Organization of the Human Cholesteryl Ester Transfer Protein Gene[†,‡]

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ABSTRACT: The plasma cholesteryl ester transfer protein (CETP) catalyzes the transfer of phospholipids and neutral lipids between the lipoproteins. Thus, this protein may be important in modulating lipoprotein levels in the plasma. We have determined the primary structure and organization of the human CETP gene. Southern blotting of cellular DNA indicated a single copy of the CETP gene exists per haploid genome. Analysis of three overlapping genomic clones showed that the gene spans approximately 25 kbp and contains 16 exons (size range 32–250 bp). Overall, the sequence and organization of the CETP gene do not resemble those of other lipid-metabolizing enzymes or apolipoproteins. However, comparison of the CETP sequence, one exon at a time, with the sequences in the sequence databases revealed a striking identity of a pentapeptide sequence (ValLeuThrLeuAla) within the hydrophobic core of the signal sequences of human CETP, apolipoproteins A-IV and A-I, and lipoprotein lipase. This pentapeptide sequence was not found in the signal sequences of other proteins, suggesting that it may mediate a specialized function related to lipid metabolism or transport.

The transport and metabolism of lipids in the plasma require the interplay of a specialized set of proteins (Tall, 1986). One of these is cholesteryl ester transfer protein (CETP).[1] CETP mediates the exchange and net transfer of cholesteryl esters, triglycerides, and phospholipids between lipoproteins in plasma (Yen et al., 1989). Cholesterol, which has been esterified within high-density lipoproteins by lecithin–cholesterol acyltransferase, is transferred by CETP to very low density lipoproteins in exchange for triglycerides. This process is thought to be part of a mechanism, termed "reverse cholesterol transport", whereby excess cholesterol from peripheral tissues is returned to the liver for reutilization and catabolism (Tall & Small, 1980).

Human CETP was recently purified and characterized (Hesler et al., 1987; Jarnagin et al., 1987; Swenson et al., 1987). It exists in plasma as a hydrophobic glycoprotein of M_r 74 000. Removal of its N-linked sugar moieties reveals a polypeptide of M_r 59 000 (Swenson et al., 1987). Monoclonal antibodies to CETP have been developed and were used to map a region of functional significance in CETP (Hesler et al., 1988; Swenson et al., 1989). The primary structures of human CETP (Drayna et al., 1987a) and its rabbit counterpart (Nagashima et al., 1988) have been deduced through cDNA cloning. A preliminary comparison of the CETP sequence with those of other proteins did not show any structural similarities (Drayna et al., 1987a). In order to make a more detailed comparison with other genes and as an initial step to analyzing the genetic basis of CETP deficiency states, we have determined the structure and organization of the human CETP gene.

Experimental Procedures

Isolation and Characterization of Genomic Clones. Two human genomic libraries were screened: (i) human germ line DNA cloned in EMBL3 (Drayna et al., 1987b) and (ii) DNA from the cell line GM1202A cloned in Charon 30 (Wood et al., 1984). Screening was done by plaque hybridization using the human CETP cDNA probe (Drayna et al., 1987a) labeled with ^{32}P by random priming (Fienberg & Vogelstein, 1983). DNA from hybridizing clones was purified and then reprobed with the CETP cDNA or with an oligonucleotide directed to the region of the CETP gene that encodes the mature NH_2 terminus of the protein. Positive clones were further characterized by restriction enzyme mapping.

DNA Blot Analysis. DNA from blood of a healthy donor was purified according to the procedure described by Kunkel et al. (1977). Cloned and cellular DNA were digested with restriction enzymes and subjected to blot analysis using portions of the CETP cDNA as probes. Hybridization was carried out at 45 °C in a solution containing 0.75 M NaCl, 0.075 M trisodium citrate, 10× Denhardt's solution, 7% $NaDodSO_4$, and 100 $\mu g\ mL^{-1}$ sonicated salmon sperm DNA. After hybridization, filters were washed at 50 °C with four changes of a solution containing 0.015 M NaCl, 0.015 M trisodium citrate, and 0.1% $NaDodSO_4$ and then autoradiographed at −70 °C.

Sequence Determination. In order to generate a preliminary transcription map and to determine which restriction fragments of the genomic clones contained exons, 10 fragments (probes 1–10, 5′ to 3′) from the CETP cDNA were prepared. A 1588-bp fragment (containing 50 nt upstream of the translation initiation codon, the entire translated region, and 58 nt downstream of the termination codon) was isolated from a clone containing the 5′ region of the CETP gene (from a genomic clone fragment) fused to a derivative of the CETP cDNA clone. This fragment, which contained probes 1–9, was digested with *Pst*I to yield probes 8 and 9 and a fragment containing probes 1–7 (fragment 1–7). After purification,

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[1] Abbreviations: CETP, cholesteryl ester transfer protein; apoA-I, apolipoprotein A-I; apoA-IV, apolipoprotein A-IV; LPL, lipoprotein lipase; PCR, polymerase chain reaction; bp, base pairs; nt, nucleotides.

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fragment 1–7 was redigested with *Eco*RV, producing probes 1 and 7 and fragment 2–6. Fragment 2–6 was recovered and further digested with *Pvu*II, producing probes 2 and 3 and fragment 4–6. Finally, fragment 4–6 was digested with *Fok*I, producing probes 4, 5, and 6. Probe 10 [containing the sequence beginning 59 nt downstream from the termination codon and extending to the poly(A) tail of the cloned CETP cDNA] was isolated from the original CETP cDNA clone. Restriction fragments of the genomic clones that hybridized to these probes were subcloned into the Bluescript vectors (Stratagene) for sequence analysis. Phagemid DNA from the subclones was purified and then sequenced with Sequenase (United States Biochemicals). The position of introns within the gene was determined by aligning the cDNA and genomic sequences.

The CETP genomic sequences were compared with sequences deposited in sequence databases (GenBank, NBRF, EMBL, and Swiss-Prot databases) through BIONET by using the FASTA (Pearson & Lipman, 1988) and FASTDB programs.

Characterization of the 5′ Region of the CETP Gene. Portions of a human liver were obtained from the liver tissue procurement and distribution system (NIH NO1 DK62274). Liver RNA was extracted by the lithium chloride/urea method (Auffray & Rougeon, 1980). The poly(A)$^+$ fraction was isolated by chromatography on oligo(dT)–cellulose. To determine the structure of the 5′ region of the CETP mRNA, a novel procedure was developed. This method employs homopolymeric tailing of the first strand cDNA and amplification by polymerase chain reaction (PCR) (Saiki et al., 1988). Since short cDNA molecules are more efficiently synthesized, an oligonucleotide (primer-1, 5′-TGCAACCCA-TACTTGACTT-3′, complementary to the CETP mRNA beginning 208 nt downstream from the initiation codon) was used to prime cDNA synthesis. After purification, a homocytidylic tract (10–15 residues) was added to the 3′ end of the cDNA by using terminal transferase. This was followed by five cycles of PCR amplification using primer-1 and a G-tailed T7 promoter primer [5′-AATACGACTCACTATA$(G)_{12}$-3′]. The PCR product was then purified and subjected to an additional 25 cycles of amplification by using an untailed T7 promoter primer (5′-AATACGACTCACTATAG-3′) and primer-2 (5′-GGATCACCTTGGCAGTC-3′, complementary to the CETP mRNA 125 nt downstream from the initiation codon). These reactions employed an internal CETP primer (primer-2) and an untailed T7 promoter primer to increase the specificity of CETP sequence amplification and to avoid random priming in internal tracts of cytidylic residues. The product of this reaction was cloned into the KS+ Bluescript vector (Stratagene) for sequence analysis. PCR amplification requires a pair of specific primers flanking the region of interest. In this procedure the use of the T7 promoter sequence, opposite to one target-specific primer, satisfies this requirement and provides the option to make synthetic transcripts of PCR-amplified sequences in vitro.

Results and Discussion

CETP Is Encoded by a Single-Copy Gene. Earlier, Lusis et al. (1987) localized the human CETP locus to a region (q12–21) in the long arm of chromosome 16. However, Southern blot analysis of cellular DNA gives rise to a complex pattern of hybridization (Drayna & Lawn, 1987; Freeman et al., 1989), suggesting that the CETP gene either spans a large region in the genome or is present in more than one copy. To address this issue, cellular DNA was subjected to Southern blot analysis using probes representing the middle and 3′ regions (codons 199–253 and 383–476, respectively) of the

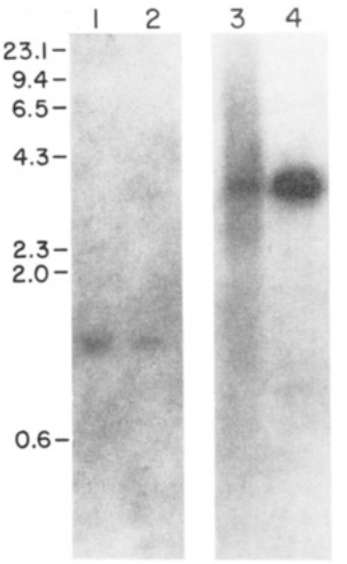

FIGURE 1: Southern blot analysis of DNA isolated from human blood and CETP genomic clones. DNA isolated from blood and cloned DNA were hybridized with ^{32}P-labeled fragments of the human CETP cDNA. Hybridization was carried out as described under Experimental Procedures. The autoradiogram is shown above. Lanes 1 and 3 contain cellular DNA. Lanes 2 and 4 contain cloned DNA. Lanes 1 and 2 show DNA cut with *Bam*HI and probed with a cDNA fragment spanning codons 199–253. Lanes 3 and 4 show DNA cut with *Bgl*II and *Hin*dIII and probed with a cDNA fragment spanning codons 383–476. Mobility of the size markers (λ *Hin*dIII fragments) is shown (in kbp) on the left.

CETP cDNA. As shown, cellular DNA digested with either *Bam*HI (Figure 1, lane 1) or *Bgl*II and *Hin*dIII (Figure 1, lane 3) yielded only single hybridizing fragments for each of the probes used. Moreover, these fragments comigrated with the predicted fragments of the cloned CETP gene digested with the same set of restriction enzymes (Figure 1, lanes 2 and 4). This simple hybridization pattern suggests that the CETP gene is present as a single copy, since more than one hybridizing fragment would be expected if multiple copies were present. In further support of this finding, PCR amplification of CETP exons, from several samples of human DNA, generated a single set of products.

Isolation of Genomic Clones and Determination of the CETP Gene Structure. Six clones were isolated from the two human genomic libraries by hybridization with the human CETP cDNA. Two of these clones also hybridized with an oligonucleotide probe directed toward the region of the CETP mRNA that encodes the mature NH_2 terminus, indicating that they contained the 5′ region of the CETP gene. Restriction enzyme mapping of these two clones confirmed that they contained the same region of cloned genomic DNA. Out of the six original isolates, three clones (designated λCG7, λCG5, and λCG2) were found to contain overlapping fragments and together spanned some 30 kbp of the contiguous DNA sequence (Figure 2). Blot analysis of cloned DNA using the 10 fragments (probes 1–10) derived from the CETP cDNA indicated that the gene spans ~25 kbp (Figure 2). Approximately 42% of the CETP gene (i.e., 10.5 kbp) was sequenced, representing the complete sequences of all exons, all exon–intron boundaries, and flanking regions (compiled sequences were submitted to the nucleic acid sequence databases). Introns 1, 3–7, 12, 13, and 15 were also sequenced completely. The remaining introns were not sequenced completely due to their large size.

The organization of the human CETP gene is depicted at the bottom of Figure 2. The coding domain of the gene is

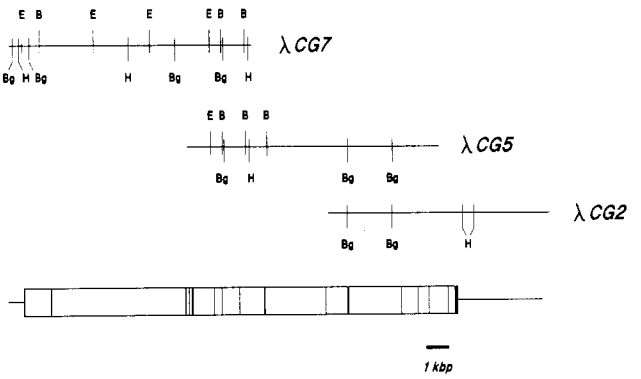

FIGURE 2: Restriction maps of CETP genomic clones and organization of the human CETP gene. Genomic clones were characterized by restriction mapping. Three clones, designated λCG7, λCG5, and λCG2, were found to contain overlapping genomic fragments. Shown below the restriction maps is the genomic organization of the CETP gene. Perpendicular lines in the bottom diagram represent exons. Open boxes represent introns. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; E, *Eco*RI. The scale bar represents 1 kbp.

comprised of 16 exons interrupted by 15 introns of variable size. The size range for exons is 32 bp (exon 13) to 250 bp (exon 16), whereas for introns, it is 87 bp (intron 3) to ~6000 bp (intron 2). The exons account for only 8% of the total gene sequence. By comparison, the exons in the gene that encodes the largest of the apolipoproteins (apolipoprotein B) account for over 30% of the total gene sequence (Cladaras et al., 1986; Ludwig et al., 1987). Thus, the CETP gene organization is relatively complex, compared to the genes that encode the apolipoproteins and other lipid-metabolizing enzymes. The sequences of the exon–intron junctions are shown in Figure 3. As shown, the splice signals in the CETP gene all conform to consensus sequences (Mount, 1982).

The coding regions of the CETP gene and the CETP cDNA sequence published previously (Drayna et al., 1987a) differ by only one residue. This change is located at the first position of codon 405 and results in a conservative substitution in the translated protein: whereas the codon specified by the gene (GUC) encodes a valine residue, the codon in the CETP cDNA (AUC) encodes an isoleucine. This minor difference may reflect allelic variation of CETP in the general population. An uncommonly used polyadenylation signal (5′-ATTAAA-3′) was found at the 3′ end of the CETP cDNA (Drayna et al., 1987a). The same signal was found in the distal end of exon 16 of the CETP gene, indicating that the variant signal is encoded by the gene.

Characterization of the 5′ Region of the CETP Gene. Mapping of the transcription initiation site was first attempted by primer extension experiments. However, results from these experiments using four oligonucleotide primers were inconclusive, probably due to the low abundance of the CETP mRNA. In order to obtain the full-length structure of the CETP mRNA, we developed a novel method to clone the 5′ region of the CETP cDNA (see Experimental Procedures). Similar approaches were reported while this work was in progress (Frohman et al., 1988; Ohara et al., 1989). This procedure yielded a single product of ~180 bp after a total of 30 cycles of amplification. Direct sequencing of PCR products and also sequence analysis of the cloned derivatives showed that they were derived from the CETP mRNA. The sequences were identical with the corresponding sequences of genomic clones up to a point 27 nt upstream from the translation initiation codon (codon +1 in Figure 4), where the synthetic homopolymeric tract was found. Thus, we have provisionally assigned the transcription initiation site of the

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                    40
Exon  1 (144 bp) GTG T   gtaagt  Intron  1 (  902 bp)  ctccctctag  TT GAA
                 Val L                                             eu Asn

                    96
Exon  2 (115 bp) CAC AA  gtgagt  Intron  2 (~6000 bp)  tccttcccag   C ATC
                 His As                                            n Ile

                   123
Exon  3 (135 bp) TGG TG  gtaagc  Intron  3 (   87 bp)  ctctttccag   G CTG
                 Trp Tr                                            p Leu

                   147
Exon  4 ( 71 bp) CTG A   gtatgt  Intron  4 (  229 bp)  ctccctgcag  CC TGT
                 Leu T                                             hr Cys

                   158
Exon  5 (188 bp) CGA GA  gtaagt  Intron  5 ( 1022 bp)  ctgccttcag   G CCT
                 Arg Gl                                            u Pro

                   199
Exon  6 ( 70 bp) GGA CAG gtgagt  Intron  6 (  217 bp)  tctgccccag ATC TGC
                 Gly Gln                                          Ile Cys

                   220
Exon  7 ( 61 bp) GCT G   gtgagt  Intron  7 (  607 bp)  cctcctgcag  CC AGC
                 Ala A                                             la Ser

                   250
Exon  8 ( 92 bp) CAC AAG gtagga  Intron  8 ( ~900 bp)  cttcctccag GGT CAT
                 His Lys                                          Gly His

                   310
Exon  9 (180 bp) TTC AAG gtgagt  Intron  9 (~3000 bp)  ttcttttcag GCA GTG
                 Phe Lys                                          Ala Val

                   327
Exon 10 ( 51 bp) CAA GAG gtaact  Intron 10 (~1000 bp)  tcttccacag GTT GTC
                 Gln Glu                                          Val Val

                   383
Exon 11 (165 bp) GAA GAG gtgagg  Intron 11 (~2900 bp)  ctctccccag GAT ATC
                 Glu Glu                                          Asp Ile

                   423
Exon 12 ( 70 bp) TTC CA  gtatgt  Intron 12 (  327 bp)  tttatttcag   G ATT
                 Phe Gl                                            n Ile

                   416
Exon 13 ( 32 bp) ACT GAG gtaggt  Intron 13 (  482 bp)  ttgattgcag AGC AGC
                 Thr Glu                                          Ser Ser

                   441
Exon 14 ( 73 bp) TCT C   gtaagt  Intron 14 ( ~950 bp)  cctcgcccag  GG CTC
                 Ser A                                             rg Leu

                   469
Exon 15 ( 86 bp) CGA GAT gtgagt  Intron 15 (  180 bp)  ctcccttcag GGC TTC
                 Arg Asp                                          Gly Phe

Exon 16 (250 bp) CAACTCCTCCCTATCCTAAAGGCCCACTGGCATTAAAGTGCTGTATCC
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FIGURE 3: Exon–intron junction sequences of the human CETP gene. Nucleotide sequences of the exon and intron boundaries of the human CETP gene are shown. The numbered codons (+1, initiation codon ATG) are either preceding or interrupted by introns. Exon and intron sequences are shown in upper and lower case, respectively. The amino acids encoded by the gene are shown below the nucleotide sequences. A variant polyadenylation signal, shown underlined, is found at the distal end of exon 16.

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-360 tgtctttttc tcatagtcat tgtattttgg cctctttcta tttatggcaa cagagagaga

-300 aagcttattc ctagatatat gtatttaagt aaaaataaat gaattcatgg aaacatatta

-240 agcaattatc cagataacat aagggatggc aaaaatggtg cagatggtgg aggggagaca

-180 agtagaagtt ggggtgctctt gttgaatgt ctggctctga actctagagg aggccgcagg

-120 ggctgggcag gaaggaggtga atctctggg gccaggaaga ccctgctgcc cggaagagcc

 -60 tcatgttccg tgggggctggg cggacatac atatacgggc tccaggctga acggctcggg

  +1 CCACTTACAC ACCACTGCCTG ATAACC ATG CTG GCT GCC ACA GTC CTG ACC
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FIGURE 4: 5′ flanking region of the human CETP gene. The nucleotide sequence of the 5′ flanking region of the CETP gene is shown in lower case. A partial sequence of exon 1 (untranslated region and part of the translated region) is shown in upper case. The codons are shown as triplets (codon +1 assigned to the first ATG). Sequences in the upstream region resembling an SP1 binding site and a TATA box are shown underlined.

CETP gene to a position 27 nt upstream from the translation initiation codon. In support of this assignment, sequences resembling a "TATA" box and an SP1 binding site were found immediately upstream of this site (Figure 4). A search for sequences resembling other consensus promoter elements in

A. Amino acid sequence

CETP	Met Leu Ala Ala Thr Val Leu Thr Leu Ala Leu Leu Gly Asn Ala His Ala
apoA-IV	Met Phe Leu Lys Ala Val Val Leu Thr Leu Ala Leu Val Ala Val Ala
apoA-I	Met Lys Ala Ala Val Leu Thr Leu Ala Val Leu Phe Leu Thr
LPL	Met Glu Ser Lys Ala Leu Leu Val Leu Thr Leu Ala Val Trp Leu Glu Ser Leu Thr

B. Nucleic acid sequence

CETP	ATG CTG GCT GCC ACA GTC CTG ACC CTG GCC CTG CTG GGC AAT GCC CAT GCC
apoA-IV	ATG TTC CTG AAG GCC GTG GTC CTG ACC CTG GCC CTG GTG GCT GTC GCC
apoA-I	ATG AAA GCT GCG GTG CTG ACC TTG GCC GTG CTC TTC CTG ACG
LPL	ATG GAG AGC AAA GCC CTG CTC GTG CTG ACT CTG GCC GTG TGG CTC CAG AGT

FIGURE 5: Primary sequence similarities among the prepeptides of CETP and other proteins involved in lipid metabolism and transport. The primary structures of human CETP apoA-IV (Elshourbagy et al., 1987), apoA-I (Makrides et al., 1988), and LPL (Wion et al., 1987) were compared. Sequence similarity was found only at the prepeptide regions. Shown above are the complete amino acid sequence of the human CETP prepeptide and partial amino acid sequences of apoA-IV, apoA-I, and LPL prepeptides (A). The nucleic acid sequences that encode them are also shown (B). Residues in common with CETP are underlined. Note the core pentapeptide sequence (ValLeuThrLeuAla) common to all four prepeptide sequences.

the 5′ flanking region did not reveal any additional similarities.

Comparison of CETP to Other Lipid-Metabolizing Enzymes and Apolipoproteins. The structures of the genes that encode apolipoproteins A-I, A-II, A-IV, B, D, C-I, C-II, C-III, and E have been determined [reviewed by Li et al. (1988)]. Of these, the genes that encode A-I, A-II, A-IV, C-I, C-II, C-III, and E bear remarkable resemblance to each other (Li et al., 1988). These genes have similar genomic organization ("3-intron/4-exon" structure except for the variant apoA-IV gene), have related sequences, and contain internally repeated domains. It has been postulated that these genes have common ancestry and evolved by duplication of an ancient prototype gene. Comparison of the genes that encode all the apolipoproteins, lecithin–cholesterol acyltransferase (McLean et al., 1986), and lipoprotein lipase (Deeb & Peng, 1989) to the CETP gene shows the CETP gene organization to be distinct. In addition, the promoter regions of these genes do not appear to be homologous to that of the CETP gene (Figure 4). Thus, the CETP gene structure probably evolved independently of these genes. An earlier search of the sequence databases did not reveal the existence of genes or proteins similar to CETP (Drayna et al., 1987a). However, in a recent report a low level of overall similarity (22% amino acid sequence identity, but no stretches of identity exceeding three amino acids) between CETP and human neutrophil bactericidal protein was described (Gray et al., 1989), suggesting a possible common evolutionary origin.

In the present study, a computer search of the sequence databases was repeated one exon at a time, under the assumption that exons encode functionally distinct protein domains (Gilbert, 1978). This approach revealed an interesting similarity between human CETP and apoA-IV, apoA-I, and LPL (Figure 5). The region of identity is confined to the prepeptide region of these proteins and contains the pentapeptide sequence ValLeuThrLeuAla (VLTLA).

The existence of a common sequence in the signal peptides of CETP, apoA-IV, apoA-I, and LPL is surprising. Earlier alignments of the apolipoprotein (A-I, A-II, B, C-I, C-II, C-III, and E) signal sequences have also demonstrated significant primary structure similarity (Li et al., 1988; Gordon et al., 1984). However, this similarity is not particularly striking since the sequences are conserved throughout the protein, reflecting common ancestry. In contrast, the similarity shared by CETP, LPL, and the apolipoproteins (A-IV and A-I) is limited to the prepeptide sequences. Furthermore, the sequences are conserved not only at the protein level (Figure 5A) but also at the nucleic acid level (Figure 5B).

One explanation for this finding is the evolution of the protein sequence toward a common function. However, signal sequences of secreted proteins, despite a common function in protein targeting and translocation, generally do not show primary sequence similarity (Gierasch, 1989). An exhaustive search of the sequence databases using the core pentapeptide sequence (VLTLA) revealed that it was not present in other signal sequences apart from those of CETP, apoA-IV, apoA-I, and LPL. The probability of generating a common pentapeptide sequence, and the corresponding nucleic acid sequence, at the same position (i.e., in the hydrophobic core of the prepeptide) of CETP, apoA-IV, apoA-I, and LPL by chance during the evolution of these proteins seems low.

It seems more likely that the identical pentapeptide sequence in these signal peptides arose by convergent evolution, in fulfillment of a common function peculiar to certain proteins involved in lipid metabolism. Exactly what this function might be is an enigma. Presumably, it is somehow involved in translation and/or translocation of these proteins, since this is the general function of signal peptides. One possibility is that a special sequence is needed to guide these hydrophobic proteins through the translocation–translation machinery or to assemble them into lipoproteins in the endoplasmic reticulum. However, another intriguing possibility is that the similar nucleic or amino acid sequence is involved in translational regulation in response to changes in cellular metabolism. In this regard, it is of interest to note that some evidence has been provided showing translational regulation of apolipoproteins (A-I and A-IV but not E) (Go et al., 1988) and LPL (Ong & Kern, 1989) in response to changes in lipid or carbohydrate metabolism, respectively.

The knowledge of the normal CETP gene structure can now be used as a basis for comparative studies with other CETP genes in cases where abnormal CETP function is observed. For example, we have recently determined the genetic basis of CETP deficiency in one Japanese kindred (Brown et al., 1989). The proband of this kindred has a splice mutation characterized by a G → A change at position +1 of the splice donor of intron 14. Given the relatively large size and complexity of the CETP gene, it seems likely that a variety of mutations that result in CETP deficiency will be found.

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