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Isolation and Characterization of a cDNA Encoding a Horse Liver Butyrylcholinesterase

EVIDENCE FOR CPT-11 DRUG ACTIVATION

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ABSTRACT. Butyrylcholinesterases (BuChEs; acylcholine acylhydrolase; EC 3.1.1.8) have been demonstrated to convert the anticancer agent CPT-11 (irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) into its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin). In addition, significant differences in the extent of drug metabolism have been observed with BuChEs derived from different species. In an attempt to understand these differences, we have isolated the cDNA encoding a horse BuChE. Based upon the NH_2-terminal amino acid sequence of a purified horse BuChE, we designed degenerate primers to amplify the coding sequence from horse liver cDNA. Following polymerase chain reaction and rapid amplification of the cDNA ends, we generated an 1850-bp DNA fragment, containing an 1806-bp open reading frame. The cDNA encodes a protein of 602 amino acid residues, including a 28-amino-acid NH_2-terminal signal peptide. Furthermore, the DNA sequence and the deduced amino acid sequence revealed extensive homology to butyrylcholinesterase genes from several other species. *In vitro* transcription–translation of the cDNA produced a 66-kDa protein, identical to the size of native horse serum BuChE following removal of carbohydrate residues with endoglycosidase F. Additionally, transient expression of the cDNA in Cos-7 cells yielded extracts that exhibited cholinesterase activity and demonstrated a K_m value for butyrylthiocholine of 106 ± 9 nM. This extract converted the anticancer drug CPT-11 into SN-38, demonstrating that this drug can be activated by enzymes other than carboxylesterases. BIOCHEM PHARMACOL **59**;7:773–781, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. butyrylcholinesterase; CPT-11; SN-38; topoisomerase; drug metabolism

CPT-11 (irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) is a widely used chemotherapeutic prodrug that is cleaved by esterases to produce the potent topoisomerase I poison SN-38 (7-ethyl-10-hydroxycamptothecin; [1]). However, the enzyme that efficiently activates CPT-11 in humans has not been identified. Thus, the isolation and characterization of esterases that can cleave CPT-11 and generate SN-38 with high efficiency are of interest to both clinicians and pharmacokineticists involved in patient studies with this agent.

Previous reports have indicated that CEs† are responsible for the cleavage of CPT-11 [2]. Thus far, CEs from rabbit, pig [3], and human [4] liver have been shown to metabolize CPT-11. In a direct comparison, the rabbit CE enzyme was found to be at least 100-fold more efficient than a human CE at converting CPT-11 to SN-38 *in vitro* [5]. Furthermore, when expressed in human tumor cell lines, the rabbit CE increased the drug sensitivity 8- to 50-fold as compared with cells expressing a human CE. These observations indicate that enzymes from other species demonstrate significantly higher levels of drug activation than their human counterpart. Therefore, studying the structure and function of related proteins from several different organisms might provide essential information about the mechanism and specificity of drug activation.

Mouse plasma is very proficient at converting CPT-11 to SN-38.‡ Since mouse plasma contains high levels of several different esterases, the possibility exists that CPT-11 is metabolized by enzymes other than CEs. This idea was substantiated by our report that BuChE from humans, horse, and mouse can convert CPT-11 to SN-38 *in vitro* [6]. We also demonstrated that purified horse BuChE was ~200-fold more efficient than its human counterpart. This is the first report indicating that enzymes other than CE may be involved in CPT-11 activation. Based on these results, we believe that BuChEs from several different species can be used for structure–function comparisons in

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† *Abbreviations*: BCHE, butyrylcholinesterase gene; BuChE, butyrylcholinesterase enzyme; BuSCh, butyrylthiocholine; CE, carboxylesterase; endo F, endoglycosidase F; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; knt, kilonucleotide; ORF, open reading frame; PCR, polymerase chain reaction; and RACE, rapid amplification of cDNA ends.

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‡Potter PM, unpublished data.

an effort to identify the specific amino acids responsible for the recognition and activation of CPT-11.

Since a commercially available, purified horse BuChE demonstrates CPT-11 activation *in vitro* [6], we decided to isolate the cDNA encoding this enzyme. Here we report on the isolation and characterization of a horse liver cDNA that encodes an enzyme with cholinesterase activity, and activates CPT-11 when expressed in mammalian cells.

MATERIALS AND METHODS

Enzymes, Drugs, and Cell Lines

Horse BuChE (Cat. No. C1057) was purchased from the Sigma Chemical Co., and endo F was obtained from Boehringer Mannheim. CPT-11 was provided by Dr. J. P. McGovren (Pharmacia & Upjohn) and dissolved in methanol. Cos-7 cells were obtained from the American Type Culture Collection, and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 2 mM glutamine in an atmosphere of 10% CO_2 at 37°. Cells were checked routinely for mycoplasma contamination.

Protein Sequencing

Proteins were separated by SDS–PAGE, and NH_2-terminal amino acid sequence analysis was performed as described previously [3].

RNA and cDNA Preparations

Total RNA was isolated from horse liver using RNAzol B (TEL-TEST, Inc.). This was used to prepare cDNA with the cDNA cycle kit and oligo dT primers (Invitrogen) or to generate poly(A)$^+$ mRNA using the mRNA Separator Kit (Clontech). For RACE analysis, cDNA was prepared from poly(A)$^+$ mRNA with the Marathon cDNA Amplification Kit (Clontech).

Oligonucleotide Synthesis

Oligonucleotides were prepared on a Perkin-Elmer Corp./Applied Biosystems, Inc. (PE/ABI) 3948 DNA synthesizer and then purified.

PCR Reactions

PCR reactions were performed in a final volume of 50 μL containing 20–50 ng of DNA template, a 200 μM concentration of each dNTP, a 800 nM concentration of each primer, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). The full-length horse liver BCHE cDNA was amplified using Advantage-HF polymerase (Clontech).

Plasmid Constructions

PCR products were ligated into pCRII-TOPO or pCR-BluntII-TOPO vectors according to the manufacturer's instructions (Invitrogen). The full-length horse BCHE cDNA flanked by *Not*I restriction sites was ligated into the mammalian expression vector pCIneo (Promega) and the *in vitro* transcription vector pCITE-4b(+) (Novagen). pCI-horse and pCITEhorse contained the horse BCHE cDNA in the correct orientation for transcription and translation. All plasmids were propagated in the *Escherichia coli* strain Top10.

DNA Sequencing

DNA sequencing was performed using an automated dye termination protocol, and data were determined by a PE/ABI 373 sequencer. Sequence analysis was performed using the GCG suite of programs on a Dec Alpha Server 8200 [7].

Cos-7 Transfections

For transient transfection assays, 10^7 Cos-7 cells were electroporated in 200 μL of PBS with 20 μg of plasmid DNA using a Bio-Rad electroporator and capacitance extender (Bio-Rad). Optimal transfection efficiencies were achieved using 260 V and 960 μF. Following transfection, cells were plated in 75-cm^2 flasks in fresh DMEM containing 10% heat-inactivated fetal bovine serum and 2 mM glutamine. After 48 hr, the medium was removed and saved, and the cells were harvested by trypsinization.

Northern Analysis

Total RNA was separated in 17% formaldehyde-1% agarose gels and transferred to Hybond N$^+$ membrane (Amersham) as previously described [8]. The horse BCHE transcript was identified by hybridization with a 425-bp [^{32}P]dCTP-labeled PCR fragment from the horse BCHE cDNA using ExpressHyb (Clontech). The integrity of the RNA was confirmed by hybridization with a 500-bp fragment of the human G3PDH cDNA.

In Vitro *Transcription and Translation*

In vitro transcription and translation reactions were performed using the Single Tube Protein System (Novagen) containing [^{35}S]methionine (Amersham). Equal amounts of radioactivity from each sample were separated on an 8% SDS–polyacrylamide gel and visualized by autoradiography.

Endo F Digestion

Horse BuChE (3 μg) was denatured by heating to 100° in 1 M β-mercaptoethanol containing 0.1% (w/v) SDS for 15 min. Following chromatography through a Micro Bio-Spin

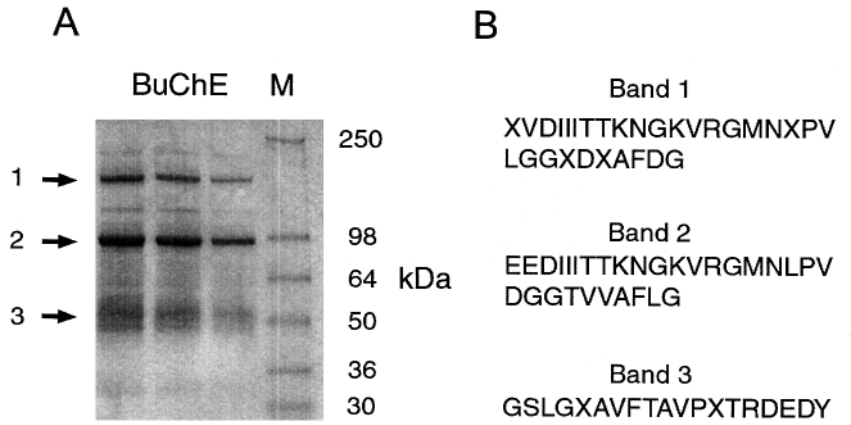

FIG. 1. Sequence analysis of the NH_2 terminus of the horse BuChE. (A) SDS–PAGE analysis of the commercially available horse BuChE. (B) NH_2-terminal amino acid sequences of the proteins indicated by the arrows in A.

6 column (Bio-Rad), the sample was made up to 50 mM potassium acetate (pH 5.5), and 0.5% (v/v) Triton X-100 and 4 mU of endo F were added. After incubation at 37° overnight, the reaction was terminated by the addition of SDS–PAGE loading buffer, and samples were subjected to electrophoresis. Gels were stained using a Colloidal blue staining kit (Novex).

Cholinesterase Assays

Cholinesterase assays were carried out in quadruplicate for each sample, using 4 mM propionylthiocholine as a substrate in the presence of 0.25 mM 5,5′-dithiobis-2-nitrobenzoic acid. Product formation was monitored spectrophotometrically by measuring absorbance at 405 nm at 15-sec intervals for 2 min. Cholinesterase activity was calculated from the rate of change of absorbance and the ϵ_m for 5-thio-2-nitrobenzoic acid (1.36×10^4). Protein concentrations of cell extracts were determined using the Bio-Rad Protein Assay reagent (Bio-Rad) with bovine serum albumin as a standard.

BuSCh Kinetics

K_m values for BuSCh were assessed using the spectrophotometric assay described above except that propionylthiocholine was replaced by different concentrations of BuSCh. Data were fitted to a one-site binding hyperbolic non-linear regression using the GraphPad Prism program.

CPT-11 Conversion Assays

Cell extracts were prepared by sonication in 50 mM HEPES buffer (pH 7.4) on ice. Appropriate amounts of extracts were incubated with 25 μM CPT-11 in a final volume of 200 μL of 50 mM HEPES (pH 7.4) at 37° for 24 hr. Reactions were terminated by the addition of 200 μL of cold acid-methanol and centrifuged for 15 min at 16,000 *g*. The conversion of CPT-11 to SN-38 was monitored by HPLC in 20-μL sample volumes as previously described [5, 9].

RESULTS

Amino Acid Sequence Analysis of a Commercially Available Horse BuChE

To determine the N-terminal amino acid sequence of the horse BuChE, proteins were separated by SDS–PAGE and transferred to Immobilon-P membrane by electroblotting. Following staining of the membrane with Coomassie Blue, two major and several minor proteins were visible (Fig. 1A). Proteins of molecular masses of ~200, 95, and 55 kDa were subjected to N-terminal amino acid analysis.

Figure 1B indicates the derived amino acid sequence for the proteins present within the horse BuChE. The samples representing the 200- and 95-kDa bands generated very similar sequences, with 22 identical matches out of 30 residues. We presumed, therefore, that the 200-kDa species represented a dimer of the 95-kDa protein. Additionally, BLAST searches of the SwissProt and TREMBL databases with either of these sequences demonstrated that the greatest homology was identified in BCHEs derived from rabbit (P21927), human (P06276), and mouse (Q03311). In contrast, data derived from the 55-kDa protein did not match any sequences within the protein databanks and currently is unknown.

Isolation of a Horse Liver BuChE cDNA

From the derived amino acid sequence of the 200- and 95-kDa proteins, degenerate oligonucleotide primers were designed that could encode residues 9–17 and 21–28 (Fig. 1B). The sequence of the C-terminal primer was based on the conserved amino acid residues (NQFNDYTSKKESC) present at positions 559–571 in BuChEs from human, cat, and mouse. The oligonucleotide primer sequences are shown in Table 1. cDNA prepared from horse liver total RNA was used as a template in PCR reactions with primer

TABLE 1. Nucleotide sequences of the oligonucleotide primers used in the amplification of the horse butyrylcholinesterase cDNA

Oligonucleotide	Sequence 5′–3′
N1a	AA(G/A)AA(C/T)GGIAA(G/A)GTIAG(A/G)GGIATGAA
N1b	AA(G/A)AA(C/T)GGIAA(G/A)GTICGIGGIATGAA
N2a	GA(C/T)GGIGGIACIGTIGTIGCITT
N3	GT(A/G)TA(A/G)TC(G/A)TT(G/A)AA(T/C)TG(G/A)TT
horse5	GTGTTACATGATTTCCACTCCTTGC
horse3	GGTACACACGCGCCGTCTTTG
horseNK	ACTGAAGCTTGCGGCCGCCACCATGCAGAGCTGGGGTACAATC
horseC	CGTGCGGCCGCAAGCTTCTTCAAAAATCTGAACAG

combinations N1a-N3, N1b-N3, and N2a-N3. Amplification conditions were: 45 sec of denaturation at 95°, 1 min of annealing at 48°, and 2 min of polymerization at 72° for 45 cycles. Using the primer combination N1b-N3, a 1.7-kb PCR product was identified by agarose gel electrophoresis.

The DNA sequence and the derived amino acid sequence of the PCR product revealed significant homology to several vertebrate BCHEs reported in the GenBank and SwissProt databases. The sequence alignment with BCHEs from human (P06276), mouse (Q03311), cat [062760], and rabbit (P21927), however, indicated that this DNA fragment was incomplete. The coding sequences for the first 37 N-terminal and 10 C-terminal amino acids were missing.

To determine the sequence of the 5′ and 3′ ends of the horse BCHE cDNA, we performed RACE reactions and ligated the products into pCRII-TOPO. Sequence analysis identified a 452-bp 5′ RACE fragment and an 842-bp 3′ RACE product that contained the termini of the horse BCHE coding sequence and several hundred bases of the immediate flanking regions.

We amplified the complete horse BCHE cDNA using high fidelity Advantage-HF polymerase in two steps. First, we amplified a DNA fragment with primers horse5 and horse3 (Table 1), which annealed 64 bp upstream of the ATG initiation codon and 26 bp downstream of the UGA termination codon. Then the product of this reaction was used as a template for PCR reactions with horseNK and horseC primers (Table 1). These oligonucleotides were designed to generate *Hin*dIII and *Not*I sites 10 and 1 bp upstream of the ATG codon, and 2 and 9 bp downstream of the stop codon, respectively. Additionally, a Kozak consensus sequence [5′-CCACC-3′] was engineered into horseNK immediately upstream of the ATG codon for efficient protein translation [10, 11]. Using 15 cycles of amplification with a 57° annealing temperature, these primers amplified an 1850-bp DNA fragment including an 1806-bp ORF.

Sequence Analysis of the Full-Length Horse BCHE cDNA

The complete sequence of the horse BCHE cDNA and the deduced amino acid sequence are shown in Fig. 2. The coding sequence has been deposited with the GenBank database (Accession No. AF178685). The composition of the cDNA sequence, 59% A+T and 41% G+C, is characteristic of BCHEs [12]. The amino acid sequence of the derived horse protein shows the highest level of homology with the cat (90.1%), rabbit (89.8%), and human (89.3%) BuChEs. Figure 3 demonstrates the sequence alignments of BuChEs from different species that have been isolated to date. Residues Ser-198, Glu-325, and His-438 form the catalytic triad, and are required for enzymatic activity [13]. Furthermore, Trp-82 is the critical choline-binding site [14], and Asp-70 is essential for the hydrolysis of succinylcholine [15, 16]. The cysteine residues at positions 65 and 92, 252 and 263, and 400 and 519 form intramolecular disulfide bonds [17]. Additionally, the cysteine at position 571 has been demonstrated to link monomers of the human BuChE by an intermolecular disulfide bond [18]. Eight conserved asparagine residues that are specifically glycosylated in the human BuChE are present in all BuChEs (Fig. 3).

Recently, the complete amino acid sequence of the horse BuChE has been determined by automated Edman degradation; however, the cDNA encoding the enzyme had not been isolated [19]. The derived amino acid sequence from the cDNA described in this manuscript demonstrated three differences to the previously published data: K407R, K458R, and S485N. Direct sequencing of PCR fragments amplified from horse genomic DNA confirmed that the residues at these positions were consistent with the data derived from the cDNA sequence. These substitutions may be attributable to strain differences between the animals.

Computer analysis of the protein sequence indicated that the first 28 amino acids are consistent with a signal peptide that is responsible for targeting the protein to the secretory pathway. NH_2-terminal amino acid sequencing of the purified horse BuChE revealed that the signal peptide was missing (Fig. 1B), indicating that these residues are probably cleaved from the mature protein, during or after secretion. The predicted molecular mass of the protein after such modification is 65.5 kDa, consistent with the reports of other BuChE sequences [20, 21].

Northern Analysis of the Horse BuChE Transcript

Since the RACE analysis predicted an mRNA transcript considerably larger than the size of the predicted ORF, we

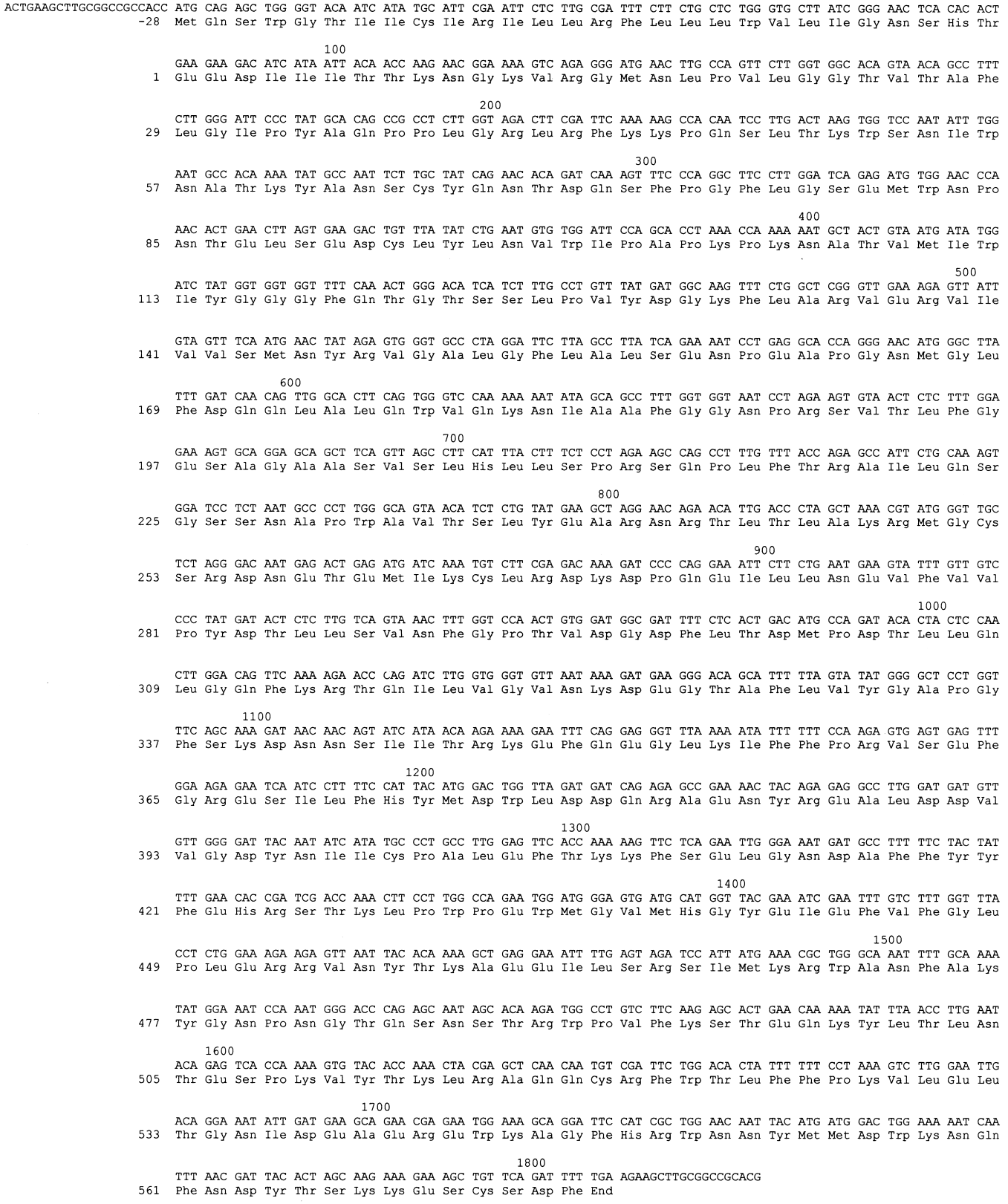

FIG. 2. Complete protein coding sequence of the horse BCHE (Accession No. AF178685). The 1806-bp ORF encodes a 602-amino-acid protein with a predicted molecular mass of 69 kDa. The amino acid sequence is labeled from residue 29, since this is the start of the mature protein.

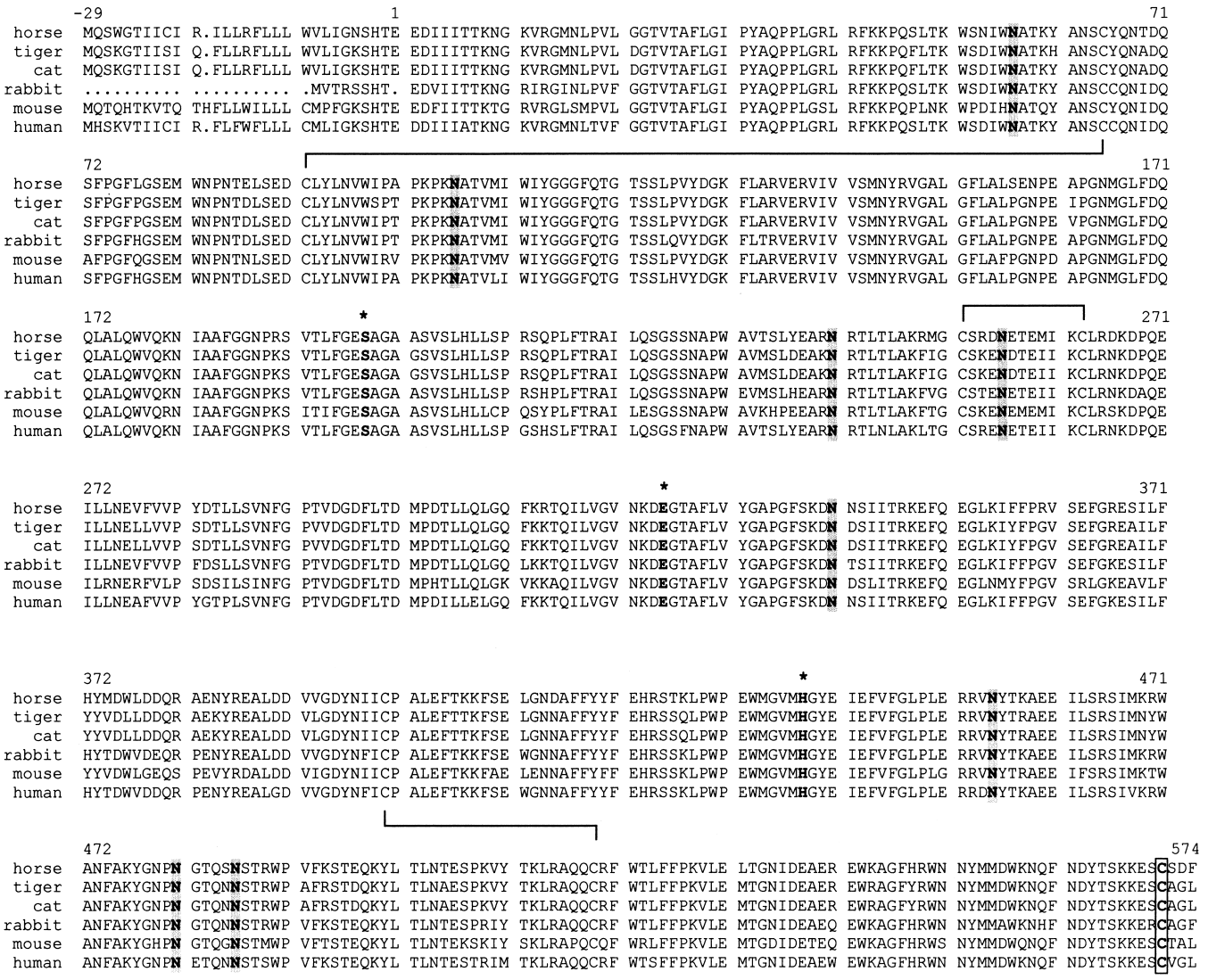

FIG. 3. Alignment of the horse, tiger, cat, rabbit, mouse, and human BuChE proteins. The active site amino acids, Ser-198, Glu-325, and His-438 (*) and the conserved Asn residues (gray box) are indicated. Intramolecular cysteine bridges are indicated by solid lines, and Cys-571 (open box) is involved in intermolecular disulfide bonding.

performed northern analysis using total RNA derived from horse liver. Figure 4A demonstrates that two RNA species, a major band of 2.9 knt and a minor band of 3.6 knt, were detected. As both of these transcripts are large enough to encode an 1800-bp ORF, we presume that they represent the mature forms of the horse BCHE mRNA. To confirm the integrity of the RNA, we hybridized the membrane with a radiolabeled 500-bp fragment of the human G3PDH gene. We detected a single 1.3-knt transcript consistent with the size of G3PDH mRNA from other species (Fig. 4B; [22]).

In Vitro *Translation of the Horse BuChE cDNA*

To determine the size of the protein encoded by the horse BCHE cDNA, we ligated the 1.8-kb cDNA into the plasmid pCITE-4b(+) to create pCITEhorse. After *in vitro* transcription and translation in the presence of [^{35}S]methionine, we detected a 66-kDa protein by autoradiography (Fig. 5A). The control plasmid pCITElacZ, which encodes β-galactosidase, generated a protein of the predicted molecular mass (119 kDa). As expected, no protein was produced by pCITE-4b(+) or by pCITEesroh, which contains the cDNA in the incorrect orientation for protein expression.

Since there was a significant difference in the size of the native horse BuChE and the protein encoded by the cDNA, we presumed that the native enzyme was glycosylated. To remove N-linked carbohydrate chains, we incubated the horse BuChE with endo F. Figure 5B demonstrates that following incubation with the glycosidase, the molecular mass of the protein was reduced from ~95 to 66 kDa, yielding a protein similar in size to that observed in the *in vitro* translation reactions (Fig. 5A).

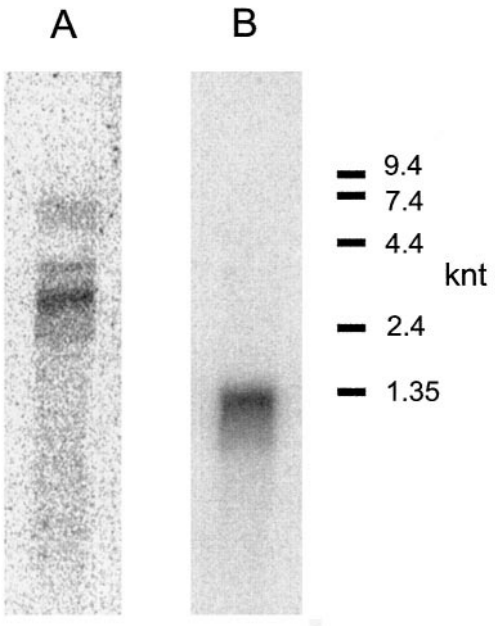

FIG. 4. Northern analysis of horse liver RNA using either the horse BCHE cDNA (A) or a human G3PDH cDNA (B) as a probe. A 9.5- to 0.24-knt ladder was used to determine transcript size.

Expression of the Horse BuChE in Cos-7 Cells

To confirm that the 1.8-kb ORF present in the cDNA encoded a biologically active protein, we ligated the horse BCHE cDNA into the mammalian expression vector pCIneo to create pCIhorse. Following transfection of pCIhorse and pCIneo into Cos-7 cells, cell extracts and culture medium were assayed for cholinesterase activity using propionylthiocholine as a substrate.

Table 2 demonstrates that extracts from pCIhorse transfected cells had ~175-fold more cholinesterase activity than extracts of pCIneo transfected cells. Thus, the cDNA encodes a *bona fide* BuChE. Additionally, approximately 22-fold more cholinesterase activity was detected in the culture medium of pCIhorse expressing cells as compared with cells transfected with pCIneo. Therefore, we conclude that the horse BuChE produced in Cos-7 cells was secreted. Indeed, the total amount of enzyme activity present in the medium was 2-fold greater than that present in cell extracts (Table 2). Kinetic analysis using Cos-7 cell extracts indicated that the recombinant protein had a K_m for BuSCh of 106 ± 9 nM, consistent with previous reports for the native horse serum BuChE [23].

To determine that the horse BuChE expressed in Cos-7 cells could activate CPT-11, we incubated equal amounts of protein from Cos-7/pCIneo and Cos-7/pCIhorse extracts with CPT-11 and measured the amount of SN-38 produced by HPLC. Table 2 indicates that cell extracts derived from Cos-7 expressing the horse BuChE cDNA converted ~11 times more CPT-11 than extracts of pCIneo transfected cells.

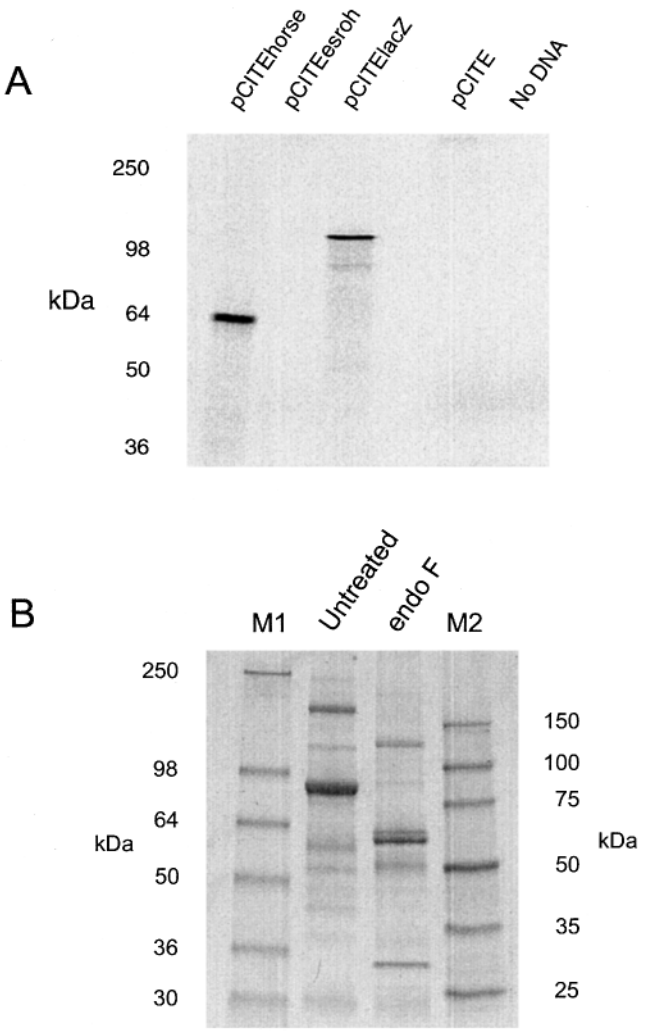

FIG. 5. (A) *In vitro* transcription of the horse BCHE cDNA. PCITEhorse: pCITE-4b(+) containing the horse BCHE cDNA; pCITEesroh: pCITE-4b(+) containing the horse BCHE in the incorrect orientation with respect to the T7 promoter; pCITElacZ: pCITE-4b(+) containing the β-galactosidase gene; and pCITE: pCITE-4b vector. (B) Endo F treatment of native horse BuChE. M1: SeeBlue markers (Novex); untreated: denatured horse BuChE; endo F: horse BuChE following overnight incubation with endo F; and M2: Perfect Protein 150- to 10-kDa ladder (Novagen).

DISCUSSION

We have reported previously that horse BuChE can activate CPT-11 more efficiently than the human enzyme [6]. In an attempt to understand the reason for this difference in drug metabolism, we decided to isolate a cDNA encoding the horse BuChE. Analysis of purified horse protein revealed that the 95- and ~ 200-kDa components (Fig. 1) contained almost the same NH_2-terminal amino acid sequences. Therefore, we presumed that these two proteins represent a monomer and dimer of the same subunit. This assumption is supported by the finding that identical human BuChE subunits assemble into dimers via intermolecular disulfide bonds [17]. These bonds form between

TABLE 2. Cholinesterase activity and metabolism of CPT-11 by Cos-7 cell extracts

Plasmid	Cholinesterase activity (μmol/min/mg or mL)		Total cholinesterase activity (μmol/min)		SN-38 produced (pg/hr/mg)
	Extract	Medium	Extract	Medium	
pClneo	8.4 ± 1.1	7.5 ± 1.3	63.9	588	34.2
pClhorse	1,483 ± 7.1	168.1 ± 6.9	9,877	22,189	373

Cholinesterase activity values are means ± SD (N = 4), and complete experiments were repeated 4 times.

cysteine residues at position 571 in each monomer. Since Cys-571 is conserved among all known BuChEs, we predict that subunit association in these proteins will occur by the same mechanism.

The horse BCHE cDNA encodes a protein of 602 amino acids. However, computer analysis predicted that a 28-residue signal peptide is present, which probably is cleaved from the mature protein. Thus, the active protein contains 574 amino acids, with a predicted molecular mass of 65.5 kDa. Lockridge *et al.* [20] sequenced human serum cholinesterase and reported 574 amino acids and a predicted molecular mass of 65 kDa for this protein. Such similarity, however, is not surprising, considering the 89% amino acid identity between the human and horse BuChEs. Since BuChEs are abundant in plasma, we anticipate that they would be secreted from cells; hence, these proteins do not contain the HXEL motif at their C-termini, which has been shown to be responsible for intracellular sequestration within the endoplasmic reticulum [24, 25].

Northern analysis of horse liver total RNA revealed the presence of 2.9- and 3.6-knt BCHE-specific transcripts. Similar observations were reported by Jbilo *et al.* [12, 26], who demonstrated the presence of 3.2- and 3.5-knt BCHE transcripts in several different rabbit tissues. These authors proposed that the two transcripts were derived from a single gene using alternative polyadenylation sites. They based their assumption on two previous observations. First, McTiernan *et al.* [27] demonstrated the existence of two functional polyadenylation sites in the human BCHE cDNA sequence, and Arpagaus *et al.* [28, 29] reported that BCHEs are coded by a single copy gene in 11 different vertebrates. Since we observed transcripts of very similar sizes in horse RNA, we presume that alternative polyadenylation sites would be present within the horse BCHE gene.

In vitro transcription–translation of the horse BCHE cDNA generated a protein of 66 kDa; however, the purified BuChE monomer appears as a 95-kDa protein following denaturing SDS–PAGE. Thus, there is approximately a 24% difference between the molecular masses of the horse BuChE proteins determined by the two methods. Treatment of the 95-kDa protein with endo F, which removes Asn-linked carbohydrate residues, reduced the molecular mass to ~66 kDa. Hence, we conclude that N-linked glycosylation is the major modification present within the protein.

Thus far, only two types of esterases, CEs [2] and BuChEs [6], have been shown to activate CPT-11. However, the same enzymes from different species display very different efficiencies in drug metabolism. For example, the CEs from rabbit and human liver share 81% amino acid homology, but the rabbit enzyme is at least 100-fold more efficient at CPT-11 conversion than the human CE [5]. Similarly, the horse and human BuChEs are 90% identical, but the purified horse protein activates CPT-11 approximately 200-fold more proficiently than the human enzyme [6]. Thus, sequence homology itself cannot predict the capability of an enzyme to activate CPT-11.

Determination of the three-dimensional crystal structure and computer modeling are a more reliable predictor of enzyme–substrate interactions. Unfortunately, to date, none of the known CEs that can metabolize CPT-11 have been crystallized; thus, the difference in the behavior of the human and rabbit CE is difficult to rationalize. Acetylcholinesterase, which is closely related to BuChEs, however, has been crystallized from *Torpedo californica* [14]. Since CPT-11 is a potent inhibitor of this enzyme, it is not converted to SN-38 by AcChE [6]. The availability of the crystal structure of a closely related protein and the high level of amino acid sequence identity among the BuChEs from different species make this group of enzymes an attractive model system to study structure–function relationships regarding CPT-11 metabolism. To this end, we are currently modeling CPT-11 into the active site of acetylcholinesterase and the predicted active sites of both human and horse BuChEs in an attempt to understand the difference in drug metabolism by these enzymes.

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References

1. Kawato Y, Aonuma M, Hirota Y, Kuga H and Sato K, Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res* **51:** 4187–4191, 1991.
2. Satoh T, Hosokawa M, Atsumi R, Suzuki W, Hakusui H and Nagai E, Metabolic activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. *Biol Pharm Bull* **17:** 662–664, 1994.

3. Potter PM, Pawlik CA, Morton CL, Naeve CW and Danks MK, Isolation and partial characterization of a cDNA encoding a rabbit liver carboxylesterase that activates the prodrug irinotecan (CPT-11). *Cancer Res* **58:** 2646–2651, 1998.
4. Kojima A, Hackett NR, Ohwada A and Crystal RG, *In vivo* human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors. *J Clin Invest* **101:** 1789–1796, 1998.
5. Danks MK, Morton CL, Krull EJ, Cheshire PJ, Richmond LB, Naeve CW, Pawlik CA, Houghton PJ and Potter PM, Comparison of activation of CPT-11 by rabbit and human carboxylesterases for use in enzyme/prodrug therapy. *Clin Cancer Res* **5:** 917–924, 1999.
6. Morton CL, Wadkins RM, Danks MK and Potter PM, CPT-11 is a potent inhibitor of acetylcholinesterase but is rapidly catalyzed to SN-38 by butyrylcholinesterase. *Cancer Res* **59:** 1458–1463, 1999.
7. Devereux J, Haeberli P and Smithies O, A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12:** 387–395, 1984.
8. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (Eds.), Preparation and analysis of RNA. In: *Current Protocols in Molecular Biology*, pp. 4.0.1–4.10.11. John Wiley, New York, 1993.
9. Guichard S, Morton CL, Krull EJ, Stewart CF, Danks MK and Potter PM, Conversion of the CPT-11 metabolite APC to SN-38 by rabbit liver carboxylesterase. *Clin Cancer Res* **4:** 3089–3094, 1998.
10. Kozak M, At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol* **196:** 947–950, 1987.
11. Kozak M, An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* **15:** 8125–8148, 1987.
12. Jbilo O, Bartels CF, Chatonnet A, Toutant JP and Lockridge O, Tissue distribution of human acetylcholinesterase and butyrylcholinesterase messenger RNA. *Toxicon* **32:** 1445–1457, 1994.
13. Shafferman A, Kronman C, Flashner Y, Leitner M, Grosfeld H, Ordentlich A, Gozes Y, Cohen S, Ariel N, Barak D, Harel M, Silman I, Sussman JL and Velan B, Mutagenesis of human acetylcholinesterase. Identification of residues involved in catalytic activity and in polypeptide folding. *J Biol Chem* **267:** 17640–17648, 1992.
14. Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L and Silman I, Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science* **253:** 872–879, 1991.
15. Neville LF, Gnatt A, Padan R, Seidman S and Soreq H, Anionic site interactions in human butyrylcholinesterase disrupted by two single point mutations. *J Biol Chem* **265:** 20735–20738, 1990.
16. McGuire MC, Nogueira CP, Bartels CF, Lightstone H, Hajra A, Van der Spek AF, Lockridge O and La Du BN, Identification of the structural mutation responsible for the dibucaine-resistant (atypical) variant form of human serum cholinesterase. *Proc Natl Acad Sci USA* **86:** 953–957, 1989.
17. Lockridge O, Adkins S and La Du BN, Location of disulfide bonds within the sequence of human serum cholinesterase. *J Biol Chem* **262:** 12945–12952, 1987.
18. Chatonnet A and Lockridge O, Comparison of butyrylcholinesterase and acetylcholinesterase. *Biochem J* **260:** 625–634, 1989.
19. Moorad DR, Garcia GE and Doctor BP, Amino acid sequence of horse serum butyrylcholinesterase. In: *Structure and Function of Cholinesterases and Related Proteins* (Eds. Doctor BP, Taylor P, Quinn DM, Rotundo RL and Gentry MK), pp. 145–146. Plenum Press, New York, 1998.
20. Lockridge O, Bartels CF, Vaughan TA, Wong CK, Norton SE and Johnson LL, Complete amino acid sequence of human serum cholinesterase. *J Biol Chem* **262:** 549–557, 1987.
21. Gentry MK and Doctor BP, Alignment of amino acid sequences of acetylcholinesterases and butyrylcholinesterases. In: *Cholinesterases: Structure, Function, Genetics and Cell Biology* (Eds. Massouilie J, Bacou F, Barnard E, Chatonnet A, Doctor BP and Quinn DM), pp. 394–398. American Chemical Society, Washington, DC, 1991.
22. Hanauer A and Mandel JL, The glyceraldehyde 3 phosphate dehydrogenase gene family: Structure of a human cDNA and of an X chromosome linked pseudogene; amazing complexity of the gene family in mouse. *EMBO J* **3:** 2627–2633, 1984.
23. Main AR, McKnelly SC and Burgess-Miller SK, A subunit-sized butyrylcholinesterase present in high concentrations in pooled rabbit serum. *Biochem J* **167:** 367–376, 1977.
24. Munro S and Pelham HRB, A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48:** 899–907, 1987.
25. Potter PM, Wolverton JS, Morton CL, Wierdl M and Danks MK, Cellular localization domains of a rabbit and a human carboxylesterase: Influence on irinotecan (CPT-11) metabolism by the rabbit enzyme. *Cancer Res* **58:** 3627–3632, 1998.
26. Jbilo O, Toutant JP, Vatsis KP, Chatonnet A and Lockridge O, Promoter and transcription start site of human and rabbit butyrylcholinesterase genes. *J Biol Chem* **269:** 20829–20837, 1994.
27. McTiernan C, Adkins S, Chatonnet A, Vaughan TA, Bartels CF, Kott M, Rosenberry TL, La Du BN and Lockridge O, Brain cDNA clone for human cholinesterase. *Proc Natl Acad Sci USA* **84:** 6682–6686, 1987.
28. Arpagaus M, Chatonnet A, Masson P, Newton M, Vaughan TA, Bartels CF, Nogueira CP, La Du BN and Lockridge O, Use of the polymerase chain reaction for homology probing of butyrylcholinesterase from several vertebrates. *J Biol Chem* **266:** 6966–6974, 1991.
29. Arpagaus M, Kott M, Vatsis KP, Bartels CF, La Du BN and Lockridge O, Structure of the gene for human butyrylcholinesterase. Evidence for a single copy. *Biochemistry* **29:** 124–131, 1990.