in Mice Containing the mt.CETP(−E9) Transgene and in mt.CETP(−E9)/NFR.CETP(−E9) Transgenic Mice—Three founders containing the mt.CETP(−E9) transgene were obtained. Lines 1 and 2, derived from two of these founders, expressed the CETP mRNA, the third line did not. Following zinc induction, lines 1 and 2 displayed massive accumulation of E9-deleted CETP mRNA in the liver and small intestine, and much smaller amounts in the spleen (Fig. 2, Tg8). By contrast, in NFR.CETP(±E9) Tg mice (line 5203) the full-length (FL) CETP mRNA was expressed at much lower levels in the spleen and liver and in trace amounts in the small intestine (Fig. 2, 5203, FL).
Transgene—Analysis of plasma lipids showed no changes in total plasma phospholipid, cholesterol, or triglyceride concentrations in mice expressing the mT.CETP(−E9) transgene (Jiang et al., 1995). This suggests that E9-deleted CETP does not bind to HDL efficiently.

Plasma Lipoproteins in Mice Expressing the mT.CETP(−E9) Transgene—Analysis of plasma lipoproteins showed no changes in total plasma phospholipid, cholesterol, or triglyceride concentrations in mice expressing the mT.CETP(−E9) transgene (basal or zinc-induced) compared to nontransgenic littersmates (not shown). Analysis of plasma lipoprotein cholesterol by fast protein liquid chromatography of pooled plasma showed that the profile of mT.CETP(−E9) animals (zinc-induced) was very similar to control (Fig. 6, nTg versus Tg8). By contrast, the
decreased in several separate experiments. However, small differences in the HDL fraction (fractions 12–14) were not reproducible. The lack of effect of the mT.CETP(−E9) transgene on plasma or HDL cholesterol, in mice expressing the transgene singly or in compounds, was confirmed when HDL cholesterol was determined by precipitation techniques, before and after zinc treatment (Table II). There was also no change in HDL phospholipid as a result of mT.CETP(−E9) transgene expression or induction (not shown). Both lines of mT.CETP(−E9) Tg mice gave similar results, whether studied alone or as compounds with NFR-CETP mice.

Thus, despite massive mRNA accumulation, the E9-deleted form of CETP appears in plasma in relatively small amounts, is inactive in lipid transfer, binds HDL poorly, and has no apparent influence on the plasma lipoprotein profile. Subsequent studies were thus carried out with mice expressing an NFR.CETP(±E9) transgene to see if factors increasing CETP gene expression also increased the ratio of FL to E9-deleted CETP mRNA.

Expression and Regulation of Alternatively Spliced CETP mRNAs in Mice Expressing NFR.CETP(±E9) Transgene—Four founders were obtained incorporating the NFR.CETP±E9 transgene (Fig. 1, transgene 12). Two of the founders expressed appreciable levels of CETP activity in plasma; however, only one of these established a line. The response of this line to hypercholesterolemia, or developmental changes was characterized in detail. In adult mice the main sites of expression were the liver and spleen, where both FL and E9-deleted species of mRNA were found, with the FL form predominant (Fig. 7, day 60).

To see if hypercholesterolemia altered the splicing of E9, the NFR.CETP(±E9) Tg mice were fed a high fat, high cholesterol diet. The transgene was also bred into the apoE0 background. There was a small nonsignificant increase in hepatic FL CETP mRNA in response to the high cholesterol diet or the apoE0 background (Table III). This response was less pronounced than that obtained in other lines of NFR-CETP Tg mice, such as 5203, probably reflecting positional effects (Jiang et al., 1992). However, when compound NFR.CETP(±E9) Tg/apoE0 mice were placed on the high cholesterol diet, there was a marked induction of the full-length CETP mRNA in both liver and spleen, about 4.5- and 13-fold, respectively. The E9-deleted CETP mRNA did not increase in response to the high cholesterol diet or the apoE0 background (Table III). This response was less pronounced than that obtained in other lines of NFR-CETP Tg mice, such as 5203, probably reflecting positional effects (Jiang et al., 1992). However, when compound NFR.CETP(±E9) Tg/apoE0 mice were placed on the high cholesterol diet, there was a marked induction of the full-length CETP mRNA in both liver and spleen, about 4.5- and 13-fold, respectively. The E9-deleted CETP mRNA did not increase in response to the high cholesterol diet or the apoE0 background, and increased less than the FL CETP mRNA in the compound mice on the high cholesterol diet. Thus, the ratio of FL/E9-deleted mRNA was significantly increased in liver and spleen in NFR.CETP(±E9) Tg mice on the high cholesterol diet (Table III), and was increased further in the compound mice on the high cholesterol diet. Thus, the induction of CETP gene expression by hypercholesterolemia favors the FL form of CETP mRNA over the E9-deleted form. Paralleling the mRNA changes, plasma CETP activity was increased from 5.8 ± 3% in NFR.CETP(±E9) Tg mice on chow to 9.5 ± 3% on the high cholesterol diet to 59 ± 9% in NFR.CETP(±E9) Tg/apoE0 mice on the high cholesterol diet.

Next we studied CETP mRNA expression in NFR.CETP(±E9) Tg mice during development (Fig. 7). Hepatic expression of full-length CETP mRNA increased at the end of embryonic development, peaked in suckling mice, and decreased in adults. In the small intestine, there was substantial expression of the CETP mRNA at birth (day 0), but expression was extinguished in adults. In contrast to these findings, in the spleen CETP mRNA was not detected in fetal mice, was low in the neonatal period, and increased to highest levels in adult animals.

Both full-length and E9-deleted forms were present at all times that the CETP mRNA was detected, but there were subtle differences in the pattern of expression. The develop-
The hypothesis that omission of E9 sequences represents a mechanisms to limit the formation of active plasma CETP. Mice expressing a CETP transgene with E9 sequences omitted had no detectable plasma lipid transfer activity and no changes in plasma lipoprotein profile, showing that E9-deleted sequences are inactive. Furthermore, studies in mice expressing a CETP transgene with optional splicing of exon 9 indicated that inclusion of E9 was favored under circumstances where CETP mRNA was increased.

Studies of mice containing the mT-CETP transgene with E9 sequences omitted (mT.CETP(-E9) Tg mice) suggested that expression of E9-deleted transcripts was curtailed on several levels. Following zinc induction, there was massive expression of E9-deleted CETP mRNA to levels more than 100-fold that of the FL CETP mRNA in the NFR.CETP(+E9) Tg mice (Fig. 2). However, compared to the FL CETP protein, there was no corresponding overexpression of E9-deleted protein in cell homogenates or in plasma (Figs. 3 and 4). Most likely, the E9-deleted mRNA is poorly translated. An alternative explanation, i.e. that the E9-deleted protein is unstable, seems less likely, since pulse-chase experiments in cell lines stably expressing both forms of CETP show that the intracellular half-life of the E9-deleted protein is much longer than the full-length form.2 As in the cell culture studies (Quinet et al., 1993; Inazu et al., 1992), the E9-deleted protein appeared to be relatively poorly secreted, since it was more abundant than the FL form in cell homogenates but less abundant in plasma (cf. Figs. 3 and 4). The E9-deleted form of CETP has not been detected in human plasma (Inazu et al., 1992); its presence in the mT.CETP(-E9) Tg mice probably results from overexpression. Finally, even though these mice had some E9-deleted protein in plasma, it bound to HDL poorly and was inactive in lipid transfer. Site-directed mutagenesis studies show that two positively charged amino acids contained within E9 are essential for HDL binding and optimal lipid transfer activity (Jiang et al., 1995).

One aspect of the cell culture studies which was apparently not confirmed in the Tg mice was the dominant inhibition of the secretion of full-length active CETP by the E9-deleted protein (Quinet et al., 1993). Perhaps in the cell transfection studies full-length and E9-deleted proteins were more highly concentrated in the endoplasmic reticulum than in vivo, leading to formation of poorly secreted hetero-dimers (Quinet et al., 1993). The negative studies using compound mT.CETP (=E9)/NFR.CETP(+E9) mice do not completely exclude this as potential regulatory mechanism, because it is possible that the temporal and spatial expression of the two forms of CETP mRNA was different in the compound mice, since the two mRNAs were being formed off separate promoters. In this regard, the 10-fold increase in plasma CETP activity induced by hypercholesterolemia in the NFR.CETP(+E9) mice exceeded the 4.5-fold increase in hepatic CETP mRNA, whereas in NFR.CETP(+E9) mice changes in plasma CETP closely parallel CETP mRNA changes (Masucci-Magoulas et al., 1996). Thus, the alternative splicing mechanism may have contributed to the induction of plasma CETP activity in NFR.CETP(+E9) mice.

* NFR.CETP(+E9)mT.CETP(-E9).

**TABLE II**

| Group                | TC before Zn²⁺ | TC after Zn²⁺ | HDL-C before Zn²⁺ | HDL-C after Zn²⁺ |
|----------------------|----------------|---------------|--------------------|------------------|
| Nontransgenic (n = 5) | 77.1 ± 5.3     | 74.9 ± 4.7    | 18.5 ± 1.7         | 18.7 ± 1.9       |
| mT.CETP(-E9) (n = 9) | 75.8 ± 8.8     | 75.0 ± 8.8    | 19.7 ± 2.1         | 20.9 ± 2.7       |
| NFR.CETP(+E9) (n = 6) | 50.9 ± 9.8     | 47.3 ± 6.4    | 11.2 ± 4.7         | 13.3 ± 4.3       |
| Compounds* (n = 4)   | 51.2 ± 3.9     | 43.7 ± 5.6    | 22.8 ± 12.4        | 24.4 ± 9.9       |

mg cholesterol/dl plasma

mg cholesterol/dl plasma

A

Days: -6 -1 0 7 17 60

**LIVER**

FL [ ]

∆E9 [ ]

B

Days: -1 0 17 60

**SPLNEEN**

FL [ ]

∆E9 [ ]

C

Days: 0 17 60

**SMALL INTESTINE**

FL [ ]

∆E9 [ ]

**Fig. 7. Changes in CETP mRNA during development.** RNA was obtained from pooled embryos and newborn or adult mice (three to four mice for each time point) and analyzed by RNase protection assay. Day 0 represents birth.

mental changes were quantitated by PhosphorImager and are depicted in Fig. 8; the data are averaged for three separate developmental studies in pools of three to four mice/study. In the liver, the peak of both FL and E9-deleted CETP mRNAs was seen in day 7 suckling mice. Prior to this the full-length form increased while the E9-deleted form did not, so that the FL/E9-deleted mRNA ratio was more than 100-fold that of the FL mRNA expression. In the spleen, there was a sharp increase in CETP mRNA prior to the peak of FL mRNA expression. In the small intestine, both forms were expressed in neonatal mice and extinguished following the suckling period. Expression levels after day 0 were too low to allow accurate quantitation of ratios of the two mRNA forms.

**DISCUSSION**

In general, alternative splicing of genes either serves as a regulatory device to modulate levels of gene expression or as a way to produce variant protein isoforms with differing functions. The present findings suggest that alternative splicing of the CETP gene falls into the first category and support the hypothesis that omission of E9 sequences represents a mechanism to limit the formation of active plasma CETP. Mice expressing a CETP transgene with E9 sequences omitted had no detectable plasma lipid transfer activity and no changes in plasma lipoprotein profile, showing that E9-deleted sequences are inactive. Furthermore, studies in mice expressing a CETP transgene with optional splicing of exon 9 indicated that inclusion of E9 was favored under circumstances where CETP mRNA was increased.

Studies of mice containing the mT-CETP transgene with E9 sequences omitted (mT.CETP(-E9) Tg mice) suggested that expression of E9-deleted transcripts was curtailed on several levels. Following zinc induction, there was massive expression of E9-deleted CETP mRNA to levels more than 100-fold that of the FL CETP mRNA in the NFR.CETP(+E9) Tg mice (Fig. 2). However, compared to the FL CETP protein, there was no corresponding overexpression of E9-deleted protein in cell homogenates or in plasma (Figs. 3 and 4). Most likely, the E9-deleted mRNA is poorly translated. An alternative explanation, i.e. that the E9-deleted protein is unstable, seems less likely, since pulse-chase experiments in cell lines stably expressing both forms of CETP show that the intracellular half-life of the E9-deleted protein is much longer than the full-length form.2 As in the cell culture studies (Quinet et al., 1993; Inazu et al., 1992), the E9-deleted protein appeared to be relatively poorly secreted, since it was more abundant than the FL form in cell homogenates but less abundant in plasma (cf. Figs. 3 and 4). The E9-deleted form of CETP has not been detected in human plasma (Inazu et al., 1992); its presence in the mT.CETP(-E9) Tg mice probably results from overexpression. Finally, even though these mice had some E9-deleted protein in plasma, it bound to HDL poorly and was inactive in lipid transfer. Site-directed mutagenesis studies show that two positively charged amino acids contained within E9 are essential for HDL binding and optimal lipid transfer activity (Jiang et al., 1995).

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C. Marinos and A. Tall, unpublished results.
Alternative Splicing of CETP Gene

TABLE III

Relative abundance of full-length and ΔE9 CETP mRNA in the liver and spleen of NFR.CETP(+E9) and NFR.CETP(+E9)/apoE0 mice. Data are based on RNase protection assay using 50 or 100 \( \mu \)g total RNA. The data are shown as a ratio of CETP mRNA/actin mRNA (normalized to liver/chow sample) or the ratio of FL/ΔE9 CETP mRNA.

|                  | NFR.CETP(+E9), chow \( n = 12 \) | NFR.CETP(+E9), high fat \( n = 6 \) | NFR.CETP(+E9)/E0, chow \( n = 6 \) | NFR.CETP(+E9)/E0, high fat \( n = 6 \) |
|------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Liver**        |                                 |                                 |                                 |                                 |
| FL/β-actin       | 1.00 ± 0.33*                    | 1.46 ± 0.49*                    | 1.25 ± 0.66*                    | 4.56 ± 0.92**                   |
| ΔE9/β-actin      | 0.67 ± 0.18*                    | 0.72 ± 0.19*                    | 0.66 ± 0.28*                    | 1.48 ± 0.21*                    |
| FL/ΔE9           | 1.48 ± 0.24*                    | 2.01 ± 0.29**                   | 1.78 ± 0.37**                   | 3.13 ± 0.74**                   |
| **Spleen**       |                                 |                                 |                                 |                                 |
| FL/β-actin       | 0.76 ± 0.50*                    | 0.86 ± 0.26*                    | 0.73 ± 0.16*                    | 10.11 ± 6.33*                   |
| ΔE9/β-actin      | 0.44 ± 0.32*                    | 0.33 ± 0.10*                    | 0.35 ± 0.08*                    | 2.97 ± 1.80*                    |
| FL/ΔE9           | 1.84 ± 0.50*                    | 2.63 ± 0.39*                    | 2.09 ± 0.19*                    | 3.38 ± 0.27**                   |

\( ^{a,b,c} \) are significantly different from each other at \( p < 0.05 \). * Indicates a significant difference with NFR.CETP(+E9), chow control, i.e. \( p < 0.001 \) and NFR.CETP(+E9), high-fat, \( p < 0.01 \). ** Indicates a significant difference with the NFR.CETP(+E9), chow, \( p < 0.01 \).

Regulatory studies in NFR.CETP(+E9) transgenic mice showed an increased ratio of FL/E9-deleted CETP mRNA when CETP gene expression was increased in response to hypercholesterolemia and during development. In the developmental studies the increase in ratio of hepatic FL/E9-deleted mRNA preceded the peak of mRNA expression, suggesting that changes in splicing may occur more rapidly than overall changes in mRNA abundance. The results of the studies in NFR.CETP(+E9) Tg mice are consistent with a regulatory role of alternative splicing. However, the changes in the splicing pattern were moderate and the largest change in ratio of FL/E9-deleted mRNA was only about 2-fold. However, the change in ratio of FL/E9-deleted mRNA was the most sensitive index of diet response (Table III). Thus, alternative splicing may be a subtle mechanism to control the amount of active CETP that is formed, perhaps with different kinetics to transcriptional or other control mechanisms, as suggested in the developmental studies (Fig. 8). Our studies do not differentiate whether altered ratios of full-length/E9-deleted mRNA arise from regulation of splicing per se, or altered stability of the different mRNA forms.

A major novel finding of the present study was that CETP gene expression was found to be markedly influenced by developmental changes. In the line of mice expressing the NFR.CETP(+E9) transgene, hepatic and intestinal expression peaked in suckling or newborn mice, while splenic expression was highest in adults. Although these findings will need to be confirmed in a species that naturally expresses CETP, they imply strong developmental control of CETP gene expression. The intestinal expression could indicate a role in cholesterol absorption or chylomicron metabolism in the newborn animal. Hepatic expression may coincide with the induction of enzymes of lipid biosynthesis and increased secretion of VLDL. The different time course of expression in the spleen suggests a distinctive role. In hamsters and humans the CETP mRNA appears to be expressed primarily in lymphocytes; in humans splenic lymphocytes contain about equal amounts of FL and E9-deleted mRNA (Jiang et al., 1991). Thus, the increase in expression in the spleen could parallel maturation of the immune system. Implicit in this discussion of tissue-specific functions during development is the idea that CETP must somehow be locally concentrated and active within organs, analogous to apoE (Mahley, 1988).

Recent studies showed decreased atherosclerosis in hypertriglyceridermic CETP transgenic mice (Hayek et al., 1995) and increased coronary heart disease in humans with genetic CETP deficiency (Zhong et al., 1995). Thus, increasing CETP expression in humans could be an antiatherogenic treatment. Marked changes in the formation of active plasma CETP, reflecting coupled changes in transcription and splicing, suggest that modulation of CETP gene expression could be a therapeutic option.

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