Glycosylation-Dependent Activity of Baculovirus-Expressed Human Liver Carboxylesterases: cDNA Cloning and Characterization of Two Highly Similar Enzyme Forms‡

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ABSTRACT: A cDNA, designated hCE, encoding the entire sequence of a carboxylesterase, was isolated from a human liver λgt11 library. The hCE-deduced protein sequence contained 568 amino acids, including an 18 amino acid signal peptide sequence, and had a calculated molecular mass of the mature protein of 60 609 Da. A second cDNA, designated hCE_v, was isolated from the same λgt11 library and contained a 3-bp deletion resulting in the loss of the final amino acid in the signal peptide sequence (Ala_{-1}) and a second 3-bp deltion leading to an in-frame loss of Gln_{345}. Expression of mRNA corresponding to both hCE and hCE_v was detected in eight adult human liver samples, with individual levels varying 5-fold (hCE) and 12-fold (hCE_v). A single immunoreactive protein was detected in 13 adult human liver samples when probed with antibody directed against a rat carboxylesterase. Based on allele-specific oligonucleotide hybridizations, we believe that the hCE and hCE_v cDNAs represent two distinct members of the carboxylesterase family. The carboxylesterase genes were localized to human chromosome 16 using a somatic cell hybrid mapping strategy. Baculovirus expression of hCE in Sf9 cells produced a protein with an estimated molecular mass of 59 000 Da. This enzyme was able to hydrolyze aromatic and aliphatic esters but possessed no catalytic activity toward amides or a fatty acyl CoA ester. Baculovirus-mediated expression of the hCE_v cDNA yielded a second protein of 56 000 Da resulting from inefficient N-glycosylation of the hCE_v protein. Although the substrate specificity for the hCE_v protein was identical to that of expressed hCE for any given substrate, the specific activity for the hCE protein was always higher than that for the hCE_v protein. Tunicamycin inhibition studies provided the first evidence that N-glycosylation of these luminal enzymes is essential for maximal catalytic activity.

The nonspecific serine esterase family is comprised of many distinct esterases characterized by broad substrate specificity and differential patterns of inhibition by organophosphates (Heymann, 1980). Carboxylesterases are members of the serine esterase family and are widely distributed in different tissues with the highest activity found in hepatic microsomal fractions (Satoh, 1987). The hydrolysis of carboxyl ester, amide, and thioester bonds in a variety of drugs and environmental chemicals by microsomal carboxylesterases is generally considered a detoxification reaction which results in loss of biological activity. However, hydrolysis of certain aromatic amides, herbicides, and insecticides may lead to increased toxicity (Heymann, 1980, 1982; Satoh, 1987). The generation of biologically active hydrolytic metabolites has been exploited in the design of ester and amide prodrugs with improved absorption, bioavailability, and duration of action (Heymann, 1982; Leinweber, 1987; Svensson & Tunek, 1988). Roles for carboxylesterases in the storage and mobilization of physiological as well as xenobiotic esters and in the regulation of such events as hormone activation and deactivation are suggested by their ability to hydrolyze medium-chain mono- and diacylglycerols, fatty acyl esters of carnitine and coenzyme A, and retinyl esters (Leinweber, 1987). Egasyn is a 64-kDa luminal endoplasmic reticulum glycoprotein identified in rats and mice as a member of the serine esterase family of enzymes. In addition to its function as a hydrolytic enzyme, a portion of the egasyn protein binds to β-glucuronidase via its carboxylesterase active site, resulting in sequestration of bound β-glucuronidase within the endoplasmic reticulum of tissues that express this form of esterase (Medda et al., 1986, 1987; Ovnic et al., 1991a). Carboxylesterases have also been implicated in the immunotoxicity of halothane, following the identification of a 59-kDa carboxylesterase as one of the target proteins covalently modified by the reactive trifluoroacetyl metabolite of halothane (Satoh et al., 1989).

Although they overlap in their substrate specificities, carboxylesterase isozymes can be classified on the basis of their biochemical, immunological, and electrophoretic properties. Isozymic forms have been identified in experimental animals (Mentlein et al., 1980; Hosokawa et al., 1987; Korza & Ozols, 1988; Ozols, 1989) and humans (Ketterman et al., 1989). However, an understanding of the tissue distribution, regulation, and physiological significance of these enzymes has been hindered by the experimental limitations associated with their broad overlapping substrate and inhibitor selectivities and biochemical properties. Molecular approaches have therefore been undertaken for studying the structure and function of carboxylesterases. Several cDNAs encoding rat and mouse liver isozymes have been isolated (Long et al.,

‡ The nucleotide sequences reported in this manuscript have been submitted to GenBank and given the accession numbers L07764 and L07765.

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1988; Takagi et al., 1988; Robbi et al., 1990; Ovnic et al., 1991a,b), and this approach has recently been extended to studies of human liver carboxylesterases. Two very similar partial cDNAs have been reported for human liver (Long et al., 1991; Riddles et al., 1991), both of which are missing the 5′-coding regions of the protein. A serine esterase from human alveolar macrophages has also recently been cloned and identified as a member of the liver microsomal carboxylesterase family (Munger et al., 1991).

In the present report, we describe the isolation of two distinct full-length human liver carboxylesterase cDNAs and their expression in human liver samples. The human carboxylesterase gene was localized to chromosome 16. Baculovirus was used to functionally express the proteins encoded by the two cDNAs, both of which exhibited glycosylation-dependent hydrolytic activity toward simple aromatic and aliphatic esters.

EXPERIMENTAL PROCEDURES

Materials. Human liver samples were obtained from organ transplant donors and have been described previously (Yamano et al., 1989). Baculovirus transfer vector pAc373 was kindly provided by Dr. Max Summers at Texas A&M University. Sf9[1] cells and wild-type baculovirus were from Invitrogen, San Diego, CA. The DyeDeoxy Terminator cycling kit was from Applied Biosystems and Taq polymerase from Perkin-Elmer Cetus. Isocarboxazide was kindly provided by Hoffmann-LaRoche, Nutley, NJ, and malathion was a gift from Cheminova, Lemvig, Denmark.

Cloning and Sequencing of Human Liver Carboxylesterases. A λgt11 cDNA library, designated K19, previously constructed from human liver mRNA (Yamano et al., 1990), was screened by plaque hybridization using a 230-bp *Bam*HI fragment from the 5′-region of a partial human liver carboxylesterase cDNA (Long et al., 1991). The largest cDNA inserts obtained from library screening were subcloned into pUC9 and then sequenced using the DyeDeoxy terminator cycling kit with Taq polymerase and the Applied Biosystem 370A DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Each cDNA base was sequenced at least twice in each direction, and sequence data were aligned using the Beckman Microgenie Program.

Chromosomal Localization of the Carboxylesterase Gene Locus. The somatic cell hybrid strategy was used to determine the chromosome location of the human carboxylesterase. Isolation and characterization of the human–mouse and human–hamster cell lines have been described previously (McBride et al., 1982a,b,c). These cell lines were characterized by karyotypic analysis and electrophoretic analysis of human biochemical markers. The carboxylesterase genes were localized by Southern blotting and hybridization of DNA using the carboxylesterase cDNA as a probe and conditions described previously (McBride et al., 1986).

Protein and mRNA Analysis. Human liver microsomes were prepared as described by Aoyama et al. (1989). Microsomes (40 μg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970) followed by Western immunoblotting (Towbin et al., 1979) using rabbit polyclonal antibodies against rat liver carboxylesterase (Satoh et al., 1989). Western blots were incubated with a 500-fold dilution of rabbit anti-carboxylesterase serum followed by alkaline phosphatase-conjugated goat anti-rabbit IgG and stained using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase substrate system.

[1] Abbreviation: Sf9, *Spodoptera frugiperda.*

Table I: Primers and Probes for Genomic Amplification and Allele-Specific Hybridization of Carboxylesterases[a]

primer	5′	3′
DEL1	2 TGCACAGAGACCTCGCAGGCCCCG 25	161 ACGAACTTCCCCAGCACTTTG 141
DEL2	1058 CTGAAAGGAATTTCCACACTG 1078	1202 GACTTCCACAGGAGTGACATG 1182

probe	deletion 1	deletion 2
WT	88 GCGGCTTGGGCAGGGCATCCG 108	1124 TTCCAATGCAGTTGATGAGC 1143
VAR	109 CGCGGCTTGGGGGCATCCGTC 129	1141 GATTCCAATGTTGATGAGC 1159

[a] The oligonucleotides are shown 5′ to 3′. Numbers correspond to the nucleotide positions in hCE and hCE_v. Primers DEL1 and DEL2 were used for amplification of human genomic DNA samples using the polymerase chain reaction. DNA samples amplified with the DEL1 primers were subsequently hybridized with the wild-type (WT) and variant (VAR) probes for deletion 1 and DNA samples amplified with the DEL2 primers were hybridized with the WT and VAR probes for deletion 2.

The carboxylesterase cDNA inserts were subcloned into the *Eco*RI site of the vector pGEM-3Z for use in RNase protection assays. Plasmids were linearized with *Bal*I, and RNA transcripts were generated from nucleotide 1597 through the remaining 3′-end of the cDNA using T7 RNA polymerase. Removal of the 1715-bp *Pst*I fragment and religation yielded a second construct containing 257 bp of the 5′-region of the carboxylesterase cDNA. This plasmid was linearized with *Eco*RI and antisense RNA transcribed from the SP6 promoter. Antisense RNA transcripts of human β-actin (Ponte et al., 1984) were used as a control.

RNA probes were prepared with the Riboprobe transcription system using [α-^{32}P]CTP essentially as described (Gilman, 1989). The specific activity of the carboxylesterase probes was designed to be 4-fold higher than that of the actin probe to account for the difference in abundance of their respective mRNAs. Total RNA was isolated as described by Chirgwin et al. (1979), except cesium trifluoroacetic acid was used in place of cesium chloride. Labeled carboxylesterase (4×10^5 cpm) and actin (1×10^5 cpm) transcripts were hybridized with 10 μg of total RNA, digested with ribonucleases A and T_1, and electrophoresed on a 6% acrylamide/8 M urea gel, followed by autoradiography. Autoradiograms were scanned with a computing densitometer, and the intensity of the protected fragments was analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The amount of carboxylesterase mRNA is expressed relative to the amount of β-actin mRNA.

Allele-Specific Oligonucleotide Hybridization. Sequence differences between the carboxylesterase cDNAs were investigated using allele-specific PCR. Oligonucleotides were synthesized on an Applied Biosystem 380B DNA synthesizer and purified by Centricon-10 filtration (Amicon Corporation) for amplification of the region surrounding base pairs 97–99 (deletion 1) and 1131–1133 (deletion 2) of the hCE cDNA. The specific locations and composition of the primers are listed in Table I. All PCR reactions were carried out in a volume of 100 μL containing 10 mM Tris-HCl, pH 8.3, 1 mM $MgCl_2$, 50 mM KCl, 100 μg/mL gelatin, 200 μM each dATP, dCTP, dGTP, and dTTP, 100 pmol of each primer, 2 units of Taq DNA polymerase, and human genomic DNA as template. DNA was initially denatured at 94 °C for 5 min before addition of Taq polymerase. Reactions were carried out for 30 cycles on a Perkin-Elmer Cetus DNA thermal cycler at an annealing temperature of 60 °C for 1 min, a polymerization temperature of 72 °C for 30 s, and a heat denaturation temperature of 94

°C for 1 min. An aliquot (5 μL) from each completed reaction was subjected to electrophoresis in a mixture of 3% NuSieve agarose and 1% standard agarose with ethidium bromide staining for detection of amplified products. The remaining reaction mixture was prepared according to published procedures (Kogan & Gitschier, 1990) and loaded as two aliquots onto a nylon filter (Nytran, Schleicher and Schuell, Keene, NH) in a slot blot apparatus. Hybridization with ^{32}P-labeled oligonucleotide probes was at 30 °C, and filters were washed at T_m −5 °C. Probes specific for wild-type allele and both possible 3-bp deletions were included in the analysis, as described in Table I.

Expression of Carboxylesterase using Baculovirus. Recombinant baculovirus was constructed essentially as described previously (Summers & Smith, 1987; Gonzalez et al., 1991). Briefly, *Eco*RI inserts of the carboxylesterase cDNA were removed from pUC9 and made blunt-ended with the Klenow fragment of DNA polymerase I. The transfer vector pAc373 was digested with *Bam*HI, made blunt-ended, and treated with calf intestinal alkaline phosphatase prior to ligation with the carboxylesterase insert. The pAc373 plasmid containing the carboxylesterase cDNA and wild-type baculovirus DNA were cotransfected into *Spodoptera frugiperda* (Sf9) cells by calcium phosphate precipitation. Recombinant viruses were selected and purified by four rounds of plaque purification using a ^{32}P-labeled carboxylesterase cDNA probe. Following amplification and determination of the viral titer, recombinant virus was used to infect Sf9 cells at a multiplicity of infection of two plaque-forming units per cell. Cells were routinely harvested 72 h following infection and washed twice with PBS, and cell pellets were stored at −70 °C until further analysis. In some instances tunicamycin was added to the culture media during the expression period, as indicated in the figure legends.

Analysis of Baculovirus-Expressed Carboxylesterase. Cell pellets were resuspended in PBS and lysates prepared by sonication. Protein concentrations were determined using the Pierce BCA assay. Expression of carboxylesterase was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970). In some instances, Western immunoblotting was carried out using antiserum against a rat carboxylesterase (Satoh et al., 1989) as described above. Endoglycosidase H treatment of expressed protein was carried out as described previously (Shimokawa & Smith, 1992).

Purification of Carboxylesterase. For purification of baculovirus-expressed carboxylesterase, 10 culture dishes at a density of 6×10^7 Sf9 cells were infected with recombinant virus. Cells were harvested 72 h after infection as described above and suspended in 10 mM potassium phosphate buffer, pH 6.8 (buffer A). Following sonication, the homogenate was centrifuged at 10000*g* for 20 min. A solution of 10% sodium cholate was added dropwise to the supernatant such that the final concentration was 1%. The solution was stirred for 2 h and dialyzed in buffer A overnight. All operations were performed at 4 °C. The resulting mixture of soluble cytosolic and microsomal fractions was used to purify carboxylesterase using DEAE–Sepharose chromatography. The carboxylesterase was eluted from the column with a linear 0–400 mM NaCl gradient in buffer A. Carboxylesterase activities were monitored by hydrolysis of *p*-nitrophenyl acetate as described below.

Enzymatic Activity Assays. The substrate specificity of expressed carboxylesterases was investigated for total cell lysates and purified protein samples. *p*-Nitrophenyl acetate, *p*-nitrophenyl propionate, and *p*-nitrophenyl butyrate hydrolysis were measured at a final concentration of 1.6 mM, according to the procedure of Krisch (1966). Malathion, clofibrate, and diethyl succinate hydrolysis (0.3 mM) were coupled to the reduction of *p*-iodonitrophenyltetrazolium violet as described previously (Talcott, 1979). Hydrolysis of acetanilide (10 mM), phenacetin (5 mM), and paracetamol (10 mM) was measured fluorometrically (Heymann et al., 1981). Methyl butyrate hydrolysis was followed by measuring methanol release from 20 mM substrate solutions (Tsujita & Okuda, 1983). Isocarboxazide (Moroi & Satoh, 1975) and palmitoyl CoA (Berge & Farstad, 1979) hydroxylase activities were measured spectrophotometrically at concentrations of 1 and 0.04 mM, respectively, as described elsewhere. Inhibition of carboxylesterase activity by diethyl-*p*-nitrophenyl phosphate followed the procedure of Krisch (1966).

RESULTS

Isolation and Sequencing of Human Carboxylesterase cDNAs. Using a 230-bp fragment from the 5′-region of a partial clone (Long et al., 1991) as a probe, several full-length cDNAs were isolated from the K19 (Yamano et al., 1989) human liver cDNA library. One cDNA, designated hCE, spanned 1972 bp, including a 216-bp 3′-untranslated region containing a polyadenylation signal, and encoded a protein of 568 amino acids with a calculated molecular mass of 62 571 Da (Figure 1). An 18 amino acid hydrophobic leader sequence was predicted on the basis of the position of the first ATG initiation codon upstream from the sequenced amino terminus of a mature human liver carboxylesterase (Ozols, 1989) and sequence similarity to other signal peptides (von Heijne, 1983). The predicted molecular mass of the mature carboxylesterase protein was 60 609 Da. The deduced amino acid sequence of hCE displayed 99.8% identity to the partial human liver sequence reported by Long et al. (1991) and included an additional 30 amino acids on the NH_2-terminal end. Similarity to rat liver carboxylesterases was 68% to form E1 (Long et al., 1988; Takagi et al., 1988) and 77% to form ES-10 (Robbi et al., 1990). A 78% similarity to rabbit liver carboxylesterase form 1 (Korza & Ozols, 1988) and 36% to rabbit liver carboxylesterase form 2 (Ozols, 1989) was found. In contrast, only a 30% identity to human brain and serum cholinesterase (Lockridge et al., 1987; McTiernan et al., 1987) and cholesterol esterase (Anderson & Sando, 1991) was noted.

Another cDNA, designated hCE_v, was isolated from the same λgt11 library and was identical within the coding region of the hCE cDNA except for two 3-bp deletions at positions 97–99 and 1131–1133 (Figure 1). The first 3-bp deletion resulted in the loss of the final amino acid in the hydrophobic signal peptide sequence (Ala_{-1}), while the second deletion resulted in an in-frame loss of Gln_{345}. Differences in the 5′-untranslated region between the two clones were also noted. The overall nucleotide and amino acid similarity between the two clones was 98.4 and 99.6%, respectively.

Chromosome Mapping of the Carboxylesterase Gene. Human–rodent somatic cell hybrids were used to determine the location of the carboxylesterase gene and other related genes. The human gene was detected as a major 4.5-kb *Eco*RI fragment and a weaker 6.0-kb *Eco*RI fragment following Southern hybridization with a 494-bp *Apa*I fragment from the 3′-region of hCE cDNA (data not shown). A major 4.5-kb and minor 6.0-kb fragment were also previously detected on Southern hybridization of *Eco*RI-digested human liver genomic DNA probed with a 1700-bp cDNA spanning all but the 5′-region of the hCE sequence (Long et al., 1991), suggesting that the *Eco*RI fragments detected in these mapping studies might represent distinct carboxylesterase genes.

Alternatively, the two fragments might result from regions of the probe sequence being divided between two exons and the presence of intronic *Eco*RI sites. The location of the carboxylesterase locus was determined by correlating the presence or absence of these fragments with the presence or absence of specific human chromosomes previously characterized for each hybrid cell line (McBride et al., 1982a,b,c). Both human sequences hybridizing with the hCE probe (4.5 and 6 kb) appeared to segregate concordantly. The carboxylesterase fragments could be unambiguously assigned to human chromosome 16 since it segregated discordantly (≥29%) with all other human chromosomes (Table II). No RFLPs were detected on examining DNAs from 10 unrelated individuals digested with 12 different restriction enzymes.

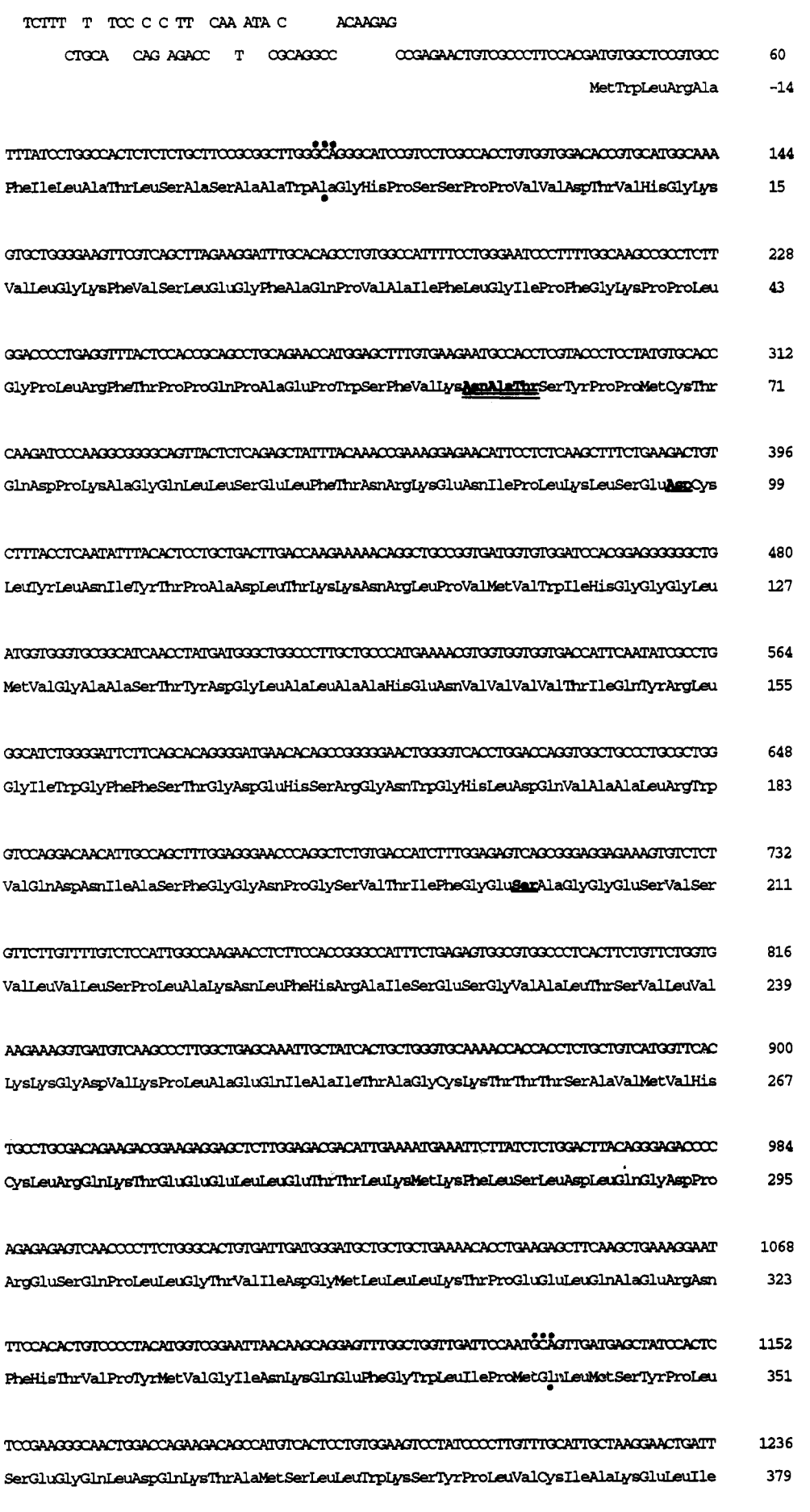
Analysis of hCE mRNA Expression in Human Liver. Based on their cDNA sequences, no difference in the sizes of mRNA for hCE and hCE_v would be expected. Therefore, RNase protection assays were used to determine the level of each carboxylesterase mRNA in human liver samples. All experiments were carried out under conditions in which the labeled probe was known to be in excess over the target mRNA species to permit its quantitation. Using an antisense RNA transcript synthesized from the 3′-terminal region of either the hCE or hCE_v cDNA which included 160 bp of coding and 216 bp of noncoding sequence, a 376-bp protected fragment was excepted. A single protected fragment of expected length was detected in 12 adult human liver samples (Figure 2A). Based on complete sequence similarity between hCE and hCE_v in the probe region, both mRNA species should be detected with these probes. A second pair of probes was designed for RNase protection analysis of the 5′-region of the mRNA which would distinguish between the messages corresponding to the hCE and hCE_v cDNAs. An antisense RNA transcript synthesized from this region of the hCE cDNA hybridized with human liver RNA to give an expected protected fragment of 257 bp in all samples (Figure 2B). In addition, a protected fragment of 158 bp resulting from hybridization with mRNA specific for hCE_v was also detected in all samples (a second protected fragment of 74 bp expected from RNase digestion of the hCE probe/hCE_v mRNA hybrid was too small to be detected with the electrophoresis conditions employed). Likewise, both hCE and hCE_v mRNAs were detected in all samples with an antisense RNA transcript synthesized from the 5′-region of the hCE_v cDNA. The hCE_v probe protected a fragment of 276 bp corresponding to hCE_v mRNA and a 232-bp fragment corresponding to mRNA for hCE (Figure 2C). Unlabeled sense transcripts were synthesized as positive controls to confirm the protection pattern observed with the hCE and hCE_v probes. In general, protection of the 5′-end of the RNA was more difficult than for the 3′-end, perhaps due to differences in secondary structure or stretches of sequence particularly sensitive to RNase treatment. Unidentified fragments of varying intensities and multiple bands were detected in some individuals irrespective of hybridization and RNase digestion conditions, suggesting that other related mRNAs might also be recognized by these probes.

The 236- (Figure 2A) and 147-bp fragments (Figures 2B,C) represent mRNA for human β-actin used as a control. The multiple bands observed with the smaller actin probe most likely result from protection of related genes since human β-actin is known to be a multigene family (Ponte et al., 1983). When the amount of carboxylesterase mRNA was expressed relative to the amount of β-actin mRNA, a more than 5-fold difference in expression of hCE mRNA (Figure 2B) and a 12-fold difference in expression of hCE_v mRNA (Figure 2C) were found. In several individuals, one carboxylesterase mRNA was expressed at a high level while the second mRNA was expressed at a relatively low level (e.g., K10, K12, K18, K20, K21). In contrast, both mRNAs were expressed at low levels in samples K19 and M2 and at relatively high levels in liver K14.

Expression of Carboxylesterase Proteins in Human Liver. To determine the variability in carboxylesterase protein expression in these human liver samples, Western immunoblotting was performed using antibody generated against rat carboxylesterase. The antibody reacted strongly with human liver microsomal carboxylesterase protein to give a single immunoreactive protein band of relative molecular mass 59 000 Da in all 13 adult liver samples probed (Figure 3). The calculated molecular weights of the mature carboxylesterase proteins encoded by hCE and hCE_v differ by less than 200 Da, so detection of these two proteins as distinct species would not be expected. No immunoreactive protein bands were detected in two fetal liver microsome samples. The amount of carboxylesterase protein varied less than 8-fold in this group of liver samples, similar to the variation in mRNA levels.

Allele-Specific Hybridization. The high nucleotide similarity between the hCE and hCE_v clones suggested that these two cDNAs might represent different alleles from the same gene. Oligonucleotide probes specific for hCE and hCE_v spanning the region of deletion 2 were hybridized with human genomic DNA samples which had been amplified in the sequence surrounding this deletion (Table I). Hybridization and washing conditions were controlled such that the 3-bp difference between the hCE and hCE_v DNA could be detected with the respective oligonucleotide probes as demonstrated with plasmid DNA samples (Figure 4). In genomic DNA samples from nine Caucasian and six Japanese subjects, a signal with both the wild-type and variant probe was observed (Figure 4). Differences in the intensity of the signal reflect differences in the amount of product from the amplification reaction. These results indicate that if the hCE and hCE_v cDNAs correspond to different alleles from the same gene, then all 15 subjects studied are heterozygotes. Since this is unlikely, it appears as though the two clones might represent distinct but highly related carboxylesterase genes. A similar approach using oligonucleotide probes specific to the region containing deletion 1 confirmed the presence of both hCE and hCE_v sequences in the 15 genomic samples analyzed (data not shown).

Expression of hCE and hCE_v cDNAs Using Baculovirus. The hCE and hCE_v cDNAs were inserted into baculovirus, and Sf9 cells infected with the recombinant viruses produced major protein species detectable in SDS polyacrylamide gels (Figure 5A). The recombinant hCE virus produced a single species of estimated molecular mass 59 000 Da, consistent with the calculated molecular mass of a mature protein based on the cDNA sequence in Figure 1. The variant carboxylesterase recombinant virus produced two distinct proteins of approximately equal intensity, corresponding to estimated molecular mass of 56 000 and 59 000 Da. The protein bands identified by Coomassie staining of SDS polyacrylamide gels for both recombinant viruses were confirmed to be carboxylesterase-related on the basis of their reaction with antibody against rat carboxylesterase (Figure 5B). The 59 000-Da protein comigrated with protein detected in the K19 liver from which the λgt11 library was made (Figure 5B). The recombinant protein was associated with the cell lysate, and no detectable carboxylesterase protein was found in the culture media, indicating that the protein is not secreted.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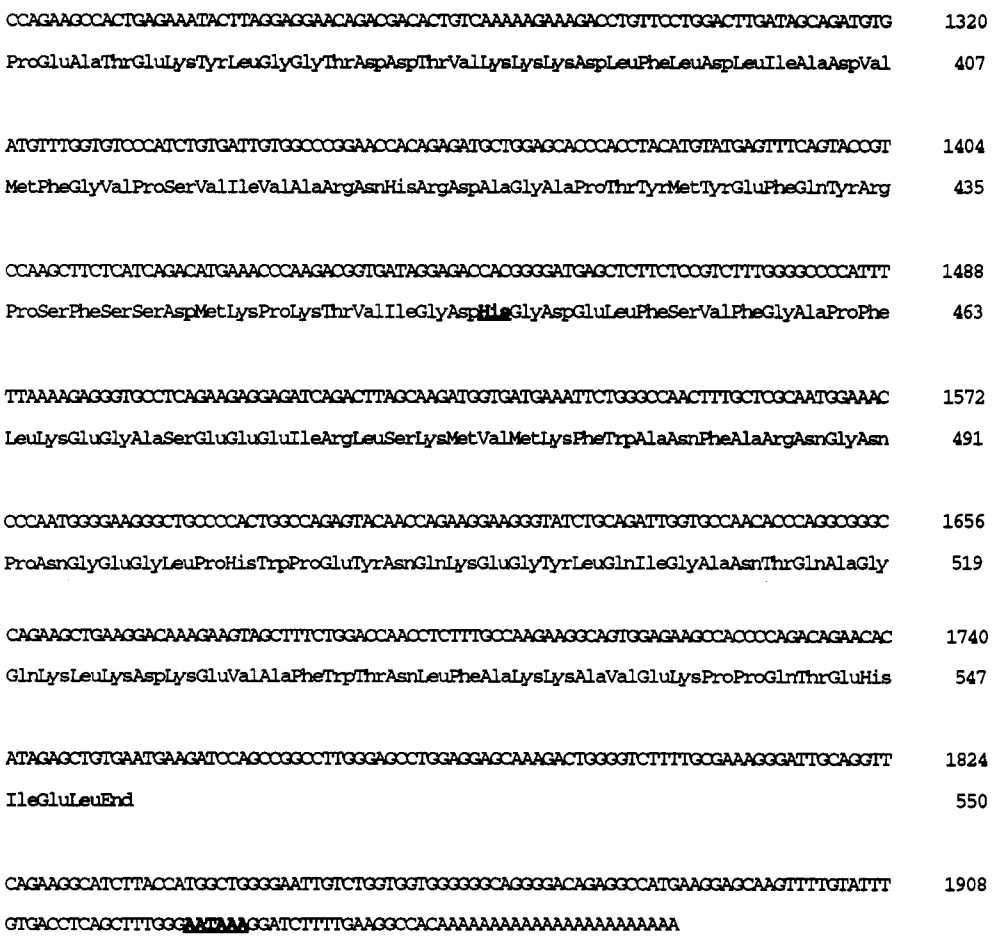

FIGURE 1: Sequence of the hCE and hCE_v cDNAs. The complete cDNA and deduced amino acid sequences of hCE are presented. Only nucleotide and amino acid sequences in hCE_v that differ from hCE are displayed above and below the hCE DNA and amino acid sequences, respectively. Deletions in hCE_v are marked with ●. Active-site residues and the polyadenylation signal are underlined. A putative site for N-linked glycosylation is double underlined. The nucleotide and amino acid residues are numbered at the right. Amino acid number 1 corresponds to the first amino acid in the mature protein.

Infection of Sf9 cells with the hCE_v recombinant virus reproducibly led to expression of two distinct protein species, the larger of which corresponded to the recombinant protein produced from hCE as well as the mature protein detected in human liver samples. A third protein species with an even lower molecular weight was sometimes detected by Western blotting and most likely represents a proteolytic degradation product (Figure 5B). Densitometric scans of the SDS polyacrylamide gels indicated that the amount of the 59 000-Da carboxylesterase protein varied between experiments from 30 to 70% of total carboxylesterase protein. To determine if the hCE_v protein was incompletely glycosylated, experiments were carried out with tunicamycin to block the formation of *N*-glycosidic linkages by inhibition of *N*-acetylglucosamine 1-phosphate transfer to dolichol monophosphate. Addition of tunicamycin to the culture media following infection of Sf9 cells with either hCE or hCE_v recombinant baculovirus resulted in a loss of the 59 000-Da carboxylesterase protein and a parallel increase in the 56 000-Da carboxylesterase protein (Figure 6A). The effect of tunicamycin was dependent on its concentration as well as the period of time in which it was included in the culture media following infection; however, glycosylation of the carboxylesterase could not be completely inhibited with nontoxic concentrations of tunicamycin. Furthermore, carboxylesterase protein produced in the absence of tunicamycin could be completely deglycosylated in vitro by incubation with endoglycosidase H (Figure 6B), indicating the presence of a high-mannose-type carbohydrate chain.

Purification of recombinant hCE and hCE_v proteins was achieved using DEAE–Sepharose chromatography. A single band of estimated molecular mass 59 000 Da was detected following electrophoresis of the purified samples on an SDS polyacrylamide gel (Figure 7). In the case of expression of the hCE_v cDNA, in which two distinct proteins differing in their glycosylation state are produced (Figures 5 and 6), only the higher molecular weight glycosylated form is purified using anion-exchange chromatography. A small amount of carboxylesterase activity was detected in the flow-through fraction of the anion-exchange column, suggesting that the less glycosylated protein did not bind to this column.

Substrate Specificity of Baculovirus-Expressed Carboxylesterases. The hydrolytic activity of hCE and hCE_v recombinant proteins toward a spectrum of known carboxylesterase substrates was measured in total cell lysates and preparations of purified carboxylesterase protein. The best substrates for both forms of carboxylesterase were methyl butyrate and the *p*-nitrophenyl esters, simple aliphatic and aromatic esters (Table III). These results are consistent with what has been reported for a purified human liver carboxylesterase (Ketterman et al., 1989; Hosokawa et al., 1990). The esters diethyl succinate and clofibrate were hydrolyzed at a rate more than 1 order of magnitude less than that of the *p*-nitrophenyl esters, and methyl butyrate and malathion at an even lower rate. Amides and a fatty acyl CoA ester were poor substrates for the carboxylesterases, with hydrolysis rates for acetanilide, phenacetin, paracetamol, isocarboxazide, and palmitoyl CoA not significantly different than background

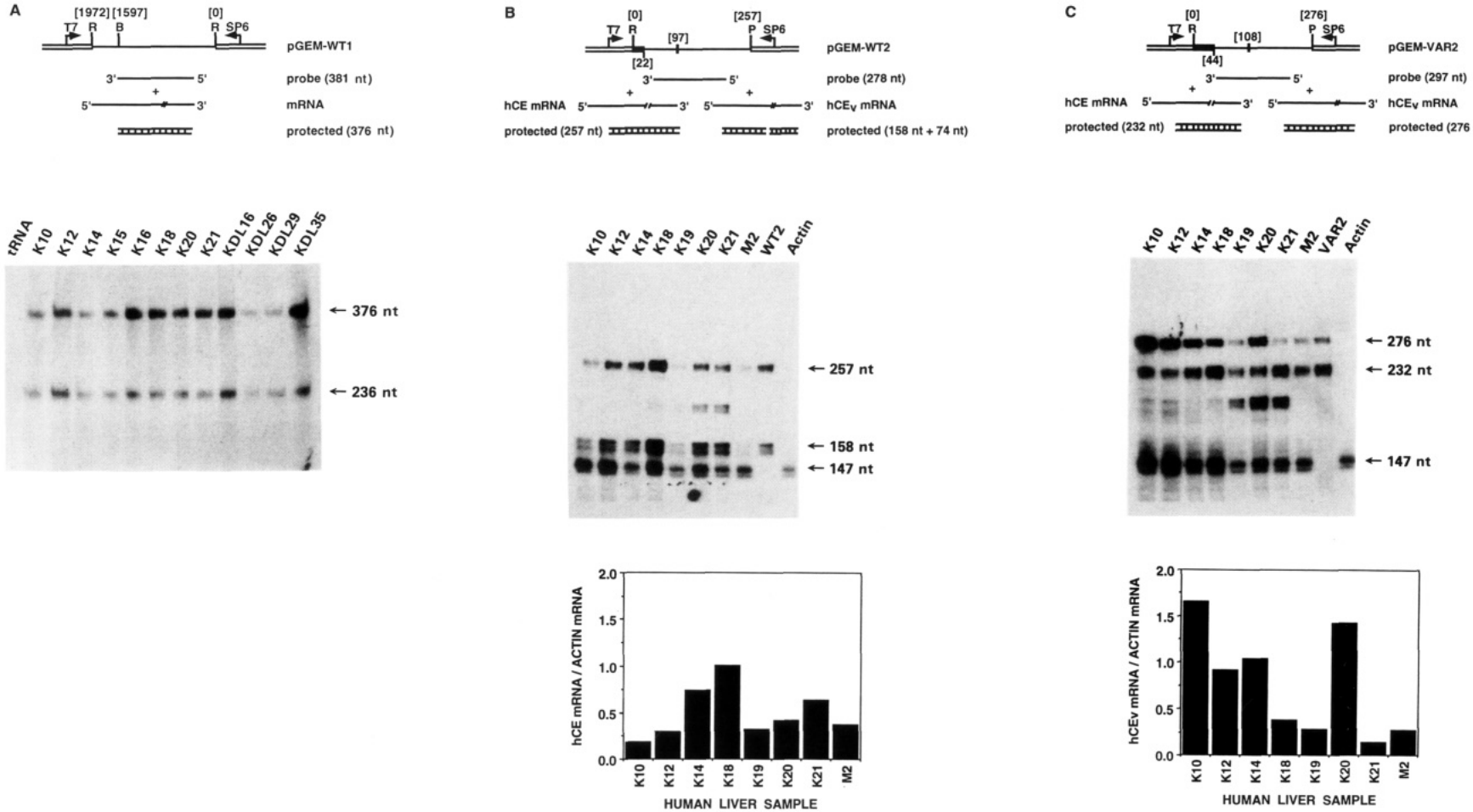

FIGURE 2: RNase protection assay of human liver RNA. Total human liver RNA was isolated, and 10 μg from each sample was hybridized with ^{32}P-labeled hCE and hCE$_v$ antisense RNA probes, digested with RNase, and electrophoresed on a polyacrylamide/urea gel. (A) A 1972-bp hCE *Eco*RI cDNA insert was subcloned into pGEM-3Z vector (═) to produce pGEM-WT1. A 381-nt, ^{32}P-labeled, antisense RNA probe was synthesized by linearizing pGEM-WT1 with *Bal*I (position 1597) and transcribing with T7 RNA polymerase. Following hybridization with hCE or hCE$_v$ mRNA, a protected fragment of 376 nt is predicted. Each lane contains either a single human liver sample or an equal amount of tRNA. The 236-nt protected fragment corresponds to β-actin. (B) A 257-bp *Eco*RI (position 0)–*Pst*I (position 257) hCE cDNA insert was subcloned into pGEM-3Z vector (═) to produce pGEM-WT2. The first 22 base pairs and base pairs 97–99 differ from that of hCE$_v$. A 278-nt, ^{32}P-labeled, antisense RNA probe was synthesized by linearizing pGEM-WT2 with *Eco*RI (position 0) and transcribing with SP6 RNA polymerase. Following hybridization with hCE mRNA, a protected fragment of 257 nt is predicted, and following hybridization with hCE$_v$ mRNA, protected fragments of 158 and 74 nt are expected. Each lane contains a single human liver sample probed with both hCE and actin. The 147-nt protected fragment corresponds to β-actin. In the lanes marked WT2 and actin, K14 RNA was hybridized with only the indicated probe. Ratios of hCE mRNA to β-actin mRNA determined by densitometric measurement of the autoradiograms are plotted for each liver sample. (C) A 276-bp *Eco*RI (position 0)–*Pst*I (position 276) hCE$_v$ cDNA insert was subcloned into pGEM-3Z vector (═) to produce pGEM-VAR2. The first 44 base pairs and base pairs 108–110 differ from that of hCE. A 297-nt, ^{32}P-labeled, antisense RNA probe was synthesized by linearizing pGEM-VAR2 with *Eco*RI (position 0) and transcribing with SP6 RNA polymerase. Following hybridization with hCE$_v$ mRNA, a protected fragment of 276 nt is predicted, and following hybridization with hCE mRNA, a 232-nt protected fragment is expected. Each lane contains a single human liver sample probed with both hCE$_v$ and actin. The 147-nt protected fragment corresponds to β-actin. In the lanes marked VAR2 and actin, K14 RNA was hybridized with only the indicated probe. Ratios of hCE$_v$ mRNA to β-actin mRNA determined by densitometric measurement of the autoradiograms are plotted for each liver sample.

Table II: Segregation of the Carboxylesterase Gene with Human Chromosome 16[a]

human chromosome	gene/chromosome +/+	+/−	−/+	−/−	discordancy (%)
1	18	17	16	43	35
2	11	24	15	44	41
3	11	24	25	34	52
4	26	9	32	27	44
5	12	23	12	47	37
6	20	15	19	40	36
7	14	21	26	33	50
8	17	18	9	50	29
9	15	20	16	43	38
10	7	28	3	56	33
11	19	16	13	46	31
12	17	18	11	48	31
13	11	24	15	44	41
14	23	12	18	41	32
15	15	20	30	29	53
16	35	0	0	59	0
17	26	9	31	28	43
18	14	21	22	37	46
19	14	21	15	44	38
20	21	14	19	40	35
21	25	10	36	23	49
22	13	22	15	44	39
X	19	16	28	31	47

[a] The human carboxylesterase gene was detected as 4.5- (strong) and 6.0-kb (weak) bands in *Eco*RI-digested human–rodent somatic cell hybrid DNAs after Southern hybridization with a 494-bp hCE probe. The two human bands cosegregated. Detection of the human band is correlated with the presence or absence of each human chromosome in the group of somatic cell hybrids. The human–hamster hybrids contained 28 primary clones and 13 subclones (23 positive of 41 total), and the human–mouse hybrids represented 19 primary clones and 34 subclones (12 positive of 53 total).

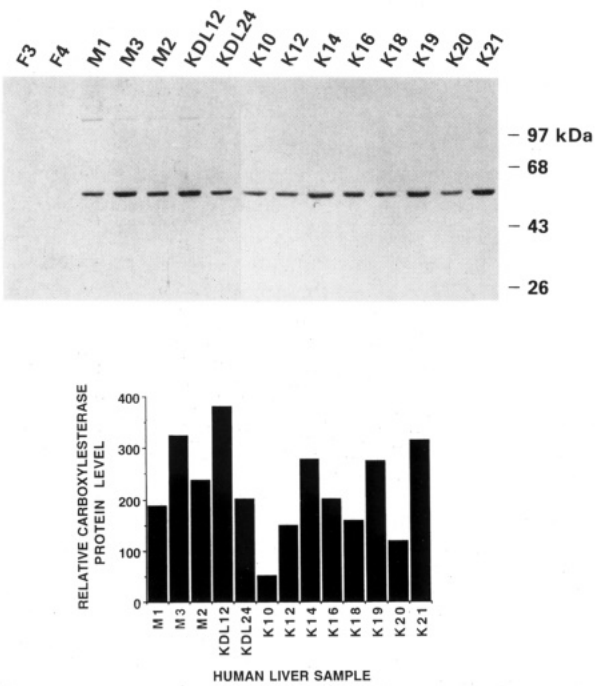

FIGURE 3: Western immunoblot of carboxylesterase protein expressed in human livers. Thirteen adult (lanes 3–15) and two fetal (lanes 1–2) human liver microsome samples (40 μg/well) were electrophoresed and transferred to nitrocellulose paper. The carboxylesterase protein was stained using rabbit anti-rat carboxylesterase and alkaline phosphatase conjugated goat anti-rabbit IgG. Relative levels of carboxylesterase protein determined from densitometric scanning of the immunoblots are plotted for each liver sample.

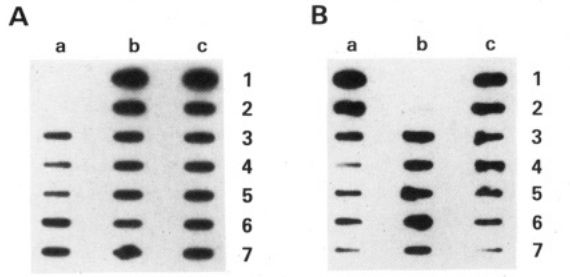

FIGURE 4: Allele-specific hybridization of carboxylesterase in human genomic DNA samples. Amplified DNA was denatured and slot-blotted onto Nytran membranes, probed with the designated oligonucleotide, and exposed to X-ray film for 2 h at room temperature. The control samples are overexposed to illustrate the specificity of the probes. Rows 1 and 2 in both panels contain either unamplified (row 1) or amplified (row 2) control plasmid DNA. Control DNAs are hCE_v (1a, 2a), hCE (1b, 2b), and a 1:1 mix of hCE and HCE_v (1c, 2c). Individual Caucasian (rows 3–5) and Japanese (rows 6 and 7) genomic DNA samples were amplified, divided into two aliquots, and probed with an oligonucleotide specific for hCE (panel A) or hCE_v (panel B).

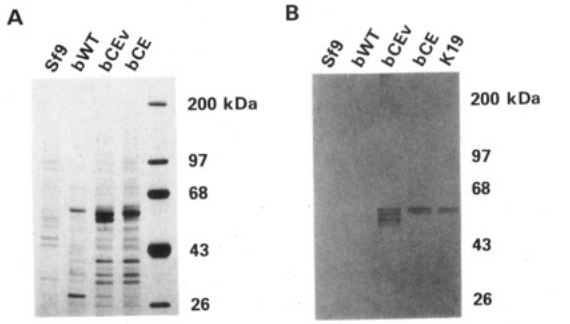

FIGURE 5: Analysis of Sf9 cells infected with recombinant hCE and hCE_v. Wild-type baculovirus (bWT) or recombinant baculovirus containing either the hCE (bCE) or hCE_v (bCE_v) cDNA was used to infect Sf9 cells. Seventy-two hours after infection, total sonicated cell protein (100 μg) was subjected to electrophoresis on SDS-containing polyacrylamide gels and Coomassie staining (panel A). A second sample of total cell protein (25 μg) was electrophoresed in a similar fashion and subjected to immunoblot analysis using rabbit antibody against rat carboxylesterase and phosphatase-conjugated goat anti-rabbit IgG (panel B). A sample of microsomal protein (20 μg) from human liver K19 was also examined in parallel. Molecular weight standards are labeled on the side.

levels measured in mock-infected Sf9 cells. For those esters which were hydrolyzed by the carboxylesterases, the specific activity for the wild-type protein was significantly higher than that of the variant carboxylesterase. No examples of selective metabolism of a given substrate by a single protein were found. Inhibition of all hydrolytic activities by 0.1 mM diethyl-*p*-nitrophenyl phosphate confirms the involvement of the highly conserved Ser residue in the catalytic reaction (Krisch, 1966). The enzymatic activity of the expressed carboxylesterases was also shown to be affected by the degree of glycosylation. In fact, a linear relationship was found between *p*-nitrophenyl acetate hydrolysis and the percentage of carboxylesterase protein which was glycosylated (Figure 8).

DISCUSSION

Two distinct, highly similar cDNAs encoding carboxylesterases have been isolated from a human liver cDNA library. The hCE and hCE_v cDNAs represent the first full-length clones for human liver carboxylesterases. Interestingly, a 99% deduced amino acid sequence similarity between an alveolar macrophage esterase (Munger et al., 1991) and hCE suggests that these enzymes might be encoded by the same gene, despite evidence for production of the former in alveolar macrophages. The alveolar macrophage cDNA has the 3-bp deletion resulting in loss of Ala_{-1} as in hCE_v but not the second deletion at bp

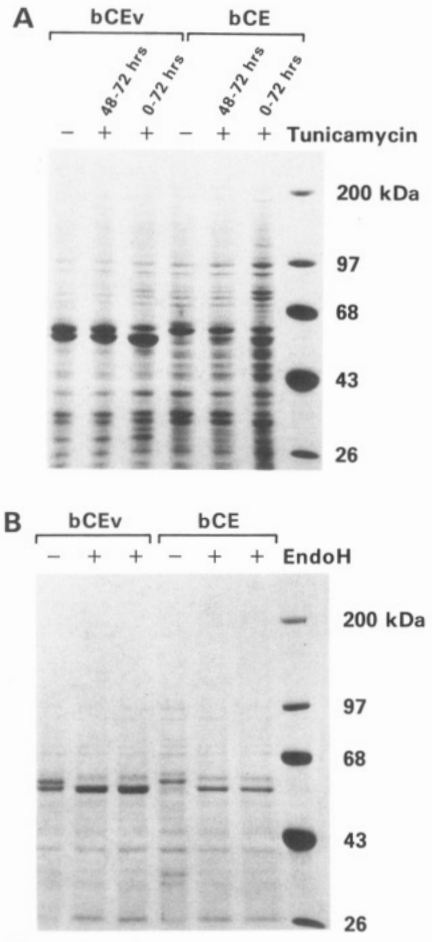

FIGURE 6: Expression of recombinant carboxylesterases as glycoproteins. Recombinant baculovirus containing the hCE (bCE) or hCE_v (bCE_v) cDNA was used to infect Sf9 cells. (A) Tunicamycin was included in the culture media for the indicated period, and cells were harvested at 72 h following infection. Total sonicated cell protein (100 μg) was subjected to electrophoresis on SDS-containing polyacrylamide gels, and proteins were detected by Coosmassie Blue staining. (B) Total cellular protein (50 μg) harvested 72 h after infection and culture in the absence of tunicamycin was denatured and incubated with 20 munits of endoglycosidase H (Endo H) at 37 °C for 12 h. Following incubation, the protein was subjected to gel electrophoresis as described above. Molecular weight standards are labeled on the side.

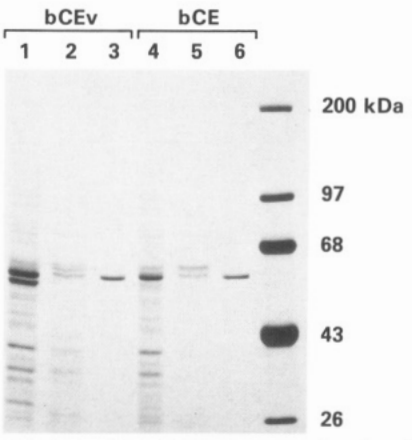

FIGURE 7: Purification of baculovirus-expressed carboxylesterase. Seventy-two hours following infection of Sf9 cells with recombinant baculovirus containing the hCE (bCE) or hCE_v (bCE_v) cDNA, total cell protein was solubilized and subjected to DEAE–Sepharose chromatography. Total cellular protein (50 μg; lanes 1 and 4), soluble protein (80 μg; lanes 2 and 5), and purified carboxylesterase (3 μg; lanes 3 and 6) were subjected to SDS polyacrylamide gel electrophoresis followed by Coomassie Blue staining. Molecular weight standards are labeled on the side.

1131. The 5′-noncoding region of the alveolar macrophage cDNA is also more similar to hCE than to hCE_v. Except for differences in trafficking, with detection of at least a portion of the alveolar esterase as a secretory form, the alveolar esterase displays properties common to liver carboxylesterases (Munger et al., 1991).

A comparison of amino acid sequences either directly sequenced or deduced from cDNAs indicates that liver carboxylesterases have been highly conserved throughout evolution. All liver carboxylesterases sequenced to date have a consensus octapeptide containing the active-site Ser residue (G-E-S-A-G-G/A-X-S), Glu and His residues forming a putative charge relay system with the active-site Ser (Cygler et al., 1993), identical cysteine binding pairs, at least one N-linked glycosylation site, and a signal peptide sequence of varying length (Long et al., 1988; Takagi et al., 1988; Robbi et al., 1990; Korza & Ozols, 1988; Ozols, 1989). The biggest difference between species is the number of potential glycosylation sites, ranging from five in one rat form (Long et al., 1988; Takagi et al., 1988) to only one in the human liver sequences (Figure 1). While these structural features are also highly conserved in members of a second family of serine esterases, the cholinesterases, the remaining protein sequence shows limited similarity to the liver carboxylesterases (Lockridge et al., 1987). Similarities also exist between carboxylesterases and serine proteases. Namely, these two proteins show similar patterns of inhibition by organophosphates and carbamates, an overlapping substrate specificity, inactivation by irreversible binding of diisopropyl fluorophosphate to active-site serines, and a three-in-eight match between consensus active-site sequences (Myers et al., 1988). However, comparisons of their primary sequences revealed no significant overall similarity between carboxylesterases and the functionally related serine protease multigene family, and these enzymes are likely derived from separate gene families (Myers et al., 1988).

Although retained in the lumen of the endoplasmic reticulum, many carboxylesterases contain a hydrophobic signal peptide sequence which initially directs protein sorting down a secretory pathway. For the human liver carboxylesterases encoded by hCE or hCE_v, this consists of an 18 or 17 amino acid sequence, respectively. In most cases, a bulky aromatic residue followed by a small neutral residue directly precedes the cleavage site (von Heijne et al., 1983). However, in hCE_v the neutral Ala residue is deleted. While it is possible that such a deletion could result in altered recognition of the cleavage site and therefore altered processing of the protein, both carboxylesterase proteins produced in the baculovirus system were completely retained in the cells. The tetrapeptide Lys-Asp-Glu-Leu (KDEL) was initially identified at the carboxyl terminus of several soluble endoplasmic reticulum proteins and determined to be essential for their retention in this compartment (Munro & Pelham, 1986, 1987). Others have since demonstrated that variants of the KDEL sequence can also direct intracellular retention of proteins, with the dipeptide Glu-Leu being the major requirement for retention (Andres et al., 1990; Robbi & Beaufay, 1991). The human carboxylesterases described here conform to this requirement (COOH-terminal sequence of His-Ile-Glu-Leu), consistent with the detection of these proteins in the microsomal fraction of human liver as well as cell lysates infected with recombinant carboxylesterase virus. Recent evidence suggests that secretory forms of liver carboxylesterases which lack a recognized retention signal exist in rats and mice (Medda & Proia, 1992).

Table III: Substrate Specificity of Baculovirus-Expressed Carboxylesterases[a]

substrate	cell lysate (nmol/min/mg protein)			purified enzyme (μmol/min/mg protein)	
	hCE	hCE_v	Sf9	hCE	hCE_v
p-nitrophenyl acetate	541 ± 64.6	336 ± 15.2	18.9 ± 2.10	60.7 ● 4.03	39.6 ± 4.10
p-nitrophenyl propionate	1262 ± 131	1211 ± 129	59.4 ± 6.23	141 ± 16.3	131 ± 13.5
p-nitrophenyl butyrate	1592 ± 147	1385 ± 174	50.2 ± 3.44	155 ± 11.9	108 ± 0.90
methyl butyrate	1159 ± 96.4	652 ± 76.3	0	156 ± 13.8	69.3 ± 6.78
diethyl succinate	119 ± 13.4	77.7 ± 11.2	0	4.30 ± 0.21	3.41 ± 0.22
clofibrate	112 ± 17.1	101 ± 17.3	0	3.47 ± 0.21	2.50 ± 0.17
malathion	24.6 ± 7.4	15.7 ± 5.5	38.4 ± 2.27	0.73 ± 0.11	0.44 ± 0.08
acetanilide	9.62 ± 0.50	7.63 ± 1.19	5.08 ± 2.43	0.067 ± 0.011	0.068 ± 0.017
phenacetin	3.70 ± 0.60	2.53 ± 0.47	4.10 ± 1.88	0.075 ± 0.006	0.062 ± 0.002
paracetamol	<1.00	<1.00	<1.00	<0.001	<0.001
isocarboxazid	6.08 ± 6.16	6.79 ± 3.46	11.1 ± 5.61	0.065 ± 0.014	0.197 ● 0.062
palmitoyl CoA	13.5 ± 1.92	12.1 ± 0.67	17.0 ± 8.78	0.089 ± 0.009	0.049 ± 0.008

[a] Sf9 cells were infected with recombinant baculovirus containing hCE or hCE_v cDNA, and cells were harvested 72 h after infection. The infected cells were sonicated and in some instances used for purification of carboxylesterases as described in Figure 7. Hydrolysis rates for cell lysates and purified enzyme preparations were measured as described under Experimental Procedures. Hydrolysis rates for mock-infected Sf9 cells are indicated. Values are the mean ±SD of 3–5 determinations.

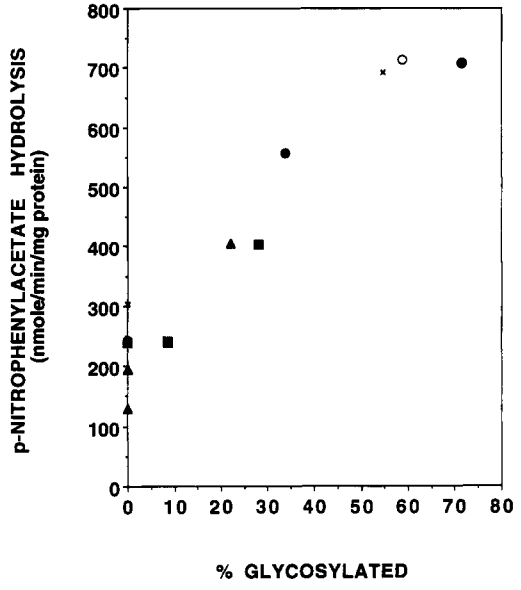

FIGURE 8: Effect of N-glycosylation on the enzymatic activity of baculovirus-expressed carboxylesterases. The hydrolysis of *p*-nitrophenyl acetate was measured in cell lysates following infection of Sf9 cells with recombinant baculovirus containing the hCE or hCE_v cDNA. During the 72 h after infection, tunicamycin was present in the media at concentrations of 0 (●), 1 (○), 2.5 (×), 5 (■), or 50 (▲) μg/mL. The percentage glycosylated was calculated from densitometric measurement of the 56- and 59-kDa protein bands on SDS polyacrylamide gels loaded with 50 μg of cell lysate. Each point represents one determination. Linear regression of the combined data: $Y = 163 + 10.9*X$; $r^2 = 0.947$.

It would not be surprising if secretory forms were also found for the human carboxylesterases, since it has been shown that carboxylesterase hydrolytic activity can be significantly increased by combining the cytosolic and microsomal subcellular fractions of human hepatic tissue (Ketterman et al., 1989).

Despite only a 2 amino acid difference between the proteins encoded by hCE and hCE_v, these two products are likely derived from different genes. RNase protection assays specific for the two mRNAs indicated that both hCE and hCE_v RNA were expressed in all human liver samples probed. Further evidence for separate genes was obtained from detection of genomic DNA fragments with oligonucleotides specific for hCE and hCE_v. While both hCE and hCE_v were consistently expressed in all investigated human liver samples, their expression patterns were often not in parallel. In many cases, preferential expression of one mRNA over the second mRNA was evident, which might be expected if the two genes evolved such that the function of their respective proteins were essentially duplicated. The lack of substrate specificity between the hCE and hCE_v proteins suggests that the gene products have similar, if not identical, functions. The range of expression of the variant gene was much greater than that of the wild-type gene, which may reflect high levels of hCE_v necessary for compensation of low levels of the more active hCE in certain individuals. It is of interest to determine if expression of these genes follows similar patterns in other tissues.

The human carboxylesterase gene was localized to chromosome 16. Esterase B3 was previously mapped to human chromosome 16 by its activity toward α-naphthyl butyrate and umbelliferyl acetate (Astrin et al., 1982). More recently, a human monocyte/macrophage serine esterase-1 with α-naphthyl acetate activity (esterase CES-1) was also mapped to this chromosome at 16q13-q22.1 as well as to mouse chromosome 8 (Becker-Follmann et al., 1991). Although the specific substrates used to map the location of B3 and CES-1 were not investigated in the present study, these esters closely resemble the simple aliphatic and aromatic esters metabolized by the hCE and hCE_v proteins, and it is likely that the esterases localized on chromosome 16 are encoded by the same gene. Based on the somatic cell hybrid mapping of hCE, these carboxylesterases may be members of a small subfamily, since only 4.5- and 6.0-kb fragments were detected.

Expression of both the wild-type and variant forms of the human liver carboxylesterase using baculovirus represents the first report of functional expression of these proteins in a eukaryotic system. The major protein from expression of both cDNAs displayed electrophoretic and immunological properties consistent with known carboxylesterases. Efficient glycosylation of hCE protein was demonstrated in the baculovirus system, in agreement with the expression of this protein in human liver as a high-mannose-type glycoprotein (Hosokawa et al., 1990). In contrast, the hCE_v protein could not be completely glycosylated, irrespective of the culture conditions. Although the deletions in the hCE_v protein do not involve the putative glycosylation site, it is possible that if processing is affected by the loss of Ala_{-1}, such changes might be accompanied by alterations in the posttranslational modifications of the protein. Alternatively, the loss of Gln_{345} in hCE_v protein could lead to conformational changes which affect glycosylation.

Expression of the proteins encoded by hCE and hCE_v allowed for assignment of specific hydrolytic activities. The cloned

carboxylesterases metabolized only ester bonds of relatively simple aliphatic and aromatic compounds, similar to the specificity reported for a purified mid pI human liver carboxylesterase (Ketterman et al., 1989). The specific activity for *p*-nitrophenyl acetate hydrolysis of 60.7 ± 4.03 μmol/min/mg protein was similar to the values of 142.7 and 67.3 μmol/min/mg protein reported for a purified carboxylesterase from two different human livers (Ketterman et al., 1989), indicating that the baculovirus system is a good model for characterizing human carboxylesterases. The substrate specificity was identical for the two proteins; however, for those compounds significantly hydrolyzed by the expressed carboxylesterases, specific activities associated with the hCE enzyme always exceeded the corresponding specific activity for the variant form. This finding cannot be accounted for by differences in the extent of glycosylation for these proteins, a factor shown to be directly correlated to catalytic activity, since in purified enzyme preparations only glycosylated protein is present. Therefore, in addition to being inefficiently glycosylated, the hCE_v protein is also less active than hCE toward any given substrate. The fact that no differences in substrate specificity were found for the two proteins suggests that these carboxylesterase genes evolved such that their functions were duplicated.

Proposed roles for glycoprotein oligosaccharide moieties include the promotion of glycoprotein transport from the endoplasmic reticulum to the Golgi compartment and stabilization of protein three-dimensional structure (Collet & Fielding, 1990). In the present study, tunicamycin had no effect on the localization of carboxylesterase proteins expressed in the baculovirus system, consistent with earlier observations that glycosylation is not responsible for the retention of several forms of rat liver carboxylesterase in the endoplasmic reticulum (Robbi & Beaufay, 1986, 1987, 1988). However, N-linked oligosaccharides were necessary for maximal catalytic activity of these enzymes. Tunicamycin inhibition studies have yielded similar findings for other enzymes, including glucuronosyltransferases (MacKenzie et al., 1990), 11β-hydroxysteroid dehydrogenase (Agarwal et al., 1990), lipoprotein lipase (Ong & Kern, 1989), lecithin:cholesterol acyltransferase (Collet & Fielding, 1991), and alkaline phosphatase (DiLorenzo et al., 1991). Site-directed mutagenesis of the putative N-linked glycosylation site would provide a more definitive answer to the importance of oligosaccharides on the catalytic activity of the carboxylesterases as well as to any role in the stability of the protein.

This report is an initial attempt at characterization of the human liver carboxylesterase family of enzymes. Isolation and expression of individual cDNAs encoding these proteins is necessary for assignment of specific hydrolytic activities to the various carboxylesterases. The present studies clearly indicate that additional carboxylesterases must exist to account for the hydrolysis of the wide variety of known substrates. Identification of their respective genes will also provide insight into the structural requirements for a given substrate as well as the basis for the large interindividual variation in carboxylesterase hydrolytic activity (Ketterman, 1991). Of perhaps even greater interest is an understanding of the physiological roles that carboxylesterases play. Except for sequestration of β-glucuronidase by a carboxylesterase referred to as egasyn (Medda et al., 1986; 1987; Ovnic et al., 1991a), direct involvement of these enzymes in physiological processes, such as lipid metabolism, is only speculative. A complete understanding of such functions will require isolation of the human carboxylesterases responsible for hydrolysis of endogenous compounds. Finally, the regulation of the carboxylesterases can begin to be studied at a molecular level. In the rat, carboxylesterases are regulated by steroid hormones in an isozyme-specific manner (Hosokawa & Satoh, 1988). Isolation of 5′-flanking regions of the human carboxylesterase genes will permit the study of such effects on the human carboxylesterases.

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