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GENOMIC STRUCTURE AND TRANSCRIPTIONAL REGULATION OF THE RAT, MOUSE, AND HUMAN CARBOXYLESTERASE GENES

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The mammalian carboxylesterases (CESs) comprise a multigene family which gene products play important roles in biotransformation of ester- or amide-type prodrugs. Since expression level of CESs may affect the pharmacokinetic behavior of prodrugs* in vivo*, it is important to understand the transcriptional regulation mechanism of the* CES *genes. However, little is known about the gene structure and transcriptional regulation of the mammalian* CES *genes. In the present study, to investigate the transcriptional regulation of the promoter region of the* CES1 *and* CES2 *genes were isolated from mouse, rat and human genomic DNA by PCR amplification. A TATA box was not found the transcriptional start site of all* CES *promoter. These* CES *promoters share several common binding sites for transcription factors among the same* CES *families, suggesting that the orthologous* CES *genes have evolutionally conserved transcriptional regulatory mechanisms. The result of present study suggested that the mammalian* CES *promoters were at least partly conserved among the same* CES *families, and some of the transcription factors may play similar roles in transcriptional regulation of the human and murine* CES *genes.

Key Words: Carboxylesterase (CES); Transcriptional regulation; Promoter; Prodrug.

INTRODUCTION

The mammalian carboxylesterases (CES, EC 3.1.1.1) comprise a multi-gene family whose gene products are localized in the endoplasmic reticulum (ER) of many tissues. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals, as well as drugs (including prodrugs), to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, environmental toxicants, and carcinogens. CES also catalyze the hydrolysis of endogenous compounds, such as short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-coenzyme A (CoA) esters.

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We have reviewed the characteristics of CES in relation to the metabolism of xenobiotics (Satohand Hosokawa, 1998). Multiple isozymes of hepatic microsomal CES exist in various animal species (Hosokawa. et al., 1990; Satoh and Hosokawa, 1998), and some of these isozymes are involved in the metabolic activation of certain carcinogens, as well as being associated with hepato-carcinogenesis (Maki et al., 1991).

Mammalian CES belong to a protein superfamily termed the α, β-hydrolase-fold family in which members may have highly specialized functions, as is the case for ACHE and juvenile hormone esterase (Hosokawaet al., 1990; Satoh and Hosokawa, 1998; Satoh et al., 2002). The expression of CES is ubiquitous, with high levels in the liver, small intestine, kidney, and lung. CES show such a broad range of substrate specificity that they can be involved in detoxification or biotransformation of many kinds of drugs, as well as endogenous fatty acid esters. It has been suggested that CES can be classified into four major groups according to the homology of the amino acid sequence (Hosokawa. et al., 1990; Satohand Hosokawa, 1998; Satoh et al., 2002), and the majority of CES that have been identified belong to the CES1 or CES2 family. It has also been shown that striking species differences exist (Inoue et al., 1979; Hosokawa et al., 1990; Hosokawa et al., 1994; Prueksaritanont et al., 1996; Zhuet al., 2000). For example, Inoue et al. (1979) showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in a disc electrophoresis coupled with staining of esterase activity. On the other hand, esterase activities were observed in the intestines of other species (human, rat, mouse, guinea pig, and rabbit) and found to produce a few active bands in an electrophoretic assay. Since pharmacokinetic and pharmacological data of ester prodrugs obtained from preclinical experiments using various animals are generally used as references for human studies, it is important to clarify the biochemical properties, such as substrate specificity, tissue distribution, and transcriptional regulation, of each CES isozyme.

Recent developments have included more detailed biochemical characterization of mammalian CES enzymes and genes, leading to a better understanding of the biochemical significance and physiological role of CES. This chapter deals primarily with the characteristics and the molecular cloning of the individual, recently identified CES isozymes.

Role of CES in Drug Metabolism of Ester- or Amide-Type

Drug-metabolizing enzymes that are present predominantly in the liver are involved in biotransformation of both endogenous and exogenous compounds to polar products to facilitate their elimination. These reactions are categorized into phase I and phase II reactions. CES are categorized as phase I drug-metabolizing enzymes that can hydrolyze a variety of ester-containing drugs and prodrugs, such as angiotensin-converting enzyme inhibitors (temocapril, cilazapril, quinapril, and imidapril) (Takai et al., 1997; Mori et al., 1999; Furihata et al., 2004a), anti-tumor drugs (CPT-11 and Capecitabin) (Satoh et al., 1994; Tabata et al., 2004), and narcotics (cocaine, heroin, and meperidine) (Pindel. et al., 1997; Zhang et al., 1999). In this regard, it is thought that CES are one of the major determinants for pharmacokinetics and pharmacodynamics of ester drugs or ester prodrugs (Fig. 1). Actually, it has been shown that dog CES1 isozyme was involved in a pulmonary first-pass effect in the disposition of a propranolol ester prodrug (Imai et al., 2003). It has also been shown that the expression level of human CES isozyme was correlated with the conversion ratio of CPT-11 to SN-38, the active metabolite, which is thought to be a key step for the chemotherapeutic action of this anti-tumor drug (Pindel et al., 1997; Zhang et al., 1999; Ohtsuka. et al., 2003; Sanghani. et al., 2003).

The CES and the 5-diphosphate (UDP)-glucuronosyltransferase (UGT) families, the catalytic domains of which are localized in the luminal sides of the ER membrane, are two

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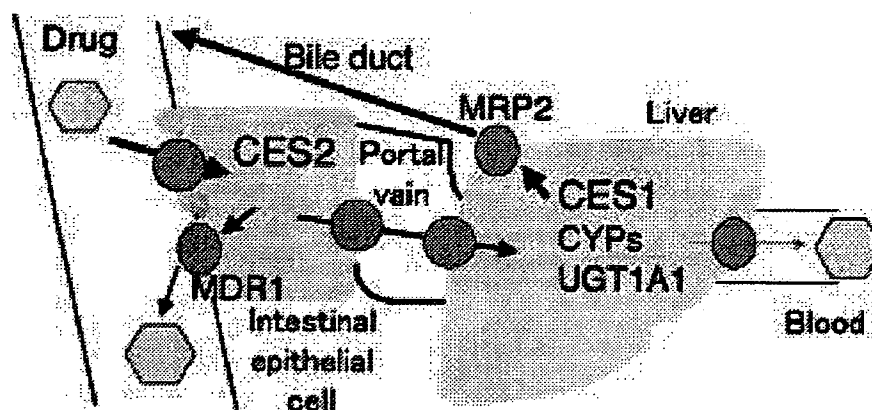

Figure 1 Role of CES isozyme in drug metabolism. CES, carboxylesterase; CYP, cytochrome P450, UGT, UDP, glucuronosyltransferase; MDR, multidrug resistance; MRP2, multidrug resistance-associated protein 2. Round shapes indicate membrane transporters.

major enzyme groups responsible for phase I and II reactions (Fig. 2). The hydrolyzed products by CES are also substrate for UGT, such as SN-38 from CPT-11. Thus, we think that the CES-UGT interaction in the luminal sides of ER membrane is important for drug metabolism. Furthermore, the hydrolyzed product of CES are two kinds of chemical properties: 1) the alcohol or phenol that is a substrate for UGT and 2) organic anions that are

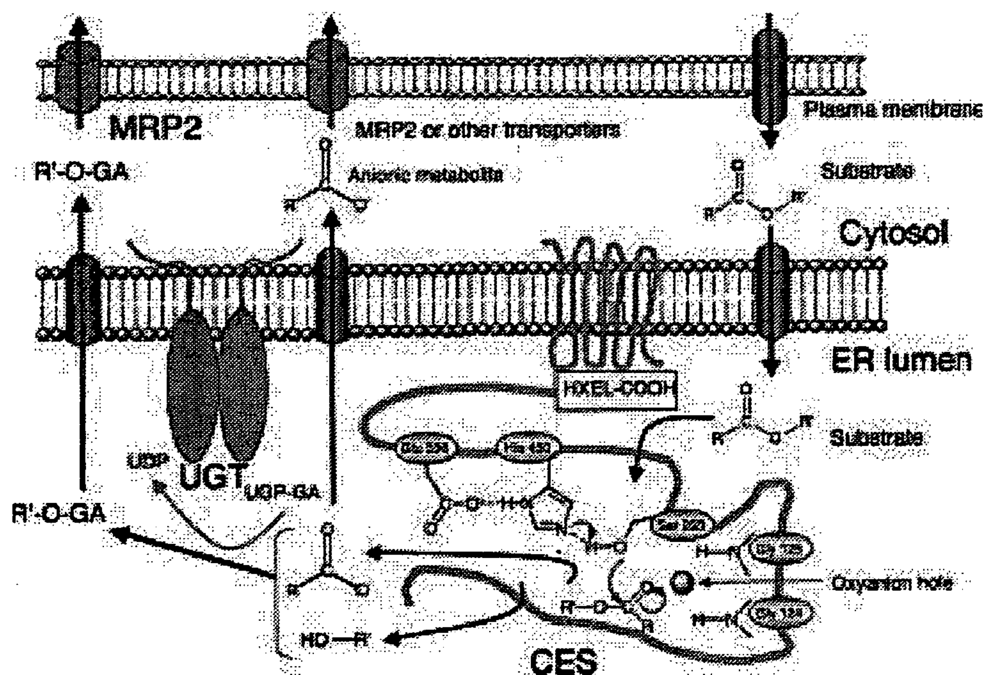

Figure 2 CES-UGT interaction in the luminal sides of endoplasmic reticulum (ER) membrane and CES-transporter interaction in the cell.

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substrates for organic anion transporter, such as multidrug resistance-associated protein 2 (MRP2) (Fig. 2). In this regard, we think CES are one of the major drug-metabolizing enzymes for enzyme-enzyme interaction and enzyme-transporter interaction.

Classification and Nomenclature of CES Superfamilies

According to the classification of esterase by Aldridge (Aldridge, 1993), the serine superfamily of esterase, i.e., acetylcholine esterase, butyrylcholin esterase, and CES, falls into the B-esterase group. CES have very broad substrate specificity. It is becoming increasingly clear that esterase tends to have a broad and overlapping substrate specificity toward amides and esters. A single esterolytic reaction is frequently mediated by several kinds of enzyme. Recent studies on esterase, as with other enzymes concerned with xenobiotic metabolism, have afforded evidence of multiple forms. It seems almost impossible to classify these CES isozymes based on their substrate specificity along the lines of the International Union of Biochemistry (IUB) classification because the individual hydrolases exhibit properties of CES, lipase, or both. Mentlein et al. (1984) proposed classifying these hydrolases as "unidentified" CES (EC 3.1.99.1 to 3.1.99.x). We think CES comprises a superfamily that is based on the high homology and similarity of the characteristics. Therefore, we tried to classify CES isozymes into five subfamilies: CES 1, CES 2, CES 3, CES 4, and CES 5 (Fig. 3) (Satoh and Hosokawa, 1998). The CES 1 family includes the major forms of CES isozymes (more than 60% homology of human CES). Thus, they could be divided into eight subfamilies, through CES 1A to CES 1H. The CES 1A subfamily includes the major forms of human CES and the major isoforms of rat, dog, rabbit, and mouse CES. The CES 1H subfamily includes RL1, ML1, and hydrolase B and C, which catalyze the long-chain acyl-CoA hydrolysis. As shown in Fig. 1, several proteins of ER lumen have the common carboxyl-terminal recognition sequence HXEL-COOH which is a structural motif essential for retention of the protein in the luminal site of the ER. In a case of CES 1G family, the carboxyl terminal amino acid sequence is HTEHK-COOH, which differs from the consensus sequence of ER retention signal. Thus, the member of the CES 1G family are all the secretary type of CES. In contrast, the CES 2 family includes human intestinal CES (hCE2), rat RL4 (rCES2), rat intestinal CES, mouse ML3 (mCES2), rabbit form2, and hamster AT51, which is mainly expressed in the small intestine. CES 3 includes ES-male and human hCE3. The CES 4 families were currently identified as CES families (Miyazaki et al., 2003). CES4 families are excreted as a major urinary protein in the domestic cat. Since urinary CES is derived from the kidney proximal straight tubules, these CES isozymes are named the CAUXIN (carboxylesterase-like urinary excreted protein). The CES 5 family includes the 46.5-kDa CES isozymes, which have a different structure from those of other CES families. The 46.5 = kDa ES from mouse liver (Watanabe et al., 1993) and the amido hydrolase of monkey liver (Kusano et al., 1996) probably belong to this family. These groupings are similar to the results of phylogenetic analysis (Fig. 3).

Structure and Catalytic Mechanism of CES Isozymes

It has been shown that several proteins of ER lumen have the common carboxy-terminal sequence KDEL-COOH, and the structural motif is essential for retention of the protein in the luminal site of ER through KDEL receptor bound to ER membrane (Pelham, 1990; Tang and Kalow, 1995).

Korza and Ozols (1988) and Ozols (1989) have established the primary structures of two microsomal esterases purified from rabbit liver and designated them as 60-kDa esterase form 1 and form 2. These two forms of CES isozymes have the consensus sequence for the ER

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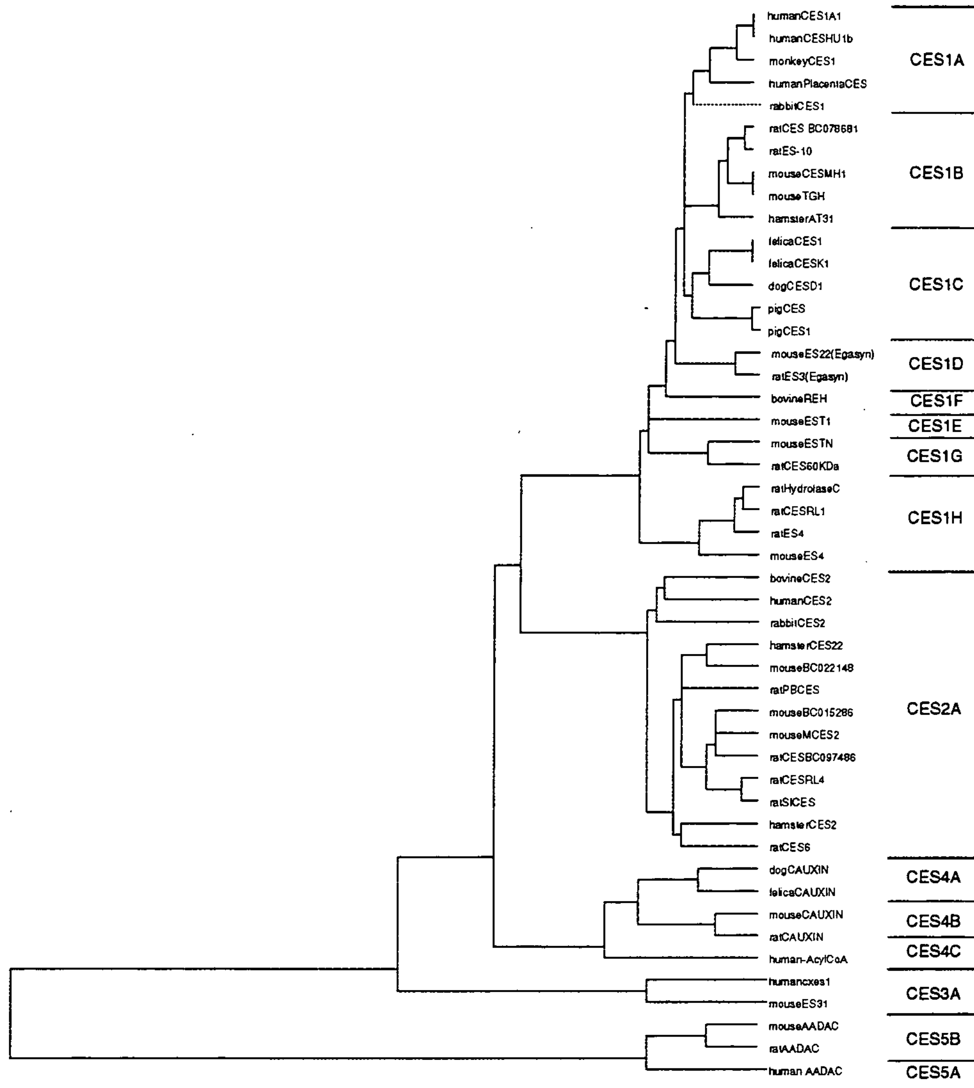

Figure 3 Phylogenetic tree of the carboxylesterase (CES) superfamily, using a simple unweighted pair-group method of analysis (UPGMA).

retention tetra-peptide that recognized with the KDEL receptor in the luminal side of ER. Robbi et al. (1990) reported cDNA cloning of rat liver pI 6.1 esterase (ES-10) and pI 5.5 esterase (ES-3, egasyn). This was the first report to show that cDNA of liver CES has the consensus sequence of the ER retention tetrapeptide (HVEL-COOH). Later, Robbi and Beaufay (1994) isolated a cDNA clone of another rat liver pI 5.5 esterase (ES-3, egasyn), which has the consensus sequence of the ER retention tetrapeptide (HTEL-COOH). In a case of mouse liver microsomal CES, the carboxyl terminal amino acid sequence of clone Es-N is HTEHK-COOH, which differs from the consensus sequence of ER retention signal. The other clone encoded egasyn, an accessory protein of beta-glucuronidase in the liver microsomes (Medda et al., 1987). Egasyn is identical to CES, and it binds beta-glucuronidase via its CES active site.

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Thus, it contains the consensus sequence of the ER retention signal (HTEL-COOH). Ovnic et al. (1991a) conducted genetic mapping and confirmed the location of an egasyn cDNA fragment in cluster 1 of the esterase region on chromosome 8. Shibata et al. (1993) found that the human *CES* gene spans approximately 30 kb and has 14 small exons. Many CES have a signal peptide of 17 to 20 amino acid residues, including hydrophobic amino acid, for retention in the lumen of the ER. In general, a bulky aromatic residue, followed by a small neutral residue, directly precedes the cleavage site (von Heijne, 1983).

Many CES have four Cys that may be involved in specific disulfide bonds. Among them, Cys98 is the most highly conserved residue in many CES isozymes. Recently, Cygler et al. (1993) reported an important alignment of a collection of related amino acid sequences of esterase, lipase, and related proteins based on X-ray structures of *Torpedo californica* acetylcholinesterase and *Geotrichum candidum* lipase. According to these authors, Ser203, Glu336, and His450 form an catalytic triad, and Gly124–Gly125 may be part of an oxyanion hole. These residues are also highly conserved among CES isozymes. Thus, we have started mutation analysis (Satoh and Hosokawa, 1998). Site specific mutagenesis for Ser203 to Thr203, Glu336 to Ala336, or His450 to Ala450, greatly reduced the CES activity toward several substrates. Therefore, the mutagenesis confirmed the role for Glu336 and His450 in forming a putative charge relay system with active site Ser203 (Satohand Hosokawa, 1998). Recently, Frey et al. (1994) investigated the formation of low barrier hydrogen bonds between His and Asp (Glu for CES) and found that it facilitates the action of nucleophilic attack by the β-OH group of Ser on the acyl carbonyl group of peptides in chymotrypsin. The catalytic triad in the tetrahedral addition intermediate is stabilized by the low barrier hydrogen bonds. According to their theory, we thought the low barrier hydrogen bond between Glu336 and His450 facilitates the action of nucleophilic attack by the β-OH group of Ser203 on the carbonyl group of substrates in CES (Fig. 4). In the tetrahedral intermediate, the formation of low barrier hydrogen bonds is between His450 and Glu336, and the transition state is stabilized by the low barrier hydrogen bonds. The low barrier hydrogen bonds facilitated a mechanism that includes weak hydrogen bonds between the tetrahedral oxyanion and peptide N-H bonds contributed by Gly123 and Gly124, which stabilize the tetrahedral adduct on the substrate side of the transition state. Formation of the acyl-enzyme complex in the next step requires removal of a proton from His450 so that the tetrahedral intermediate is disrupted in the acyl-enzyme intermediate. When the unbound portion of the alcohol group of the first product of the substrate has diffused away, a second step occurs in which the deacylation step is essentially the reverse of the acylation step, with water molecules substituting for the alchole group of the original substrate. To clarify the catalytic mechanism of CES, mutation analysis of other structural domains, such as the site of salt bridges, the substrate binding site, and the glycosylation site, would be worthwhile.

It is of interest that the sequences required for the hydrolytic capability at the catalytic triad (Glu, His, Ser) of CES, acetylcholine esterase, butyrylcholine esterase, and cholesterol esterase are highly conserved. The catalytic triad is a common structure of α, β-hydrolase-fold families, which are responsible for the hydrolysis of endogenous and exogenous compounds.

Furthermore, these amino acid residues as previously described are strongly conserved among orthologous CES from mouse, rat, rabbit, monkey, and human. A three-dimensional model for human CES has been proposed based on the crystal structure coordinates of acetylcholine esterase and overlapping active sites with pancreatic lipase (Alamet al., 2002) and CES. The modeled structure shares the overall folding and topology of the proteins identified in the recently published crystal structures of the rabbit (Bencharit et al., 2002) and human CES (Bencharit et al., 2003a; Bencharit et al., 2003b).

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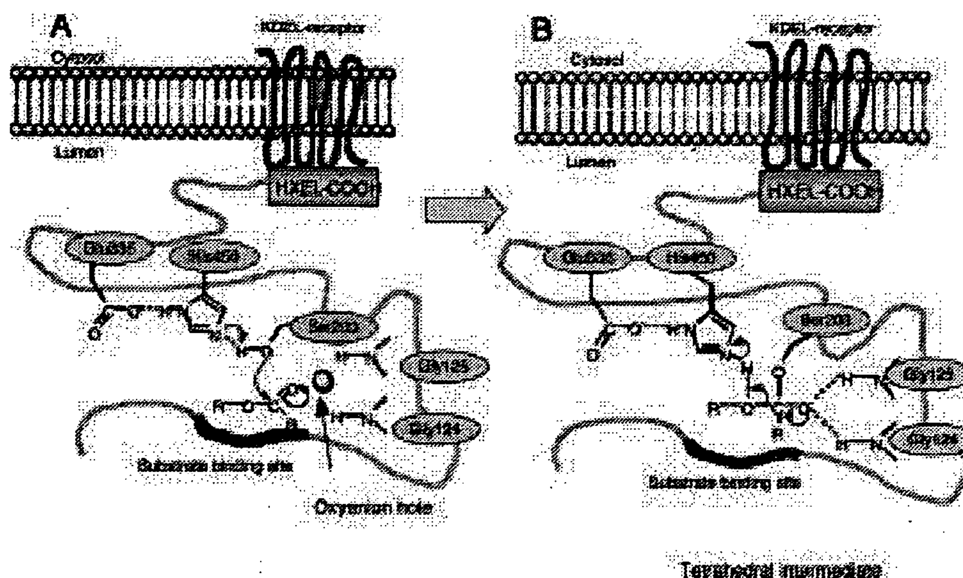

Figure 4 A proposed mechanism for the formation of the tetrahedral intermediate. (A) Hydrolysis of the substrate starts with an attack by the oxygen atom of the hydroxy group of Ser 203 on the carbonyl carbon atom of the substrate. (B) The carbon-oxygen bond of this carbonyl group becomes a single bond, and the oxygen atom acquires a net negative charge. The four atoms now bonded to the carbonyl carbon are arranged as a tetrahedron shape. This transient tetrahedral intermediate from a substrate is possibly formed by hydrogen bonds between the oxygen anion (called an oxyanion) and the main-chain NH group. This site is called an oxyanion hole.

CES has a three-dimensional α–β hydrolase fold that is a structural feature of all lipases (Wongand Schotz, 2002). In general, the structure of CES may be viewed as comprising a central catalytic domain surrounded by α–β and regulatory domains (Bencharit et al., 2002; Bencharit et al., 2003a; Bencharit et al., 2003b). In essence, the α–β hydrolase fold consists of a central β sheet surrounded by a variable number of α helices and accommodates a catalytic triad composed of Ser, His, and a carboxylic acid. The residues that compose the catalytic domain of human CES1 are very highly conserved among orthologous CES1 proteins from different species. This suggests that the catalytic function of these proteins is conserved across species. The catalytic triad is located at the bottom of a deep active site cleft approximately in the of the molecule and comprise a large flexible pocket on one side of Ser203 and a small rigid pocket on the opposite side. The orientation and location of the active site provide an ideal hydrophobic environment for the hydrolysis of a wide variety of hydrophobic substrates. The small rigid active site pocket is adjacent to the oxyanion hole formed by Gly123–124 and is lined by several hydrophobic residues (Bencharitet al., 2003a). Short acyl chains would be easily accommodated within the small rigid pocket. The larger flexible active site pocket is lined by several nonpolar residues and could accommodate larger or polycyclic molecules such as cholesterol. The large pocket is adjacent to a side door secondary pore that would permit small molecules (substrates and reaction products) to enter and exit the active site (Bencharit et al., 2003a). Longer acyl chains may be oriented for catalysis in such a way that they extend through the side door. Indeed, the presence of a hydrophobic residue at position 423 in mouse and 425 in human is necessary for efficient hydrolysis of hydrophobic substrates, because mutation of Met present in position 423 of the related rat lung CES to Ile increased the CES activity toward a more hydrophobic

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substrate without affecting activity toward short-chain esters (Wallace et al., 1999). According to the X-ray crystal structure of the human CES1, this residue lines the flexible pocket adjacent to the side door (Bencharit et al., 2003a). Given the wide range of substrates that CES are known to hydrolyze, the large flexible pocket confers the ability to hydrolyze many structurally distinct compounds, whereas the rigid pocket is much more selective with regard to the substrates that may be accommodated.

Structure-Activity Relationships of Substrates with CES1 and CES2 Families

It has been suggested that CES can be classified into five major groups according to the homology of the amino acid sequence as previously described (Satoh and Hosokawa, 1998), and the majority of CES that have been identified belong to the CES1 or CES2 family. Recent studies have shown that there are some differences between these families in terms of substrate specificity, tissue distribution, immunological properties, and gene regulation (Satoh and Hosokawa, 1998). For example, the preferential substrates for CES1 (also called hCE1, hCE, or CES HU1) (Kroetz et al., 1993; Satoh and Hosokawa, 1998), a human CES1 family isozyme, are thought to be compounds esterified by small alcohol, whereas those for hCE-2, a human CES2 family isozyme, are thought to be compounds esterified by relatively large alcohol. For drugs of abuse, heroin shows the highest rates of catalysis by both enzymes. CES1, but not CES2, hydrolyzed the methyl ester of cocaine and the ethyl esters of meperidine and delapril (Kroetz et al., 1993; Pindel et al., 1997; Takai et al., 1997; Satoh and Hosokawa, 1998; Takayama et al., 1998; Zhang et al., 1999). In contrast to the specificity of CES1 for the methyl ester of cocaine, only CES2 hydrolyzed the benzoyl ester of cocaine. For the remaining substrates that could be hydrolyzed by both enzymes, CES2 exhibited higher catalytic efficiency than CES1 for heroin; enzymatic conversion of 6-acetylmorphine to morphine was not known before the isolation and characterization of CES2 (Kamendulis et al., 1996). We reported that mouse MH1, a mouse CES1 family isozyme, hydrolyzed temocapril, which esterified a small alcohol, similar to human CES1 isozyme (Mori et al., 1999; Furihata et al., 2004a).

On the other hand, we also reported that rat rCES2 (also called CES RL4), a rat CES2 family isozyme, hydrolyzed methylprednisolone hemisuccinate (MPHS), which esterified a large alcohol as same as human CES2 family (Furihata et al., 2005). A comparison of the biochemical properties of rCES2 and those of rat CES1 isozymes shows that they are strikingly different. Regarding the preferential substrates, rat CES1 do not seem to be involved in the hydrolysis of MPHS in the rat liver, on the contrary, temocapril is a substrate for CES1 but not for rCES2. Knowledge of these substrate structure-activity relationships and the tissue distribution of CES isoenzymes is critical to predicting the metabolism and pharmacokinetics and pharamcodynamics of ester drugs or prodrugs.

Induction of CES Isozymes

Much interest has been shown by both clinicians and researchers in the induction of expression of drug-metabolizing enzymes by chemicals, including medical agents, since it is one of the main reasons for drug-drug interaction, causing adverse effects, and for the reduction in pharmacological potencies of drugs. As for CES, it has been shown that rodent CES isozymewas induced by phenobarbital (Hosokawa et al., 1987), aminopyrine (Hosokawa et al., 1988), or peroxisome proliferators (clofibrate, di (2-ethylhexyl)phthalate, and

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perfluorinated fatty acids) (Hosokawa et al., 1994; Howarthet al., 2001; Furihata et al., 2003; Furihata et al., 2004a).

The mouse is one of the most widely used experimental animals in the process of development of a drug, and several mouse CES isozymes have been identified (Hosokawa et al., 1990; Ovnic et al., 1991a; Ovnicet al., 1991b; Aida et al., 1993; Satoh and Hosokawa, 1998; Dolinsky et al., 2001; Furihata et al., 2003; Xie et al., 2003; Furihata et al., 2004a; Furihata et al., 2005). However, information on the involvement of mouse CES in drug metabolism is limited. We have reported that exposure of C57BL/6 mice to di (2-ethyl-hexyl) phthalate (DEHP), a peroxisome proliferator, in their diet resulted in a significant increase in the amount of CES protein, concomitant with an increase in the level of hydrolytic activity toward xenobiotics in mouse liver microsomes (Hosokawa et al., 1994). We have also recently shown that one of the mouse CES isozymes induced by DEHP is mCES2/microsomal acylcarnitine hydrolase, a CES2 family isozyme (Furihata et al., 2003). Our immunochemical study also suggested that mouse CES isozymes related to CES1 were induced by DEHP treatment, but they remained to be identified. More recently, we identified a mouse CES1 isozyme, mCES1, that was induced by DEHP. Purification, cDNA cloning, and baculovirus-mediated expression of mCES1 revealed that mCES1 plays an important role in temocapril metabolism and that it belongs to the CES1A subfamily. Collectively, our results showed that mCES1 is very similar to hCE-1. Therefore, mCES1 is thought to be one of the critical determinants for pharmacokinetics and pharmacodynamic actions of ester prodrugs as well as ester drugs. This work provides useful information for study of metabolism and dispositions of ester prodrugs as well as ester drugs.

Zhu et al. (2000) reported that dexamethasone caused a slight increase in human CES isozymes. Among the inducers, dexamethasone possesses a potent and interesting ability to affect CES expression in the rat liver. Hattori et al. (1992) reported that MPHS was hydrolyzed to methylprednisolone via CES in rat liver microsomes and that several clinically used glucocorticoids, including dexamethasone, caused a remarkable increase in the level of MPHS hydrolase activity. In contrast to the report of induction of CES activity, some researchers have shown that the level of microsomal *p*-nitrophenylacetate hydrolase activity was significantly decreased in rat liver microsomes. The apparent contradiction in the same animal is probably due to the different methods for determination of CES activity by different substrates. Therefore, it is hypothesized that the CES isozyme contributing to *p*-nitrophenylacetate hydrolysis in rat liver microsome is different from the one contributing to MPHS hydrolysis. It has been reported that dexamethasone decreased the expression of rat CES1 isozymes (CES RH1, also known as ES-10 or hydrolase A, and CES RL1, also known as ES-4 or hydrolase B) in the rat liver and that the decrease in the expression levels of those enzymes was linked to the reduction in the level of *p*-nitrophenylacetate hydrolase activity (Furihata et al., 2005). On the other hand, rat CES isozymes responsible for MPHS hydrolysis in rat liver microsomes have not been identified. But more recently we identified a dexamethasone-induced CES isozyme that can hydrolyze MPHS in the rat liver and plasma as a member of the CES2 family, rCES2 (Furihata et al., 2005). The different biochemical properties of rCES2 from those of known rat CES1 isozymes, including its unique existence in plasma, will be useful information for studies aimed at elucidation of functions of CES in drug metabolism. In addition, we also identified the gene encoding rCES2 by cDNA cloning and functional expression in Sf9 cells. Since we demonstrated that the level of the corresponding mRNA expression was markedly increased, the identification of the coding gene is valuable for studies aimed at elucidation of the molecular mechanisms by which dexamethasone induces rCES2 expression (Furihata et al., 2005).

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Gene Structure and Regulation of CES Isozymes

Both the murine (Satohand Hosokawa, 1998) and human (Shibata et al., 1993; Langmann et al., 1997; Satoh et al., 2002) *CES1* genes span about 30 kb and contain 14 small exons. Recently, sequencing of the mouse and human genomes was completed, enabling detailed sequence comparisons. The previously published sequences of the individual exons, splice junctions, size of the introns, and restriction sites within the murine and human *CES* genes are consistent with their respective genes sequenced by the mouse and human genome projects. Therefore, the organization of the *CES* gene is evolutionarily conserved in mice and humans. Previous studies have mapped the human carboxylesterase gene to chromosome 16 at 16q13–q22.1 (Zschunke et al., 1991; Kroetz et al., 1993). This region is syntenic to a region of mouse chromosome 8 at 8C5 (Zschunke et al., 1991). The murine CES Es22 (Ovnic et al., 1991a) and Esl (Ovnic et al., 1991b) have been previously mapped to chromosome 8. The mouse genome sequencing project unambiguously demonstrated that the murine *CES* gene was located on the minus strand of chromosome 8 at 8C5 in a cluster of six *CES* genes that span 260.6 kb in total. These six *CES* genes are presumed to have originated from repeated gene duplications of a common ancestral gene that encoded a CES (Shibata et al., 1993), and subsequent evolutionary divergence occurred.

Recent studies have shown that there are some differences between these families in terms of substrate specificity, tissue distribution, immunological properties, and gene regulation. Therefore, the 5′-flanking regions of *CES1* and *CES2* genes were isolated from mouse, rat, and human genomic DNAs by PCR amplification. Two individual mouse *CES* genes (*mCES MH1* and *mCES ML1*) (Furihata et al., 2004a) and two individual human *CES* genes (*CES HU1a* and *HU1b*) were found to belong to the CES1 family, and mouse *mCES2* (Furihata et al., 2003), rat *rCES2* (Furihata et al., 2004a) and human *CES HU3* genes were found to belong to the CES2 family. A TATA box does not precede the transcription start site of any of the *CES* promoters. *CES* promoters share several common binding sites for transcription factors among the same CES families, suggesting that orthologous *CES* genes have evolutionally conserved transcriptional regulatory patterns. Potential binding sites of *CES* promoters for transcriptional factors include Sp1, Sp3, C/EBP, USF1, NF-1, NFkB, PPAR, GR, SREBP, HNF1, HNF3, and HNF4 binding sites. In the case of human *CES1* genes, we isolated two *CES* genes encoding human CES HU1, which were tentatively designated as *CES HU1a* and *CES HU1b* (Fig. 5). These genes are identical except for exon 1 and *cis* elements. Electrophoretic mobility shift assays and reporter gene assays demonstrated that SP1, C/EBP, and NF-1 could bind to each responsive element of the *CES HU1a* promoter, but that C/EBP could not bind to responsive elements of the *CES HU1b* promoter. On the other hand, the structure of the *CES2* gene promoter was different from that of the *CES1* gene promoter (Fig. 5). Recently, we have shown tissue expression profiles of mCES2 and parts of the mechanism by which transcription of the *mCES2* gene is regulated (Furihata et al., 2004b). mCES2 is expressed in the liver, kidney, small intestine, brain, thymus, lung, adipose tissue, and testes. We have also shown that Sp1, Sp3, and USF1 contribute to synergistic transactivation of the *mCES2* promoter. Although the possibility of involvement of other transcription factors in the regulation of *mCES2* gene expression cannot be ruled out and further studies are needed to elucidate the mechanism fully, our data indicate that Sp1, Sp3, and USF1 are indispensable factors for transcription of the *mCES2* gene. More recently, we have demonstrated that HNF-4α can strongly enhance *mCES2* gene transcription and that the

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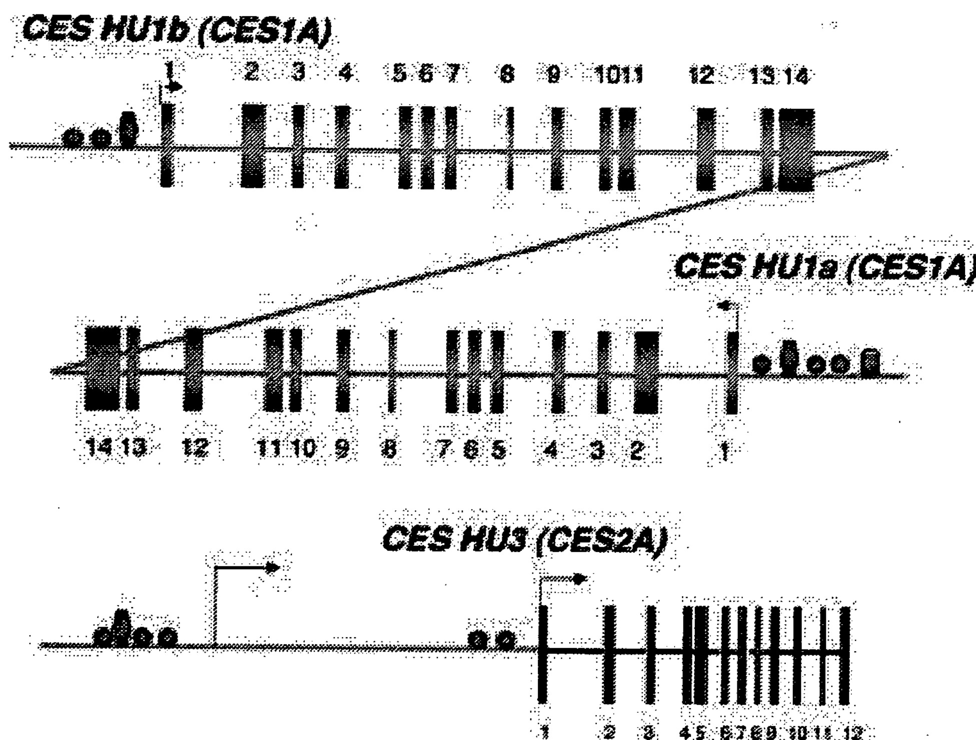

Figure 5 Gene structure and 5′ regulatory element of *CES HU1a (CES1A1), CES HU1b(CES1A2), and CES HU3(hCE2)(CES2A1)* genes.

involvement of HNF-4α accounts for the high expression level of mCES2 in the liver. These findings are notable when the physiological roles of mCES2 are studied, since HNF-4α is involved in various hepatic functions, such as glucose and cholesterol metabolism and drug metabolism. In addition, we found that bile acid can repress *mCES2* gene transcription by repressing HNF-4α-mediated transactivation. What is significant in the repression of mCES2 expression? We currently have no answer to this question. Since mCES2 can hydrolyze not only acylcarnitines, but also various mono- and diacylglycerols and acyl-CoAs, and since it is predominantly expressed in the liver, kidney, and small intestine, the repression is likely to reflect physiological functions of mCES2 in lipid metabolism in these tissues. Further detailed study aimed at elucidation of the physiological roles of mCES2 will provide important insights into lipid metabolism. The results of our current study have provided some clues for understanding the molecular mechanisms regulating *CES* gene expression and is an important step toward elucidation of physiological functions of mCES2 (Furihata et al., 2004b).

CONCLUSIONS

Multiple CES play an important role in the hydrolytic biotransformation of a vast number of structurally diverse drugs. These enzymes are a major determinant of the pharmacokinetic behavior of most therapeutic agents containing an ester or amide bond. There are several factors that influence CES activity, either directly or at the level of enzyme

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regulation. Exposure to environmental pollutants or to lipophilic drugs may cause induction of CES activity. Several drug-metabolizing enzymes, such as cytochrome P450, UGT, and sulfotransferase, have been extensively studied to clarify the substrate specificity using molecular cloning and cell expression systems. Consequently, the novel findings obtained reveal that the substrate specificity of CES is, at least in part, explained by the differences in the nucleotide sequences of the individual CES isozymes. In addition, it becomes clear that membrane-bound type CES isozymes in microsomes are required to possess the KDEL-tetrapeptide motif at the carboxy terminal of the molecule. Mammalian CES have been found to have acyl-glycerol, acyl-CoA, and acyl-carnitine hydrolyzing activities. To clarify the substrate specificity of each CES isozyme, we have begun to study for search the substrate recognition site of each isozyme. The substrate specificity of CES toward newly developed prodrugs under consideration can be examined using purified CES, mammalian cell expression systems, and specific inhibitors. However, such *in vitro* experiments may not be possible to predict *in vivo* results, except in particular cases. Therefore, we need to obtain sufficient information for different pharmocokinetic parameters of prodrugs among mammalian species. Finaly, we should clarify the inter-individual difference in human CES for the study of the prediction of pharmacodynamics.

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Regular Article

Structural Organization and Characterization of the Regulatory Element of the Human Carboxylesterase (*CES1A1* and *CES1A2*) genes

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Summary: Mammalian carboxylesterases comprise a multigene family, the gene products of which are localized in the endoplasmic reticulum. The carboxylesterases catalyze the hydrolysis of various xenobiotics and endogenous substrates such as ester, amide and thioester bonds and are thought to function mainly in drug metabolism. We have suggested the possibility that individual variation of human liver carboxylesterase activity causes the difference in expression levels of CES1A isozymes. However, little is known about the transcriptional regulation of human carboxylesterase genes. In the present study, we isolated two CES genes encoding human carboxylesterase CES1A, which were designated as *CES1A1* (AB119997) and *CES1A2* (AB119998). These genes were identical except for exon 1 and the 5′ regulatory element. We investigated the transcriptional regulation of these two CES genes. A reporter gene assay and electrophoretic mobility shift assay demonstrated that Sp1 and C/EBPα could bind to each responsive element of the *CES1A1* promoter but that the Sp1 and C/EBP could not bind to the responsive element of the *CES1A2* promoter. Thus, CES1A1 mRNA expression level is much higher than the expression level of CES1A2 mRNA in the liver and lung. It is thought that these results provide information on individual variation of human carboxylesterase isozymes.

Keywords: CES1A1, CES1A2; carboxylesterase, Sp1, C/EBP, nuclear receptor

Introduction

Mammalian carboxylesterases (EC 3.1.1.1) comprise a multigene family whose gene products are localized in the endoplasmic reticulum (ER) of many tissues. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs (including prodrugs) to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, environmental toxicants and carcinogens. Carboxylesterases also catalyze the hydrolysis of endogenous compounds such as short-chain and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters. We have reviewed the characteristics of carboxylesterases in relation to the metabolism of xenobiotics.[1] Multiple isozymes of hepatic microsomal carboxylesterase exist in various animal species.[1-5]

Mammalian carboxylesterases are members of the α,β-hydrolase-fold family and are found in various mammals.[1,5,6] The expression of carboxylesterases is ubiquitous, with high levels in the liver, small intestine, kidney, and lung. Carboxylesterases show such a broad range of substrate specificity that they can be involved in detoxification or biotransformation of many kinds of drugs or prodrugs as well as endogenous fatty acid esters. It has been suggested that carboxylesterases can be classified into four major groups according to the homology of the amino acid sequence,[1,5,6] and the majority of carboxylesterases that have been identified belong to the CES1 or CES2 family. It has also been shown that striking species

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differences exist.[6–11] For example, Inoue et al.[10] showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in a disc electrophoresis coupled with staining of esterase activity. On the other hand, esterase activities were observed in the intestines of other species (human, rat, mouse, guinea pig and rabbit). Since pharmacokinetic and pharmacological data of ester-prodrugs obtained from preclinical experiments using various animals are generally used as references for human studies, it is important to clarify the biochemical properties of each carboxylesterase isozyme such as substrate specificity, tissue distribution and transcriptional regulation.

Recently, we have identified a mouse liver microsomal acylcarnitine hydrolase, mCES2, as a member of the carboxylesterase 2 family.[12] It has been revealed that this enzyme is significantly induced by di(2-ethylhexyl)phthalate and shows medium- and long-chain acylcarnitine hydrolase activity. In addition, we have found that mCES2 is expressed in various tissues with higher levels of expression in the liver, kidney and small intestine.[12] It was shown that three transcription factors, specificity protein (Sp) 1, Sp3 and upstream stimulatory factor 1, could bind to the promoter region of the *mCES2* gene, leading to a synergistic transactivation of the promoter. Although this mechanism may explain the ubiquitous tissue expression profiles of mCES2, it is unlikely to contribute to the higher levels of mCES2 expression in the liver, kidney and small intestine. Therefore, it is thought that there exists another mechanism controlling this tissue-specific transcription of the *mCES2* gene. More recently, we have shown that hepatocyte nuclear factor-4alpha (HNF-4α) can strongly enhance *mCES2* gene transcription and that the involvement of HNF-4α accounts for the high expression level of mCES2 in the liver.[13] These findings are notable when physiological roles of mCES2 are studied, since HNF-4α is involved in various hepatic functions, such as glucose and cholesterol metabolism and drug metabolism. In addition, we found that bile acid can repress *mCES2* gene transcription by repressing HNF-4α-mediated transactivation.[13]

We have already suggested the possibility that individual variation of human liver carboxylesterase activity causes the difference in expression levels of CES1A isozymes.[2] However, little is known about the transcriptional regulation of human carboxylesterase genes. In the present study, we isolated two CES genes encoding human carboxylesterase CES1A, which were designated as *CES1A1* and *CES1A2*. These genes were identical except for exon 1 and the 5′ regulatory element. We investigated the transcriptional regulation of these two CES genes. A reporter gene assay and electrophoretic mobility shift assay demonstrated that Sp1 and CCAAT/enhancer binding protein(C/EBP) could bind to each responsive element of the *CES1A1* promoter but that the Sp1 and C/EBP could not bind to the responsive element of *CES1A2* promoter. Thus, CES1A1 mRNA expression level is much higher than the expression level of CES1A2 mRNA in the liver, kidney, testis, lung, small intestine, brain and heart. It is thought that these results provide information on the individual variation of human carboxylesterase isozymes.

Materials and Methods

Genomic DNA isolation

Genomic DNA was isolated from Human Genomic Library HL1067j (Clontech Laboratories, Palo Alto CA, U.S.A.) by screening with a CES1A1 cDNA probe. K802 cells were infected with EMBLE3 SP6/T7 and incubated in 37°C. The plaques were transferred to a Hybond-N+ Nylon Membrane (GE Healthcare, Buckinghamshire, England) followed by fixing with 0.05 M NaOH. Positive clones were detected by an ECL direct nucleic acid labeling and detection system, and genomic DNA in the clones was extracted. Polymerase chain reaction (PCR) was performed with the extracted DNA and a set of primers, CES1A1(−953/−934)F and CES1A1(+46/+65)R (**Table 1**). The product was cloned into the pTarget vector (Promega, Madison, WI, U.S.A.) and then transferred to the pGL3-basic vector (Promega) with Kpn I and Sma I digestion. This genomic clone is referred to as p1A1(−896/+122). Similarly, the other genomic DNA clone was obtained with primers CES1A2(−949/−930)F and CES1A2(+46/+65)R (**Table 1**). The product subcloned into the pGL3-basic vector is referred as p1A2(-892/+122). The procedure used for DNA sequencing was described previously.[14]

Determination of the transcription start sites of the two *CES1A* genes

RNA ligase-mediated and oligocapping rapid amplification of 5′-cDNA ends (RLM 5′-RACE) was performed to determine the transcription start site of the two *CES1A* genes by using a GeneRacer kit (Invitrogen, Carlsbad, CA, U.S.A.) as previously described.[15] The gene-specific antisense primers are shown in **Table 1**. Products of RACE were subcloned into the pCR-Blunt II TOPO vector (Invitrogen) and sequenced by using a Dye Terminator Cycle Sequencing-Quick Start Kit and a CES 2000 DNA Analysis System (Beckman Coulter, Fullerton, CA, U.S.A.).

Construction of reporter plasmids

Deletion reporter plasmids of the *CES1A1* gene promoter were constructed with the pGL3-basic vector and a series of sense primers, CES1A1 (−953/−934)F, CES1A1 (−681/−662)F, CES1A1 (−432/−413)F, CES1A1 (−300/−279)F, CES1A1 (−218/−198)F, and CES1A1 (−112/−93)F (**Table 1**). In the same way, deletion reporter plasmids of the *CES1A2* gene promoter were constructed with a series of sense primers, CES1A2 (−949/−930)F, CES1A2 (−678/−659)F, CES1A2 (−432/−413)F, CES1A2 (−300/−281)F, CES1A2 (−217/−197)F, and CES1A2 (−111/−92)F (**Table 1**).

To obtain a reporter plasmid harboring mutations, site-directed mutagenesis was performed by the methods described previously.[15] The primers for the plasmid with mutations in a C/EBP binding site were CES1A1-C/EBPmutF and CES1A1-C/EBPmutR (**Table 1**). The primers for the plasmid with mutations in an SP1-A binding site were CES1A1-SP1AmutF, CES1A1-SP1AmutR, CES1A2-SP1mutF and CES1A2-SP1mutR,

Table 1. Primers and probes used for cloning, sequencing and mRNA expression analysis

(For sequencing)		(For site-directed mutagenesis)	
CES1A1(−300/−279)F	5′-TGA ACC CTT ATG TAA CAA GTA G-33′	CES1A1-C/EBPmutF	5′-TGT AAC AAG TAG AGA TCT AAG TTT ACA G-3′
CES1A1(+365/+384)R	5′-TCT CCG TGA TCC AGC CGT AA-3′	CES1A1-C/EBPmutR	5′-CTG TAA ACT TAG ATC TCT ACT TGT TAC A-3′
CES1A1(−111/−93)F	5′-TGG CAG CGC AGG GCC GTA AC-33′	CES1A1-SP1AmutF	5′-TAA ACT GTG GGT AGA TCT GGC CTG AGG CCC-3′
CES1A1(+246/+265)R	5′-GGG CGA TCT CAG GAT GTT CA-33′	CES1A1 SP1AmutR	5′-GGG CCT CAG GCC AGA TCT ACC CAC AGT TTA-3′
CES1A2(−300/−281)F	5′-TGA CAC CGT TAT GCC ACA AG-3′	CES1A1-SP1BmutF	5′-CGG TAA CTC TAG ATC GGG CTG GGC TCC-3′
CES1A2(+373/+396)R	5′-TCT CCT GAG CCG CTA TCC GTT ATC-3′	CES1A1-SP1BmutR	5′-GGA GCC CAG CCC GAT CTA GAG TTA CCG-3′
CES1A2(−111/−93)F	5′-TGG CAG TGC AGG GCG GTA AC-33′	CES1A2-SP1mutF	5′-TAA ACT GTG AGT AGA TCT GGC TTG AGG CCC-3′
CES1A2(+302/+321)R	5′-TCT TGT GTG CGG AGT GAG GT-3′	CES1A2-SP1mutR	5′-GGG CCT CAA GCC AGA TCT ACT CAC AGT TTA-3′
(For cDNA cloning)		(For gel mobility shift assay)	
CES1A1cDNA-F	5′-GAG ACC TCG CAG GCC CCG A-3′	CES1A1(−293/−264)GM	5′-TTA TGT AAC AAG TAG TTG GGC AAG TTT ACA-3′
CES1A2cDNA-F	5′-GAG ACC TCG CAG GCC CCC G-3′	CES1A2(−292/−263)GM	5′-TTA TGC CAC AAG CAG TTG GGC GAG TTT ACA-33′
CES1AcDNA-R	5′-AAC CTG CAA TCC CTT TCG CAA-3′	CES1A1(−211/−182)GM	5′-ACT GTG GGT GGG CGT GGC CTG AGG CCC CAC-3′
(For reporter gene deletion analysis)		CES1A2(−210/−181)GM	5′-ACT GTG AGT GGG CGT GGC TTG AGG CCC CAC-3′
CES1A1(−953/−934)F	5′-CCC AGC AGC TTG TAA ATG AC-3′	CES1A1(−96/−73)GM	5′-TAA CTC TGG GCG GGG CTG GGC TCC-3′
CES1A1(−681/−662)F	5′-GGA CGG AGT TCA TTT TTA CA-3′	CES1A2(−95/−73)GM	5′-TAA CTG GGG GCC AGG GTG GCG CC-3′
CES1A1(−432/−413)F	5′-GAG TGG CTC TAA CAT TTT CC-3′	SP1	5′-ATT CGA TCG GGG CGG GGC GAG C-3′
CES1A1(−300/−279)F	5′-TGA ACC CTT ATG TAA CAA GTA G-3′	SP1mut	5′-ATT CGA TCG GTT CGG GGC GAG C-3′
CES1A1(−218/−198)F	5′-AGG GTA AAC TGT GGG TGG GCG-3′	C/EBP	5′-TGC AGA TTG CGC AAT CTG CA-3′
CES1A1(−112/−93)F	5′-TGG CAG CGC AGG GCG GTA AC-3′	C/EBPmut	5′-TGC AGA GAC TAG TCT CTG CA-3′
CES1A1(+46/+65)R	5′-CAG AAG GAC TCA CCC CAA GC-3′	(For real-time PCR)	
CES1A2(−949/−930)F	5′-CCC AGC AGC TTG TAA ATG AC-3′	CES1A-RT-F	5′-GAG ACC TCG CAG GCC CC-3′
CES1A2(−678/−659)F	5′-GAG ATG GAA TCA TTT TTA CA-3′	CES1A-RT-R	5′-GAC GAA CTT CCC CAG CAC TT-3′
CES1A2(−432/−413)F	5′-GAG TGG CTC TAA CAT TTT CC-3′	CES1A1-RT-Taq	5′-(FAM)-TCC GTG CCT TTA TC-3′
CES1A2(−300/−281)F	5′-TGA CAC CGT TAT GCC ACA AG-3′	CES1A2-RT-Taq1	5′-(FAM)-CTC CCT GCT CTT GTC-3′
CES1A2(−217/−197)F	5′-AGG GTA AAC TGT GAG TGG GCG-3′	CES1A2-RT-Taq2	5′-(VIC)-CTC CCT GCT CTT GTC-3′
CES1A2(−111/−92)F	5′-TGG CAG TGC AGG GCG GTA AC-3′		
CES1A2(+46/+65)R	5′-CAG AAG GAC TCA CCC CAA GC-3′		

and those for the plasmid with mutations in an SP1-B site were CES1A1-SP1BmutF and CES1A1-SP1BmutR (**Table 1**).

An Sp1 mammalian expression plasmid, pCMVSp1, and an Sp3 *Drosophila* expression vector, pPacUSp3, were generous gifts from Dr. Robert Tjian (University of California, Berkeley, Berkeley, U.S.A.) and Dr. Guntram Suske (Philipps-Universitat Marburg, Marburg, Germany), respectively. The procedures for construction of an Sp1 *Drosophila* expression plasmid, pAcSP1, were previously described.[15] C/EBPα and C/EBPβ mammalian expression plasmids, pMSV/EBPα and pMSV/EBPβ were generous gifts from Dr. Steven L. McKnight (University of Texas South-Western Medical Center at Dallas, Texas, U.S.A.). C/EBP cDNAs were subcloned into the pTarget vector and are named pC/EBPα or pC/EBPβ.

Cell culture, transient transfection and luciferase assay

FLC7 cells, human hepatoma cells, were kindly provided by Dr. Seishi Nagamori (Kyorin University, Tokyo, Japan) and were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium/F-12 (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin. Transient transfection to FLC7 cells was performed by using a Trans IT (Mirus Bio, Madison, WI, USA) as described previously[13] with 200 ng/well of the reporter plasmid and 4 ng/well of phRL-TK (Promega). Either a C/EBP expression plasmid (pC/EBPα or pC/EBPβ) or pTarget empty plasmid (50 ng/well) was added to the transfection mixture in a cotransfection assay. After 24 h of incubation, the cells were harvested and the luminescence was determined as described previously.[13]

Drosophila SL2 cells, purchased from American Type Culture Collection (Rockville, MD, U.S.A.), were grown at 24°C without CO_2 in *Drosophila* Schneider's medium (Invitrogen) supplemented with 10% fetal bovine serum and 50 units/mL penicillin and with 50 μg/mL streptomycin. Transient transfection to SL2 cells was performed with 150 ng of the reporter plasmid and various amounts of a mixture of an expression plasmid (pAcSp1 and/or pPacUSp3) using Cellfectin reagent (Invitrogen) as described previously.[15] The total amount of DNA of the mixture was adjusted to 300 ng by adding an empty pAc5.1/V5-His vector (Invitrogen). Complete growth medium was added to each well 5 h after transfection. The luciferase activity was determined using the same system as that described above with normalization by total protein amount. The protein concentration was determined by using a Bio-Rad *Dc* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Experiments were performed in duplicate, and each value presented is the mean (±S.D) from three independent assays. Student's *t*-test was performed to determine significance of the difference between groups in mutation analysis.

Preparation of nuclear extracts from cells and gel mobility shift assay (GMSA)

Nuclear extracts were prepared from the FLC7 cells by using a CelLytic Nuclear Extraction Kit (Sigma-Aldrich Co., St. Louis, MO., U.S.A.) as described previously.[15] The nuclear extracts were stored at −80°C until use. Probe double-stranded (ds) DNA was generated by annealing the complementary oligonucleotides listed in **Table 1**. Consensus and mutated consensus oligonucleotides for the Sp1 and CAAT/enhancer binding proteins (C/EBP) were designed by the directions of Santa Cruz Biotechnology (Santa Cruz, CA., U.S.A.). dsDNA

Table 2. Quantitation of CES1A1 and CES1A2 mRNA expression levels in 20 human liver samples (HHL)

Sample	CES1A1 (× 10^4 copies/μg total RNA)	CES1A2 (× 10^4 copies/μg total RNA)
HHL1	7.04	ND
HHL3	19.0	3.91
HHL6	63.6	12.5
HHL8	90.0	0.318
HHL9	32.1	ND
HHL11	96.1	ND
HHL12	39.8	0.048
HHL13	23.1	ND
HHL14	26.8	4.58
HHL16	5.61	1.15
HHL17	102	0.018
HHL24	8.82	1.56
HHL25	86.9	0.005
HHL26	199	ND
HHL27	13.6	1.65
HHL28	51.6	ND
HHL30	1.13	0.222
HHL32	10.2	ND
HHL33	1.11	ND
HHL34	54.7	7.59

ND, not detected

was end-labeled with [γ-^{32}P]dATP (Amersham Pharmacia Biotech) using T4-polynucleotide kinase (Promega). The methods used for GMSA were described in detail in our previous report.[15] Samples were held for a further 20 min at room temperature with the probe, and the mixtures were electrophoresed with 6% polyacrylamide gel in 0.5 × 45 mM Tris-borate buffer with 1 mM EDTA at 4°C. Gels were dried and autoradiographed by BASS 2000 II (Fuji Film, Tokyo, Japan).

Competition experiments were carried out as described above except that cold competitive dsDNA (**Table 1**) was added to the binding reaction mixture at a 50-fold excess of the probe amount before addition of the probe. For supershift assays, 2 μg of antibodies against Sp1 (sc-59x, Santa Cruz Biotech) was added to the binding reaction mixture at room temperature for 30 min prior to addition of the probe.

Preparation of total RNA, cDNA, microsomes, and cytosol of human liver

Caucasian liver tissues (HHL, **Table 2**) were obtained from the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Tokyo, Japan). These liver tissues had been rejected for liver transplantation. All of the patients were negative for hepatitis B virus and hepatitis C virus. The liver tissues were snap-frozen in liquid nitrogen and stored at −80°C until used. Full permission was obtained from the Ethics Committee of Chiba University, Japan, based on the Helsinki declaration.

Total RNA was isolated from liver tissue by using TRIzol reagent (Invitrogen) as described previously.[12] After confirming no genomic DNA contamination, total RNA was used for cDNA synthesis with Ready-To-Go RT-PCR Beads and random hexamer (GE Healthcare).

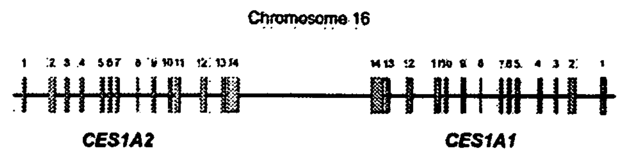

Fig. 1. Illustration of the genomic structures of *CES1A* genes Both *CES1A1* and *CES1A2* are located on chromosome 16 with a tail-to-tail structure. The numbers and lengths of introns and exons in the two genes. The two genes share a common intergenic region spanning 9 kbp and have their own regulatory sequences. Boxes indicate exons and the horizontal line indicates the genomic DNA with the direction from left to right set to the sense strand. The numbers indicate exon numbers.

Quantitative real-time PCR

Quantification of the expression levels of CES1A1 mRNA and CES1A2 mRNA in human liver was performed by using an ABI PRISM 7000 (Applied Biosystems, Foster, CA, U.S.A) with specific primers and fluorescent probes for each gene (**Table 1**). These primers and probes were custom-made (Applied Biosystems). Standard curves for CES1A1 and CES1A2 expression levels were prepared by using CES1A1 cDNA and CES1A2 cDNA, respectively, with stepwise concentration points (from 5 × 10^2 copies to 5 × 10^6 copies).

Results

Molecular cloning of the *CES1A1* and *CES1A2* genes from a human genome library and human genome

To clarify the regulation mechanism of human CES isozymes, the genome DNA and 5′- flanking region of CES1A1 was screened with human genomic library HL1067j (Clonteck) using a CES1A1-specific probe by plaque hybridization. From 1.3 × 10^5 plaques tested, 7 positive clones were isolated, and the genome DNA insert was subcloned into the pGEM plasmid and sequenced. These clones contained DNA inserts of 0.9–6.0 Kb. Among them, a relatively long clone designated as *CES H2B* showed a sequence similar to that of exon1 of CES1A1 cDNA. However, the exon1 sequence of *CES H2B* was not completely identical to CES1A1 cDNA (AB 119995). Therefore, to obtain a completely identical sequence of the human *CES1A1* gene, PCR was performed with the human genome DNA from human liver, and a final PCR product designated as *CES GHL3* was isolated. The open reading frame of *CES GHL3* was completely identical to CES1A1 cDNA. When the nucleotide sequence of the CES clone, as described above, was compared to the results of a genome project, complete *CES* gene was obtained. The open reading frame of *CES H2B* was completely identical to CES1A2 cDNA (AB119996). Therefore, the two complete CES genes were designated *CES1A1* gene (AB119997) and *CES1A2* gene (AB119998), respectively, corresponding to *CES GHL3* and *CES H2B*. It is noteworthy that both the *CES1A1* gene and *CES1A2* gene are located on chromosome 16q13-q22 with a tail-to-tail structure (**Fig. 1**). Both genes span about 30 kb and consist of 14 exons of 39 to 379 bp in length. A comparison of the nucleotide se-