Human plasma CETP deficiency: identification of a novel mutation in exon 9 of the CETP gene in a Caucasian subject from North America

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Abstract Human plasma cholesteryl ester transfer protein (CETP) is a 476-residue hydrophobic glycoprotein that catalyzes the heterotransfer of cholesteryl esters and triacylglycerols among lipoproteins: Mutations in the CETP gene have been identified, mostly in the Japanese population. These mutations result in hypercholesterolemia due to the presence of large cholesteryl ester-rich HDL particles, elevated plasma apoA-I and apoE, and reduced apoB levels. Here we report the plasma lipoprotein phenotype and molecular defect in a 57-year-old female Nova Scotian subject lacking Japanese ancestry who is homozygous for a novel mutation in the CETP gene. Her total plasma cholesterol was 7.3 mmol/l with an LDL cholesterol of 2.9 mmol/l and HDL cholesterol of 4.4 mmol/l. She was mildly hypertriglyceridemic (1.6 mmol/l) and had markedly elevated apoA-I (256 mg/dl) and apoE (14.4 mg/dl) with only slightly reduced apo/B levels (94 mg/dl). Her VLDL and LDL were cholesteryl ester-poor (1.8 and 37.2% of lipids, respectively) and triacylglycerol-rich (67.3 and 18.9% of lipids, respectively) while her HDL was cholesteryl ester-rich (40.2–45.7% of lipids) and triacylglycerol-poor (3.3–2.5% of lipids). No plasma CETP activity or mass was detected. Bi-directional DNA sequence analysis of PCR products from all 16 exons showed a single base substitution (C→T at nucleotide 836 in exon 9 resulting in 268 Arg→STOP) in both alleles. No other mutation was detected. A single base mismatched, 26 bp reverse PCR primer that produced a single Mae III RFLP site upon amplification of the mutated DNA sequence was designed for rapid population screening. This subject is, we believe, the first Caucasian North American patient reported to have CETP deficiency.—**Teh, E. M., P. J. Dolphin, W. C. Breckenridge, and M–H. Tan.** Human plasma CETP deficiency: identification of a novel mutation in exon 9 of the CETP gene in a Caucasian subject from North America. *J. Lipid Res.* 1998. **39:** 442–456.

Supplementary key words cholesteryl ester transfer protein • hyperalphalipoproteinemia • mutation • HDL • cholesteryl ester and phospholipid fatty acids

High levels of plasma HDL have been implicated as a negative risk factor for coronary artery disease (CAD) in prospective epidemiological studies (1). However, a precise understanding of the causal mechanism(s) underlying the inverse relationship between HDL and CAD remains unclear. The putative antiatherogenic roles of HDL arise first from its observed ability to stimulate the efflux of cholesterol from peripheral cells and, after esterification by plasma lecithin:cholesterol acyltransferase (LCAT), facilitate the transfer of this tissue-derived cholesterol to the liver via the reverse cholesterol transport pathway (2). Second, HDL contains naturally occurring lipophillic antioxidants such as vitamin E which are available to help prevent the oxidation (3) and subsequent uptake of LDL by macrophages and vascular tissues. Because HDL particles are intimately involved in reverse cholesterol transport, an increase in HDL cholesterol is thought to reflect the effectiveness of this pathway or the efficiency of the metabolism of the other atherogenic lipoproteins such as very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Several environmental factors that are able to influence HDL levels act indirectly through the lipases and lipid transfer proteins (2). The female sex, alcohol consumption, and exercise are positive effectors for increasing HDL levels while the male sex, smoking, and diets high in cholesterol and saturated fats decrease HDL levels. Previously, it has been shown that the absence of cholesteryl ester transfer activity mediated by

Abbreviations: CETP, cholesteryl ester transfer protein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; CE, cholesteryl ester; CAD, coronary artery disease; ACAT, acyl-CoA:cholesterol acyltransferase; RFLP, restriction fragment length polymorphism.

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the cholesteryl ester transfer protein (CETP) also increases HDL levels (4).

CETP is responsible for the net transfer and heteroexchange of triglycerides (TG) and cholesteryl ester (CE) between the high density lipoprotein (HDL) fractions and the very low (VLDL) and low density lipoproteins (LDL) fractions (5). Plasma CETP is a highly hydrophobic glycoprotein containing 476 amino acids and four N-linked glycosylation sites (4). The human CETP gene has 16 exons encompassing 25 kbp and is located close to the LCAT gene locus (16q22) on chromosome 16q21 (6). In humans, plasma CETP mRNA is highly expressed in the liver as well as in spleen and adipose tissue, with lower levels expressed in the small intestine, adrenal, kidney, and heart (7, 8).

The first human genetic CETP deficiency identified was a splicing defect in a Japanese subject with markedly elevated HDL and low levels of cholesteryl ester transfer activity (9, 10). The G→A transition at intron 14(+1) altered the strictly conserved G–T intron splice donor to an A–T resulting in the splicing defect. A few other CETP mutations have also been described in the Japanese population (11–14) in which CETP deficiency appears to be particularly prevalent in the region of Omagari city (15). To our knowledge, the only other reports of familial CETP deficiency have been in the German population (16). Phenotypically, familial CETP deficiency results in an increased CE content of the HDL with decreasing CE content in the VLDL and LDL, increased levels of plasma HDL (17), apoA-I, apoA-IV, and apoE (18) and decreased apoB-100 due to an apparent increase in the LDL catabolic rate (19). Whereas a decrease or an absence of plasma CETP activity creates an anti-atherogenic lipoprotein profile, there is presently controversy concerning the potentially beneficial effects of CETP deficiency. Some authors have associated CETP deficiency with longevity (4, 20) while others report an increased prevalence of coronary heart disease in CETP-deficient subjects with HDL cholesterol levels between 41 and 60 mg/dl. (21). Recently a large population-based study in the Omagari city region of Japan (15) provided additional evidence that the hyperalphalipoproteinemia resulting from this disorder may not represent a longevity syndrome.

In the present study, we report a novel mutation (C→T transition) at nucleotide 836 in exon 9 of the CETP gene that results in a premature termination of the CETP protein at residue 268 (268Arg→Stop). Furthermore, this is also the first report of a CETP gene mutation in a Caucasian North American subject lacking Japanese ancestry and thus affords an opportunity to study CETP deficiency in a different, non-Japanese, genetic background.

METHODS

Subject

The proband, who presented to her family physician with hypercholesterolemia, is a 57-year-old female resident of Nova Scotia with no known Japanese ancestry. At the time her total plasma cholesterol was in excess of 9.4 mmol/L and she was placed on Pravachol®, a lipid lowering agent, 40 mg once daily. Pharmacological intervention had little effect upon her total plasma cholesterol and she was referred to one of us (MHT) for lipoprotein analysis which revealed an LDL-cholesterol (LDL-C) of 4.23 mmol/L, HDL-cholesterol (HDL-C) of 4.9 mmol/L, triglycerides of 1.4 mmol/L. She had never had a myocardial infarction and did not suffer from angina, dyspena, or claudication on exertion. She did not have diabetes mellitus nor thyroid, hepatic, or renal dysfunction and had no history of hypertension or xanthoma. Her family members tested all had normal plasma lipid values except for one sibling who was also hypercholesterolemic. Her other siblings and family members were not prepared to participate in this study. The proband's father died of heart disease of an unknown cause and her mother is presently aged 90 years. The patient was taken off all lipid lowering medication for the duration of this study.

Lipoprotein isolation and analysis

Plasma lipoproteins were isolated and subfractionated using a discontinuous density gradient and ultracentrifugation by a slight modification of the method of Chapman et al. (22) as described by us (23). When necessary, individual subfractions were dialyzed against normal saline for 24 h at 4°C prior to analysis of their lipid or apolipoprotein content. The lipid components of lipoproteins, whether present in plasma or as fractions isolated by density gradient ultracentrifugation, were analyzed using either enzyme kits (Boehringer Mannheim) for total cholesterol, free cholesterol, triacylglycerol, and phospholipids or by the gas chromatographic total lipid profiling technique of Kuksis et al. (24). Total protein was determined by a sodium dodecyl sulfate–modified Lowry procedure (25) using bovine serum albumin (Sigma, St. Louis, MO) as a standard. Electroimmunoassays to quantitate apoA-I, apoB, and apoE were performed as previously described by us (26).

Determination of cholesteryl ester and phosholipid fatty acid composition

The lipid classes present within individual lipoprotein fractions separated by density gradient ultracentrifugation were fractionated by thin-layer chromatography on silica gel H plates (Mandel Scientific) using a

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solvent system of hexane–diethyl ether–glacial acetic acid 80:16:14 (v/v) after extraction by the method of Folch, Lees and Sloane Stanley (27). All procedures were conducted in a nitrogen atmosphere. After fractionation, the isolated phospholipids and cholesteryl esters were immediately transmethylated (28) and the fatty acid methyl esters were analyzed by gas chromatography using a 30 m × 0.25 mm Supelco SP-2330 capillary column. Fatty acid methyl ester standards were obtained from Supelco Inc., Bellefonte, PA.

Determination of plasma CETP mass and activity

Plasma CETP mass was determined using the double antibody sandwich immunoradiometric assay of Clark, Moberly, and Bamberger (29). The capture monoclonal antibody was 2F8 and the detection monoclonal antibody was ^{125}I-labeled 2E7. All reagents, including CETP standards, were most generously provided by Drs. Clark and Bamburger of the Department of Cardiovascular and Metabolic Diseases, Central Research Division of Pfizer Inc, Groton, CT. Reciprocal lipid transfer mediated by CETP was measured in the proband and normal subjects after incubation of 3-ml aliquots of plasma at either 4°C or 37°C for 5 h. All incubations contained 1 mm phenylmethylsulfonyl fluoride (Sigma) to inhibit plasma LCAT activity (30). After incubation, the plasma samples were fractionated into VLDL and LDL + HDL by a single ultracentrifugation at d 1.006 g/ml for 17 h at 40,000 rpm using a Beckman 50.3Ti rotor and L5-50 ultracentrifuge. The lipid mass composition of each fraction was then quantitated enzymatically as described above.

Determination of plasma LCAT activity

LCAT activity was measured using the exogenous synthetic proteoliposome substrate method of Chen and Albers (31) as described by Jauhiainen and Dolphin (30).

DNA analysis

Genomic DNA was purified from white blood cells according to Miller, Dykes, and Polesky (32) with some modifications. In vitro amplifications of genomic DNA were performed by PCR using paired intronic oligonucleotide primers for all exons. Single strand conformation polymorphism (SSCP) was performed as described by Orita et al. (33) with a slight modification. Diluted ^{32}P-α[dCTP] (DuPont, Missisauga, Ontario)-labeled PCR products were resolved on 0.5 × MDE gels (J. T. Baker Inc., NJ) containing different percentages of glycerol (0–10%). Single stranded conformers were visualized by exposure to Kodak X-ray film (Interscience Inc., Markham, Ontario). All PCR products were confirmed to be single bands as resolved on agarose gel electrophoresis before performing SSCP analyses. PCR single strand conformation polymorphism analysis was performed on all 16 exons including exon/intron junctions.

PCR and sequencing

PCR amplifications were carried out using the same synthetic primers used for SSCP. All amplifications consisted of 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 74°C for 3 min followed by a 10-min extension at 70°C in a total volume of 50 μL. PCR products were separated by agarose gel electrophoresis and isolated with the Sephaglas™ BandPrep Kit (Pharmacia, Uppsala, Sweden). Purified products were cloned into DH5α using the TA cloning vector, pCR 2.1 (Invitrogen, San Diego, CA). Sequencing of double stranded templates was performed by dideoxy chain termination using T7 polymerase (Pharmacia, Uppsala, Sweden). At least two different PCR products were cloned and all clones were sequenced in triplicate to discern any errors introduced by Taq polymerase. Direct sequencing of the PCR products of exon 9 in both directions was also performed to confirm the mutation and the genotype of the patient.

Primer-mediated restriction fragment length polymorphism (RFLP) modification for rapid diagnosis

An RFLP site for Mae III (Pharmacia, Uppsala, Sweden) was created only in the presence of a mutation by 26-bp reverse mismatched primer 5′-CTACCTTGGC CAGCGAGTGGAAGAGT-3′ and an 18-bp forward primer 5′-GACACACAGGGTCCAGCCAG-3′ to yield a 168 bp PCR product. Diluted ^{32}P - α[dCTP] (DuPont, Missisauga, Ontario) PCR product was digested with 1 μL of Mae III (Pharmacia, Uppsala, Sweden) for 1 h at 55°C as recommended. Controls were performed in a similar manner. The restriction digests were resolved by 12% non-denaturing polyacrylamide gel electrophoresis (PAGE). A 138 bp and a 29 bp fragment were generated in the mutated DNA sequences.

RESULTS

Table 1 shows the plasma lipid values of the proband while still taking Pravachol® (40 mg daily) and upon initial referral. Her total plasma cholesterol was markedly elevated (7.3 mmol/l) as were her plasma esterified cholesterol and phospholipid values. She was also mildly hypertriglyceridemic. Her plasma apoA-I level was 2-fold elevated and her apoE level was almost 3-fold elevated while her apoB level was slightly lower than

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TABLE 1. Plasma lipids and apolipoproteins in CETP deficiency

Lipid	Whole Plasma	VLDL	LDL	HDL
		mmol/liter		
Total cholesterol	7.34	0.24	2.92	4.38
Free cholesterol	2.20	0.20	1.10	1.00
Esterified cholesterol	5.14	0.04	1.82	3.38
Triglycerides	1.59	1.11	0.59	<0.01
Phospholipids	4.60	0.28	1.32	30.5
Apolipoprotein	*mg/dl*			
ApoA-I	256.0			
ApoB	94.0			
ApoE	14.4			

All lipid values were determined enzymatically as described in Methods. Plasma apolipoproteins were quantitated by electroimmunoassay (26). Lipoproteins were isolated by sequential ultracentrifugation at the following densities: VLDL, d 1.006 g/ml; LDL, d 1.006–1.063 g/ml; HDL, d 1.063–1.21 g/ml.

control subjects. Much of the increase in total plasma cholesterol and phospholipids was accounted for by a very pronounced increase in HDL lipid mass. Her plasma LCAT activity was 29 nmol CE formed/ml plasma per h which was significantly lower than control values (46.4 nmol CE formed/ml plasma per h) and in conjunction with a decreased substrate efficiency of her plasma lipoproteins most probably accounted for the increased ratio of free cholesterol to esterified cholesterol present in her plasma when compared to normal subjects. Analysis of the proband's lipoprotein lipid composition after isolation by density gradient ultracentrifugation is shown in **Table 2**. VLDL and LDL show a marked decrease in their weight % content of cholesteryl esters, whereas their triacylglycerol content is significantly elevated. The reverse pattern is observed within the HDL fraction where both the HDL_2 and HDL_3 subspecies have an elevated content of cholesteryl esters and a decreased triacylglycerol content when compared to normal subjects. Significantly, the relative content of HDL phospholipids is decreased, which indicates the presence of a higher proportion of larger HDL particles than normal. The distributions of

TABLE 2. Lipoprotein composition in CETP deficiency

Lipid	VLDL	LDL	HDL_2	HDL_3
		wt%		
Free cholesterol	9.3	14.4	10.4	6.1
Cholesteryl esters	1.8	37.2	40.2	45.7
Triglycerides	67.3	18.9	3.3	2.5
Phospholipids	21.6	29.5	46.1	45.6
CE/TG mass	0.027	1.97	12.34	18.43
C/PL mass	0.43	0.49	0.23	0.13

Lipoproteins were isolated by density gradient ultracentrifugation as described (22, 23) and the lipids were quantitated by gas chromatographic total lipid profiling (24).

plasma lipids and apolipoproteins in a control subject and the proband are shown in **Fig. 1**. The control subject exhibited a normal lipoprotein distribution (Fig. 1A) with VLDL present in fractions 1 and 2, (d < 1.019 g/ml) LDL in fractions 5–12 (d 1.024–1.063 g/ml) with the peak in fraction 7 (d 1.031 g/ml) coincident with the peak in apoB (Fig. 1B). The HDL lipid spanned fractions 12–24 (d 1.058–1.190 g/ml) with a peak in fraction 17 (d 1.107 g/ml), (Fig. 1A) coinciding with the peak in apoA-I (Fig. 1B). ApoE was detected in fractions 1–5 (d 1.006–1.026 g/ml) and 11–18 (d 1.050–1.121 g/ml) in the density range normally occupied by HDL_2. The lipoprotein distribution in the CETP-deficient proband's plasma (Fig. 1C) was markedly different from that of the control subject. Most notably, the HDL fraction was elevated and represented the predominant plasma lipoprotein species and the majority of the HDL was within the HDL_2 density range (fractions 10–17; d 1.044–1.112 g/ml). The apo-A-I peak had also shifted to lower density and was present in fraction 13 (d 1.068 g/ml) (Fig. 1D). Similarly, the peak in apoE had shifted from fraction 14/15 (d 1.072–1.091 g/ml) in the control subject (Fig. 1B) to fraction 11 (d 1.054 g/ml) in the CETP-deficient subject (Fig. 1D). These data are consistent with the previously reported hyperalphalipoproteinemia of CETP-deficient subjects and the presence of a higher proportion of large HDL_2-like particles than in control subjects. The molar ratios of cholesteryl esters to apoB for the apoB-rich lipoprotein fractions isolated by ultracentrifugation (Fig. 1) from our subject and a normal control female are shown in **Table 3**. This ratio for the VLDL and IDL fractions of the CETP-deficient subject was much lower than that observed for the normal female. However, LDL fractions from the CETP-deficient subject had cholesteryl ester to apoB molar ratios essentially equivalent to the control. These data suggest that CETP-deficiency has a marked effect upon the VLDL CE/apoB ratio but relatively little effect upon this ratio in LDL.

We next evaluated the ability of plasma from a control subject and the proband to catalyze the temperature-dependent reciprocal transfer of cholesteryl esters and triacylglycerols among lipoprotein species. Comparing the post-incubation values at 4°C with those obtained after incubation at 37°C, the control subject's VLDL showed the expected augmentation in esterified cholesterol content and a decrease in the triglyceride content with reciprocal changes in the LDL + HDL fraction. Significantly, there was no augmentation of VLDL total or esterified cholesterol or significant decrease in VLDL triacylglycerol mass when the proband's plasma was incubated at 37°C (data not shown). These data are consistent with an absence of CETP-

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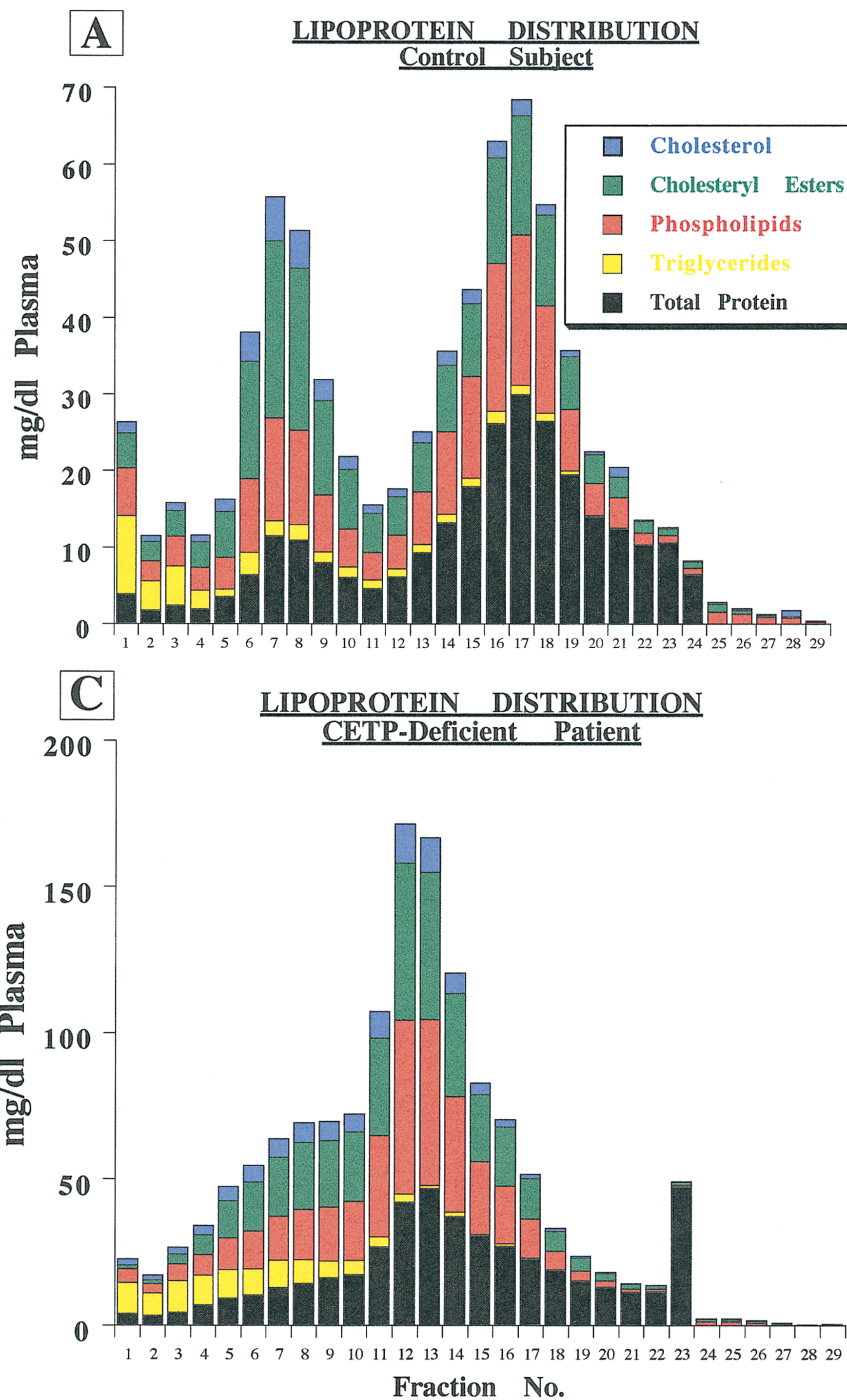

Fig. 1.

mediated reciprocal transfer of cholesteryl esters and triacylglycerols in the plasma of the proband.

In the absence of CETP-mediated lipid transfer it would be expected that VLDL would contain cholesteryl esters predominantly derived through esterification of cholesterol by hepatic ACAT, whereas the LDL and HDL, both of which are to varying degrees substrates for LCAT, would contain a significant proportion of LCAT-derived cholesteryl esters. Human plasma LCAT exhibits a preference for the transesterification

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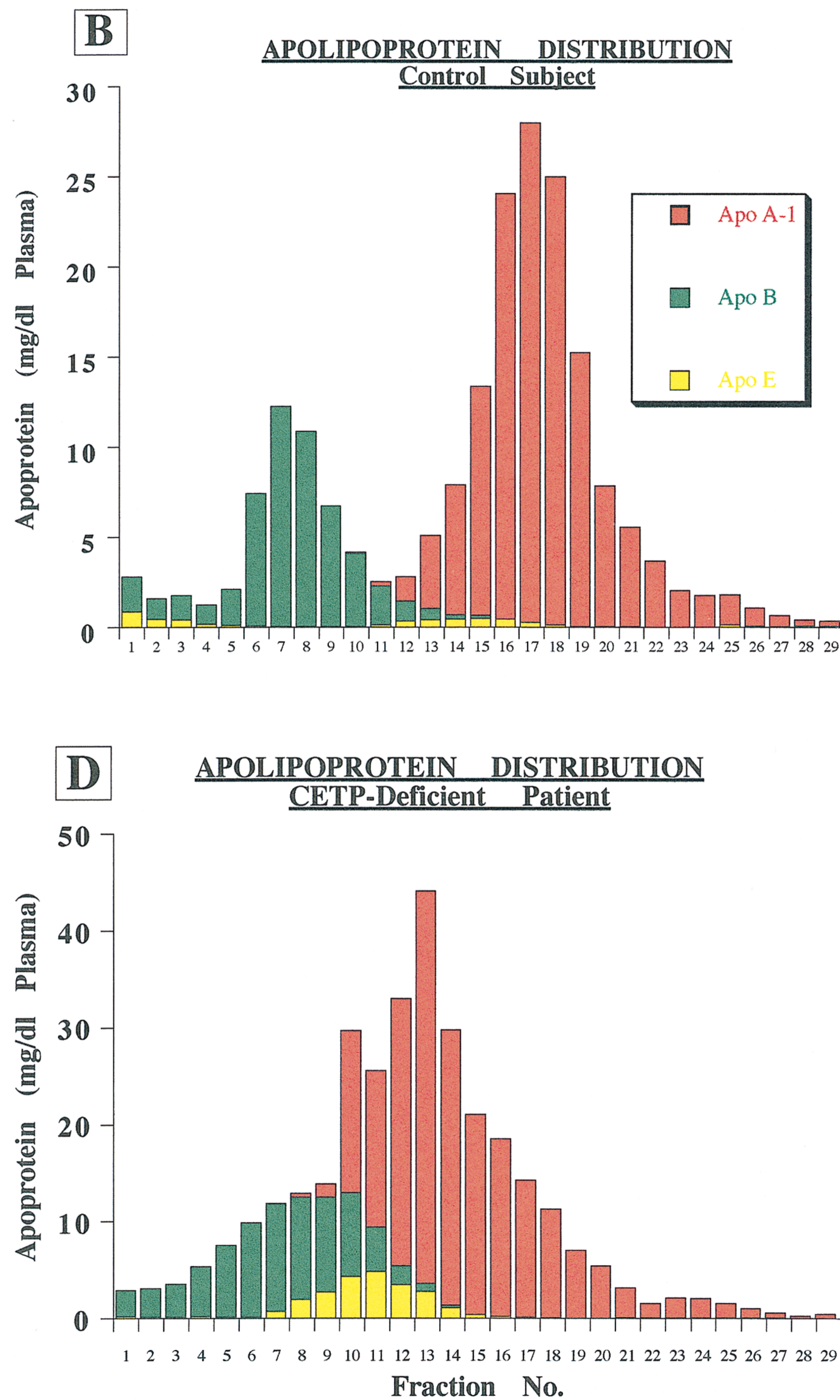

Fig. 1. Ultracentrifugal distributions of plasma lipids (A and C) and apolipoproteins (B and D) from a control female (A and B) and female CETP-deficient (C and D) subject. Ultracentrifugation, using a discontinuous density gradient, was performed as described (22, 23). Lipids were quantitated by gas chromatographic total lipid profiling (24), and apolipoproteins were quantitated by electroimmunoassay (26). Total protein was measured by a modified Lowry procedure (25).

of linoleic acid (C18:2) from the *sn*-2 position of phosphatidylcholine to cholesterol forming cholesteryl linoleate. Hepatic ACAT, in contrast, prefers to utilize the Co-A esters of stearic and oleic acids. Hence some indication of the origin of plasma cholesteryl esters can be obtained in the absence of exchange. **Table 4** shows

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analyses of the fatty acid compositions of the cholesteryl esters and phospholipids isolated from the major lipoprotein classes present in control plasmas and that of the proband. Owing to the pronounced shift in hydrated density of the HDL in the CETP-deficient subject, care was taken to exclude fractions containing significant amounts of apoA-I from the LDL fraction when preparing lipoprotein fractions for fatty acid analysis. Thus fractions 1 + 2 (d < 1.019 g.ml) (Fig. 1) constituted the VLDL, fractions 5–9 (d 1.023–1.044 g/ml) constituted the LDL fraction, and fractions 12–22 (d 1.058–1.167 g/ml) constituted the HDL fraction used for analyses in Table 4. Almost 50% of the cholesteryl ester present in the VLDL of the patient is cholesterol-C18:0, which is in marked contrast to the control subject VLDL where cholesterol-C18:2 predominates, followed by cholesterol-C18:l and cholesterol-C16:0. The cholesterol-C18:1/C18:2 ratio is thus markedly different in the patient's VLDL (2.36) when compared with the controls (0.58 ± 0.08). The fatty acid composition of the LDL and HDL from the control subjects was similar to that of the control VLDL as was the cholesterol-C18:1/C18:2 ratio. Inspection of the cholesteryl ester fatty acid composition in the LDL and HDL of the patient showed that cholesterol-C18:2 and cholesterol-C18:1 predominated as in the control LDL and HDL and a similar cholesterol-C18:1/C18:2 ratio was found; however, the proportion of cholesterol-C18:0 was significantly reduced in the patient's LDL and HDL when compared to her VLDL. The fatty acid compositions of the phospholipids present within the lipoproteins of the control subjects and the patient showed, with the exception of C18:2 and C20:4 in the patient's VLDL, a significant degree of similarity. The phospholipid fatty acid composition of the control VLDL, LDL, and HDL were not significantly different, the major species being C16:0, C18:2, C18:1, and C18:0. No significant difference between the patient's LDL and HDL phospholipid fatty acid composition and that of the corresponding control fractions was evident. The only significant differences noted between the phospholipid fatty acids of the patient and control VLDL were for C18:2 and C20:4.

In order to further evaluate whether the CETP protein was present in or absent from the plasma of the proband, we performed a radioimmunometric, double antibody sandwich assay utilizing monoclonal antibodies that had been previously shown to inhibit human plasma CETP activity. The recommended 1 in 40–50 dilution of plasma was utilized for three control plasmas whereas undiluted plasma from the proband was used in the assay. The values for CETP mass obtained for the control subjects was 1.45 to 3.26 mg/ml and fell within the previously reported normal range (29). No detectable CETP mass was observed in the proband's undiluted plasma indicating that the epitopes recognized by one or both of the monoclonal antibodies utilized in this assay were absent.

The data presented above provided strong evidence for the possibility that the proband had a genetic mutation resulting in either a lack of secretion of the CETP protein or the secretion of a dysfunctional protein lacking activity which was either not recognized by the monoclonal antibodies used in the radioimmunometric assay or was rapidly catabolized and removed from the plasma compartment.

The previously reported mutations in the human CETP gene are shown in **Table 5**. Of these the most common appear to be the G→A transition at intron 14(+1) which results in a splicing defect. The single A→G substitution in exon 15 resulting in 442Asp→Gly also appears relatively common in the Japanese population. In an attempt to rapidly identify the location of any mutation in the CETP gene of our proband, all 16 exons of her CETP gene including the intron/exon junctions were amplified by PCR and subjected to single strand conformational polymorphism analysis (SSCP). Upon no occasion, using three different concentrations of glycerol in the running gel, were differences in migration between PCR products derived from the proband's CETP gene and those of a control subject observed (data not shown). The lack of identification of a mutation using SSCP is not conclusive evidence of the lack of any mutation as this approach is only successful in approximately 80% of cases. We next screened for the presence of the known mutations listed in Table 5 by sequencing of the cloned PCR products of the relevant exons. The most common mutations, G→A transition at intron 14(+1) and the A→G substitution in exon 15 resulting in 442Asp→Gly were not present in our proband nor were any of the other previously reported mutations in exons 1, 6, 10, 12, or 14 (data not shown). All exons were then sequenced and a single C→T substitution was identified at position 836 in exon 9 which would result in 268Arg→Stop as shown in **Fig. 2**. Five clones from each of two PCR products of exon 9 from control subjects and the proband were sequenced and all showed the single C→T substitution at position 836. The entire coding region including the exon/intron junctions of the proband's CETP gene was sequenced twice and no additional mutations were identified. In order to evaluate whether the proband was homozygous or heterozygous for this new CETP gene mutation, direct sequencing of the PCR products from exon 9 was performed. The results, shown in **Fig. 3**, demonstrate the C→T substitution at position 836 when sequencing in the forward direction and the corresponding G→A substitution in

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TABLE 3. Molar proportions of cholesteryl esters and apoB in apoB-rich fractions from density gradient ultracentrifugation

Density Gradient Fraction	CE/ApoB Molar Ratio	
	CETP-Deficient Subject	Normal Female Subject
1 } VLDL	330	1825
2 } VLDL	307	1668
3	739	1920
4	1015	2479
5	1362	2353
6	1345	1636
7	1427	1489
8	1707	1535
9	1813	1448

Molar proportions of cholesteryl esters to apoB were calculated from the lipid and apolipoprotein analyses of the density gradient ultracentrifugal fractions of the CETP-deficient subject and the normal control female shown in Fig. 1. VLDL is present in fractions 1 and 2, IDL in fractions 3 and 4, and LDL in fractions 5–9. The major apoB-containing fraction was fraction 7 in both subjects.

the reverse sequence. The normal nucleotide (C or G) was not evident at position 836 of the proband's CETP gene; thus we conclude that the patient is homozygous for this mutation.

The C→T substitution at position 836 does not result in a loss or gain of a restriction fragment length polymorphism (RFLP) site; however, by use of a 26 bp mismatched reverse primer and an 18 bp forward primer, the 168 bp PCR product from the mutated DNA sequence contains an RFLP site for Mae III whereas the PCR product from the normal gene does not. **Figure 4** shows the results of applying this approach for the rapid screening of the novel exon 9 mutation. Mae III cleaves the 168 bp product from the mutated sequence into two fragments of 139 bp and 29 bp and no cleavage products are observed when the 168 bp PCR product is derived from the normal gene. These data also confirm that the proband is homozygous for the mutation as there was no evidence of an uncut 168 bp band when the PCR product from her gene was exposed to Mae III.

DISCUSSION

The proband investigated in this study is a 57-year-old Caucasian female without Japanese ancestry who presented with hypercholesterolemia later shown to be due to a marked hyperalphalipoproteinemia (Table 1). In concert with other reports of CETP-deficient subjects (4, 14, 18) her apoA-I levels were twice normal and her plasma apoE levels were 3-fold elevated. Her apoB levels, however, were only slightly reduced at 94 mg/dl (normal value 100 mg/dl) and her LDL-C was within the normal range when compared to controls.

TABLE 4. Lipoprotein cholesteryl ester and phospholipid fatty acid compositions

Fatty Acid	VLDL		LDL		HDL	
	Control	Patient	Control	Patient	Control	Patient
			wt%			
Cholesteryl ester fatty acids						
16:0	14.1 ± 2.0	19.4	12.7 ± 3.6	14.5	15.8 ± 1.4	17.3
16:1	3.1 ± 2.3	0.0	4.0 ± 0.8	4.0	5.6 ± 2.4	3.8
18:0	11.6 ± 8.8	59.8	3.9 ± 1.7	4.5	5.3 ± 1.9	2.0
18:1	19.3 ± 5.6	9.9	22.3 ± 4.6	21.7	27.9 ± 6.1	29.4
18:2	33.6 ± 7.4	4.2	41.7 ± 5.6	48.6	41.6 ± 4.4	43.0
18:3	8.1 ± 3.4	4.0	1.8 ± 0.5	3.1	3.8 ± 2.0	1.2
20:4	3.3 ± 1.6	2.7	2.8 ± 1.2	3.7	4.4 ± 0.5	3.2
18:1/18:2	0.58 ± 0.08	2.36	0.57 ± 0.06	0.45	0.68 ± 0.2	0.68
Phospholipid fatty acids						
16:0	37.2 ± 3.5	30.2	38.5 ± 1.8	35.4	38.6 ± 4.0	32.8
16:1	0.9 ± 1.1	0.0	1.9 ± 0.2	0.0	1.4 ± 0.9	1.7
18:0	17.1 ± 1.8	26.2	15.6 ± 1.0	15.8	15.5 ± 1.9	14.5
18:1	17.2 ± 2.4	15.5	17.2 ± 2.0	19.7	17.4 ± 2.7	20.5
18:2	21.2 ± 0.8	6.3	21.4 ± 1.8	21.9	20.7 ± 0.8	22.1
18:3	0.0 ± 0.0	0.0	0.2 ± 0.2	0.0	0.0 ± 0.0	0.0
20:4	6.3 ± 1.6	21.8	5.4 ± 1.5	7.2	6.5 ± 1.5	8.4
18:1/18:2	0.82 ± 0.13	2.47	0.83 ± 0.16	0.90	0.84 ± 0.14	0.93

Lipoprotein fractions were isolated from plasma by density gradient ultracentrifugation. Fractions 1 and 2 (d 1.019 g/ml) (Fig. 1) constituted the VLDL, fractions 5–9 (d 1.023–1.044 g/ml) constituted the LDL fraction, and fractions 12–22 (d 1.058–1.167 g/ml) constituted the HDL fraction and were used for analyses. Lipids were extracted from the lipoprotein fractions by the method of Folch, Lees, and Sloane Stanley (27), separated by thin-layer chromatography as described in Methods. The isolated cholesteryl esters and phospholipids were transmethylated (28) and analyzed by gas chromatography.

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TABLE 5. Mutation in the human plasma CETP gene

Mutation	Location	Type	Effect	Reference
ΔC (German)	exon 1	38-STOP (frameshift)	CETP Act. = 0	Funk et al. (16)
G→T (Japanese)	exon 6	181Gly→STOP	CETP Act. = 0	Arai et al. (14)
C→A (Japanese)	exon 10	309Gln→STOP	CETP Act. = 0	Gotoda et al. (13)
T→G (Japanese)	intron 10	splice donor site mutation	CETP Act. = 0	Sakai et al. (12)
G→C (German)	exon 12	373Ala→Pro	HDL ↗	Funke et al. (16)
G→A (Japanese)	intron 14	splice junction site mutation	CETP Act. = 0	Inazu et al. (4)
Insert T (Japanese)	intron 14	splice junction site mutation	CETP Act. = 0	Inazu et al. (11)
A→G (Japanese)	exon 15	442Asp→Gly	CETP Act. ↘	Inazu et al. (11)
G→A (German)	exon 15	451Arg→Gln	HDL ↗	Funke et al. (16)

Previously reported mutations in the human plasma CETP gene located on chromosome 16q21 and containing 16 exons and 15 introns coding for a 476 residue polypeptide (M_r 53,108 Da) with four N-linked glycosylation sites for a total M_r of 74,000 Da.

This is in contrast with other reports of Japanese subjects with this genetic defect (4, 9, 14, 19) where apoB levels of 60–70 mg/dl were observed in conjunction with LDL-C levels of 1.99 ± 0.8 mmol/l (4) and an enhanced LDL catabolic rate (19). To date we have not conducted LDL turnover studies in our proband. The apparently elevated apoB level in our patient does, however, raise the possibility that the observed hypercatabolism of LDL in the Japanese subjects may be polygenic in nature and not totally dependent upon the absence of plasma CETP activity. Inspection of the lipoprotein compositional data (Table 2) for the proband reveals a cholesteryl ester-poor, triacylglycerol-enriched VLDL and LDL and a cholesteryl ester-enriched, tria-

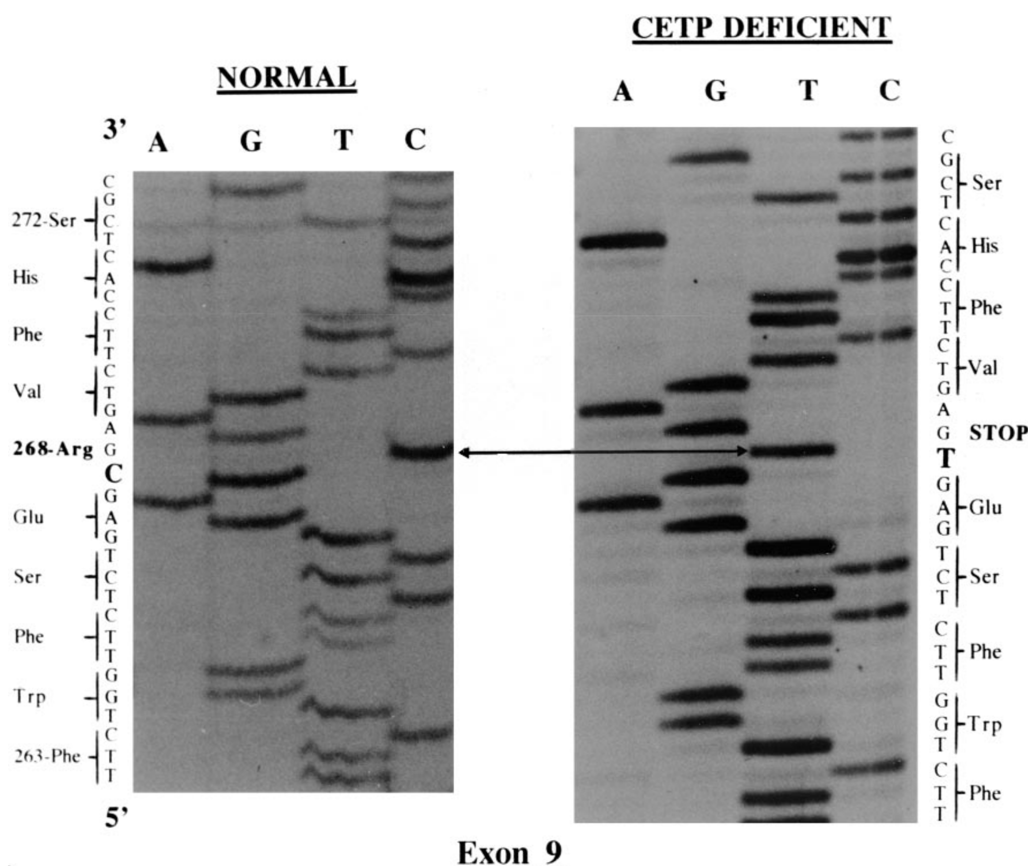

Fig. 2. DNA sequence analysis of the cloned PCR product of exon 9 from the CETP gene of a control subject and the proband. Sequencing was performed as described in Methods. A single C→T substitution at nucleotide position 836 resulting in the conversion of 268-Arg to a STOP codon is clearly observed. Five clones from each of two PCR products of exon 9 from control subjects and the proband were sequenced and all gave the above result.

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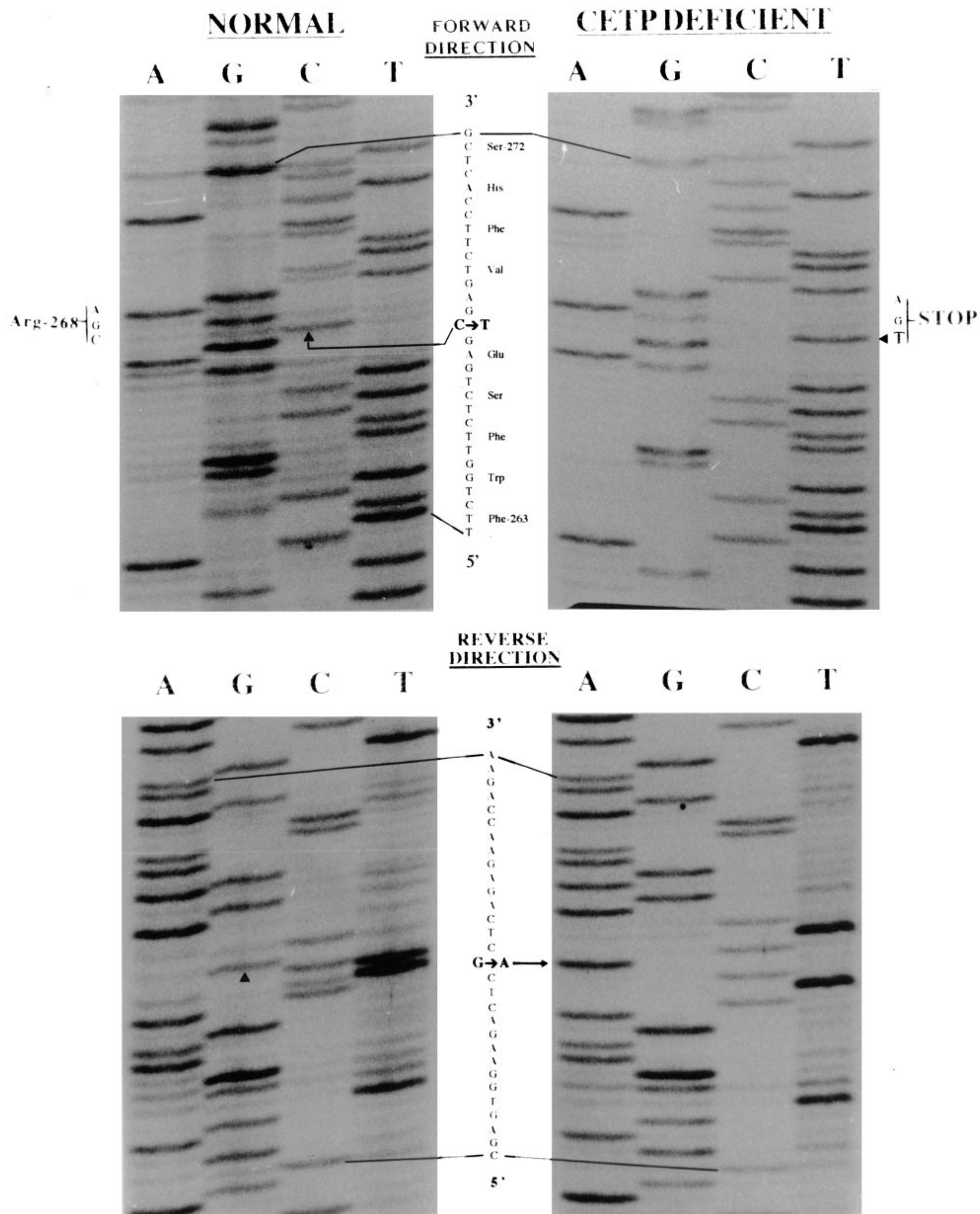

Fig. 3. Direct DNA sequencing of the PCR products of exon 9 of the CETP gene from a control subject and the CETP-deficient proband. After isolation of the PCR products they were directly sequenced as described in Methods. The top half of the figure shows the forward sequence and the C→T substitution at position 836. The reverse sequence in the lower half of the figure shows the corresponding G→A substitution in exon 9 from the CETP-deficient proband. No normal nucleotides (C in the forward or G in the reverse sequence) were observed at position 836 in exon 9 from the CETP-deficient proband indicating that this subject is homozygous for the single base substitution at this position.

cylglycerol-poor HDL when compared with control values. These data are indicative of the lack of reciprocal lipid transfer and are consistent with the previous findings (4, 9) in homozygous CETP-deficient subjects. Similarly, the aberrant lipoprotein and apolipoprotein ultracentrifuge profiles (Fig. 1) noted for our subject

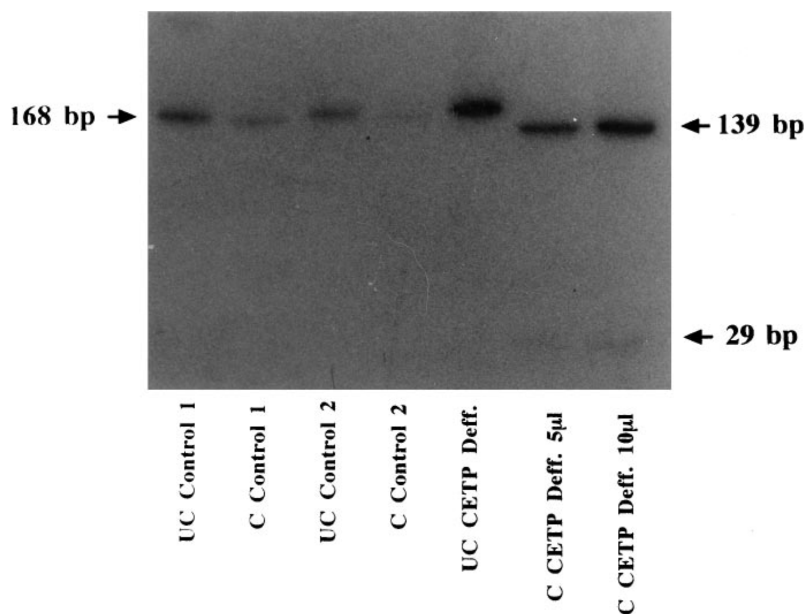

Fig. 4. Rapid detection of the novel exon 9 mutation using a mismatched reverse primer to generate a Mae III RFLP site specific for the mutated exon 9 sequence. The C→T substitution at position 836 does not result in the formation or loss of an RFLP site. A 26 bp, single mismatch reverse primer (see Methods) was therefore designed to generate a 168 bp PCR product containing a Mae III site when generated from the mutated DNA sequence of the proband. Mae III had no effect upon the PCR product generated from the control subject. Exposure of the PCR product from the proband's DNA to Mae III resulted in the complete digestion of the 168 bp fragment to form a 139 bp and a 29 bp fragment. The complete absence of a 168 bp fragment after digestion of the proband's PCR product confirms that this subject is homozygous for the mutation.

are similar to those previously reported for gel filtration of plasma from CETP-deficient homozygotes (9, 18).

Analysis of the fatty acid composition of the lipoprotein cholesteryl esters and phospholipids proved of interest (Table 4) in evaluating the possible origins of individual cholesteryl esters. Based upon similar analyses, and in particular the C18:1/C18:2 ratio of lipoprotein cholesteryl esters, Bisgaier et al. (18) have argued that the majority of CE in the VLDL and LDL of CETP-deficient subjects arises from the action of intracellular (presumably mostly hepatic) acyl-CoA:cholesterol acyl-

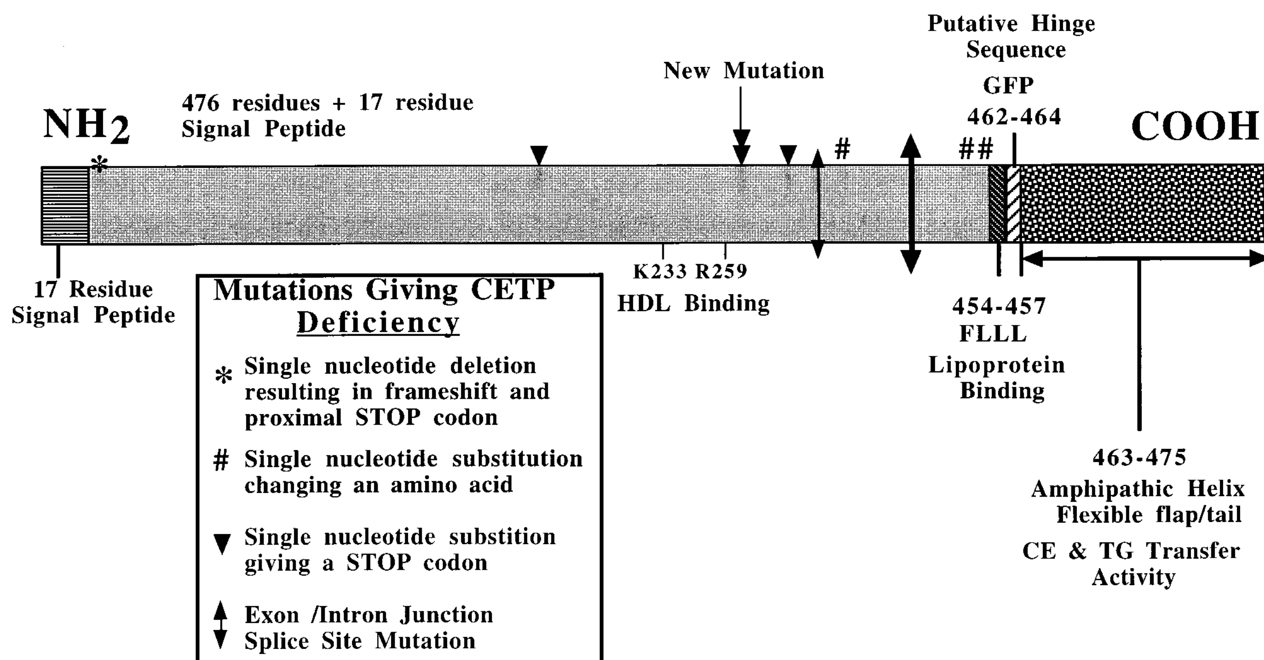

Fig. 5. Functional domains of the human plasma cholesteryl ester transfer protein (CETP). The type and relative positions of previously reported mutations are as indicated. The C→T substitution at nucleotide 836 in exon 9 resulting in 268Arg→STOP reported here is indicated by ▼.

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transferase (ACAT) and that LDL is utilized as an LCAT substrate only when the preferred substrate, HDL, is exhausted. The data presented in Table 4 only partially substantiate this hypothesis. The predominant CE fatty acids present in lipoproteins from control subjects are C18:2 > C18:1 > C16:0, their relative proportions are very similar, and there is no significant difference in the C18:1/C18:2 ratio between lipoprotein fractions. We have previously shown in vitro that plasma VLDL is qualitatively the best acceptor of HDL-CE, whereas LDL is quantitatively the major acceptor of HDL-CE in normolipidemic subjects and that the transfer of HDL-CE to the apoB-containing lipoproteins is proportional to their relative concentrations in plasma (23). However, in addition to catalyzing a net mass transfer of CE, CETP also mediates CE exchange (23) and, with time, a complete equilibration of CE species between lipoproteins would be expected and is indeed observed in Table 4. This equilibration is not as evident in the data reported by Bisgaier et al. (18), particularly with respect to C18:0 which ranged from 6.4 ± 0.4 to 19.7 ± 4.6% of CE fatty acid across the lipoprotein spectrum and approximated the lower value in the d 1.050–1.063 g/ml fraction. The predominant CE fatty acids in the VLDL of our CETP-deficient proband were C16:0 and C18:0 with all other fatty acids present at less than 10% of the total. In the absence of exchange and net mass transfer of CE from HDL, it would therefore appear that the cholesteryl esters of ACAT origin are cholesteryl palmitate and cholesteryl sterate. The CE fatty acid contents of the proband's LDL and HDL are similar to the control fractions but different from that of her VLDL in that the predominant fatty acids are C18:2 > C18:1 > C16:0 with a relatively minor contribution from C18:0 (2.0–4.5%). We suggest that this similarity between LDL and HDL in the proband is due to the action of plasma LCAT on both HDL and LDL.

The phospholipid fatty acid analysis reported in the lower half of Table 4 shows a great deal of similarity among all lipoprotein fractions from the control subjects with C16:0 < C18:2 > C18:1 > C18:0. Although significant differences in phospholipid molecular species among lipoprotein fractions have been noted in plasma samples that are rapidly analyzed after phlebotomy and also in human umbilical cord blood samples (34), neither situation is applicable to the analyses in Table 4 where all samples were from adults and were not analyzed immediately. A full equilibration of the phospholipid molecular species due to the action of the plasma phospholipid transfer proteins and CETP would therefore be expected, at least in the control subjects. The phospholipid fatty acid contents of the proband's LDL and HDL are very similar to those of the control fractions and given the more than adequate content of C18:1 and C18:2, which tend to occupy the *sn*-2 position in phosphatidylcholine and are preferred by LCAT, ample substrate would be available for the formation of cholesteryl oleate and linoleate by the action of LCAT upon the proband's LDL and HDL. The fatty acid composition of the phospholipids from the proband's VLDL is significantly different from that of her other lipoprotein fractions and that of control subjects in that the relative content of C18:2 is much reduced and that or C20:4 significantly elevated. While dietary factors may have influenced her VLDL phospholipid composition it is evident that a full equilibration of the phospholipid molecular species among the plasma lipoproteins in the CETP-deficient subject has not occurred. This may, in part, be due to the absence of functional CETP which is believed to catalyze up to 50% of all phospholipid transfer in human plasma (35).

The amount of CE synthesized and secreted by human liver remains an area of controversy as VLDL isolated from plasma has been modified by the action of CETP. The absence of CETP, however, should allow an estimate of the amount of CE secreted with VLDL. The proband has very low amounts of CE as VLDL lipid mass and the CE/apoB molar ratio in her VLDL and IDL fractions is considerably lower than in a normal female subject (Table 3). These data indicate that the amount of CE secreted with VLDL in this particular subject may be relatively small and the low VLDL CE/apoB ratio suggests that a considerable net CE transfer (approximately 1,500 moles of CE per particle) from HDL would have to occur to bring this particle to a composition comparable to that of plasma VLDL from a normal subject. It is of interest to note that the LDL fractions from the CETP-deficient subject have CE/apoB molar ratios similar to those of equivalent fractions from the normal subject. If the CETP-deficient VLDL were simply catabolized to LDL without transfer of CE from HDL, it would be anticipated that the CE/apoB ratio would remain at 300–400 rather than the observed 1700–1800. There are two possible explanations for the increase in the CE/apoB molar ratio in the LDL fraction from the CETP-deficient subject. First, there may be some contamination of the LDL in density gradient fractions 8 and 9 with apoE-HDL and apoA-I-HDL as these apoproteins are present in small amounts in these fractions (Fig. 1). Second, as discussed above, LCAT shows a significant activity with LDL as a substrate. The fatty acid composition of the LDL-CE from the CETP-deficient subject is consistent with the concept that a significant proportion of her LDL-CE is derived from the action of LCAT whereas the fatty acid composition of her VLDL-CE suggests that they may be derived from ACAT activity. Thus the data from this CETP-deficient subject are consistent

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with the concept that the CE content of nascent VLDL is low and that VLDL are the major recipient of CE transferred from HDL. The data also suggest that the action of LCAT upon LDL may be sufficient to generate a normal complement of LDL cholesteryl ester when CETP is absent from the plasma.

The molecular analysis of the proband's DNA by PCR and DNA sequencing has, we believe, clearly demonstrated the presence of a novel C→T substitution at position 836 in exon 9 of her CETP gene that results in the conversion of 268Arg into a Stop codon. The subject is homozygous for this single base substitution as evidenced by the bi-directional sequencing of the PCR products of exon 9 prior to cloning (Fig. 3) and also the complete cleavage of the 168 bp product of the single mismatch 26 bp reverse primer by Mae III (Fig. 4). No other mutation was found within the coding sequence or at any exon/intron junction of her gene. A schematic diagram of the CETP protein indicating its putative functional domains and the positions and types of previously reported mutations is shown in **Fig. 5**. With the aid of specific monoclonal antibodies (36) and site-directed mutagenesis (37) the lipid transfer domain of CETP has been localized to the C-terminal 12 amino acids. A putative hinge sequence (38), lipoprotein binding (39) and HDL binding sites (40) have also been identified towards the C-terminus of the protein. Clearly, any mutation such as the one reported here, which leads to a premature truncation of the CETP protein prior to these functional domains, would result in a dysfunctional, catalytically inactive protein. The monoclonal antibodies used in this study to detect plasma CETP were C-terminal directed and thus would not be expected to react with a CETP protein truncated at residue 268. This would explain our inability to detect CETP in the plasma of our proband. Currently we unaware whether this truncated CETP variant is expressed and present in the proband's plasma. We are presently attempting to evaluate the CETP mRNA levels in blood cells from this patient.

In order to rapidly screen subjects for the presence of this novel mutation we used a single mismatched 26 bp reverse primer that introduced a Mae III site after amplification of the mutated but not the normal exon 9 sequence (Fig. 4). We have, to date, screened DNA samples from 60 subjects from the same region of Nova Scotia as the proband and have not detected the mutation in any additional individuals.

In conclusion, we have identified a Nova Scotian patient with most of the phenotypic characteristics of familial CETP deficiency. Molecular analysis of her CETP gene revealed the presence of a new mutation resulting in the conversion of 268Arg→STOP which, if expressed, would result in a truncated CETP protein lacking the lipid transfer and lipoprotein binding domains. To rapidly screen for this mutation in the general population we have designed a single mismatch reverse PCR primer that generates a Mae III RFLP site when utilized to amplify the mutated sequence. The proband is homozygous for the mutation, is Caucasian lacking Japanese and ancestry, and is, we believe, the first such patient in North America reported to have CETP deficiency. The identification of Caucasian subjects with CETP deficiency will afford the opportunity to study the absence of CETP activity in a different (non-Japanese) genetic environment.

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