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Alternative Splicing of the mRNA Encoding the Human Cholesteryl Ester Transfer Protein†

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ABSTRACT: The plasma cholesteryl ester transfer protein (CETP) is known to facilitate the transfer of lipids between plasma lipoproteins. The human CETP gene is a complex locus encompassing 16 exons. The CETP mRNA is found in liver and small intestine as well as in a variety of peripheral tissues. While the CETP cDNA from human adipose tissue was being cloned, a variant CETP cDNA was discovered which excluded the complete sequence encoded by exon 9, but which was otherwise identical to the full-length CETP cDNA, suggesting modification of the CETP gene transcript by an alternative RNA splicing mechanism. RNase protection analysis of tissue RNA confirmed the presence of exon 9 deleted transcripts and showed that they represented a variable proportion of the total CETP mRNA in various human tissues including adipose tissue (25%), liver (33%), and spleen (46%). Transient expression of the exon 9 deleted cDNA in COS cells or stable expression in CHO cells showed that the protein encoded by the alternatively spliced transcript was inactive in neutral lipid transfer, smaller, and poorly secreted compared to the protein derived from the full-length cDNA. Endo H digestion suggested that the inactive, cell-associated protein was present within the endoplasmic reticulum. The experiments show that the expression of the human CETP gene is modified by alternative splicing of the ninth exon, in a tissue-specific fashion. The function of alternative splicing is unknown but could serve to produce a protein with a function other than plasma neutral lipid transfer, or as an on-off switch to regulate the local concentration of biologically active protein.

The plasma cholesteryl ester transfer protein (CETP)[1] is a hydrophobic glycoprotein, which facilitates neutral lipid and phospholipid transfer between the plasma lipoproteins (Tall, 1986; Hesler et al., 1987). The CETP appears to be a member

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[1] Abbreviations: CETP, cholesteryl ester transfer protein; bp, base pair(s); PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); VLDL, very-low-density lipoprotein(s); PMSF, phenylmethanesulfonyl fluoride.

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of a family of proteins which bind hydrophobic lipids, and includes various lipopolysaccharide binding proteins (Tobias et al., 1988; Gray et al., 1989). The importance of CETP in lipoprotein metabolism is illustrated by human genetic CETP deficiency where markedly increased HDL and decreased LDL are found (Brown et al., 1989; Inazu et al., 1990). In families with genetic CETP deficiency, plasma CETP concentration is inversely related to the levels of HDL-2 (Inazu et al., 1990), which is suspected to be an anti-atherogenic factor.

The cDNA of human CETP has been described (Drayna et al., 1987), and the primary structure and organization of the CETP gene were recently determined (Agellon et al., 1990). The CETP cDNA encodes an M_r 53K polypeptide which appears as a 74-kDa band in SDS-PAGE due in part to the presence of several N-linked sugar moieties (Hesler et al., 1987; Drayna et al., 1987). The CETP mRNA was detected in a number of tissues including liver, small intestine, adrenal gland, and spleen (Drayna et al., 1987); more recently, adipose tissue and muscle have also been found to contain relatively abundant CETP mRNA (Jiang et al., 1991). The widespread tissue distribution of the CETP mRNA raises the possibility that synthesis of CETP in various peripheral tissues may have a local function in lipid metabolism, perhaps promoting transfer of lipids in a pericellular environment (Morton & Zilversmit, 1979; Hashimoto et al., 1984; Stein et al., 1985, 1986; Morton, 1988; Granot et al., 1987; Rinninger & Pittman, 1989).

The widespread availability of cDNA cloning has revealed the frequent occurrence of diverse products of a single gene, in different tissues, often achieved by an alternative splicing mechanism (Smith et al., 1989). We have investigated the possibility that alternative forms of CETP might be produced in different tissues. We have isolated a novel CETP cDNA from mRNA of human adipose tissue, in which the ninth exon of the CETP gene has been removed by alternative splicing. The alternatively spliced variant of the CETP mRNA is present in all tissues containing the CETP mRNA, but is most highly expressed in spleen.

Experimental Procedures

Synthesis and Enzymatic Amplification of CETP cDNA. Total cellular RNA was isolated from human omental adipose tissue by the acid guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). Complementary DNA was synthesized from 8.0 μg of total RNA by incubating the RNA for 40 min at 37 °C in 20 μL of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 100 ng of dT_{13-19}, 40 units of RNasin (Promega), and 200 units of MLV reverse transcriptase (United States Biochemical Corp., Cleveland, OH). A 1603 bp region (nucleotides 128–1730) of the CETP cDNA (Drayna et al., 1987) spanning its entire coding sequence was first amplified with 50 pmol each of primers P1 (5′-TTGCTAGCACCATGCTGGCTGCCA-3′, spanning the 5′-untranslated region to the signal sequence) and P2 (5′-CTCCATCTCCGTACTCCTAACCCAACTTCC-3′, located in the 3′-untranslated region; see Figure 1) using *Taq* DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT), as described previously (Brown et al., 1989). The amplification program was 30 cycles of 0.5 min at 94 °C, 1 min at 55 °C, and 3 min at 72 °C on a Microcycler (Eppendorf, Fremont, CA). A second amplification reaction with primer P2 and a nested primer, P3 (3′-GCTAGCCCATGCCTGCTCCAAAG-3′, located at nucleotides 173–191), was performed with 1 μL of the Centricon 30 (Amicon, Danvers, MA) purified DNA of the initial amplification mixture under the same conditions. After the second amplification, two distinct cDNA fragments of 1.4 and 1.6 kb were observed. Southern blot analysis was performed with the full-length CETP cDNA (Drayna et al., 1987) as described previously (Agellon et al., 1990).

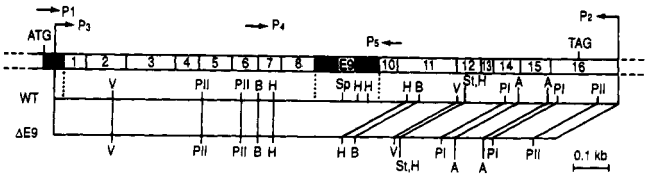

FIGURE 1: Restriction map of full-length human CETP cDNA (WT) and the alternatively spliced cDNA (ΔE9). P1, P2, and P3 represent primers used to enzymatically amplify human adipose tissue CETP cDNA. Amplified DNA was characterized by restriction mapping. V, *Eco*RV; PII, *Pvu*II; B, *Bgl*II; H, *Hae*III (not all sites shown); Sp, *Sph*I; St, *Stu*I; PI, *Pst*I; A, *Ava*I. The scale bar represents 0.1 kb. P4 and P5 represent primers used for DNA sequence analysis.

Cloning and Sequence Analysis of Human Adipose Tissue CETP cDNA. Amplified DNA was digested with *Nhe*I at an artificially introduced restriction site on the 5′ end of primer P3 and subcloned into pGEM3Zf− vector. All individual clones were sequenced with *Taq* DNA polymerase (Taq Track, Promega) using the ^{32}P-labeled sense primer P4 (5′-TCCTGAAGGGACAGATCTGCAAAGAGATCA-3′, located at nucleotides 714–743; spanning exon 6 to exon 7) or the antisense primer P5 (5′-GATTTCCTGGTTGGTGTTGAAGCC-3′, located at nucleotides 1079–1102 of exon 10) (Drayna et al., 1987; Agellon et al., 1990). Automated sequence analysis of the 1.4-kb cDNA clone (designated pGEMΔE9) and its *Eco*RV fragment (nucleotides 308–1279) was performed by the chain termination method using an Applied Biosystems 370A DNA sequencer with universal primers.

RNA Isolation and RNase Protection Assays. Total cellular RNA was isolated from surgical samples and immediately frozen in liquid N_2. The tissues examined were liver, spleen, heart and abdominal wall, gluteal, and omental adipose tissue. RNase protection assays were performed as described previously (Quinet et al., 1990). A *Bgl*II fragment of normal human cDNA spanning exons 6 to 11 (444 bp, nucleotides 727–1170) was subcloned into pGEM3Zf+ (Promega Biotek, Madison, WI) and used to prepare radiolabeled CETP cRNA probes. A second probe was prepared for quantitation purposes from a *Pvu*II–*Sph*I fragment (nucleotides 674–957) of the human CETP cDNA subcloned into pGEM4Z. This probe extended only 82 nucleotides into exon 9. Total RNA (50 μg) from five different tissues was analyzed on either 4 or 6% polyacrylamide–urea sequencing gels depending on the expected sizes of protected fragments.

Construction and Expression of the Exon 9 Deleted CETP cDNA Vector. The full-length exon 9 deleted CETP cDNA (designated pGEMFLΔE9) was constructed by exchanging *Bgl*II fragments spanning exons 6 to 11 between the full-length wild-type cDNA (designated pGEMFLWT) and the original exon 9 deleted cDNA (pGEMΔE9) derived from human adipose mRNA. Full-length cDNA inserts of pGEMFLΔE9 and pGEMFLWT were isolated and cloned into the multiple cloning sites located downstream of human cytomegalovirus (hCMV) early promoter in an expression vector pLAY (designated pLAYΔE9 and pLAYWT, respectively). The pLAY vector was kindly provided by Dan Leahey, Department of Biochemistry, Columbia University. The pLAY vector was a derivative of pRSV which contains a simian virus 40 (SV 40) origin of replication and early promoter region, a long terminal repeat (LTR), a mouse dihydrofolate reductase

(dhfr), and an ampicillin resistance gene. The pLAY vector was constructed by replacing the LTR of pRSV with the hCMV early promotor. In other experiments, the *Bgl*II fragment (ΔE9) was cloned into the *Bgl*II-digested pCMV4WT expression vector (Wang et al., 1991). Monkey kidney COS-7 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (Hyclone, Logan, UT), and cells were split at 1:5, approximately 16 h prior to transfection. Transient expressions of pLAYWT, pLAYΔE9, and pCMV4WT and pCMV4ΔE9 were performed by the DEAE-dextran method using a transient expression transfection kit (Stratagene, La Jolla, CA) as described previously (Wang et al., 1991). DNA [10–15 μg/plate, prepared with the poly(ethylene glycol) precipitation method] was diluted to 170 μL with PBS and then mixed with 85 μL of PBS and 85 μL of DEAE-dextran (2 mg/mL) and 0.9% (w/v) NaCl to form transfection solution. Medium on the cells was removed, and cells were washed with PBS. The transfection solution was added dropwise onto cells. The cells were bathed for 45 min at 37 °C with swirling at 15-min intervals. The transfection solution was aspirated; cells were rinsed with PBS and further incubated in 3 mL of the DME medium containing 100 μM chloroquine for 3 h at 37 °C. After removal of this medium, the cells were washed with PBS, and 8 mL of Opti-medium (Gibco, BRL) was added to the cells. For every set of transfections, plates transfected with no DNA were included. Multiple plates were transfected for WT, ΔE9, or no DNA and pooled to reduce variations between cells in different plates. From each plate, both cells and cell culture supernatant were collected and stored at −70 °C for subsequent determination of cholesteryl ester and triglyceride transfer activity and immunoreactive mass (see below). To obtain stable cell lines expressing the WT or ΔE9 CETP, pLAY WT or pLAYΔE9 cDNAs were transfected into dhfr minus CHO cells, and cells were selected for survival in HAT medium. After 3–6 weeks, clones of cells were isolated, expanded, and assayed for expression of CETP mRNA and protein.

Western Immunoblot Analysis. Media proteins from transiently transfected cells were dialyzed, lyophilized, and subjected to anti-CETP monoclonal antibody (TP2) affinity chromatography as described previously (Hesler et al., 1988). The retained fractions were analyzed by SDS–PAGE, transferred to nitrocellulose, and incubated with ^{125}I-labeled TP2. Cells were lysed in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.1% SDS, 0.1% deoxycholate, 0.1% Triton X-100, 1 μM PMSF, 0.1 μM aprotinin, and 1 μM leupeptin. CETP was immunoprecipitated from the cell lysate supernatants with monoclonal antibody TP-2 attached to Sepharose beads (Hesler et al., 1988). Immunoprecipitation was carried out overnight at 4 °C. The TP2–Sepharose–CETP complexes were isolated by centrifugation, washed with 0.1 M ammonium bicarbonate, lyophilized, and then either resuspended in buffer for *N*-glycosidase F or Endo H treatments or prepared directly for SDS–PAGE and immunoblot analyses as above. In the former case, immunoprecipitates in 0.10 M sodium phosphate buffer, pH 8.0, containing 10 mM EDTA, 1% Triton X-100, 0.02% SDS, 3 mM DTT, and 1 mM PMSF were treated with 0.75 unit of *N*-glycosidase F and allowed to incubate under N_2 at 37 °C for 18 h. For Endo H digestion, immunoprecipitates were treated with 2 milliunits (endoglycosidase H of *Streptomyces plitacus*) in 100 μL of 50 mM sodium phosphate buffer, pH 5.75, containing 1% Triton, 0.01% SDS, 3 mM DTT, and 1 μM PMSF under the same conditions of incubation.

Analytical Methods. [^{3}H]Cholesteryl linoleate HDL_3 (*d* 1.11–1.21 g/mL) and [^{3}H]cholesteryl linoleate/[^{14}C]triolein HDL_3 were prepared as described previously (Morton & Zilversmit, 1979; Swenson et al., 1988). In order to detect cholesteryl ester transfer activity, the Opti-MEM I medium (200 μL) was incubated for 15 h at 37 °C with labeled HDL_3 (10000 cpm) and LDL (*d* 1.020–1.063 g/mL, 100 μg of protein) in a total 300-μL incubation volume. LDL was precipitated by the addition of bovine serum albumin, heparin, and $MnCl_2$ [final concentrations 4%, 75 mM, and 140 mM, respectively (Hesler et al., 1989)]. All assays were performed in the linear range, using conditions such that transfer of the radiolabeled cholesteryl ester or triglyceride was <15%. The resulting transfer activities were estimated by triplicate assays. To measure the CETP mass in media, the remaining medium (6–8 mL) was dialyzed against 2 mM Tris-HCl, pH 7.5, for 24 h at 4 °C, lyophilized, and analyzed by RIA using ^{125}I-mAb TP2 (Marcel et al., 1990).

Results

Isolation of a Novel CETP cDNA from Human Omental Adipose Tissue mRNA. Recently, it was discovered that human adipose tissue is a rich source of the CETP mRNA, with an abundance similar to liver (Jiang et al., 1991). To assess the possibility that CETP might exist in a variant form in adipose tissue, the CETP cDNA was obtained from human omental adipose tissue mRNA using polymerase chain reaction (PCR) amplification. Agarose gel electrophoresis showed two distinct cDNA fragments of sizes 1.6 and 1.4 kb. The 1.6-kb fragment corresponds to the size expected for the previously reported CETP cDNA (Drayna et al., 1987). The smaller fragment was also shown to be an authentic CETP cDNA by Southern blot hybridization (not shown). To determine the location of the approximately 0.2-kb deletion in the 1.4-kb cDNA, mapping analysis was performed with eight different restriction enzymes (Figure 1). The deletion was located between two *Bgl*II sites (Drayna et al., 1987) and resulted in the loss of a unique *Sph*I site contained within sequences corresponding to exon 9 of the CETP gene. The restriction map of the variant cDNA suggested the absence of sequences derived from exon 9 (180 nucleotides) in the 1.4-kb CETP cDNA (Figure 1). The amplified cDNA was cloned, and nine individual clones were isolated; five clones had the 1.4-kb insert, and four clones had the normal 1.6-kb insert.

To determine if there was, in fact, deletion of exon 9, all clones were sequenced with an exon 6–7 sense primer (P4) or an exon 10 antisense primer (P5) (Figure 1). This revealed that the variant cDNA was derived by direct connection of sequences corresponding to exon 8 to those corresponding to exon 10, with the complete exclusion of exon 9 derived sequences (Figure 2). Several independent clones containing the smaller 1.4-kb CETP cDNA showed perfect exclusion of exon 9 sequences. Complete automated sequencing of one of the 1.4-kb cDNA clones (pGEMΔE9) confirmed that the sequence between nucleotides 173 and 1730 is identical to the reported CETP cDNA sequence (Drayna et al., 1987) except for the absence of exon 9 derived sequence and two nucleotide substitutions. One substitution is at nucleotide 1696 (CGTG → CATG) in the 3′-untranslated region, generating a new *Nla*III restriction site, and the other is a silent substitution at nucleotide 1117 (GTC → GTT), encoding a valine residue.

The human CETP gene exists as a single copy per haploid genome, and exon 9, consisting of 180 bp, can be spliced out without interruption of the reading frame (a "+O" splicing mechanism) (Agellon et al., 1990). Thus, this exon can be included or excluded independently of other exons (as a cas-

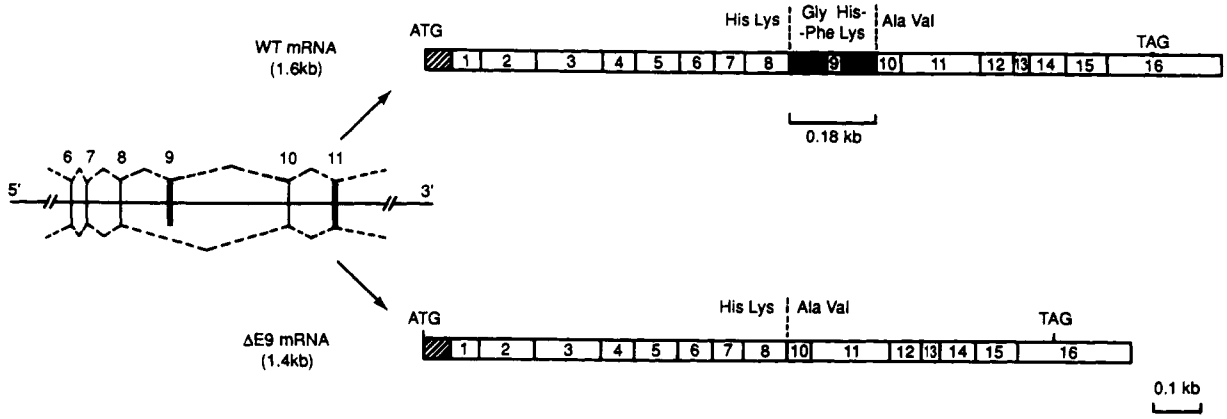

FIGURE 2: Schematic representation of two forms of CETP transcripts. Splicing pathways are shown by the diagonal lines on exons of human CETP gene. The upper panel shows the constitutively spliced transcript encoding normal CETP (WT mRNA); the lower panel shows the alternatively spliced transcript encoding exon 9 deleted CETP (ΔE9 mRNA). The amino acids are shown at the junction between exons 8/9 and exons 9/10 for the WT, and between exons 8/10 for the ΔE9 form.

sette exon) while maintaining the translational reading frame (Figure 2). The absence of the sequence encoded by exon 9 from the CETP transcript would generate a polypeptide lacking 60 amino acids compared with the wild type.

Distribution of Alternatively Spliced CETP Transcripts in Various Human Tissues. The human CETP mRNA appears as a single broad band in Northern blots of RNA from various tissues (Drayna et al., 1987). However, an alternatively spliced mRNA, only 0.18 kb different from the full-length transcript, might not be resolved by Northern blots. To confirm the existence of an exon 9 deleted CETP transcript and to determine its abundance relative to the full-length mRNA, RNase protection assays were performed on total RNA isolated from various human tissues. A radiolabeled riboprobe corresponding to the antisense DNA strand of the pGEM vector containing the normal *Bgl*II fragment of the CETP cDNA was synthesized and hybridized to RNA isolated from human liver, spleen, and omental and abdominal wall fat. Subsequent to RNase T2 digestion, the protected fragments were resolved by polyacrylamide gel electrophoresis (Figure 3A,B). Normally spliced transcripts protect a fragment of 444 nucleotides, and an alternatively spliced transcript, lacking the sequences encoded by exon 9, will protect fragments of 110 and 154 nucleotides (Figure 3C). RNA from each of the tissues examined protected a fragment of 444 nucleotides, consistent with the presence of the full-length CETP transcript. Protected fragments of 110 and 154 nucleotides were also observed in every tissue, indicating that all tissues also express the alternatively spliced CETP transcript (Figure 3A,B). The size of the protected fragments (110 and 154 nt), resulting from the exclusion of exon 9 derived sequences, was estimated by comparison with molecular weight standards and was confirmed by RNase protection analysis of RNA isolated from COS cells transiently transfected with pLAYΔE9 (see Experimental Procedures) (Figure 3A). The reason for the doublet in the 154-nt fragment is unknown. The additional fainter fragments (e.g., in liver) were similar to nonspecific bands in the tRNA control, or minor partial degradation products seen in the WT. These data confirm several different tissues contain an alternatively spliced CETP mRNA, in which exon 8 sequences are joined to exon 10 sequences.

In order to estimate the relative abundance of the exon 9 deleted transcript in different human tissues, a smaller riboprobe spanning the exon 8/9 junction was employed (Figure 4). Using this probe, the ΔE9 transcript appeared as a single 207-nt fragment, somewhat smaller than the 289-nt fragment produced by the full-length fragment. The relative abundance of the two forms of mRNA was determined by densitometry, employing tissues from several different donors. In different subjects, the exon 9 deleted mRNA (as a percentage of total CETP mRNA) represented 25 ± 3% in gluteal adipose tissue (mean ± SEM, $n = 8$), 24 ± 5% in omental fat ($n = 3$), 26% in abdominal wall fat ($n = 1$), 14% in heart ($n = 1$), 33% in liver ($n = 3$), and 46 ± 6% in spleen ($n = 4$). These results indicate a similar percentage of the alternatively spliced mRNA in adipose tissue from different regions. However, there appeared to be significant differences between various tissues, with the highest levels in spleen.

Characterization of Protein Encoded by the Alternatively Spliced Transcript. The functional significance of the alternatively spliced transcript was evaluated by transient expression of wild-type and exon 9 deleted CETP cDNAs. On six consecutive occasions (involving two to four transfections of WT and ΔE9 cDNA per experiment), the ΔE9 cDNA gave rise to no neutral lipid transfer activity in cellular media. In contrast to these results, the WT cDNA gave rise to readily detectable CE and TG transfer activity [4000 cpm transferred $(200\ \mu L \text{ of medium})^{-1}\ (6\ h)^{-1}$], as reported previously (Wang et al., 1991). Since the sensitivity of the assay is about 150 cpm, this indicates that the ΔE9 cDNA gives rise to <4% of the activity of the WT. Quantitation of the mass of ΔE9 CETP in media indicated that it was poorly secreted compared to the WT (~12% of the concentration of WT protein in media as determined by immunoassay). Thus, the specific activity of the ΔE9 protein was <33% of that of the WT. In cell lysates, the WT gave rise to readily detectable activity [~1200 cpm transferred $(200\ \mu L \text{ of cell lysate})^{-1}\ (6\ h)^{-1}$], whereas the ΔE9 protein showed no activity. Estimates of mass showed similar concentrations for WT CETP and ΔE9 CETP in cell lysates. Thus, the specific activity of ΔE9 CETP in cell lysates was <13% of that of WT.

To further assess the potential activity of the ΔE9 CETP, stable lines of CHO cells were prepared, expressing either the WT or the ΔE9 cDNAs. Abundant CE transfer activity and protein were detected in media prepared from cells transfected with WT cDNA, but no activity or protein was detected in the media from the ΔE9 cDNA, indicating activity <2% of WT. Analysis of cell lysates showed readily detectable protein for WT and ΔE9 cDNA (Figure 5); the former gave rise to abundant CE transfer activity, whereas the latter was completely inactive. In order to determine the cellular localization of the cell-associated forms of CETP, immunoprecipitates of cell lysates were digested with Endo H. As expected, plasma CETP (Figure 5) was totally resistant to Endo H digestion.

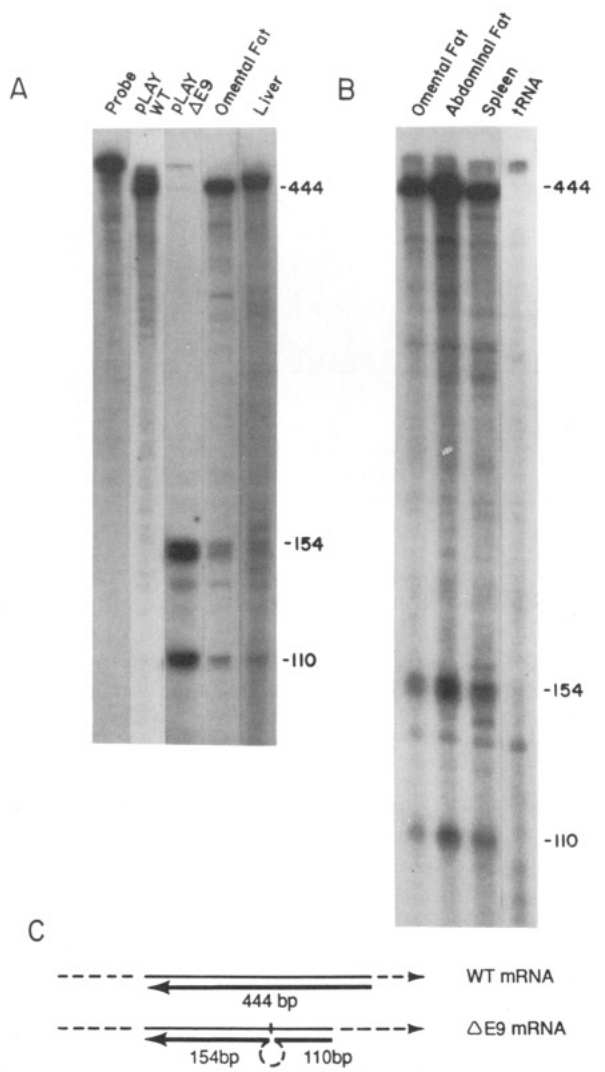

FIGURE 3: Tissue distribution of the two forms of CETP transcript, shown with a riboprobe spanning the exon 8/9 and exon 9/10 junctions. The figure shows an autoradiogram of an RNase protection assay performed as described under Experimental Procedures. In panels A and B, 50 μg of total RNA from the human tissues indicated or from cells transiently transfected with pLAY WT and pLAYΔE9 (i.e., containing either wild-type or exon 9 deleted CETP cDNAs) was hybridized to a 500-nucleotide-long probe, corresponding to the *Bgl*II fragment (exons 6–11) of the wild-type CETP cDNA. Human liver and pLAYΔE9 RNAs were assayed at 100 and 5 μg, respectively, and 50 μg of tRNA was hybridized as a control. The sizes (in nucleotides) of the protected fragments were determined by comparison to ^{32}P-labeled ϕX*Hae*III restriction fragments and are indicated on the right side of the panel. Additional small bands not present in tRNA are probably caused by incomplete digestion or unstable regions in the RNA–RNA hybrids. (C) Diagrammatic representation of hybridization products formed between the normal and alternatively spliced CETP transcripts and the probe RNA. The numbers indicate the expected sizes (in nucleotides) of the protected fragments for either transcript.

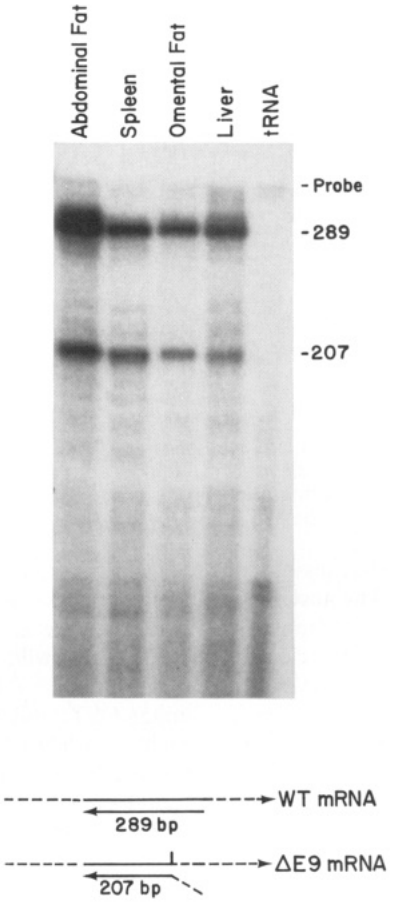

FIGURE 4: Quantitation of the two forms of CETP mRNA, as shown with a shorter riboprobe, spanning the exon 8/9 junction. RNase protection analysis was performed on total RNA (50 μg) from abdominal fat, spleen, and omental fat or 100 μg of human liver RNA. Hybridization was to probe spanning exons 6–8 and a portion of exon 9 (82 nucleotides) as described under Experimental Procedures. The protected fragment sizes indicated diagrammatically are 289 bp for the normal transcript and 207 bp for the alternatively spliced transcript.

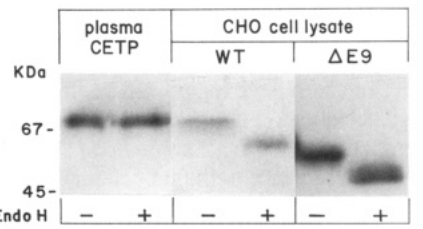

FIGURE 5: Western blot of immunoprecipitates from plasma and CHO cell lysates subjected to endoglycopeptidase H digestion. Plasma (200 μL) or cell lysates ($\sim 5 \times 10^6$ cells) were immunoprecipitated in duplicate, incubated with Endo H (+) or without enzyme in the same buffer solution (–), and analyzed by SDS–PAGE and immunoblotting with ^{125}I-TP-2. Lanes 1 and 2, plasma CETP; lanes 3 and 4, wild-type CETP cDNA stably expressed in CHO cells; lanes 5 and 6, exon 9 deleted CETP cDNA stably expressed in CHO cells.

However, both WT and ΔE9 proteins were completely converted to lower molecular weight forms upon digestion with Endo H (Figure 5), indicating that they are high-mannose forms of the proteins, probably within the endoplasmic reticulum. In order to estimate the contribution of the N-linked sugar, immunoprecipitates of COS cell lysates were treated with glycopeptidase F and then analyzed with 6% SDS–PAGE (not shown). This converted the WT CETP from M_r 65K to 58K and the ΔE9 CETP from 53K to 48K. The smaller molecular weight difference for the ΔE9 form also indicates that the N-linked site in E9 is occupied in the WT protein.

In earlier studies, a single protein of M_r 74 000 was found when CETP was purified from fasting plasma in the presence of protease inhibitors (Hesler et al., 1987, 1988; Brown et al., 1989). To evaluate the possibility that an additional smaller form of CETP (i.e., exon 9 deleted) might appear in plasma in the postprandial state, CETP was immunopurified from plasma of two normolipidemic subjects 2 and 5 h after ingestion of a single high-fat, high-cholesterol meal. No evidence was obtained for the appearance of a truncated form of CETP in postprandial plasma (the results in one subject are shown in Figure 6). Since the glycosylation of CETP may be incomplete in cultured cells, we considered the possibility that

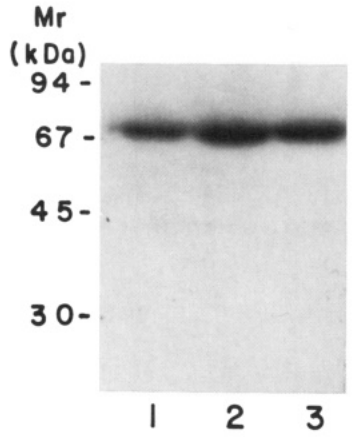

FIGURE 6: Western blot of immunoprecipitates from plasma before and during alimentary lipemia. Plasma (200 μL) was immunopurified and analyzed by SDS–PAGE and immunoblotting with ^{125}I-TP-2, as described under Experimental Procedures. Lanes 1, 2, and 3 represent samples taken at 0, 2, and 5 h, respectively, after ingestion of a single high-fat, high-cholesterol meal (containing approximately 100 g of fat and 100 mg of cholesterol).

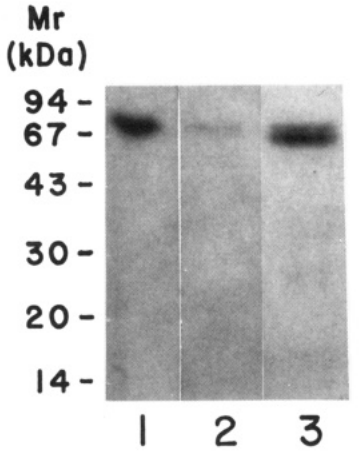

FIGURE 7: Western blot of immunoprecipitates of tissue homogenates. Tissues (1–2 g) were homogenized on ice in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% SDS, 0.1% deoxycholate, 0.1% Triton X-100, 1 mM PMSF, 0.1 μM aprotinin, and 1 μM leupeptin using a Polytron homogenizer. CETP was immunoprecipitated from the homogenates with monoclonal antibody TP-2, in the clear supernatant obtained after a 15K*g*, 20-min centrifugation, and detected by SDS–PAGE and immunoblotting as described under Experimental Procedures. Lane 1, CM-cellulose-purified CETP; lane 2, CETP isolated from adipose tissue; lane 3, CETP isolated from human spleen from the same individual.

the two forms might be superimposed in plasma, due to greater glycosylation of the ΔE9 form in vivo. However, glyco F treatment of plasma and recombinant WT CETP produced identically migrating 60-kDa forms, with no additional faster migrating bands seen in plasma (not shown).

Since we could find no evidence for a truncated form of CETP in plasma, CETP was immunoprecipitated from fresh tissue homogenates in the presence of protease inhibitors and analyzed by SDS gels and Western blotting (Figure 7). No smaller form of CETP was seen in homogenates of adipose tissue (Figure 7, lane 2; $n = 3$). However, the amounts of CETP that could be recovered from adipose tissue were probably too little to detect a smaller protein that was similar in abundance to the ΔE9 mRNA. By contrast, in homogenates of human spleen ($n = 3$), CETP appeared as a doublet (Figure 7). The lower molecular weight band migrated with an apparent molecular weight of 61K, about 7K less than the larger, presumably full-length protein. This form of CETP was identical in molecular weight to the ΔE9 CETP produced in CHO cells (M_r 61 000, Figure 5), suggesting that the more abundant smaller version of CETP (57% of protein mass as determined by scanning the Western blot) is the product of the alternatively spliced CETP mRNA. Consistent with this suggestion, RNase protection analysis indicated that the ΔE9 mRNA represented 65% of CETP mRNA in spleen and 25% in omental adipose tissue in the subject whose tissues were analyzed in Figure 7.

Discussion

The CETP gene is a complex locus encompassing 16 exons and spanning over 24 kbp of genomic DNA (Agellon et al., 1990). We have demonstrated the existence of a novel transcript lacking sequences corresponding to exon 9 of the CETP gene. Exon 9 consists of 180 bp and can be spliced out of the primary transcript without interruption of the reading frame. The two forms of transcripts are most likely generated by an alternative splicing mechanism (Figure 2). The abundance of exon 9 deleted transcripts was relatively high, representing approximately 15–35% of total CETP mRNA in liver and adipose tissue and 35–65% in spleen. Transient or stable expression experiments showed that the polypeptide encoded by the alternatively spliced CETP transcript was inactive in neutral lipid transfer. Thus, the function of alternative splicing might be to produce a protein with different activities than those normally attributed to plasma CETP or to serve as a switch, modulating lipid transfer activity within specific tissues.

The isolated cDNA sequences excluding the sequence encoded by exon 9 were identical to the reported CETP cDNA (Drayna et al., 1987) between nucleotides 173 and 1730 except for two conserved nucleotide substitutions which do not change amino acid residues. There are no definite sequence data between the 5′-untranslated region and the end of the signal peptide sequence in the exon 9 deleted transcript. However, the ΔE9 transcript was initially amplified using primer (P1) in the 5′-untranslated region of the CETP cDNA and then primer (P3) which overlaps nine nucleotides at the 3′ end of the signal sequence (Figure 1). Furthermore, the sequences of the CETP gene encoding the signal peptide are found within exon 1, and a unique leader sequence and transcription start site were identified in numerous clones obtained by anchored PCR of the G-tailed 5′ end of the liver CETP cDNA (Agellon et al., 1990). Thus, an alternative first exon appears unlikely. Together, the data strongly suggest that the exon 9 deleted transcript has an identical leader sequence and signal peptide region to those of the full-length transcript.

The molecular weight difference between the wild-type CETP and the exon 9 deleted CETP was approximately 13K (Figure 5), which is consistent with the absence of both 60 amino acids (6.6 kDa) and 1 N-linked carbohydrate moiety (~5 kDa). Four potential N-linked glycosylation sites exist in the CETP sequence, and one of these sites is located in exon 9 (Drayna et al., 1987). The larger change in molecular weight upon glyco F digestion of WT CETP (7K), compared to ΔE9 CETP (5K), also suggests that the N-linked site in exon 9 is occupied in the WT CETP, contributing ~2 kDa. Thus, the N-linked site in exon 9 is occupied and contributes about 2K–5K to the molecular weight of WT CETP. As with most linker insertion or deletion mutants of CETP (Wang et al., 1991, and unpublished results), the secretion of the ΔE9 CETP was much less than that of WT. This could be due to lack of the N-linked site in exon 9, an increased overall index of hydropathy (Kyte & Doolittle, 1982) of the exon 9 deleted CETP polypeptide compared to full-length CETP (ΔE9, +0.15, vs FL, +0.13), or conformational change. Although

the physiological relevance of decreased secretion by COS or CHO cells is somewhat tentative, poor cellular secretion may explain the absence of the ΔE9 CETP from plasma (Figure 6).

In earlier studies, several laboratories reported at least two different polypeptides of M_r 55K–69K in their most purified preparation of plasma CETP (Ihm et al., 1982; Morton & Zilversmit, 1979; Abbey et al., 1985; Ohnishi et al., 1990). However, when adequate care was taken to inhibit proteinases, a single broad band (72–76 kDa) was isolated, and there was no evidence for a distinctly smaller protein corresponding to the exon 9 deleted CETP in plasma (Hesler et al., 1987, 1988); this observation has been made repeatedly with at least 10 different donors of plasma [e.g., Figure 1 in Brown et al. (1989)] and also in the postprandial state in the present study. There is an obvious discrepancy between the relatively abundant transcripts encoding exon 9 deleted polypeptides and the failure to detect a corresponding smaller polypeptide in plasma. There are several potential explanations for this finding.

Alternative splicing may also be used as an on–off switch for biological activity, regulating such fundamental processes as sex determination (Bell et al., 1988) or nuclear localization of oncogenes (Weber et al., 1990). Alternative splicing of the CETP mRNA may regulate the release of active WT CETP from cells. In this scenario, the ΔE9 CETP would be largely retained within the endoplasmic reticulum and eventually degraded, as presumably occurs in cells transfected with the cDNA. Plasma CETP activity is known to be regulated by chronic diet-induced changes in tissue mRNA levels (Quinet et al., 1990, 1991). Alternative splicing might represent a means for more rapid modulation of CETP activity. This would be analogous to the regulation of LPL activity, in which long-term regulation tends to alter mRNA abundance whereas short-term regulation may be mediated by changes in cellular secretion (Semenkovich et al., 1989; Eckel, 1989), sometimes reflecting different levels of intracellular degradation (Doolittle et al., 1990).

Alternative splicing sometimes produces proteins which have novel biochemical activities or are targeted to different cellular locations (Smith et al., 1989). Thus, the ΔE9 form of CETP might have specialized functions unrelated to its known extracellular neutral lipid transfer activities. The natural expression of CETP in specialized cells might have different consequences to the results obtained by transfection of CHO or COS cells. It is interesting that the alternatively spliced mRNA is most abundant in spleen. In hamster spleen, the CETP mRNA is present in a rim around the edge of germinal follicles (Jiang et al., 1991), suggesting its presence in lymphocytes or antigen-presenting macrophages. Thus, the ΔE9 form of CETP could play a specialized role in specific cell types in spleen.

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