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Structure of the Human Pancreatic Cholesterol Esterase Gene

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ABSTRACT: The gene for human pancreatic cholesterol esterase consists of 11 exons and 10 introns and is 9.2 kb in length. The last and longest exon (841 nucleotides) is unique to the human gene. Functional amino acids are encoded on separate exons. The leader sequence is encoded by a single exon which carries two additional N-terminal amino acids of the mature functional protein. A positive TATA element is identified 43 nucleotides from the start codon. Pulse-field gel electrophoresis and hybridization with various cDNA probes and direct sequence data revealed the existence of a CEase-like gene. Partial sequence analysis of this gene from a human cosmid library and human genomic DNA showed a premature stop signal in exon 10, shortly after the codon for the active-site histidine. Both the functional gene and the CEase-like gene have a polyadenylation signal in the 3′-untranslated region. Thus, the complex gene structure for this intestinally active enzyme may provide in part a potential molecular explanation for the well-known heterogeneity of the intestinal absorption of cholesterol.

Pancreatic cholesterol esterase (CEase)[1] (EC 3.1.1.13) promotes the intestinal absorption of cholesterol esters by catalyzing hydrolysis of the ester bond, an obligatory step in the uptake of this form of dietary sterol (Vahouny & Treadwell, 1964). Recent studies have shown that a serine residue and a histidine residue play a key role in the hydrolytic mechanism (Kissel et al., 1989; DiPersio et al., 1991). Moreover, Bosner et al. (1988) described a heparin binding site on pancreatic CEase that serves to bind this enzyme to the absorptive membrane of the enterocyte to facilitate cholesterol absorption. Kyger et al. (1989) reported the first cDNA cloning of the enzyme, and genomic cloning of rat

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[1] Abbreviation: CEase, cholesterol esterase.

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pancreatic CEase followed soon thereafter (Fontaine et al., 1991).

Despite these advances, understanding of the human pancreatic enzyme has been slower to emerge. Recently, cDNA cloning of this enzyme was reported (Nilsson et al., 1990), and it was demonstrated that unlike any other mammalian CEase that has been studied, the human protein is much larger because of a C-terminal tail containing repetitive proline-rich units (Hui & Kissel, 1990; Nilsson et al., 1990). Because of the established heterogeneity of cholesterol absorption (McNamara, 1982), and because of the striking difference between the human enzyme and that from rat, cow, and pig, we now report the complete gene structure of human pancreatic CEase. Consistent with the unique C-terminus of the human enzyme, the final exon is much larger than that of the rat gene. Additionally, a CEase-like gene was identified that may code for a shorter but functional enzyme, the significance of which remains under study.

Materials and Methods

[γ-^{32}P]dATP and [α-^{32}P]dCTP were purchased from Amersham Radio-Chemical Co. Primers for sequencing the gene were obtained either from the Washington University Protein Facility Laboratory at St. Louis or from the Midland Certified Reagent Co., Midland, TX. Restriction enzymes were supplied by Promega Biotechnologies; nitrocellulose membranes used for hybridization were from MSI Micron Separations Inc., Westboro, MA. For construction of cDNA libraries, a kit was obtained from Bethesda Research Labs, Grand Island, NY. Random primer labeling kits were obtained from Boehringer Mannheim, Germany, while sequencing and pulse-field gel equipment from Bio-Rad, Melville, NY, were used. Agarose LE from Midwest Scientific was used for running gels. Hybridization buffers were prepared as described by Sambrook et al. (1989). Direct gel hybridization photoradiographs were developed after 24 h using X-OMAT film supplied by Kodak.

A cDNA library in a λ gt11 vector was made using mRNA from human pancreas, and the library was screened with a random primer labeled bovine CEase clone (Kyger et al., 1989). Since the human cDNA clone showed sequence identity to human milk bile salt stimulated lipase (Nilsson et al., 1990), it was used to screen two different human cosmid libraries, PTCF (Grosveld et al., 1982) and pWE 15 (Wachl et al., 1987). Cosmid DNA from the positive clones was prepared (Liszewiski et al., 1989) and mapped by digestion with various restriction endonucleases. Oligonucleotides corresponding to various cDNA regions were used both as probes and as sequencing primers. Intron–exon splice junctions were established by double-stranded DNA sequencing using Sanger's dideoxy sequencing method (Sanger et al., 1977). Pulse-field gel electrophoresis was performed by restricting the DNA from 5×10^6 SK Hep 1 cells in a low-melt agarose plug (Poustka et al., 1987), which was then subjected to pulse-field gel electrophoresis for 14 h at 198 V at 4 °C using ramp times between 1:4 and 5:20.

Results and Discussion

Identification of Genomic Clones for Human Pancreatic CEase. To identify the human pancreatic CEase gene and to determine the sequence of its intron and exon splice junctions, we used two different cosmid libraries, pWE 15 and PTCF, which were screened with the [α-^{32}P]dCTP-labeled cDNA sequence of human and (or) bovine pancreatic CEase cloned in our laboratory (Kyger et al., 1989). Cosmid genomic DNAs were digested by *Eco*RI and *Bam*HI, subjected to Southern blot analysis, and hybridized to probes recognizing

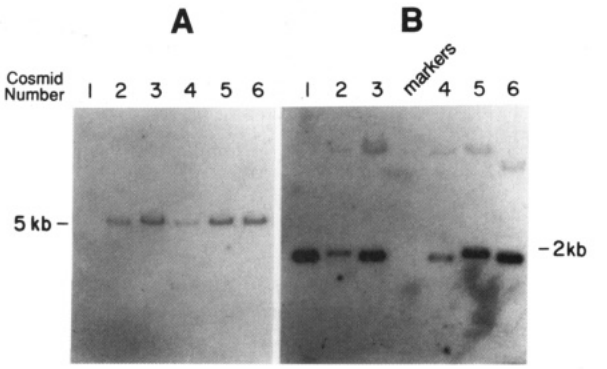

FIGURE 1: Hybridization pattern of restriction fragments of six genomic CEase clones. Six positive cosmid clones obtained from PTCF and pWE 15 human genomic libraries were digested with *Eco*RI and *Bam*HI; the DNA was Southern-blotted and hybridized to end-labeled probes from the 5′ end (A) and 3′ end (B) of the human pancreatic cDNA for CEase. Panel A shows that the 5′-end probe hybridized to a 5-kb *Bam*HI fragment. Panel B shows that the 3′-end probe hybridizes to a 2-kb *Eco*RI fragment.

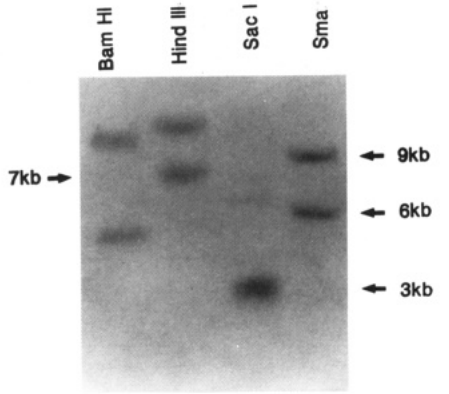

FIGURE 2: Restriction fragment analysis of cosmid pW-15-2. Cosmid 6 of Figure 1 (pW-15-2) which showed hybridization to both the 5′ and 3′ ends of the cDNA was further restricted with *Bam*HI, *Hin*dIII, *Sac*I, and *Sma*I, then Southern-blotted, and hybridized with a 30-mer (see the text) corresponding to a sequence from exon 11 and represented in the human pancreatic cDNA. Double band patterns were obtained for *Bam*HI, *Hin*dIII, and *Sma*I, suggesting gene duplication. Only a single band can be observed for *Sac*I, suggesting the preservation of *Sac*I sites in both genes in that region.

either the 5′ (5′-GGGCGCCGTGTACACAGA) or the 3′ (5′-CCTCTTGATACCAAGGCTC) end of the cDNA (Figure 1). The results show that, except for cosmid 1 which hybridized only to the 3′ end of the cDNA, the remaining five cosmids hybridized to both the 3′ and 5′ ends of the cDNA, suggesting that these five carry the full complement of the gene (Figure 1).

Separation of the CEase Gene from a CEase-like Gene. Cosmid 6 (pW-15-2) was analyzed further. Figure 2 shows the hybridization pattern of pW-15-2 digested with various restriction enzymes and probed with the central portion of the cDNA sequence (5′-TACCGGCCCAAGACAGGACA-GTCTCTCTAA). *Bam*HI, *Hin*dIII, and *Sma*I digests of the cosmid DNA showed a double-band pattern while a single 3-kb *Sac*I fragment was found. Of these bands, a 7-kb *Hin*dIII and a 9-kb *Sma*I fragment carried almost the entire functional CEase gene, while the 6-kb *Sma*I fragment carried a partial sequence of another CEase-like gene (see below). The *Sma*I and *Hin*dIII fragments were subcloned into pUC 19 and subjected to structural analysis. pW-15-2 yielded another 3-kb *Sma*I fragment, which when hybridized with a probe (5′-ACCGAGATTGCCCTAGCC) that is 170 nucleotides upstream from the first completed the total CEase gene.

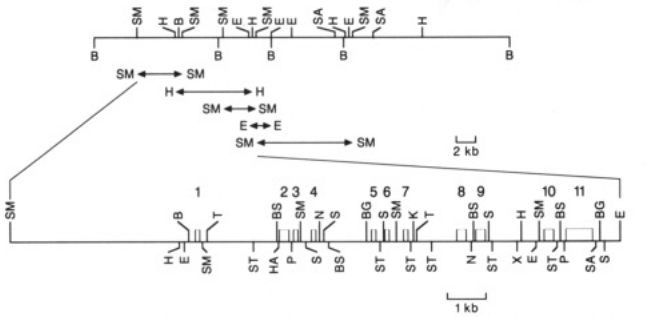

FIGURE 3: Structure of the human pancreatic CEase gene. The overlapping fragments that carry the functional gene of CEase were identified by Southern blot hybridization of cosmid DNA after restriction enzyme digestion. The specified fragments were subcloned either into pUC 19 or into M13 Mp18 and then subjected to sequencing. The intron–exon junctions were established by using forward and reverse primers chosen from various regions of the cDNA. (B = *Bam*HI; BG = *Bgl*I; BS = *Bst*EI; E = *Eco*RI; H = *Hin*dIII; N = *Nci*I; P = *Pst*I; S = *Sty*I; SA = *Sac*I; SM = *Sma*I; ST = *Stu*I; X = *Xba*I; T = *Taq*I; HA = *Hae*III; K = *Kpn*I.)

Structure of the Human Pancreatic CEase Gene. A restriction map of the CEase gene is shown in Figure 3. Essentially the whole gene is present in the 7-kb *Hin*dIII fragment which ends in intron 9 and a 9-kb *Sma*I fragment obtained from the same cosmid which begins in intron 9, with an overlap contributed by a 3-kb *Sma*I fragment. The 5′-upstream region is carried by a 4.6-kb *Sma*I fragment that ends at the beginning of first intron. The 3′ end of the human gene representing exons 10 and 11 is carried by a 2-kb *Eco*RI fragment which, due to its GC-rich content, was subcloned into M13 MP18 and subjected to single-stranded sequencing. The total size of the gene on the basis of these sequencing studies is 9.2 kb, with 11 exons and 10 introns present.

The first and smallest exon, which comprises the 5′-flanking and upstream sequence, is only 85 nucleotides long and codes for the signal peptide plus the first 2 N-terminal amino acids of the functional protein. This is in contrast to most secretory proteins whose signal peptides are coded alone by single exons (Early et al., 1980; Scheele & Jacoby, 1983; Walhaus & Hogness, 1983, Liscun et al., 1985), including the genes for trypsin (Bell et al., 1984), chymotrypsin (Craik et al., 1983), elastase (Swift et al., 1984), and pancreatic lipase (Mickel et al., 1989). Analysis of the other segments of the CEase gene showed that exons 2, 4, 6, 8, and 9 vary in size from 150 nucleotides to 204 nucleotides and contain no known functional sites. The third exon is 123 nucleotides long and has the tentative consensus heparin binding site "KKRCLQ" present in animal and human CEases alike (Martin et al., 1987; Cardin & Weinstraub, 1989). The fifth exon which is 131 nucleotides long has the active-site serine sequence "GXSXG" that is common to lipolytic enzymes (Kirchgessner et al., 1987), while exon 10 (198 nucleotides long) contains the charge-relay histidine necessary for some of the hydrolytic activities of the enzyme (DiPersio et al., 1991). Exon 11 is the largest of the exons with 841 nucleotides and encodes that part of the protein which is absent in the nonhuman pancreatic CEases. The functional gene has the polyadenylation signal AATAAA at the end of this last exon. In each case, the exonic nucleotide sequence is consistent with the reported cDNA sequences (Nilsson et al., 1990).

The intron–exon boundaries of human pancreatic cholesterol esterase were determined with bidirectional sequencing (Table I). They are similar to that of the rat gene except for exons 7 and 8, and they conform to the GT-AG intron–exon splice sequence rule (Breathnach et al., 1978). The intron sizes are less than 1 kb except for 1, 4, 7, and 9. In the case of the CEase gene, the exonic nucleotide at the donor site is a "G" except at the end of exons 8, 9, and 10, where it is T, A, and A, respectively. The nucleotide sequences in the exons at the acceptor site vary as shown in Table I. 5′ clones obtained from three different libraries show variations in upstream nucleotide sequences; despite these changes, all of them showed a putative TATA promoter element. In summary, the human pancreatic CEase gene consists of 11 exons, is 9.2 kb in length, and is principally different than other human genes reported thus far.

Table I: Intron–Exon Splice Junctions of the Human Pancreatic CEase Gene[a]

Exon Number	Donor Sequence	Acceptor Sequence	Exon Size (bp)	Intron Size (Kb)
5′ UPSTREAM	ATAATACGACTCACTATAGGGATCCAGCTCCATAAATACCCGAGGCCCAGGGGGAGGGCCA			
1		* CCCAGAGGCTGATGCTCACCATG	85	
2	TGCCGCG AAG GT AAGAGCCC..... t a caagt	CCCTCGC AG CTGGGCGCCG a xa t t t	151	1.9
3	GGCTGGCAA G GT GGGAG TGG... t c	CA CCTGC AG GG ACCCTGA ctg xx a	123	0.083
4	AGGAAGCAA G GT CTGCCTCCC..... ga t	CCATCTC AG TC TCCCGGG c t g t at	198	0.275
5	AATCTGCCA G GT GCCTGGGTA.... c t atg ag	CCTGCCCC AG GT AACTATG a t t	131	1.3
6	TCTCTCTG CAG GT CTCGGATC.... gaggc	GGGTCCCGT AG ACCCTCTCC ttggtt c	108	0.171
7	GGGCCAAA AAG GT AAACAGC..... c g g ca	CTGCCCCC AG GTGGCTGAG tt t a c a	118	0.146
8	GGCCTGGAG T GT GAGTAGCTG... a a x t agttg	TCTCCCCCC AG AG CCCATG a t t	187	1.2
9	AGTCACC GA GT AGGCAGGGGG... cacaga g x tgct t	ATTCCCCC AG G GAGGACTT g aggg x xxx	204	0.184
10	CCAATGCC AA GT GAGGATCT..... c a t	CTCCCTCC AG G AGTGCCA t ca	198	1.4
11	CCAAAACA GG GT AAGAGCTGGG.... g gt tg t	ACTCTGC AG G GACCCCA	841	0.330

[a] The underlines and spaces at the donor and acceptor sites denote the amino acid at which splicing occurs. Overlined represents the typical TATA element. The double underline is the protein start codon. Asterisk = RNA start site; lower case letters = rat gene exon–intron splice-junction sequence difference; x = deletion.

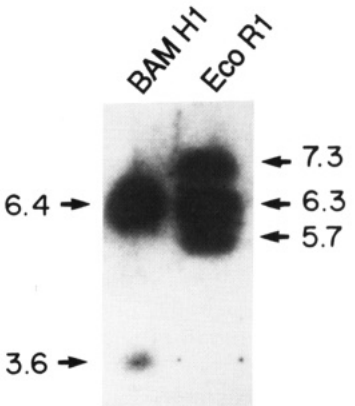

FIGURE 4: Pulse-field gel electrophoresis of genomic DNA from SK-Hep-I cells (ATCC HTB 52). Low-melt agarose plugs with 5 × 10^6 cells were prepared in 0.5% agarose and digested with 100 units of the specified restriction enzyme. Pulse-field electrophoresis was done with ramp times between 1:4 and 5:20, 198 V at 4 °C for 14 h. *Bst*EII and *Hin*dIII digests of λ DNA and *Hae*III digest of φX174 DNA were used as molecular weight markers. After electrophoresis, DNA fragments were transferred to a nylon membrane and hybridized to random primer labeled human CEase cDNA. Membrane was washed with 0.2× SSC in 0.1% SDS at 65 °C and exposed to Kodak X-O-mat film for 1 week.

CEase-like Gene. Pulse-field gel electrophoresis of total cellular DNA from SK-Hep-I (ATCC HTB 52) cells digested with *Bam*HI and *Eco*RI and hybridized with human CEase cDNA (Figure 4) showed the gene size to be 10 and 19.3 kb, respectively, suggesting gene duplication. This was further confirmed by sequence analysis of a 6-kb *Sma*I fragment

Table II: Comparison of Nucleotide and Amino Acid Changes in CEase and CEase-like Genes in Exon 10[a]

CAT	GCA	GAT	GAC	ATT	CAG	TAC	GTT	TTC	GGG	AAG	CCC	TTC	GCC	ACC	CCC
H	A	D	D	I	Q	Y	V	F	G	K	P	F	A	T	P
cat	aga	gat	gag	atc	cag	tac	atc	ttt	tgg	gag	ccc	ttt	gcc	acc	cca
h	a	d	e	i	q	y	t	f	w	e	p	f	a	t	p
l															
ACG	GGC	TAC	CGG	CCC	CAA	GAC	AGG	ACA	GTC	TCT	AAG				
T	G	Y	R	P	Q	D	R	T	V	S	K				
ctc	cgg	gct	acc	gcc	cca	aga	cag	gac	agt	ctc	taa				
l	r	a	t	a	p	r	q	d	s	l	Stop				

[a] Upper case letters represent the CEase gene. Lower case letters represent the CEase-like gene. The arrow indicates the position at which a frame shift occurs in the CEase gene by the introduction of TCC and a loss of "C". The first amino acid in the table is the putative active histidine (DiPersio et al., 1991). No change in the nucleotide sequence prior to this amino acid in exon 11 was observed. Downstream from the stop codon there are three base substitutions and one base deletion.

which demonstrated the presence of a CEase-like gene (data not shown). Partial sequence and restriction analysis reveals similarity to that of the native gene. A notable difference is the presence of a premature termination codon part way through the 10th exon (Table II). This unique site occurs 78 nucleotides past the putative active-site histidine (DiPersio et al., 1991), and since this gene contains all the components of the charge-relay system, it may encode an active enzyme of shorter length than the mature 100-kDa human pancreatic CEase (Kyger et al., 1990). The existence of the CEase-like gene is further supported by sequence analysis of exon 10 in genomic DNA from six individuals, all of whom showed the premature stop codon.

The translational initiation site of rat pancreatic CEase (Hui et al., 1990) and bile salt-stimulated milk lipase cDNA (Nilsson et al., 1990) sequences have been ascribed to the first ATG. This site is not the same as that described by Reue et al. (1991) for human pancreatic carboxyl ester lipase cDNA which contains two in-frame methionine codons upstream of the amino-terminal peptide sequence. The second ATG most likely represents the translational start site since the sequence around it as described here (TCACCATGG) is very similar to the concensus sequence for translation initiation (CCA/GCCATGG) described by Kozak et al. (1987).

Our primer extension studies (Figure 5) are in agreement with the results reported by Reue et al. (1991) for the 5′-untranslated portion of the human pancreatic CEase cDNA. With the exception of three cytosines at its 5′ end, this region is identical to that of human BSSL (Hui et al., 1990). However, this region is different from that reported by Nilsson et al. (1990). Both the human and rat CEases and the human BSSL (Hui et al., 1990) have short 5′-untranslated sequences (17–20 nucleotides) which are common to many mRNAs encoding other pancreatic secretory proteins, such as amylase (MacDonald et al., 1982a), trypsin (MacDonald et al., 1982b), elastases I and II (MacDonald et al., 1982c), chymotrypsin (Bell et al., 1984), and carboxypeptidase A (Quinto et al., 1982). The TATA box elements in the human gene reported here are identical to those for the rat pancreatic CEase gene (ATAAATA; Fontaine et al., 1991), and their localization with respect to the initiation ATG codon is virtually the same, −43 and −42, respectively.

We present the structure of the total human pancreatic CEase gene which is comprised of 11 exons and 10 introns. In keeping with the reported molecular weight (Bosner et al., 1988) and cDNA site (Nilsson et al., 1990) for human CEase, the coding sequence for this protein is approximately 420 nucleotides longer than that for the rat or bovine cDNA (Kyger et al., 1989). Further, a CEase-like gene exists in the human genome and is located in close proximity 3′ to the full-length CEase gene (data not shown).

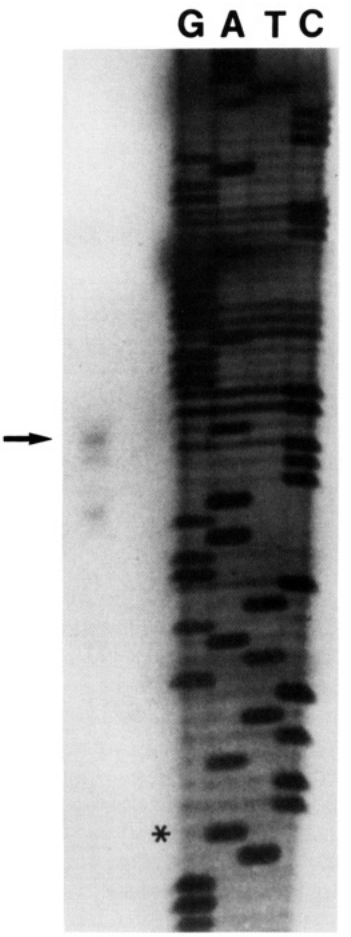

FIGURE 5: Primer extension of CEase mRNA. 5×10^3 cpm of a γ-^{32}P-labeled reverse oligonucleotide (5′-GCCCAGCAGCAGGTGAGG-3′) 26 nucleotides downstream of ATG was hybridized with 10 μg of human pancreatic total RNA at 65 °C. Primer extension was initiated by the addition of 2 μL of dNTP mix and 2 μL of AMV reverse transcriptase (Promega). The reaction mix was incubated at 37 °C for 1 h. The product was loaded onto a DNA sequencing gel along with the sequencing ladder obtained by the same oligomer with a 4.6-kb *Sma*I fragment carrying the 5′ sequence. Upon electrophoresis, the gel was subjected to autoradiography. The arrow indicates the end of the RNA (see text). The asterisk indicates the RNA cap site.

Heterogeneity in cholesterol absorption (Kesaniemi et al., 1986; Miettinen et al., 1989; Tilvis et al., 1986) may be under polygenic control similar to the variations seen in plasma cholesterol metabolism (Kesaniemi et al., 1987). Thus, small structural changes in catalytic regions of pancreatic CEase, such as the consensus heparin binding site or the charge-relay system, could produce profound changes in intestinal cholesterol uptake. Identification of a CEase-like gene adds complexity to this possibility that CEase plays a role in the heterogeneity of cholesterol absorption.

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Registry No. CEase, 9026-00-0.

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