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MOLECULAR BIOLOGICAL STUDIES ON THE BIOGENESIS
OF HUMAN CHOLINESTERASES IN VIVO AND AS DIRECTED
BY CLONED CHOLINESTERASE DNA SEQUENCES

FINAL REPORT

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HERMONA SOREQ

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Grantee: Hermona Soreq, Professor of Molecular Biology
Department of Biological Chemistry
The Life Sciences Institute
The Hebrew University
Jerusalem 91904
ISRAEL

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 Acetylcholinesterase (ACHE) is an enzyme long noted for its essential role in the termination of neurotransmission at cholinergic synapses and neuromuscular junctions. Because acetylcholinesterase is the target protein for a variety of neurotoxic compounds, including chemical warfare agents, research on this enzyme in man has profound implications for human health and well-being. Together with related but distinct protein butyrylcholinesterase (BCHE), it presents an intriguing model for the basic scientific study of tissue-specific and differential regulation of gene expression employing divergent pathways in protein biosynthesis. Molecular cloning has revealed the primary structure of human ACHE and BCHE, and analytical studies in microinjected Xenopus oocytes have demonstrated that the biochemical properties of these enzymes are most inherent to their primary amino acid sequences and may be altered by single-nucleotide changes in different in vivo variants of their corresponding

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abnormal expression of both ACHE and BCHE and the in vivo amplification of the ACHE and BCHE genes have been variously associated with abnormal megakaryocytopoiesis, leukemias and malignant tumors of the brain and ovary, implicating ACHE and BCHE in cholinergic influences on cell growth and proliferation.

Summary

Overview: Acetylcholinesterase (acetylcholine acetyl hydrolase, EC 3.1.1.7, AChE) is a type B carboxylesterase that rapidly hydrolyzes the neurotransmitter acetylcholine in neuromuscular junctions and brain cholinergic synapses. AChE may be distinguished from the closely related enzyme BCHE (acetylcholine acyl hydrolase, EC 3.1.1.8, BCHE) by its high substrate specificity and its sensitivity to selective inhibitors. Cytochemical and biochemical studies have shown that both enzymes are transiently expressed in hematopoietic, embryonic, and tumor tissue as well, and that they demonstrate distinct patterns of regulation. During the course of the work reported herein, the gene encoding AChE and that encoding variant BCHE in humans were molecularly cloned and expressed in microinjected *Xenopus* oocytes. Further, the AChE and BCHE genes were found to be amplified in leukemic blood cells, in primary ovarian carcinomas, and in individuals exposed to organophosphorous poisons, reinforcing the emerging notion that they play growth-related roles. Naturally occurring variants of the BCHE gene were demonstrated to cause defective BCHE phenotypes, and antisense oligonucleotides were employed to block BCHE gene expression, with subsequent damage in hemocytopoiesis.

Molecular cloning and oocyte expression of human AChE coding sequence: To study the primary structure of human AChE and its gene expression and amplification, cDNA libraries from human tissues expressing oocyte-translatable AChEmRNA were constructed and screened with labelled oligodeoxynucleotide probes. We isolated several cDNA clones which encode a polypeptide with $\geq 50\%$ identically aligned amino acids to *Torpedo* AChE and human BCHE. However, these cDNA clones were all truncated within a 300-nucleotide-long, G,C-rich region with a predicted pattern of secondary structure, including a high free energy (-117 kcal/mole) downstream from the expected 5'-end of the coding region. Screening of a genomic DNA library revealed the missing 5'-domain. When ligated to the cDNA and constructed into a transcription vector, this sequence encoded a synthetic mRNA translated in microinjected oocytes into catalytically active AChE with marked preference for acetylcholine over butyrylcholine as a substrate, susceptibility to inhibition by the AChE inhibitor 1,5-bis (allyldimethylammonium phenyl)-pentan-3-one dibromide (BW284C51), and resistance to the BCHE inhibitor tetraisopropylpyrophosphoramidate (iso-OMPA). Blot hybridization of genomic DNA from different individuals carrying amplified AChE genes revealed variable intensities and restriction patterns, with probes from the regions upstream and downstream from the predicted G,C-rich structure. Thus, the human AChE gene includes a putative G,C-rich attenuator domain and is subject to structural alterations in cases of AChE gene amplification.

Search for the molecular origins of BCHE polymorphism by cDNA screen, deletion mutagenesis, and *Xenopus* oocyte coinjections: Screening of cDNA libraries from various fetal and adult human tissue origins resulted in the isolation of identical cDNA clones, all coding for serum BCHE. These findings indicate that the human BCHE gene is most probably transcribed into a single transcription product in all tissues. When injected into *Xenopus* oocytes, synthetic BCHEmRNA induced the production of an extracellular-surface-associated protein displaying the characteristics of dimeric BCHE. Deletion mutagenesis experiments revealed that the entire coding sequence in BCHEmRNA is necessary for its expression in oocytes. Supplementation with total-tissue mRNAs induced the appearance of molecular forms containing four or more catalytic subunits, and associated with the oocyte surface in a manner consistent with the principal assembly patterns of cholinesterases in the native tissues.

Unusual BCHE mRNA transcripts in nervous system tumors: To study the molecular origin of the altered regulation of BCHE in nervous system tumors, BCHE cDNA sequences from human glioblastoma (Gb) and neuroblastoma (Nb) cDNA libraries were compared with BCHE cDNAs from normal fetal and adult tissues. A single 2.6-kb BCHE cDNA sequence was found in all normal tissues, whereas an additional alternatively terminated BCHE cDNA clone was found in both tumor libraries. The tumor-specific cDNA contained a 3'-0.7-kb nontranslatable extension, as well as several nucleotide alterations in the normal polyadenylation site. Single-base mutations in the coding region of this unusual BCHE cDNA imply two amino acid substitutions: Asp70--> Gly and Ser425-->Pro. The Asp70-->Gly change has recently been implicated with "atypical" BCHE, deficient in its capacity to hydrolyze succinylcholine. The 3.6-kb mRNA was less abundant in RNA blot hybridization than the 2.6-kb mRNA, in agreement with the low ratios between the 3.6-kb and 2.6-kb BCHE cDNA clones in glioblastoma and neuroblastoma libraries. Furthermore, size fractionation and microinjection of glioblastoma poly(A)⁺ RNA, followed by enzyme activity and selective inhibition measurements, demonstrated two peaks of functional BCHE mRNA, the heavier one probably reflecting the longer transcripts. Chromosomal mapping of the 0.7-kb 3'-fragment by *in situ* hybridization localized it to a unique 3q26-ter position, where we recently found an inheritably amplified defective BCHE gene in a family exposed to the cholinesterase inhibitor methyl parathion. Our findings confirm previous genetic-linkage mapping of the functional BCHE gene to the 3q26-ter position and demonstrate that extended functional mRNA transcripts encoding a BCHE form with two modified amino acids are produced from this gene in glioblastoma and neuroblastoma cells.

Structure-function relationships in human BCHE variants: The "atypical" allelic variant of human BCHE can be characterized by its failure to bind the local anesthetic dibucaine, the muscle relaxant succinylcholine, and the naturally occurring steroidal alkaloid solanidine, all assumed to bind to the charged anionic site component within the normal BCHE enzyme. A single-nucleotide substitution conferring a change of aspartate70 into glycine was recently reported in the BCHE gene from several individuals having the "atypical" BCHE phenotype, whereas in other DNA samples, this mutation appeared together with a second alteration, which conferred a change of serine 425 into proline. To separately assess the contribution of each of these mutations towards anionic site interactions in BCHE, three transcription constructs were engineered, with each of these substitutions alone or with the two of them together. *Xenopus* oocyte microinjection of normal or mutated synthetic BCHE mRNA transcripts was employed in conjunction with biochemical analyses of the resultant recombinant BCHE variants. The presence of the Gly70 mutation alone was found to render the enzyme resistant to 100 μ M solanidine and 5 mM succinylcholine; concentrations sufficient to inhibit the "normal," Asp70-containing BCHE by over 50%. Furthermore, when completely inhibited by the organophosphorous poison diisopropylfluorophosphate (DFP), Gly70 BCHE failed to be reactivated by 10 mM of the cholinesterase-specific oxime pyridine 2-aldoxime methiodide (2-PAM); a concentration restoring about 50% of activity in the "normal" Asp70 recombinant enzyme. The Pro425 mutation alone had no apparent influence on BCHE interactions with any of these ligands. However, it conferred synergistic effects on some of the anionic site changes induced by the Gly70 mutation. Thus, in addition to being resistant to solanidine and to 2-PAM reactivation, the Gly70/Pro425 variant completely failed to bind either dibucaine (1 mM) or succinylcholine (100 mM), at concentrations 20-fold higher than those inhibiting the single Gly70 mutant. In view of the evolutionarily conserved Asp70 and Ser425 peptide domains in all known cholinesterases, these findings suggest that the Asp70 residue serves as part of the anionic site responsible for binding charged substrates throughout the family of cholinesterases and that genetically linked mutations in these two peptide domains may confer selection advantage through their synergistic effects on BCHE resistance to inhibitors.

Human ACHE and BCHE are encoded by separate genes: Various hybridization approaches were employed to investigate structural and chromosomal interrelationships between the human cholinesterase genes BCHE and ACHE, encoding the polymorphic, closely related, and coordinately regulated enzymes having BCHE and ACHE activities. Homologous cosmid recombination with a 190-base pair (bp) 5'-fragment from BCHEcDNA resulted in the isolation of four overlapping cosmid clones, apparently derived from a single gene with several introns. The cosmid BCHEcDNA included the 700-bp pair fragment known to be expressed at the 3'-end of BCHEcDNA from nervous system tumors and which had been mapped by in situ hybridization to the unique 3q26-ter position. In contrast, cosmid BCHEcDNA did not hybridize with full-length ACHEcDNA, proving that the complete BCHE gene does not include ACHE-encoding sequences either in exons or in its introns. The chromosomal localization of BCHE-coding sequences was further searched for by two independent gene-mapping approaches. Filter hybridization with DNA from human/hamster hybrid cell lines revealed BCHEcDNA-hybridizing sequences only in cell lines including human chromosome 3. However, three BCHEcDNA-homologous sequences were observed at chromosomal positions 3q21, 3q26-ter, and 16q21 by a highly stringent in situ hybridization protocol that included washes at high temperature and low salt. These findings emphasize the selectivity of cosmid recombination and chromosome blots, raise the possibility of individual differences in BCHEcDNA-hybridizing sequences, and present an example for a family of highly similar proteins encoded by distinct, nonhomologous genes.

In vivo amplification of the human ACHE and BCHE genes: Cholinesterases are transiently expressed in multiple germ line, embryonic, and tumor cells. The acute poisoning effects of various organophosphorous compounds are generally attributed to their irreversible covalent interaction with cholinesterases and to the block of their catalytic activities. A de novo, apparently inheritable amplification of a BCHE-gene-encoding defective BCHE was found in a family under prolonged exposure to the agricultural organophosphorous insecticide methyl parathion. Further analysis revealed that both the BCHE and the ACHE genes are amplified in leukemias and platelet disorders and that the tumorigenic expression of these genes in ovarian carcinomas is associated with their frequent co-amplification in these tumors. The amplification of BCHE and ACHE genes in normal and tumorous tissues might potentially be analogous to the well-known amplification of other genes that encode target proteins to toxic compounds. As such, it could provide cells the survival that is endangered by organophosphorous poisons' selective advantage over other cells. Furthermore, since cholinesterases most probably play developmentally important roles in multiple cell types, the amplification and overexpression of their corresponding genes might affect fertility, be related to the progress rate of various tumor types, and have considerable implications on the ecological and clinical risks involved with the common use of organophosphorous compounds in agriculture.

Manipulations of BCHE gene expression modulate hemocytopoiesis: Megakaryocytopoiesis was selectively inhibited in cultured murine bone marrow cells by a 15-mer oligodeoxynucleotide complementary to the initiator AUG region in BCHEmRNA. Furthermore, conditioned medium from Xenopus oocytes producing recombinant BCHE stimulated megakaryocytopoiesis. These observations implicate BCHE in megakaryocytopoiesis and suggest application of oligodeoxynucleotides for modulating bone marrow development.

FOREWORD

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X In conducting research utilizing recombinant DNA technology, the investigators adhered to the current guidelines promulgated by the National Institutes of Health.

H. S.
PI Signature

Oct. 24, 1990
Date

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List of Abbreviations

ACh- Acetylcholine
ACHE- Acetylcholinesterase
BenzCh- Benzoylcholine
BuTCh- Butyrylthiocholine
BW284C51- 1:5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide
bp- Base pair
BCHE- Butyrylcholinesterase
CHE- Cholinesterase
CNS- Central nervous system
DFP-diisopropylphosphorofluoridate.
Eserine- physostigmine; 1'-methylpyrrolidino (2':3':2:3:)1,3-dimethylindolin-5-yl N-methylcarbamate
IO- Iso-OMPA
kb- kilobase pair
Km- Michaelis-Menten constant
Kn- Kanamycin
MPT- O-ethyl-S²diisopropylaminoethyl methyl phosphorothiolate
Mutein- Mutated protein
OP- Organophosphorous compound
PropCh- propionylcholine
2-PAM- 2-aldoxime methiodoide
Physostigmine- 1'-methylpyrrolidino (2':3':2:3:)1,3-dimethylindolin-5-yl N-methylcarbamate
QNB- quinuclidinylbenzoate
PI- Phosphatidyl inositol
Soman- Pinacoylmethylphosphonofluoridate
Sarin- Isopropylmethylphosphonofluoridate
SuCh- Succinylcholine

I. Introduction:

Structure-function relationships in human cholinesterase genes and their protein products

1. Overview and significance

The existence of enzymes which could hydrolyze the neuromuscular transmitter acetylcholine (ACH), was predicted in 1914 by Dale, before their actual discovery, as a result of animal reactions to injected ACH (1). In 1926, eserine (physostigmine; 1'-methylpyrrolidino (2':3':2:3)1,3-dimethylindolin-5-yl N-methylcarbamate) was employed as an inhibitor to further prove the existence of cholinesterases (CHEs;2). Within 10 years, Stedman purified crude BCHE from horse serum (3). By 1940, the existence of the two major forms of CHE was discovered by Alles and Hawes, who found that human serum and red blood cell enzymes differ in their substrate specificities and in their sensitivity to selective inhibitors (4).

Since then, much has been uncovered in relation to CHEs. The two principal, closely related carboxylesterase type B proteins from this family were termed acetylcholinesterase (acetylcholine acetyl hydrolase, EC 3.1.1.7, AChE) and butyrylcholinesterase (acylcholine acyl hydrolase, EC 3.1.1.8, BChE). Both are serine hydrolases by virtue of their biochemical properties. They exist in multiple molecular forms and can rapidly degrade choline esters, such as the ACh neurotransmitter (5,6). Both are ubiquitously distributed enzymes, with AChE being more tissue-specific than BChE; both are vulnerable to organophosphorus (OP) inhibition and can be differentiated by their relative sensitivity to particular OPs or by their affinity to various substrates (7,8).

AChE and BChE have strong (over 50%) primary structure homology, conserved cysteine residues, comparable though not identical polymorphism, and similar pharmacological and kinetic properties (9). This suggests a common mechanism of action, which may be unlike that of the trypsin family.

Although CHEs have been thoroughly studied for over 75 years, many characteristics remain unknown. Can these two enzymes be implicated with different functions? What was the evolutionary force which supported the development of various CHE forms and how many CHE-related sequences exist in the genome? Furthermore, the precise mechanism of the CHE-hydrolyzing process is still unknown. What are the functionally important domains in CHEs, and which amino acid residues are involved in their activity? The primary structure of CHEs as inferred from their DNA-coding sequences has recently been found to be different from those of other serine hydrolase sequences, such as the trypsin family (10,11), in spite of the existence of the same active-site serine residue in CHEs and in other serine hydrolases (12). This suggests a particular mechanism of function for CHEs, with specific variations between AChE and BChE. Substrate and inhibition studies further suggest the existence of additional ligand-binding subsites in CHEs. These were initially termed anionic and hydrophobic (13), though questioned by some who suggest the existence of a sole hydrophobic site (14), in addition to the serine-containing esteratic site. Structure-function relationship studies defining each of these elements are, hence, of primary importance.

The widespread finding of CHEs in species ranging from bacteria to man (see section I.6) suggests an important role for these proteins. Even so, why must there exist both AChE and BChE? Furthermore, CHEs are found in apparently non-cholinergic or noncholinergic cells, that do not express high levels of cholinergic markers, such as choline acetyl transferase, or of cholinergic receptors. These studies led to numerous proposals of diverse functions for CHEs, mostly related to cell division and/or differentiation.

Several common variants have been found for human BCHE and less abundant ones for human AChE. The phenotypically different BChEs include the "atypical" and "silent" enzymes, discovered in a minority of the general population (15). Molecular-sequencing data which emerged recently have correlated several of these mutations with specific amino acid alterations (16,17). It is important to understand how such alterations affect protein kinetics, structure, interaction with various ligands and function. What is the molecular basis for the different characteristics of these mutant proteins? Finally, can one offer an hypothesis to explain the particular vulnerability of this gene for mutagenesis? Using a molecular-cloning approach, we attempted to prepare the prerequisite tools with which some of these questions could be approached.

2. Characteristics of hydrolyzing activity

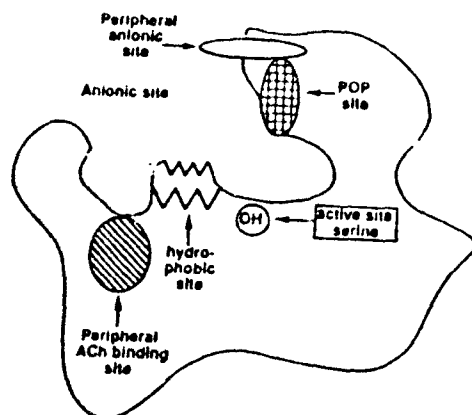
ChEs are complex molecules containing several important domains that are responsible for activity or binding of ligands within each of their catalytic subunits (13). The turnover number for ACh in these enzymes is so large ($>10^4 s^{-1}$) that diffusion of substrate to the active site is probably rate-limiting (18). The numerous domains which were suggested by various inhibitory and biophysical studies tend to cause some confusion. As such, each site warrants a separate discussion as well as a description of its interactions with other sites and with inhibitors.

There is so far no X-ray diffraction data for ChEs; however, ample biochemical evidence has been accumulated referring to the three-dimensional structure of these enzymes. The active center in ChEs is believed to contain two principal subsites, the negatively charged anionic site and the esteratic site containing the crucial serine residue (12). Based on inhibition studies, the distance between these subsites has been estimated to be approximately 4.7 Angstrom (19). The existence of an hydrophobic region has further been predicted; this would be overlapping or close to these two sites, and should play an important role in binding aryl substrates and active-site ligands. In addition, cationic ligands bind a peripheral anionic site (20), which can induce conformational changes of the active site (21). Two additional sites have been suggested: a peripheral OP-binding site which binds the inhibitor O-ethyl-S²-diisopropylaminoethyl methyl phosphorothiolate (MPT) (22) and a peripheral ACh-binding site which causes substrate inhibition of AChE, but not BCHE, by excess ACh (23). Table I lists the various sites which have been suggested to take part in ACh hydrolysis and ligand interactions for ChEs, and Figure 1 represents them in a schematic manner. It should be noted that some of these sites might be identical to or might overlap each other, and that their existence may now be re-examined by biochemical studies involving recombinant mutant proteins. The objective and significance of such studies is explained in the following paragraphs.

Table I. Putative binding sites in cholinesterases

Site	Reference
<u>Active site</u>	
Esteratic	24
Anionic	19
Hydrophobic	13
<u>Peripheral sites</u>	
Anionic	25, 26
Allosteric ACh-binding	23
Organophosphorus-binding	22

Figure 1. Cholinesterase binding sites



The various proposed binding sites are represented in a model form. Note that the peripheral organophosphorous-binding site (POP) is near or overlapping the peripheral anionic binding site (see text).

2.1 Esteratic site

The esteratic site presumably involves amino acid residues similar to those of serine hydrolases. It contains Ser¹⁹⁹, and is assumed to include the imidazol group of an histidine and the carboxyl group of an acid residue, most probably aspartate (24). Once the substrate binds, a "charge-relay" mechanism causes electron shifts, resulting in the acylation of the enzyme and liberation of choline from ACh. It has been suggested that the acylated intermediate exists in a tetrahedral conformation (13). Finally, the acylated enzyme complex is hydrolyzed, freeing the enzyme and liberating acetic acid. The rate-limiting step in vertebrate CHE hydrolysis is deacylation, whereas in the housefly it appears to be the acetylation step (23). Electron-shifting is a prerequisite in explaining the high pH dependence of this catalytic activity (24). Two highly conserved histidines, nos. 423 and 438 in the BCHE sequence, were suggested to supply part of the relay mechanism (27). Both of these exist in all of the CHE primary structures revealed to date and were included among several possible amino acid combinations, which have recently been proposed as candidates for the esteratic-site triad (11).

2.2 Active-center anionic site

The anionic site of AChE consists of multiple negative charges (25). Substrate-binding to the catalytic site is achieved via its attraction to the acidic components included in this site, which holds the substrate in position for hydrolysis. An alternative suggestion of a hydrophobic trimethyl binding site has been made (14). However, experiments using charged and uncharged ligands are strongly in favor of the classically charged anionic site (28). It is possible that anionic-site binding is supported by both Coulombic and hydrophobic interactions in which hydrophobic and charged residues in this site are involved.

The anionic site plays an important role in the induced-fit model suggested by Rosenberry (24) for CHE activity. According to this model, the binding of ACh alters the conformation of the active site, a prerequisite requirement for maximum rate of hydrolysis. Further evidence stems from binding experiments in which AChE

was reacted with closely isosteric phosphorous compounds. These varied in that one contained a charged trimethyl moiety and the second an uncharged trimethyl moiety. When added to ACHE, the phosphorous moiety was bound to the active site serine, forming either a charged substrate-enzyme complex or an uncharged one. However, anionic binding compounds could bind the uncharged though not the charged complex, suggesting that the charge is necessary to alter active-site conformation (28). Quinn (13) has proposed that the change might be caused by binding of the anionic site to the charged pole of ACH, causing the anionic and esteratic sites to become nearer to one another. Recent evidence relates to further allosteric alterations which occur upon anionic-site binding of tetraalkylammonium salts (29). As such, CHEs appear to change their conformation upon ligand binding.

2.3 Hydrophobic site

ACHE is capable of binding a variety of hydrophobic molecules, such as aromatic cations (24,25). This implies that important hydrophobic amino acid residues exist near to, or overlap, the active site. Studies on binding of hydrophobic inhibitors, quenching of tryptophan fluorescence, and chemical modifications indicated the presence of the residues lysine, tyrosine, histidine, and tryptophan at the three-dimensional proximity of the active site (30,31). The hydrophobic residues in CHE would stabilize binding of substrates by interacting with their hydrophobic region.

2.4 Peripheral anionic site

The peripheral anionic site was shown to bind cationic ligands, such as gallamine, d-tubocurarine, and decamethonium (26). It is located more than 2 nm away from the active site (20). Binding of ligands to this site has also been proposed to cause conformational changes to occur within the CHE protein (32).

2.5 Peripheral acetylcholine binding site

This site was proposed (23) as a result of ACHE inhibition by excess ACH. According to this proposition, excess ligand binds a peripheral site and causes conformational changes in the protein. It should be noted that substrate inhibition does not exist for BCHE, so that the peptide domain(s) included in this putative site should, in principle, be specific to ACHE.

2.6 Peripheral organophosphorous site

Cholinesterases react with a wide range of OP compounds (for reviews see refs. 5,15). The compound MPT (O-ethyl-S²-diisopropylaminoethyl methyl phosphorothiolate) has been shown to bind reversibly to a site other than the active site, termed the peripheral OP site (POP; 33), in addition to its irreversible binding to the active site. It has been shown that 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51), a reversible bisquaternary ammonium-specific inhibitor specific to ACHE, can protect the active site as well as the POP site of ACHE from MPT. This indicates that BW284C51 may bind to, or mask sterically, a peripheral site that overlaps or is linked to the OP peripheral site (22). Thus the protection conferred by BW284C51 uncovers some of the complexity of CHEs in relation to ligand-binding regions in the molecule.

3. Cellular and subcellular localization of cholinesterases

CHEs can be found in a wide range of tissues and cell types other than muscle fibers or neurons. Some of the many cell types containing CHEs are erythrocytes (5), adrenal medulla (6), ovarian follicles (34,35), and megakaryocytes (36). CHEs have been reported in a number of embryonic tissues (37,38). In addition, considerable levels of CHEs were detected in various neoplastic tissues, such as ovarian carcinomas (37,39) and brain tumors (40,41).

The various forms of CHEs in various microenvironments are compatible with different binding mechanisms. Large, collagen-tail-like bound forms are found in the basal lamina bound to heparin sulfate proteoglycan (42), which could potentially permit better protein flexibility and substrate accessibility. In the bovine caudate nucleus, tetramers have been found to bind the membrane via a non-catalytic peptide-lipidic subunit (43). In the mammalian erythrocyte membrane, as well as in the Torpedo electric organ, AChE dimers bind to the membrane via phosphatidyl inositol (PI) linkage (44,45,46). Other forms of CHE exist as soluble molecules, such as BCHE in human serum (47) and AChE in fetal bovine serum (48). Although BCHE has not been observed to be bound to membranes by either non-catalytic subunits or PI, it has been found to display (in muscle) asymmetric and soluble globular forms similar to those of AChE (49,50). The molecular mechanisms enabling the creation of such forms remain an enigma, as will be discussed in the following.

4. Putative biological role/s of cholinesterases

4.1 Nervous system involvement

Both AChE and BCHE serve a pivotal role in terminating muscle contraction, by rapidly hydrolyzing the neurotransmitter ACh in the neuromuscular junction. Though AChE is the predominant form performing this role, BCHE has been shown to be a sufficient substitute, as is exemplified by the sole presence of BCHE in the heart muscle of Torpedo marmorata (51). However, no physiological dysfunction has been found to date in humans having the "silent" serum BCHE phenotype, perhaps indicating that BCHE may be substituted for by AChE when necessary. The wide-range cellular distribution of CHEs ranging from oocytes to various apparently noncholinergic cells suggests additional important roles for CHEs. For example, why should human erythrocytes contain AChE dimers? Even in the central nervous system (CNS), the role of CHE in transmission should not be taken for granted. Cell types are known which receive cholinergic input yet contain no AChE, such as medium spiny neurons in the striatum (52). In contrast, there exist cholinceptive cells that can be excited by ionophoretic ACh (such as Purkinje cells in the cerebellum), but appear to lack AChE and do not receive cholinergic input in their mature state (52). It should be noted that CHEs are present in organisms lacking cholinergic innervation and even in those that have not developed nervous systems (see section 1.6), reinforcing the notion of other biologically important roles for this family of proteins.

4.2 Implications with motility

Seaman and Houlihan (53) demonstrated that cholinesterases are related to cilia coordination in Tetrahymena geleii. This was shown by inhibition of CHE activity which immobilized the organisms. In developing sea urchin eggs, an increase in CHE activity is accompanied by cilia tuft formation, while inhibition of ciliated tuft formation by lithium prevents CHE increase as well (54). AChE

has been shown to be involved in movement in the human respiratory epithelium cilia and in human spermatozoa (reviewed in ref. 55). Further evidence implicates a well-developed cholinergic system, including ACHE, and cholinergic receptors as a means of sperm cell motility (56). The cholinergic system, as such, might be necessary in cellular motility and orientation of cells by movement.

4.3 Development and growth

In addition to their involvement in cholinergic neurotransmission and cellular motility, CHES have been implicated in cell division, development, and growth. ACH promotes progesterone-induced maturation of *Xenopus* oocytes (57). ACHE in these oocytes has been shown to be seasonally regulated inversely to the reproductive cycle (58), similar to the regulation of the muscarinic ACH receptors (59). In chick embryogenesis, evidence indicates transient BCHE activity early in the development of mesenchyme tissues (60), concomitantly with cell division and DNA replication (61). Evidence for BCHE involvement in oocyte development has recently been reported in humans (35). Signals observed by *in situ* hybridization of human haploid genome oocytes with human [³²S]BCHEcDNA indicate that oocytes within follicular structures from various stages of development were specifically labelled. This was further shown to be developmentally specific, as labelling was high in preantral follicles as compared with primordial and antral follicles, while atretic follicles were negative. Cholinergic induction of polyspermy in sea urchin oocytes suggests an involvement of CHES in postfertilization mechanisms as well (55).

4.4 The role of butyrylcholinesterase as compared with that of acetylcholinesterase

The biological role of BCHE is not yet clear. Suggestions include acetylcholine hydrolysis at those synaptic cleft concentrations of transmitter liable to induce substrate inhibition of ACHE (62). In addition, a scavenging role for BCHE was proposed which implicates this enzyme in the removal of naturally occurring CHE inhibitors, including the steroidal glycoalkaloid solanine and its hydrolytic aglycone derivative solanidine, both of which may be present in toxic concentrations in potatoes (63,64).

4.5 Suggestions for peptidase activity of cholinesterases

Carboxy- and aminopeptidase activity from a site other than the esteratic site has been assigned to ACHE *in vitro* and was reported to be selective towards the substrates Substance P and enkephalin (65). In amacrine cells in the retina, Substance P and enkephalin-containing neurons are depleted of retinal peptides by light deprivation. Incubation with exogenous ACHE enhances immunocytochemical reaction for both enkephalin and Substance P, suggesting a possible extracellular role for ACHE (66). Exogenous ACHE applied to nigral neurons affects motor behaviour, and its effects are unrelated to ACH hydrolysis by ACHE (52). Results of studies such as those mentioned above as to peptidase activity or behavioural effects should be taken with precaution; others report that contamination in CHE preparations might have been the primary cause for such effects (67). Alternatively, the effect of administered ACHE may be indirect, functioning via a yet-unexplained complex feedback mechanism. It would, therefore, be advantageous to express CHES in heterologous systems, which would permit the study of the purified proteins and their physiological role under better-defined conditions.

Even if CHES are important for oocyte and other cell development, as well as for sperm motility, the question as to their precise function(s) remains open.

Silver (5) and Whittaker (15) discuss at length various roles attributed to cholinesterases in nonneuronal tissues. Among the cells and tissues were erythrocytes, platelets, vascular tissue, and the placenta. Evidence supplied therein suggests ACH-induced permeability changes to be a major component of CHE function. Interestingly enough, it has been stated that the safest conclusion is that we do not know why CHEs are localized in these various cells. It may, therefore, be necessary to compound this conclusion and to question the role of CHEs in the CNS as well.

5. Cholinesterase polymorphism and its genomic origin

5.1 General polymorphic scheme

CHEs are highly polymorphic proteins. Both size and sedimentation coefficients of CHE monomers have been studied extensively (for a review see ref. 8). The molecular weight of chick brain ACHE has been estimated at 110 kDa for an active monomer (68), whereas human brain ACHE displayed the size of 66 kDa (69), and human serum BCHE at 85 kDa (70). The globular G_1 monomers from Torpedo californica ACHE and human BCHE are glycosylated proteins of 575- and 574-amino-acid-residues in length, respectively (for a review, see 71). The G_2 dimer in Torpedo ACHE consists of two G_1 monomers, cross-linked by a disulfide bridge connecting the cysteine residues at position 572 (72), and the G_4 tetramers of both Torpedo ACHE and human serum BCHE consist of two such dimers held together by hydrophobic interactions. In addition to these globular forms, there exist asymmetric ACHE forms containing collagen-like tail structures. This tail is rich in proline and hydroxyproline and is approximately 50 nm long (73). The A_1 form contains two catalytic subunits bound to the noncollagenous region of the tail by disulfide bridges through the same cysteine residue as above, and an additional G_2 dimer bound to the first two monomers to make it into a tetramer. The A_2 form consists of two A_1 tetramers linked through their collagen tails and the largest molecule, the A_{12} , contains three sets of tetramers bound to the collagen-like tail. These structures were deciphered primarily from electron microscopy studies (74), information which was later linked to amino acid sequence data (75). However, the primary amino acid sequence of the tail molecules has not yet been published. The tail is assumed to serve as an attachment of its associated tetramers to solid matrix, such as the extracellular basal lamina (76).

5.2 Further complexity in cholinesterase polymorphism

The distinction between the various polymorphic CHE forms is by no means complete. Each molecular form may exist as different subtypes. As such, there exist two types of globular forms, amphiphilic and non-amphiphilic, such as G_4 forms, that can be distinguished by their interaction with nondenaturing detergents (77,78). Furthermore, there exist forms which contain additional noncatalytic subunits bound to the catalytic ones. Such is the case with Torpedo amphiphilic dimers found in the electric organ (79) and with the human C_2 -type serum BCHE, reported to be a dimer conjugated to albumin (70).

Phospholipase C, which cleaves phospholipids between the phosphate head group and the 1,2-diacylglycerol moiety, was shown to release dimeric ACHE from the Torpedo electric organ (80) and from human platelets (31). It was shown that these hydrophobic dimers are bound through a C-terminal polypeptide to an oligoglycan, that is, in turn, bound to glucoseamine. The glucoseamine, which is not N-acylated, is attached to phosphatidylinositol by a glycosidic link (81). Another mechanism of binding to membranes was found to be via a 20 kDa noncatalytic subunit in the bovine caudate nucleus which anchors the G_4 form to the membrane (82,83).

The 20 kDa subunit was found to be pronase-K-sensitive, suggesting that it is in part a polypeptide, and a 13-kDa fragment thereof was shown to be responsible for the hydrophobic aggregation of this form.

Another level of polymorphism relates to the finding of hybrid CHE molecules. In 1-day-old chicks, A_{1,2} forms were found to contain both ACHE and BCHE monomers as a hybrid CHE form (84). This adds a previously unperceived level of complexity to CHE polymorphism.

5.3 The genetic basis of polymorphism

With the implementation of molecular cloning techniques, some of the polymorphic CHE mysteries were uncovered. The isolation of differing Torpedo ACHE cDNAs (85) and genomic DNA sequences (86) has led to a further uncovering of the polymorphic forms origin. Evidence indicates that alternative splicing giving rise to different mRNA species. All ACHEmRNA species found are derived from several exons, of which two are present in all mRNA species, with most (90%) of the coding information included in the 3'-exon of the two. A third exon encodes the 3'-terminus and is form-specific. As such, the first exon encoding the C-terminus of ACHE to have been cloned encodes an amino acid sequence which should give rise to asymmetric forms. An alternative exon, which can replace this exon, is predicted to give rise to the hydrophobic PI-bound globular form. Thus, alternative splicing gives rise to globular PI-bound ACHE species, containing a unique carboxy terminus. The origin of soluble globular forms remains unknown. An additional exon has been found to encode a minor mRNA species, but as of yet has not been expressed or assigned to a specific molecular form (85).

In contrast with the clear linkage between ACHE polymorphism and the alternative splicing pattern at the genomic level, no evidence for alternative splicing as a cause of BCHE polymorphism has been shown. When synthetic human BCHEmRNA produced by in vitro transcription from an SP6 plasmid was injected into Xenopus oocytes, it produced functional dimeric BCHE (87). Coinjection of the synthetic mRNA with brain mRNA induced the appearance of tetramers, whereas co-injection with muscle mRNA induced production of heavier asymmetric forms, including the 16S form (88). This evidence indicates that all the necessary information for asymmetric- and globular-form synthesis is inherent in the primary amino acid structure of BCHE and that additional tissue-specific protein(s), presumably through posttranslational processing and/or assembly, are necessary to modulate subunit assembly within the system studied (87,88).

Polymorphism in the N-terminal region has not been shown for mammalian CHE primary structures, though Drosophila CHEcDNAs that have a long 5'-leader and several open reading frames have been isolated (89). 5'-alternative exons were also found for Torpedo ACHEmRNAs (85). These might be involved in regulation of translation.

It should be taken into account that the existence of PI-bound BCHE has not been proven as yet, which might explain why alternative splicing has not been found for this gene. Altogether, the molecular-form polymorphism in BCHE seems to be the result of processing of a single primary sequence and probably requires other tissue-specific mRNAs, as exemplified in the Xenopus microinjection experiments.

6. Evolutionary divergence and conservation of cholinesterases

Cholinesterases have been shown to exist in a wide range of organisms, from bacteria to human. Within this spectrum, other proteins, with strikingly similar primary structures yet different functions, have been noted. As such, the cholinesterase family is most appropriate for studying the evolution of proteins and their functions. In Table II are listed some of the organisms that contain CHEs or CHE-like proteins.

Table II. Evolutionary distribution of cholinesterases

Organism	Reference	Enzyme
<u>Bacteria</u>		
<u>Pseudomonas aeruginosa</u>	(90)	CHE
<u>Unicellular Organisms</u>		
<u>Paramecium</u>	(91)	ACHE, BCHE
<u>Physarum polycephalum</u>	(92)	ACHE
<u>Tetrahymena geleii</u>	(53)	CHE
<u>Lower Organisms</u>		
<u>Caenorhabditis elegans</u>	(93)	ACHE
<u>Sea urchin</u>	(54)	CHE
<u>Plants</u>		
<u>Pea</u>	(94)	ACHE
<u>Insects</u>		
<u>Anopheles stephensi</u>	(95)	ACHE
<u>Musca domestica</u> (housefly)	(96)	ACHE
<u>Myzus persicae</u>	(97)	ACHE, BCHE
<u>Drosophila melanogaster</u>	(98)	ACHE
	(99)	Esterase 6, Pheromone
<u>Fish</u>		
<u>Torpedo marmorata</u>	(100)	ACHE
<u>Torpedo marmorata</u>	(101)	BCHE
<u>Electrophorus electricus</u>	(102)	ACHE
<u>Birds</u>		
<u>Chick</u>	(103)	ACHE
<u>Quail</u>	(104)	ACHE, BCHE
<u>Mammals</u>		
<u>Rat</u>	(105)	Lysophospho- lipase
<u>Bovine</u>	(106)	ACHE
<u>Bovine</u>	(107)	Thyroglobulin
<u>Rabbit</u>	(108)	Microsomal esterase
<u>Human (brain)</u>	(109)	ACHE

Augustinsson (110) proposed that plasma cholinesterases and carboxylesterases evolved from a common serine hydrolase ancestor and that with time, a specific anionic site was acquired. Recently, with the molecular cloning of CHEs from various origins (10) as well as of both human ACHE- and BCHEcDNAs (88), it became apparent by sequence comparisons that both CHEs belong to a unique carboxylesterase type B family (11,111). This family appears to have evolved independently from other serine hydrolases, such as trypsin, as is evident from the observation that CHEs are completely nonhomologous to serine hydrolases in their primary sequence.

7. Chromosomal localization of human cholinesterase genes

Two genetically independent loci, CHE1 and CHE2, have been linked to altered serum BCHE properties (5,15,88). Genetic-linkage studies, focused on the generally expressed BCHE locus CHE1, have suggested that it is situated on the long arm of chromosome 3 (112), in linkage with the Tf (transferrin) gene, (3q31-q26.1; 113), the caeruloplasmin gene, and the TfRC (transferrin receptor) gene (reviewed in 114). Mutations at this locus are responsible for the normal, "atypical," and "silent" BCHE variants found in serum. Although most genetic studies of BCHE were directed towards the serum enzyme, patients with "atypical" serum BCHE were found to contain the same "atypical" form of BCHE in brain tissue (115), suggesting that the same gene is responsible for BCHE expression in all tissues.

The existence of a single BCHE gene though, was found to be insufficient to explain the finding of isozymes, such as the fast-migrating C5 variant form of cholinesterase (116). This variant is expressed in 8% of the Caucasian population and has been shown to increase activity of serum BCHE up to 48% (117). Genetic-linkage studies on the protein produced from the CHE2 locus, responsible for the C5 form, link it to the alpha-haptoglobin gene (118), found on chromosome 16, distal to the fragile site 16q22 (119). Evidence from *in situ* hybridization studies employing human BCHEcDNA and human metaphase chromosomes are in agreement with the previous genetic evidence as to Chr. 3 and 16 BCHE loci. Two chromosomes were labelled with BCHEcDNA, chromosome 3, at 3q21-q26, and chromosome 16, at 16p11-16q23(120,121). Recently, linkage analysis using all published data (122) excluded substantial portions of the genome and showed weak linkage to chromosome 16. This, though, stands in contradiction to novel linkage analysis showing tight linkage between the Γ -crystallin gene cluster and CHE2 (123). Isolation and expression of the CHE2 gene will be necessary to substantiate either of these claims.

Coates and Simpson (124) suggested the existence of two codominant alleles at a single locus, responsible for the phenotypic variants of erythrocyte AChE. However, there was no followup to these studies. Lack of information as to the chromosomal localization of AChE is probably due to the lower number of phenotypically variant AChEs. Considering the pivotal role of AChE in terminating neuromuscular transmission, mutations altering its kinetic properties might be lethal. In *Drosophila*, several mutations in the *ace* locus responsible for AChE production are indeed lethal (for a review see ref. 125).

8. Genetic variants of human butyrylcholinesterase

BCHE is less substrate-specific than AChE and as such, interacts with many ligands. Differences in such reactions between individuals has been a key element in uncovering genetic variants of BCHE in humans. As mentioned above, there exist two functional chromosomal loci, one encoding the "usual" E1⁺ and another, the E2 encoding the C5 variant form of BCHE. Individuals are either E2⁺ or E2⁻. Immunological evidence shows that E2⁻ carriers display 20-30% more serum BCHE activity in addition to an increase in antigen-binding. This indicates that such individuals may contain more BCHE protein (15).

Several variant BCHE enzymes have been associated with the E1 locus. Kalow and Genest (126) employed inhibition by dibucaine to differentiate between the relatively insensitive atypical (E1^a) and the usual (sensitive) E1⁺ individuals. They furthermore showed that the enzyme in heterozygous individuals (E1⁺ E1^a) was inhibited in a pattern compatible with the average of activities measured in individuals homozygous for the usual and the "atypical" phenotypes. The dibucaine test remains the most prominent in determining "atypical" CHE in serum. Immunological studies have further shown that the "atypical" serum enzyme displays similar specific activity using benzoylcholine as substrate, though its

concentration is 30% lower (127). This suggested that there is less enzyme in individuals carrying the "atypical" enzyme, which might be due to lower mRNA stability, more slowly synthesized enzyme, or lower protein stability.

Further studies on serum BCHE revealed individuals lacking BCHE activity altogether. These individuals were shown to be homozygous for an additional allele termed "silent" (E1^s; 128). Some attention should be paid to this allele since determination of protein activity depends on the sensitivity of the method employed. If one approach does not discover activity, another might, causing a change in allelic description. As such, re-evaluating "silent" sera resulted in the classification of two additional types of apparently silent sera with very low BCHE activity (129). It seems that true "silent" BCHE genes are very rare, and other low-activity variants may be held responsible for their mislead interpretation.

The use of sodium fluoride has enabled the reclassification of some E1^u individuals as E1^f-fluoride-resistant. The serum enzyme in such individuals displays normal dibucaine inhibition. This gene as well was shown to be allelic with the usual gene (130). Several other rare variants were reported, including some with increased or decreased catalytic activity. Others lead to electrophoretically different proteins (15). Data on the frequency of various alleles in the general population were collected and analyzed (131). The "usual" allele appears in 98% of the population, whereas the frequency of the "atypical" variants appears to vary geographically. An outstandingly high frequency of 0.075 for variant CHE1 alleles was found in Iranian Jews, and similarly high frequencies were found for Iraqi Jews (132), whereas in certain other populations no atypical genes were found (15). Individuals having the homozygous "atypical" phenotype are likely to be represented by 1 in 2000 in the European populations, whereas 31 in 2000 will be heterozygotes. It is interesting to note that E2⁻ alleles encoding the presumably chromosome-16-associated gene appear to be absent or extremely low in certain populations, whereas others, such as the Aka pygmies in Central Africa, were shown to have 13% expression of this gene. The E2 locus is particularly difficult to analyze, as it seems that nongenetic factors are able to influence the E2⁻ phenotype. Therefore, children show this phenotype even though their parents do not (133).

9. Cholinesterases and diseased states

Succinylcholine is a short-term muscle relaxant that is employed in surgery. Most of the drug is rapidly degraded by serum BCHE, though 5% reaches the neuromuscular junction. Extended apnea caused by succinylcholine use in individuals with the atypical phenotype has been shown to be the result of decreased ability of the "atypical" BCHE to degrade this drug (134). Although it is not a usual diseased state, in that the individuals are apparently normal, this phenotype may, hence, cause difficulties when succinylcholine or other BCHE-hydrolyzed substrates are employed in clinical treatment.

OP intoxication is caused by a stoichiometric (1:1) binding of OP compounds to the active-site serine of CHES (135). Treatment of OP intoxication includes prophylactic and therapeutic approaches, such as protection against the OP agent with reversible CHE inhibitors (136). Quaternary oximes are employed to reactivate the enzyme to which OPs were bound by nucleophilic displacement of the phosphoryl moiety from the active-site serine (137). This process, though, is hampered by an intramolecular reaction termed "ageing," which occurs when CHES interact with some OPs, such as soman (pinacolmethylphosphonofluoridate), sarin (isopropylmethylphosphonofluoridate), and DFP (diisopropylphosphorofluoridate). This is believed to involve dealkylation of the covalently bound OP group, rendering therapy by these compounds extremely difficult (135,138). It should be noted that

there exists a large number of CHE inhibitors used daily in treatment of a variety of disorders, such as eserine for glaucoma and myasthenia gravis (139).

Excess CHE inhibitors reaching neuromuscular junctions may block diaphragm muscles and breathing, with lethal effects. In addition to affecting the neuromuscular junction, OPs cause delayed damage to the CNS, where their exact mechanism of action needs to be revealed.

BCHE levels in serum have been shown to vary with a standard deviation of 25% (140). In addition, increased or decreased BCHE activities have been attributed to many diseased states. In patients with burn injuries, renal disease, cancer, or liver dysfunction, BCHE levels decrease, whereas increases have been observed in cases of obesity, asthma, and alcoholism (15,131,141).

ACHE has been shown to be affected in neurological or genetic disorders, such as Alzheimer's disease (142) and Down's syndrome (143). Open-neural-tube defects in human embryos are biologically characterized by the secretion of a 10S tetrameric ACHE form (144). Furthermore, BCHE and ACHE genes have been shown to be co-amplified in leukemias (145) and ovarian carcinoma tumors (39) (see section III for details). Therefore, CHE genes might be diagnostically important in the analysis of various diseased states.

In a family exposed to OP insecticides, a defective BCHE phenotype was observed. This was accompanied by a de novo, apparently inheritable, BCHE gene amplification in a father and son (146). This finding might indicate OPs as causing a selection pressure capable of leading to genomic alterations. Interestingly, DNA synthesis in glial cells transfected with specific muscarinic receptor subtypes has been shown to be induced by acetylcholine analogs, such as carbachol (147), suggesting a complex feedback mechanism triggered by cholinergic signalling. This, in turn, implies that CHEs might indeed be involved in regulating basic metabolic processes. Hence, the elucidation of their encoding sequences and structure-function relationship studies may be regarded as the first steps towards unraveling these biological roles.

II. Experimental Approaches

The methods employed throughout this study include molecular genetics and biochemical, immunocytochemical, and computer analyses. In the following, only a brief discussion will be presented for standard state-of-the-art methods, whereas novel methods will be described in full detail. All of the chemicals employed were of analytical grade, except where mentioned otherwise. Sterilization of solutions was routinely performed by 35-minute autoclave treatment or by filtration through 20- μ m-mesh millipore filters.

1. Materials

1.1 Bacterial strains

All of the bacterial strains employed were Escherichia coli (E. coli) strains, each having unique genetic qualities as detailed in the following:

AG1- recA1, EndA1 gyrA96, thi, hsdR17, supE44, relA1, (uncharacterized mutation, improves transformation efficiency). Was employed interchangeably with DH1 in the last step of cosmid propagation.

DH1- F⁻, recA1, endA1, gyrA96, thi-1, hsdR17 (r_n⁻, m_n⁻) supE44, relA1?, Lambda⁻. Cosmid library was originally prepared in DH1 cells.

LE392- F⁻, hsdR514, (r_n⁻, m_n⁻) supE44, SupF58, lacY1, galK2, galT22, metB1, trpR55, Lambda⁻. Employed in Lambda phage propagation.

MV1190- Del(Lac-proAB), thi, supE44, del(so1-recA) 306xTn10(tet^r), [F':traD36, proAB, lacI^qzdelM15]. Supports growth of M13 single-stranded phages as well as plasmids employed in synthetic mRNA synthesis.

BHB3169- RK- MK+ Rec+/1434 TS, Red-, B2, S7. Rec+ in vivo packaging host employed with cosmid (source: B.Hohn, London).

1.2 cDNA and genomic libraries

Unless otherwise indicated, cDNA was inserted into the EcoRI site of Lambda-GT10 phage DNA.

Fetal liver, brain, muscle, and glioblastoma

Human fetal liver, brain, muscle, and glioblastoma (Gb) cDNA libraries were constructed in our laboratory or by A. Ullrich, Genentech, from poly(A)⁺RNA preparations found to contain intact CHEmRNA by microinjection bioassay, using Xenopus oocytes (111, 148, 149).

Adult liver and lymphocytes

cDNA libraries from adult liver and lymphocytes were received from Dr. Y. Yarden, Rehovot, Israel.

IMR32 neuroblastoma

A cDNA library from human IMR32 neuroblastoma cells was purchased from Clonetech.

Newborn brain

A cDNA library from newborn brain was prepared by Dr. P. Lazzarini in Lambda-GT11 phages and was donated to the American Type Culture Collection (ATCC).

Cosmid libraries

Cosmid libraries were generously donated by H. Lehrach (ICRF, London) and were prepared using male blood as source and pCos2 (kanamycin-resistant) as vector in DH1 cells (150). The library employed was pooled from six independently constructed libraries, each containing 3×10^5 clones and 2×10^9 cosmid-forming units/ml.

1.3 Standard solutions

DDW- Autoclaved deionized and distilled water.

High-salt restriction buffer- 150 mM NaCl, 6 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 100 μ g/ml bovine serum albumin.

Low-salt restriction buffer- 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10mM 2-mercaptoethanol, 100 μ g/ml bovine serum albumin.

TBE- 0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA

TE- 10mM Tris-HCl pH 7.4, 1mM EDTA

STET- 8% sucrose, 5% Triton X-100, 50mM EDTA, 10 mM Tris-HCl pH 8.0.

SSC- 0.15 M NaCl and 0.015 M NaCitrate.

Carrier DNA- Salmon sperm or Herring DNA (Sigma) was dissolved to 10 mg/ml, sonicated, boiled for 20 min and stored for up to 6 months at -20°C .

1.4 Commonly used enzymes

Klenow fragment of E. coli DNA polymerase- Boehringer (Manheim, Germany).

Lysosyme- Sigma (St. Louis, Mo.) Grade I, L-6876.

Proteinase K- Sigma (St. Louis, Mo.) type XI, P-0390.

Restriction enzymes- New England Biolabs (Beverly, Mass.) or Boehringer (Mannheim, Germany).

RNase A- From Sigma or Boehringer. RNAase A was dissolved to a concentration of 1 mg/ml and boiled for 20 min to rid it of DNAase activity. Storage was at -20°C up to several months.

SP₆RNA Polymerase- Amersham International (U.K.).

Sequenase polymerase- United States Biochemicals (Cleveland, Oh.).

T4 Polynucleotide kinase- Boehringer.

T4 Polynucleotide ligase- New England Biolabs.

Unconventional chemicals- All chemicals employed in CHE bioassays were from Sigma, unless otherwise specified. Bambuterol was gratefully received from Drako (Sweden) and ecothiophate was from Ayerst laboratories (Montreal, Canada). "CAP" analog (P^1 -5'-(7-Methyl)-guanosine- P^3 -5'-guanosine triphosphate) was from Pharmacia, Sweden.

1.5 Growth media

LB (luria-Bertani) Medium -Per liter

10 g Bacto-Tryptone (Difco, Detroit, Michigan)

5g Bacto-yeast extract (Oxoid, Hampshire, England)

10 g NaCl

Mg⁺⁺ -Bacterial plates and media employed in Lambda-phage growth required the addition of 10 mM Mg⁺⁺ salt final concentration, added to LB medium from a 1 M solution of autoclaved $MgSO_4$ in DDW.

TM -10 mM Tris, pH7.0; 10 mM $MgSO_4$. Phage-dilution buffer.

Maltose -Prior to growth of bacteria to be infected by Lambda phages, 0.2% final concentration of maltose was added to LB solutions from a 20% stock maltose solution prepared in sterile DDW.

1.6 Antibiotics

Ampicillin (Amp)- Stock solution of 50 mg/ml of its sodium salt was prepared in double-distilled water (DDW), was filtered, and was employed at a final concentration of 50 µg/ml. Purchased from Sigma as the sodium salt A-1598.

Kanamycin (KN)- Stock solution of 25 mg/ml in DDW was filtered and employed at 15 µg/ml. Purchased from Sigma as kanamycin acid sulfate salt, K-1876.

1.7 Human-originated blood and tissue samples

Human originated tissues (basal nuclei from p.m. adult brain, ovarian adenocarcinoma tumors, excised at surgery) were obtained within 2 hr of surgery, having been stored at 4°C. Tissues were carefully dissected on ice, immediately frozen in liquid nitrogen and stored at -70°C until used. Human blood samples were drawn as detailed (145). Regulations of the Institutional Committee for Human Experimentation were followed at all times.

2. Methods

2.1 Generally employed molecular biology techniques

A. Plasmid miniprep

Colonies of *E. coli* containing the plasmid of interest were picked from LB-Amp plates and grown overnight (O.N.) in 5-10 ml of LB-Amp; 1.5 ml was transferred to Eppendorf tubes and spun for 1-2 min in an Eppendorf centrifuge, whereupon the supernatant was discarded and the process repeated in the same tube. The precipitate was redissolved in 75 µl of STET buffer, and was vortexed, then 6 µl of freshly prepared 10 mg/ml lysosyme was added. Samples were placed in a boiling water bath for 40 sec and centrifuged for 15 min; then, the supernatant was transferred to new tubes. To precipitate plasmid, an equal volume of isopropanol was added, and samples were placed at -20°C for 20 min, centrifuged for 5-10 min, and resuspended in 24 µl DDW. For enzymatic digestion, 8 µl was employed, and 1 µl of 1 mg/ml RNase A was added to degrade bacterial RNA.

B. Plasmid large preparation

Preparation of plasmid DNA was essentially performed by the boiling technique, as described by Maniatis et al. (151), with some important modifications. Bacteria containing the desired plasmid were mixed with 500 ml of LB containing the appropriate antibiotics and were shaken at 37°C for 12-18 hr. Cells were centrifuged in a Sorvall centrifuge at 2500Xg for 20 min at 4°C. The precipitate was resuspended in 20 ml STET buffer. One milliliter of a freshly prepared solution of 10 mg/ml lysosyme was added prior to a 1- to 1.5-min immersion in a boiling water bath. Mixtures were then cooled on ice and spun for 50 min in a Sorvall centrifuge at 9000 rpm. Supernatants were precipitated with a 0.5 M final concentration of NaClO₄ and half-volume isopropanol, spun for 15 min at 4°C, and resuspended in 1 ml DDW. Ten microliters of 1 mg/ml RNase A was added to each sample for further incubation of 30 min up to 18 hr at 37°C. Whenever plasmids served as templates for *in vitro* mRNA synthesis, DNA was treated with 100 mM proteinase K for 30 min, whereupon two half-volume phenol extractions were performed, followed by a single half-volume chloroform extraction and NaClO₄ precipitation. Samples were resuspended in DDW. Proteinase K treatment and one phenol extraction were omitted in cases where plasmid DNA did not serve as an RNA template.

C. Restriction endonuclease digestion

Digestions were performed with two to three units of each restriction enzyme per μg of DNA, with NaCl concentrations of 150 mM (high), 75 mM (medium), or 0 mM (low) for various enzymes, as recommended by the producers. Enzyme volume did not exceed 1/10 of total reaction volumes. Incubation was performed at 37°C for 2 hr up to 18 hr. High and low NaCl buffers were separately prepared (see Materials). For medium-salt-requiring enzymes, equal volumes of high and low buffers were mixed.

D. DNA fragment electroelution

To isolate size-specific inserts from plasmid and bacteriophage DNAs, DNA samples were enzymatically digested and gel-electrophoresed in 0.7%-1.2% TBE agarose gels (depending on insert size (151),) containing ethidium bromide at 1.5 $\mu\text{g}/\text{ml}$. When the fragment of interest was separated, the region it migrated to was excised with a clean razor blade, placed in a dialysis bag containing TBE buffer, and electrophoresed until all the insert DNA left the agarose gel. The electric-voltage orientation was then inverted for 30 sec. Ion-exchange columns (Prepac, BRL) were washed with 3 ml of 2 M NaCl and equilibrated in 5 ml 0.2 M NaCl. Following the addition of 0.1 M NaCl (to reach a final concentration of 0.2 M), samples were loaded using a syringe, forced through the minicolumn twice, and washed with 3 ml of 0.2 M NaCl. Insert DNAs were eluted into Eppendorf tubes with 0.5 ml of 2 M NaCl, diluted 1:1 with DDW, and were divided into two tubes. Samples were precipitated by adding 1 ml ethanol at -20°C overnight followed by 10 min of Eppendorf centrifugation. Supernatants were poured off, precipitates dried by inverting tubes for 4-5 min, and DNA inserts resuspended in DDW or TE.

E. DNA ligation

Whenever possible, double-restricted inserts with two distinct ends were employed. The ends of such inserts matched the termini of the vector DNA. This approach allowed for high efficiency in ligation and construction of novel vectors and ensured correct orientation of the ligated fragments.

Ligation was performed using up to 1 μg of DNA at 4°C overnight with 10:1 insert:vector ratios whenever doubly digested inserts were employed and a 2:1 ratio when blunt-end or singly restricted inserts were employed. Ligation mixtures contained up to 1 μg of the relevant DNA vector and insert DNA, as detailed above, in the presence of 50 mM Tris-HCl, pH 8.0, and 7 mM MgCl₂ in a volume of 10 μl . Following a 10-min heating period at 45°C, ATP and DTT were added up to final concentrations of 1.4 and 5 mM, respectively. Finally, T4-DNA Ligase (400 units, Biolabs) was added and the final volume of the reaction was adjusted to 20 μl with DDW. Samples were stored at -20°C until used.

F. Bacterial transformation

Competent *E. coli* cells were prepared by growing 10 ml cultures overnight. Starter cultures were then diluted to 0.03 OD (600 mu) into 200 ml and were grown to 0.3 OD. Cells were precipitated by centrifugation for 10 min at 3000 rpm, in a Jouan C4.11 centrifuge (Saint-Mazaire, France) at room temperature and were resuspended in 100 ml of 50 mM CaCl₂. After incubation at 4°C for 20 min, cells were precipitated as above and resuspended in 20 ml of CaCl₂. Glycerol was added to a final volume of 23 ml, and aliquots of 300 μl were frozen in liquid nitrogen for 1 min and were stored for up to 6 months at -70°C.

To transform cells, 10 μl of ligation mixture or 10 ng of circularized plasmid

was added per tube of thawed competent cells and incubated on ice for 1 hr up to several hours. Cells were then heat-shocked at 42°C for 2-3 min. 700 μ l of LB medium was added for 1 hr; this was followed by plating of 100 μ l/plate on LB-agar antibiotic plates. Plates were then incubated at 37°C overnight.

2.2 Labelling of DNA probes

A. Oligodeoxynucleotide end labelling

One hundred pmoles of the oligodeoxynucleotide in use, dissolved in 10 μ l were mixed with 100 pmoles of [^{32} P] γ -ATP (Amersham, 5000 Ci/mole). The reaction was carried out in 70 μ l of 50 mM Tris HCl, pH 7.4, 10 mM MgCl₂, 5 mM DTT, 0.1mM spermidine, 0.1 mM EDTA, and 18 units of polynucleotide kinase (New England Nuclear) for 1 hr at 37°C. The reaction was stopped by the addition of 100 μ l of 20 mM EDTA at pH 8.0. Labelled oligonucleotides were separated from non-incorporated [^{32} P] γ -ATP by ion-exchange chromatography, as follows: A 0.5-ml column of DEAE cellulose (Whatman DE52) was equilibrated with TE buffer. The reaction mixture was loaded, and the column was washed with 1ml of TE followed by 5 ml of 0.2 M NaCl. The labelled oligonucleotide was eluted with 1 ml of 2 M NaCl. Yields ranged around 1×10^8 dpm, or 50%-75% of the input of [^{32} P]ATP. Thus, the specific activity of our labelled probes was 1×10^7 dpm/pmole. For smaller amounts of probe, the procedure was scaled down, employing 20 pmoles oligonucleotide, 20 pmoles [^{32} P]ATP, 1 μ l T4 polynucleotide kinase and buffer, all in a 14- μ l reaction volume.

B. Random prime labelling of DNA fragments

Labelling of DNA probes was performed using the Boehringer random prime labelling kit, employing [^{32} P] α -dATP [3000 Ci/mole] as label. Unlabelled deoxynucleotides were separated by gel filtration on 3-ml columns of Sephadex G-50 or G-100 prepared in 0.2 M NaCl and washed with an additional 2 ml of 0.2M NaCl. Fifty micrograms of carrier DNA was added to the labelled probe and was loaded onto the column. Washing was performed with 0.2 M NaCl. Eight 350- μ l fractions were collected and the three peak fractions (detected by Beta-emission counting) were pooled to yield 1×10^8 dpm/ μ g DNA.

C. Nick translation

Fifty to one hundred nanograms of DNA were used per experiment. Reaction mixtures contained the following: 2 μ M of dGTP, dTTP, and dCTP; 50 uCi of [^{32} P] α -ATP (3000 Ci/mole, Amersham, England); 400 nM of unlabelled dATP; nick-translation buffer (50 mM Tris-HCl, pH 7.8; 5 mM MgCl₂, 10 mM beta-mercaptoethanol, 50 mg/ml bovine serum albumin (BSA); and 2 μ l of pancreatic DNAase (50 ng/ml), in a final volume of 50 μ l. The mixture was incubated at 37°C for 10 min and cooled on ice. Five units of DNA polymerase (2 μ l) were added to the mixture and reactions were incubated 1 hr at 15°C. The reaction was terminated by the addition of two volumes of 20 mM Tris-HCl, pH 7.4; 0.2 M NaCl; 20 mM EDTA, and 1% SDS. Free nucleotides were removed by passing the reaction mixture through a 3-ml G-50 sephadex column built into a polypropylene column (GS-Q, Isolab Incorporated, Akron, Ohio) as follows: The column was washed with 5 ml 0.2 M NaCl. Five microliters of 5 mg/ml sonicated and preboiled single-stranded salmon sperm DNA (ssDNA) was added to the nick translated DNA and loaded onto the column. An additional 5 ml of 0.2 M NaCl was added and collected in 330- μ l fractions. The three peak-labelled fractions were collected and specific activity determined. Yields were in the range of 1×10^8 dpm/ μ g DNA.

2.3 Hybridization analyses

A. Dot-blot hybridization

One microgram of DNA from pCosCHEDNA clones C1-C4, and 60 ng of either BCHE or ACHEcDNA were spotted onto nitrocellulose filters, with herring DNA used as a negative control. Filters were placed on Whatman 3-mm paper soaked with denaturing solution (0.5 N NaOH and 1.5 M NaCl) for 5 min and for an additional 5 min on Whatman paper soaked in neutralization solution (1M Tris-HCl, pH 8, and 1.5 M NaCl). Filters were air-dried and were prepared for hybridization as described below in DNA-blot analysis.

B. DNA-blot hybridization

One to two micrograms of cosmid or plasmid or 20 µg of genomic DNA samples were digested to completion by restriction endonucleases, separated by agarose gel electrophoresis, blotted under 0.5 N NaOH onto nylon Genescreen membranes (N.E.N), and UV- cross-linked. Hybridization was in 35% formamide, 6XSSC and 1XDenhardt's (1XDenhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% Bovine serum albumin). Where nitrocellulose filters were employed, they were denatured, neutralized, baked in vacuo for 2 hr at 80°C, and hybridized in 6XSSC, 5XDenhardt's solution, and 5 mM EDTA. Washing conditions were as detailed in the following sections.

C. Chromosomal blot hybridization

Chromosomal blots were donated by Bios Corporation (New Haven, Ct.) and included DNA from 27 different hybrid human/hamster cell lines. Each carried hamster chromosomes as well as one or more human chromosomes or fragments thereof, all restricted with HindIII. Hybridization was performed at 65°C in an incubator, as recommended in the Bios Timeframe System using the Large Bios Hybridization Cassette (Model No. TF-2500). Experiments were performed at 65°C in an incubator with 2.4 kb BCHEcDNA at 10 ng/ml. Probe DNA was multiprime-labelled (Feinberg and Vogelstein, 1983), yielding a specific activity of 6×10^8 cpm/µg. Hybridization was for 60-90 min. Washes were at 65°C in 0.1XSSC.

D. RNA-blot analysis and size fractionation

Glioblastoma and fetal liver (18-week gestation) Poly(A)⁺RNA were prepared by extraction in guanidine thiocyanate followed by two rounds of oligo(dT)-cellulose chromatography (152). Neuroblastoma-IMR32-cell RNA was gratefully received from Dr. F. Clementi (Milan, Italy). Ten microgram samples of Poly(A)⁺RNA from the same glioblastoma tumor which served for preparing the screened cDNA library were separated by gel electrophoresis, blotted onto a Genescreen filter (New England Nuclear), hybridized with the probe, and washed 60°C in 0.45 M NaCl/0.045 M sodium citrate/0.1% SDS. Exposure was for 10 days with a Cawo intensifying screen. Similarly, 300 µg of Poly(A)⁺-containing glioblastoma RNA prepared from a primary glioblastoma tumor overexpressing BCHE activity (41) was fractionated by sucrose-gradient centrifugation (148). Fractionated RNA was injected into oocytes.

E. Gene-mapping by chromosomal in situ hybridization

In situ hybridization was performed using Q-banded (153) and R-banded (154) chromosome preparations from peripheral blood lymphocytes, employing either a 2230-bp EcoRI fragment derived from a full-length BCHEcDNA of fetal liver origin (149) or an extended noncoding 3'-terminal region of 700 base pairs from this gene, isolated from human neuroblastoma and glioblastoma libraries (155). Hybridization was performed as detailed (120, 121), with some modifications.

Briefly, chromosome spreads from peripheral blood lymphocytes treated with 5-bromodeoxyuracil (154) were preincubated in 2 X SSC for 30 min at 70°C. RNA was hydrolyzed by a 60-min incubation at 37°C in 0.1 mg/ml of pancreatic ribonuclease (Sigma); this was followed by successive washes of 5 min in 2 X standard saline citrate (SSC) and 70%, 80%, and 100% ethanol. DNA was denatured by a 4-min incubation at 70°C in 70% formamide, 2 X SSC, and 10 mM potassium phosphate buffer at pH 7.0. The chromosome spreads were immediately transferred to cold ethanol at 100%, 80%, and 70% concentrations for successive washes of 5 min and were air-dried. Each spread was then covered by a 25- μ l drop of hybridization solution containing 50% formamide, 10% dextran sulfate, 1 X Denhardt's solution, and 8 ng of the preboiled DNA probe was labelled by nick-translation with (³²S)adenosine and (³²S)cytosine to a specific activity of 1X10⁸ cpm/ μ g, and was purified by three successive precipitations in ethanol in the presence of 10 W:W of salmon sperm DNA as carrier. Hybridization was for 18 hr at 37°C in a humid chamber and under cover slides. Chromosomes were then washed with 50% formamide and 2 X SSC (1hr, 37°C), 2 X SSC (15 min, 37°C), 2 X SSC and 20 mM beta-mercaptoethanol (15 min, 37°C), 0.15 X SSC (15 min, 50°C) and, where mentioned, 0.15 X SSC (15 min., 60°C), were dehydrated by successive 5-min incubations in 70%, 80%, and 100% ethanol at room temperature, and were air-dried. Exposure was under photography emulsion (Kodak NTB-2 diluted 1:1 in H₂O at 45°C) in a dry chamber at 4°C for 12-15 days, and development was for 0.5-1.5 min in HC100 Kodak developer.

Slides were stained for 15 min in 150 μ g/ml Hoechst 33258 stain (Aldrich), rinsed in distilled water, and dried. To create the R-bands, stained slides were mounted in 2 X SSC under coverslips and were illuminated for 30 min by a mercury vapor lamp at a distance maintaining a temperature of 47°-50°C, were rinsed in distilled water, and were restained in 4% buffered Giemsa (Gurr-R-66) at pH 6.8.

2.4 Screening and isolation of human CHE DNA sequences

A. Construction and screening of cDNA and genomic libraries

Tissues expressing translatable ACHEmRNA were identified by Xenopus oocyte bioassay (148). Complementary DNA libraries were then constructed from polyadenylated mRNA from fetal human muscle (18 weeks gestation) and from total RNA from adult basal brain nuclei (70 years) in the Lambda-Gt10 and Lambda-zapII-phage vectors (Stratagene, Heidelberg), respectively. Additional cDNA libraries which were screened are detailed under section III1. A human genomic DNA library was purchased from BRL (Gaithersburg, Md.). Oligodeoxynucleotides were 5'-end-labelled and differential library screening was performed as previously described (152), using the 35-mer-oligodeoxynucleotides CTACHE, d3'-ATG*TAC*TAC*GTG*ACC*TTC*TTG*GTC*AAG*CTG*GTG*AT*) 5'-complementary to the sequence that encodes the peptide N'-Tyr-Met-Met-His-Trp-Lys-Asn-Gln-Phe-Asp-His-Tyr-C' present in the C'-terminal region of Torpedo ACHE (156), and in which G or C residues were inserted in positions where codon ambiguity presented a choice between G and T or between C and A. This probe was designed not to hybridize with BCHEcDNA, as the parallel peptide of human BCHE differs in three of these twelve amino acids (149,157). Exclusion of

false positives was then accomplished with OPSYNO (149), a mixed oligonucleotide probe expected to hybridize with both BCHEcDNA and ACHEcDNA, since it encodes the peptide N'-Phe-Gly-Glu-Ser-Ala-Gly-Ala-Ser-Val-C' found in the active-esteratic-site peptide of human serum BCHE (149,157) and differs from the parallel peptide of Torpedo ACHE by only one amino acid [no. 7 in this peptide Gly in Torpedo (156)]. Screening of libraries was performed by plating 5×10^3 phages from each library on 15-cm LB-Mg plates (3×10^4 phages per plate) using Escherichia coli LE392 as host. Duplicate nitrocellulose filters were lifted, denatured, neutralized, and baked at 80°C for 2 hr (151). Upon isolation of positively hybridizing clones, sequencing analysis was performed.

To maximize reliability in sequencing the G,C-rich fragments obtained in this screening, both strands were sequenced using multiple adjacent sequencing primers. In addition, reduced nucleoside triphosphate concentrations, replacement of guanoside nucleotides by inosine, and the inclusion of manganese ions in sequencing reactions, all according to producers' recommendations, were found to be useful. Also, sequencing was repeatedly performed on the same DNA regions using the single-stranded phage M13 and the double-stranded pGEM and Puc118 plasmids. Finally, the combination of sequenase DNA polymerase with single-strand binding (SSB) protein (USB, Ohio) resolved densely packed sequence domains.

B. Isolation of cosmid cholinesterase genomic sequences by recombinant selection

B.1 General paradigm

Cosmids are basically plasmids that contain a bacterial origin of replication, and antibiotic resistance. In addition, they contain the Lambda-phage Cos sites, enabling insert DNA of 45-50 kb to be packaged in the Lambda bacterial coat. These cosmids are able to infect E. coli cells since they are phage particles and contain a bacterial origin of replication. Once inside the cell they constitute a large 50-kb plasmid.

There are advantages and disadvantages in cosmid use. Phage vectors cannot contain more than 20 kb of foreign DNA, and plasmid vectors do not transfect cells efficiently when they contain inserts larger than 10 kb. As such, isolation of human genes larger than 20 kb in one fragment necessitates cosmids. Furthermore, the recombinant screening of cosmids is performed via biological selection and does not require tedious screening with radioactively labelled probes as do other isolation procedures. Oligonucleotide probes sometimes bind sequences with nucleotide mismatches (71), whereas recombination is very specific in that it requires an exact homologous sequence of at least 60 base pairs (158).

The major difficulty in cosmid use is the same as its major advantage, its size. Since cosmids are large, they are difficult to analyze, which necessitates further in-depth restriction analysis and subcloning of interesting fragments.

Recombinant screening employs a plasmid with a fragment of DNA as bait to isolate other sequences flanking it on one or both sides. The bait sequence is inserted into a plasmid that contains antibiotic resistance (A), yet no sequence homology to the cosmid vector. This plasmid is named the probe plasmid. Partially digested human DNA is then inserted into cosmid vectors containing antibiotic resistance (B). The constructs are then packaged and used to infect E. coli. Natural recombination will occur between probe plasmid and human cosmids containing homologous genomic DNA and its flanking regions. Cells containing the new probe-plasmid-cosmid, as opposed to nonrecombined cosmid-containing cells, are now dual-antibiotic resistant (A + B).

E. coli strains employed for this procedure contain prophages that upon heat induction produce sufficient amounts of coat protein to encapsulate newly formed plasmid-cosmid phages. These, in turn, are employed to transfect new E. coli cells and are plated on dual-antibiotic (A + B) plates.

B.2 Cosmid library amplification

B.2 Cosmid library amplification

Cosmid library amplification was performed by growing 0.5-1.0 ml of cosmid library (see Materials) in DH1 cells, in 20 ml of LB-Kn (15 µg/ml at 37°C) for 14 hr.

B.3 Induction of rescue phage

Four plates of BHB3169 *E. coli* cells were plated and grown for 16 hr on LB agar, two at 42°C and two at 30°C to ensure the correct temperature-dependence of prophage induction. Cells of two BHB3169 agar plates were collected by adding 10-20 ml of LB, and OD₅₅₀ was determined. Four 1-liter Ehrlenmyer flasks containing 500 ml of LB (totalling 2l) were inoculated with the starter to an OD₅₅₀ of 0.05 and were grown at 30°C until the OD reached 0.5 (3.5 hr). Induction of rescue phage was performed for 15 min at 45°C, in a shaking water bath, whereupon it was shaken at 37°C for an additional 2.5-5 hr. To ensure induction, a few drops of chloroform was added at 37°C for 10 min to 1 ml of cells, in a clear test tube (and were compared to an equal sample without chloroform to examine if cells lyse). Bacteria were then centrifuged at 4000 g for 10 min at 4°C. Each 500-ml starter was resuspended in the smallest amount of TNM (10 mM Tris, 50 mM NaCl, and 5 mM MgCl) (5-10 ml/liter of LB) that would result in a total of 30 ml/2 liters of cells. Ten drops of chloroform/1.5 ml were then added and cultures were vortexed and incubated 30 min at room temperature. DNAase I (0.2 µg/ml) was added; the sample was centrifuged at 4000 g for 5 min; and the supernatant was collected. Helper phages were titrated on DH1 or AG1 cells. 1X10¹¹ PFU/ml was obtained.

B.4 In vitro cosmid packaging using helper phage

One hundred microliters of amplified cosmid library in DH1 cells was grown at 37°C for 16 hr in 30 µg/ml kanamycin (Kn) LB. The starter was added to four 250-ml LB-Kn-containing flasks at a final OD₅₅₀ of 0.05. Each 250 ml supplied sufficient phage cosmids for one to two cosmid screens. Bacteria were then grown at 30°C until OD₅₅₀ reached 0.3 (approximately 3X10⁷ cells/ml). For packaging, cells were grown in the presence of 5 mM final concentration of MgSO₄ and rescue phage (red S⁷ phage, see above) and helper phage added to m.o.i. of 50 (50 phage copies per cell, is approximately 1.5X10⁹ phages/ml). Flasks were incubated for 30 min at 42°C, transferred to 37°C, and shaken for 3 to 4 hr. Cells were collected, spun for 10 min at 3000 g, and resuspended in TM (10mM Tris pH 7-8, 10 mM Mg⁺⁺) in 1/20 volume. Five to ten percent chloroform was added, and tubes were shaken and incubated for 30 min at room temperature. The rescued cosmids and rescue phage was spun at 9000 rpm in a sorval centrifuge or its equivalent and titred.

B.5 Phage cosmid titration

The supernatant contains helper phage at 10¹¹/ml and cosmid phages at 10⁴-10⁶/ml. Titre at 30°C was calculated to reach values under which the helper (red S⁷) will not be induced to lyse cells, including cosmid-infected cells.

Tube	volume employed	added to TM	dilution
1)	10 µl cosmid soup	90 µl	10 ¹
2)	10 µl of 1	90 µl	10 ²
3)	10 µl of 2	90 µl	10 ³
4)	10 µl of 3	90 µl	10 ⁴

10 µl of 1,2,3, and 4 were placed into 300 µl LE392 cells at 30°C for 15 min, and 700 µl of LB was added and incubated for 1 hr at 30°C. Cells were shaken and 100 µl of tubes 1 to 4 plated. The calculation was as follows:

$$\text{cfu./ml} = \# \text{plaques} \times \text{dilution} \times 1000.$$

Titres were in the range of 10⁷ to 10⁸/ml usually 10⁷

B.6 Plasmid probe

Competent BHB3165 cells were prepared by the CaCl_2 method (see above). An EcoRI 190-bp genomic sequence containing part of the human BCHE coding sequence from nucleotides 60-250 (159) was inserted into the EcoRI site of puc118 vector (ampicillin-resistant; E. Padan, Hebrew Univ. of Jerusalem), which is not homologous to the pCos2 cosmid vector used in constructing the cosmid library. The recombinant plasmid was introduced into the BHB3165 competent cells. In general, the plasmid-DNA-containing probe fragments should be in the range of 60 bp-1 kb, and should not be homologous to the cosmid vector. Large plasmids (>5kb) will cause large recombinant molecules and that will inhibit packaging. To ensure that no fragment was homologous to the cosmid vector, the probe plasmid vector was hybridized to total cosmid library DNA prepared by the alkaline method (151).

B.7 Recombination

Two plates of probe cells BHB3165, one at 30°C and the second at 42°C were plated on LB-Amp plates and grown overnight to determine if cells were lysogenic. Cells were scraped from the BHB3165 plates and added to 200 ml of prewarmed LB containing 30 µg/ml Amp at a final OD_{550} of 0.05. Flasks were shaken at 30°C until $\text{OD}_{550} = 0.3$, whereupon MgSO_4 to 5 mM and 2×10^8 cosmids were added. Adsorption was at 30°C for 15 min, and was followed by shaking at 30°C for 1.5 hr. Kanamycin (15 µg/ml) was added and packaging was induced at 42°C in a shaking waterbath for 25 min. Flasks were transferred to 37°C for 3 hr, whereupon cells were concentrated by centrifugation at 3000 g for 10 min. One milliliter of supernatant was saved for titration and the remaining was discarded. Cells were resuspended in 10 ml of TM and 1 ml chloroform and were shaken at room temperature from time to time for 30 min. Cells were then centrifuged and supernatant containing the recombined repackaged cosmid-plasmids was saved. Titration as previously described, employing LE392 cells on Kn plates, yielded 10^8 - 10^9 cfu/ml.

B.8 Selective plating of recombined cosmids

Six 15-cm LB plates containing 15-µg/ml KN and 30-µg/ml Amp were prepared, and once again BHB3165 cells were plated by streaking out two plates at 30°C and two at 42°C to evaluate if the cells were inducible. Cells from the 30°C plates were collected and diluted in 150 ml LB to $\text{OD}_{550}=0.05$. After growth at 30°C until OD_{550} reached 0.3, BHB3165-plating cells were centrifuged and redissolved in 1/20 volume TM (7.5 ml). Traces of chloroform were evaporated from 2 ml of titrated recombined cosmids (10^8 - 10^9) by opening the tube at room temperature for 10 min, and 2 ml of BHB3165 plating cells were added. Adsorption was at 30°C for 15 min, whereupon 15 ml of LB was added and cells were shaken at 30°C for 1 hr. Cells were spun for 5 min, resuspended in 1ml LB, and plated on three Kn-Amp LB plates (330 µl/plate) on nitrocellulose filters to prevent the ampicillin from diluting into the plate. Incubation was performed at 30°C for 36 hr. Recombinant colonies were transparent and very small, whereas background colonies were thick, round, and brownish.

B.9 Repackaging of potential recombinants

Twenty-four transparent colonies were resuspended in 200 µl LB containing Kn (15 µg/ml) and Amp (30 µg/ml) and were grown at 30°C, with agitation for 3 hr. Phage production was for 15 min at 42°C, whereupon cells were shaken at 37°C for an additional 2 hr. A drop of chloroform was added, and tubes were shaken at room temperature for 15 min followed by 5 min of centrifugation in Eppendorf tubes.

Supernatants were stored at 4°C. 50 µl of BHB3165 plating cells was spotted onto a nitrocellulose filter on a 15 cm LB-mg plate and 5 µl of each of the 24 samples were spotted on the BHB3165 cells. The plate was incubated for 2 hr at 30°C and the filter transferred to an Amp-Kn plate at 30°C for 16 hr. Isolated colonies that were Kn-Amp resistant were then regrown in 200 µl LB Kn-Amp and cosmid phage supernatant was produced as mentioned above. These were employed to infect AG1 cells, as described above (with the exception that temperatures employed were only 37°C). Isolated AG1 Kn-Amp colonies were grown and the alkaline plasmid preparation (151) was employed to prepare plasmid DNA.

2.5 DNA sequencing and data analysis

Sequencing was performed, employing the Amersham M13 cloning kit. In short, isolated inserts were subcloned into the double-stranded M13 RF DNA, in both orientations by employing mp18- and mp19-phage vectors. In turn, they were transfected into *E. coli* and single-stranded DNA prepared from the M13 phages. DNA sequencing primers ranged from the universal primer to sequence-specific BCHEcDNA primers (71). Both Amersham sequencing kits and Sequenase sequencing kits were employed in the LKB and BioRad sequencing apparatus. Polyacrylamide-urea gels were constructed as company instructions required for each of the sequencing systems.

Analysis of data generated from DNA sequencing was performed using two computer software packages. The Microgenie software package, which is PC-compatible (Beckman, Palo Alto, California), was employed for DNA analysis as well as protein analysis and contains the NBRF and GenBank data banks. For more complex analysis, the sequence analysis software package of the genetics computer group (GCG; version 6.0, University of Wisconsin Biotechnology Center, Madison, WI.) which is available at the Hebrew University Medical Center, Hadassah Ein Kerem, Jerusalem, was employed. A profile, which is the composite of several homologous CHE proteins from various species, was constructed using "lineup" and "profile." Sequences included human BCHE, Torpedo ACHE, *Drosophila* esterase-6 and rat lysophospholipase, and a composite consensus sequence was determined by the program (section IV, Discussion). A data base search employing "profilesearch" led to the finding of several additional CHE-related proteins, including all of the published CHEs as well as thyroglobulin (C-terminal region) and rabbit microsomal esterase. The profile contains information defining where the family of sequences is conserved and where it varies. It can emphasize the similarity to conserved regions yet tolerate diversity in variable regions. As such, it is more sensitive in finding proteins of the same family than other conventional searches (160).

2.6 *In ovo* expression and analysis of cholinesterases from synthetic mRNA

A. Construction of transcription vectors encoding ACHE, BCHE, and BCHE variants

For ACHE expression studies, contiguous DNA fragments from several clones were ligated following digestion with the restriction enzymes HindII and SphI. Sequence continuity in the chimeric DNA product was confirmed by repeated sequencing across the ligation point, after which it was subcloned into the pGAM transcription vector having the SP6 RNA polymerase binding site (Promega, Madison, WI.). Two-nanogram samples of recombinant ACHEmRNA transcribed from this insert (2.2 kb in length) were used for microinjection experiments, in which the ability of synthetic mRNA to produce active ACHE was examined essentially as recently described (111).

"Normal" BCHEcDNA (DS) with aspartate70 and serine425 (87) and "unusual" BCHEcDNA (GP) carrying the mutations which confer the glycine70 and proline425

substitutions (ref. 155 and this document) were both subcloned into the pSP64 transcription vector (161). Enzymatic restriction of both plasmids with PstI and BamHI was employed to separate distinct DNA fragments carrying the regions with either of these mutations. Relevant fragments were isolated by gel electrophoresis and electroelution and were reconstructed into functionally complete transcription vectors by T₄ DNA ligase, creating the DP and GS constructs, each carrying a single amino acid substitution. Restriction enzyme analysis and DNA sequencing were used to confirm that each of the mutant constructs indeed contained the desired alteration and no other modifications.

B. In vitro transcription and microinjection of Xenopus oocytes

Transcription in vitro from each of the above constructs was performed with the SP6 RNA polymerase essentially as detailed elsewhere (87) and concomitantly with 5'-capping of the produced mRNA transcripts (162). The efficiency of transcription was estimated by analytical agarose gel electrophoresis. Equal amounts of each of the transcripts were injected into mature (stage 5-6) Xenopus laevis oocytes essentially as previously detailed (87). Briefly, frogs were anaesthetized on ice and oocytes dissected and gently dispersed in Barth's medium (80 mM NaCl; 1 mM KCl; 0.3 mM Ca (NO₃)₂·4H₂O; 1.6 mM MgSO₄·7H₂O; 2.4 mM NaHClO₄; 0.4 mM CaCl₂; 2 mM Tris-HCl, pH 7.4), supplemented with 400 mg/l streptomycin sulfate and penicillin G. At least 30 oocytes were injected with each mRNA preparation in several independent experiments (2 ng mRNA in 50 nl H₂O per oocyte). Parallel groups of oocytes were injected with Barth's medium and served as controls. Incubation was in 96-well microtiter plates for 18 hr at 19°C in groups of 10 oocytes incubated in 200 µl Barth's medium each.

C. Oocyte Homogenization and subcellular fractionation

Eighteen hours after injection, the incubation medium was recovered and the oocytes were homogenized on ice in 150 µl/10 oocytes fresh Barth's, PBS, or 0.1 M phosphate buffer (pH 7.0) in a glass-teflon homogenizer. The total homogenate was centrifuged for 10 min, 100,000 rpm (approx 400,000 X g), 4°C in a Beckman TL100 tabletop centrifuge (TLA100.2 fixed-angle rotor). The supernate was recovered and considered to reflect the low-salt-soluble fraction. The pellet was resuspended in 150 µl "TTNE" (10 mM Tris, pH 7.4, 1 M NaCl, 1% Triton X-100, 1 mM EGTA) and was spun for 5 min, as above. The resultant supernate was recovered and considered to represent the detergent-extractable fraction. Samples were stored at -20°C for up to 10 days. Frozen samples were rarely thawed more than once.

D. Spectrophotometric determination of CHE activities and inhibitor studies

Enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine was spectrophotometrically assessed as detailed previously (87, 163). Briefly, 5-, 10-, or 20- µl aliquots of oocyte supernatants were added to 170 µl of 0.1 M phosphate buffer (pH 7.4) containing 0.5 mM 5,5-dithionitrobenzoic acid (DTNB) in 96-well microtiter plates. Reactions were initiated by the addition of 20 µl of butyrylthiocholine (final concentration of 1 mM or 10 mM) and activities monitored by kinetically following the appearance of colored reaction product at 405 nm in a V_{MAX} kinetic microplate reader (Molecular Devices) equipped with the Softmax program for automated determination of hydrolysis rates.

For inhibitor studies, a pre-incubation period of 30 min was employed prior to the addition of substrate. Solanidine was initially dissolved in dimethylsulfoxide (DMSO) and then diluted to 16% (V:V) with H₂O to a stock concentration of 10 mM. Reaction mixtures subsequently contained 0.16% DMSO, which by itself failed to

influence BCHE activities. Final butyrylthiocholine concentrations were 10 mM in dibucaine and solanidine experiments and 1 mM in succinylcholine (SuCh) and pyridine 2-aldoxime methiodide (2-PAM) experiments. For reactivation experiments, 2-PAM was dissolved in 0.1 M NaCl at a stock concentration of 100 mM and the pH was adjusted to 7.4. Since 2-PAM directly augments the spontaneous hydrolysis of thiocholine substrates (164), a modified assay was required in the reactivation experiments. Briefly, 20 μ l oocyte homogenates were added to 30 μ l of 0.1 M phosphate buffer containing either 0.5 μ M DFP or 10 mM 2-PAM. For reactivation analysis, a 30-min pre-incubation with DFP was followed by the addition of 2-PAM for a further 2-hr period. Subsequently, 10- μ l aliquots were removed for BCHE activity measurements as detailed above.

One-microliter samples of an apparently normal, undiluted serum served as control for native CHE properties. Spontaneous hydrolysis of butyrylthiocholine was subtracted. Data represent the average of numerous experiments from several independent microinjections and unrelated transcriptions. About 80% of the microinjection experiments yielded activities in the noted range.

Michaelis-Menten constant (K_m), butyrylthiocholine (BuTCh), and inhibition coefficients (K_i) for succinylcholine (SuCh), benzoylcholine (BenzCh), propionylcholine (PropCh), and acetylcholine (ACH), all from Sigma (St. Louis, Mo.) were calculated in mM from activity assays performed as detailed above, with the exception that 1 mM BuTCh was employed as substrate for inhibition. Results are from several separate experiments from at least two unrelated transcriptions with 3.E.M. $\times$ 10%.

Fifty percent inhibition constants (IC_{50}) in μ M were determined from inhibition curves representing three experiments and two microinjections for each inhibitor.

3. Immunofluorescence and immunogold labelling by light and electron microscopy

3.1. Antibodies

Rabbit polyclonal antibodies prepared against Torpedo electric organ AChE were gratefully received from Drs. S. Camp and P. Taylor (San Diego, California) and were recently shown to interact specifically with clone-produced human BCHE peptides (163). The antibodies were preabsorbed for 2 hr on non-injected oocyte sections to prevent cross-species interaction with the amphibian enzyme (165) and were diluted 1:100 prior to incubation with injected oocyte sections.

3.2. Light-microscopic analysis of oocyte sections

a) Immunofluorescence technique with fluorochrome-conjugated second antibody

Twenty-four hours after microinjection, the oocytes were fixed for 2 hr in fresh 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After incubation in graded sucrose (5%, 10%, 20% W:V in 100 mM phosphate buffer, pH 7.3), the oocytes were embedded, animal pole up, in O.C.T. compound (Tissue-teck), and were frozen in -methylbutane cooled to -120°C with liquid nitrogen. Ten-micrometer sections were prepared in a cryotome at -20°C . The sections were collected on glass slides coated with 4% gelatin, were washed twice in PBS/0.1 M glycine, and were incubated in PBS/4% goat serum. After an overnight incubation at 4°C in primary antibody diluted in PBS/4% goat serum, the sections were washed three times in PBS/goat serum and were incubated with the fluorochrome-conjugated second antibody. After three further washes as above, the sections were mounted on coverslips in Galmount BIOMEDA non-autofluorescent medium. The sections were observed using a LEITZ fluorescence microscope.

b) Specific immunofluorescence with second phycoprobe conjugated antibody

In order to evaluate the nonspecific binding of second antibody, we used a phycoprobe-linked second antibody (anti-rabbit IgG H+L BIOMEDA), which gives a yellow signal when specifically complexed with the first antibody and a green signal when nonspecifically adsorbed to the oocyte section. The phycoprobe stock solution (1 mg/ml) was centrifuged 5 min at 12,000 X g; the supernatant was discarded; and the pellet was dissolved in 0.01 M phosphate buffer (pH 7.0) to a final concentration of 10 µg/ml. Incubations and washes were performed as described above.

E.3. Electron-microscopic techniques

Immunogold labelling was performed on 5-µm-thick cryostat sections. The technique utilized for light microscopy was performed up to the first antibody incubation, except that the PBS/goat serum was replaced by PBS/1% BSA. After three PBS/BSA washes, the sections were incubated with 5 nm colloidal gold particles conjugated to protein A (Janssen Life Sciences Products, Beese, Belgium) for 2 hr. The sections were washed three times with PBS for 1 hr at 4°C. After washing, the sections were postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.0) for 30 min at 4°C, dehydrated, and embedded in Epon. Ultrathin sections were cut, were poststained 3 min in uranylacetate 50% in acetone and 2 min in 2% lead citrate, and were then observed with a Philips EM 410 electron microscope.

III. Research Observations

1. Molecular cloning and expression of human acetylcholinesterase coding sequence

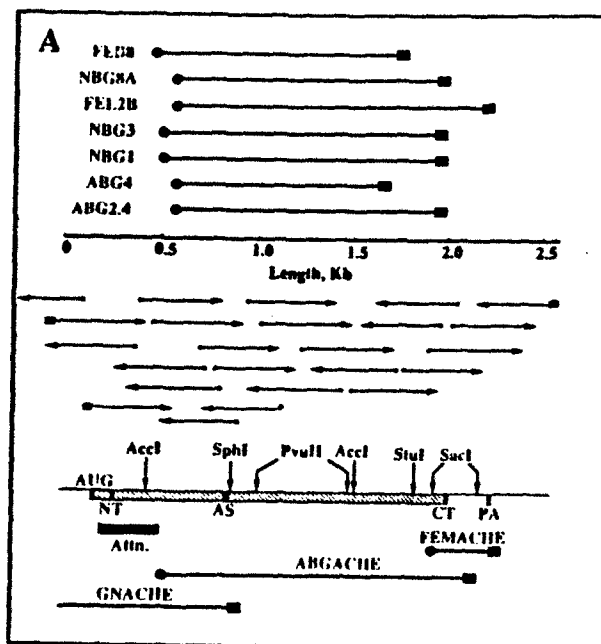
1.1 Isolation and characterization of human acetylcholinesterase coding sequence

In order to reveal the primary structure of the human ACHE and to acquire an essential tool for the study of the human ACHE gene, its structure, regulation, and amplification, molecular cloning studies were performed using oligodeoxynucleotides designed according to conserved and divergent peptide sequences of Torpedo ACHE (156) and human BCHE (149,157). The resultant DNA sequences and their inferred translation products were examined by alignment of specific residues in their primary sequences (11) and by expression studies in microinjected Xenopus oocytes (148). Finally, cloned human ACHE DNA fragments were employed as probes to study ACHE DNA amplification in vivo.

The differential screening procedure described in Methods resulted in the isolation of two similar 1.5-kb clones from neonate brain basal nuclei, designated NBG8A. Rescreening of several additional libraries (Figure 2A) with [³²P]-labelled NBG8A resulted in the isolation of over 25 positive clones, all encoding a polypeptide with the expected ACHE active-site sequence, but terminating about 500 nucleotides downstream from the expected initiator AUG codon. Figure 2A presents some of these clones. One 500 bp long muscle clone, designated FEMACHE, included a complete 3'-nontranslated region ending with a polyadenylation site and a poly(A) tail. Another, isolated from the newly constructed library from adult basal nuclei, was designated ABGACHE and included most of the coding and 3'-nontranslated sequence. Most of the isolated clones included the regions complementary to the oligodeoxynucleotide probes CTACHE and OPSYNO, which corresponded exactly to the peptide sequences used to design them. (Figure 2B, amino acid residues encoded by nucleotides 1939-1974 and 847-876, respectively). In addition, the isolated cDNA clones all contained large identical overlapping sequences, suggesting that they were derived from similar mRNA transcripts.

To derive a complete ACHE coding sequence, probe K-153, a 17-mer d[5'-CG^oGCC^oATC^oGTA^oCAC^oGTC], was designed according to the nucleotide sequence at the 5'-end of clone NBG8A. K-153 is complementary to the sequence encoding the ACHE-specific peptide Asp-Val-Tyr-Asp-Gly-Arg, and was used to screen a human genomic DNA library. The resultant genomic DNA clone, designated GNACHE, included the major exon coding for ACHE (terminated at nucleotide 1713, Figure 2B, as determined by comparative analysis of clones GNACHE and ABGACHE). It also revealed the absent upstream sequence encoding the N'-terminus of ACHE and a putative signal sequence preceded by a methionine residue. The AUG codon encoding this methionine was embedded in an appropriate consensus sequence for initiation of translation (166) and was preceded by a potential splice signal at the same position (nucleotide No. 136, Figure 2B) at which both the human BCHE gene (167) and the Torpedo ACHE gene (168) appear to be spliced. At its 3'-end, the genomic ACHE DNA clone resembled the structure of the ACHE gene from Torpedo in that it extended into two introns (ca. 1.1-kb and 0.6-kb long, respectively) at the same sites found for the electric fish gene (168), reconfirming the presumed common origin of the ACHE genes shared by these two evolutionarily remote species.

Figure 2. Isolation and characterization of the ACHE coding sequence



1. ACHEcDNA clones attenuated at the 5' region, and the construction of a complete ACHE coding sequence

Top: Several selected representative cDNA clones isolated by screening cDNA libraries from different human tissue origins and/or stages of development with labelled ACHEcDNA (145) are schematically presented. Libraries screened included FEB-Fetal Brain (149); NBG-Neonatal Basal Ganglia (donated to the American Type Culture collection by R.A. Lazzarini); FEL-Fetal liver (149); ABG-Adult Basal Ganglia, constructed in the course of this study (see section II for details). The isolated clones (FEB8; NBG1, 3, and 8A; FEL2B; ABG4 and 2.4) display variable 3'-termini and are all truncated within a narrow attenuating region (Attn.) at the 5' end of the coding sequence.

Bottom: To predict the complete ACHE coding sequence (dashed box), DNA inserts of the indicated lengths in kilobase (kb) from clones ABGACHE, FEMACHE, and GNACHE were isolated from cDNA libraries constructed from adult brain basal nuclei and fetal muscle and from a human genomic DNA library (BRL), respectively, and were subcloned either as shown or following enzymatic restriction in the EcoRI or the *Sal*I site of the M13 single-stranded phage or the sequencing plasmids pGEM or UC118 (Amersham, Stratagene). DNA sequencing was performed as detailed in section I, using the universal 17-mer primer (Amersham, No. 4511, indicated by a filled box at the beginning of arrow) or unique 17-mer primers synthesized from confirmed DNA sequences (indicated by arrows beginning with circles), as indicated by arrow length and direction. Sequence data were managed as detailed previously (149). Restriction sites for several nucleases (see arrows) were located in the sequence starting at the AUG by computer analysis of the sequence data and confirmed experimentally. Nt, N'-terminus; AS, active site; Ct, C'-terminus. An EcoRI site previously assumed to be included in this sequence (145) was found to be an experimental artifact and was omitted. Positions of the presumed initiator AUG codon, the predicted attenuating region (Attn), and the polyadenylation site (PA) are marked.

B. Primary structure of the nucleotide sequence and its inferred ACHE product

The 2.2-kb composite nucleotide sequence of clones ABGACHE, FEMACHE, and GNACHE and its encoded amino acid sequence are presented. Nucleotides are numbered (left-hand side) 5'-to-3'. The single-letter codes for the predicted amino acids are shown below the corresponding codons, starting with the first AUG. Amino acids in the predicted polypeptide product are numbered (right-hand side), starting with the initiator methionine expected by analogy with ACHEcDNA from *Torpedo* (85,156) and BCHEcDNA from man (149,157). Underlined are putative signal sequence (amino acid residues 5-25) and the region encoding the esteratic-site sequence (amino acid residues 231-240). The latter was found to match exactly the parallel peptide present in human serum BCHE (149,157). Also underlined is the C-terminal, selective 12 amino acid sequence which matched with a single nucleotide mismatch (notched) the ACHE - specific probe CTACHE (see section II) and which was expected and found to be completely different from the parallel peptide in BCHE and the 7 amino acid residues encoded by the K-153 primer (residues 161-167). Three putative sites for potential N-linked glycosylation, predicted by the sequence Asn-Xaa-Thr/Ser, in which Xaa represents any amino acid except proline, are ovally encircled. Cys residues are also circled, as are the first methionine residue, the esteratic-site serine, and the first and last amino acid residues in the mature protein based on analogies with other CHEs. Dashed underlines and arrows note putative splice signals identified in the coding sequence (nucleotides No. 136, upstream from the initiator AUG codon, and 1713 in the coding region, respectively).

In microinjected *Xenopus* oocytes, the synthetic 2.2-kb-long mRNA transcript of the recombinant sequence induced the biosynthesis of catalytically active ACHE capable of hydrolyzing 50.4 ± 5 nmoles of acetylthiocholine per hr per oocyte (average of three independent transcription and microinjection experiments). This represents a 10^4 -fold higher activity than that obtained by the microinjection of poly(A)⁺ brain mRNA (148). As expected, the recombinant enzyme appeared to hydrolyze butyrylthiocholine at a rate about 50 times lower. Furthermore, the oocyte-produced recombinant enzyme displayed substrate inhibition above 2 mM acetylthiocholine and was sensitive to inhibition by 10^{-3} M of the selective ACHE inhibitor 1,5-bis (4-Allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide (BW284C51), but not the selective BCHE inhibitor tetraisopropylpyrophosphoramidate (iso-OMPA, an OP) at the same concentration. In sucrose-gradient centrifugation (163) of products from two independent experiments, it yielded a major peak sedimenting as 3.2 ± 0.1 S, indicating monomeric units. Together, these experiments demonstrated that the combined sequence encodes for authentic human ACHE. (Table III).

Table III. Recombinant human ACHE production in microinjected *Xenopus* oocytes

Recombinant ACHEmRNA from three separate *in vitro* transcription reactions (87) was injected into 30 oocytes per experiment (2 ng mRNA per oocyte). Parallel groups of oocytes were injected with Barth's medium and served as controls. Following incubation, oocyte homogenates from three separate microinjection experiments were used for analyses of total ACHE-mediated hydrolysis of acetylthiocholine, essentially as detailed previously (87). Acetylthiocholine concentration was 1 mM. In order to ascertain sensitivity to inhibitors, either BW284C51 or iso-OMPA was added to the reaction mixtures in a final concentration of 10 μ M 40 min prior to the addition of substrate. Hydrolytic activities and percent inhibition values for the recombinant ACHE enzyme are shown in comparison with the endogenous ACHE activities in control oocytes. Spontaneous substrate hydrolysis (up to 0.6 mOD/min) was subtracted. Data shown represent mean values $\pm$ S.E.M. Note residual endogenous, BW284C51-insensitive, nonspecific hydrolytic activity accounting for approximately 2.5 nmol/hr per oocyte in all samples.

Table III: Recombinant human ACHE production in microinjected Xenopus oocytes

Oocytes injected with:	<u>ACHEmRNA</u>	<u>Barth Medium</u>
Acetylthiocholine hydrolysis rate, MOD 405/min	11.9 $\pm$ 1.9	1.1 $\pm$ 0.2
Substrate degraded, nmol/hr/oocyte	50.4 $\pm$ 5.0	5.2 $\pm$ 0.9
<u>% remaining activity</u>		
+ BW284C51	4.6 $\pm$ 0.35	48.7 $\pm$ 10.6
+ iso-OMPA	94.8 $\pm$ 1.4	88.3 $\pm$ 5.9

Comparative analysis of the amino acid sequence inferred for the deduced human ACHE sequence, human BCHE, bovine ACHE (169), Torpedo ACHE, and Drosophila CHE, Drosophila esterase 6 and Bovine thyroglobulin revealed considerable sequence similarities (Figure 3), higher than the percent of identically aligned residues observed at the nucleotide level for all of these sequences (88). The human ACHE inferred from this sequence has three potential sites for asparagine-linked carbohydrate chains (Figure 3), less than the four glycosylation sites in Torpedo ACHE (156) and the eight sites in human BCHE (149). Its lack of sequence homology to serine proteases distinguishes this protein as a type B carboxylesterase of the cholinesterase family (1) with a C'-terminal peptide that is characteristic of the soluble, asymmetric ACHE forms (87). It contains seven cysteine residues as compared with eight for both Torpedo ACHE (156) and human BCHE (Figure 3). Three intra-subunit disulfide bonds would be predicted at Cys¹⁰⁰-Cys¹²⁷, Cys²⁰⁰-Cys³⁰³ and Cys⁴⁴⁰-Cys⁵⁰⁰. A fourth predicted disulfide bridge involves Cys⁶¹¹, which in all known soluble CHEs appears to be covalently attached to the parallel cysteine residue of an identical catalytic subunit (47,87).

Computer analysis of the ACHE coding sequence presented a hypothetical stable 300-nucleotides-long secondary structure of stems and loops at the 5'-region of this sequence with, a free energy of -117.0 kcal/mole (170), higher than that recently demonstrated for the transcription-attenuating region in the genome of SV₄₀ (171). This predicted folding pattern, presented in Figure 4A, could potentially prevent reverse transcriptase and/or DNA polymerase from synthesizing this sequence, apparently explaining the 5'-attenuated cDNA clones (Figure 2A). Since tightly folded G,C-rich DNA sequences display distinct replication patterns during the cell cycle (172), we wished to examine whether this sequence domain is faithfully replicated in vivo, particularly in cases where ACHE gene amplification occurs (39, 145).

Figure 3. Amino acid homologies among CHEs from different species

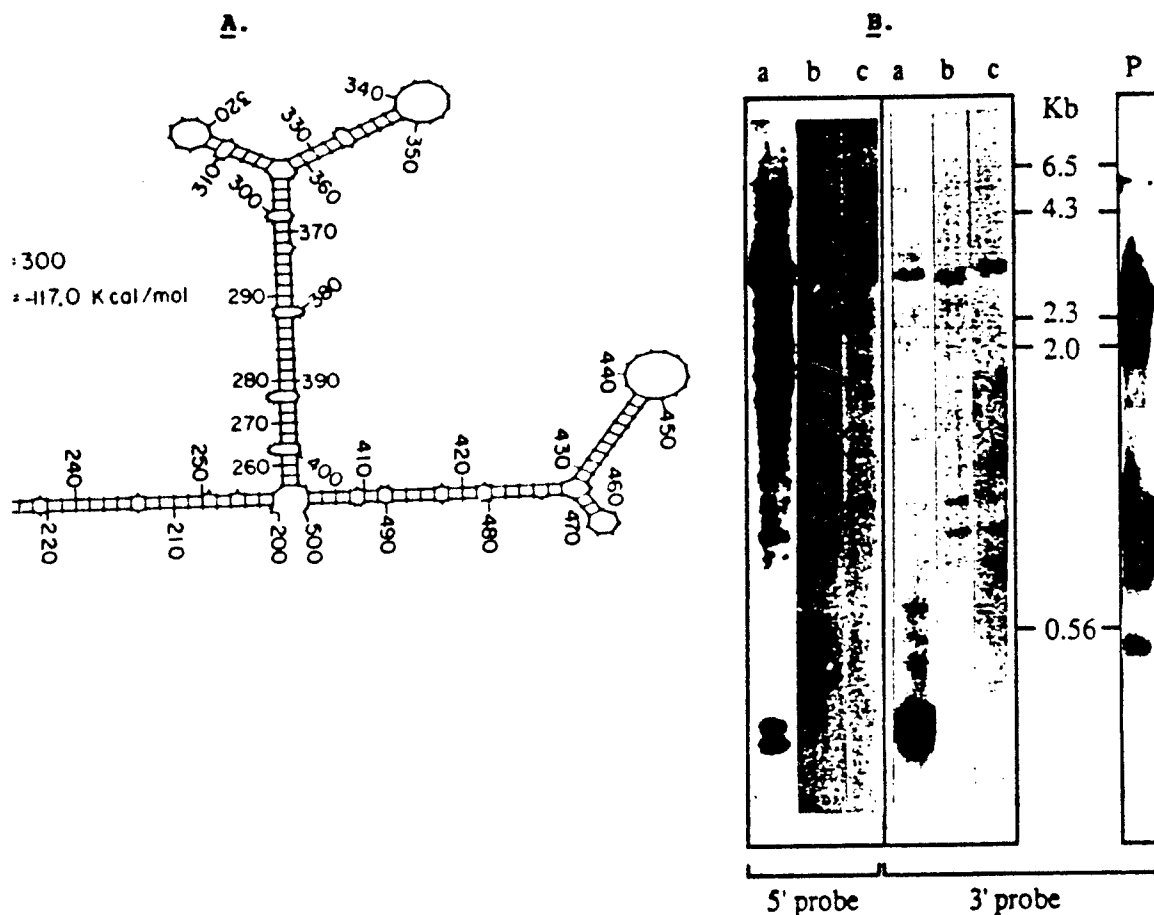
[illegible]

Amino acid sequences inferred for human ACHE (H.AChE) and BChE (149,157, H.), bovine ACHE (169, B.AChE), Torpedo ACHE (156, T.AChE), Drosophila CHE (89,

igned using the MicroGenie software program (Beckman, Geneva). Positions of teine residues (c) are noted by dots above. The putative first amino acid in mature protein (predicted by homology with the incomplete peptide sequence ilable for fetal bovine serum ACHE (169)) and the active-site serine are marked stars above. N-linked glycosylation sites are underlined. Note the high level identically aligned amino acid residues throughout the sequence, considerably her between CHEs than between the esterase 6 and thyroglobulin sequences. The al number of amino acid residues in the mature form of each protein is 583, 574, , and 2750 for H.AChE, H. BuChE, T. AChE, and Bov.Tg, respectively.

DNA blot hybridization with ACHEDNA sequences was performed using 5' and 3' ional probes. These included: (1) an SphI fragment including the putative enuator sequence which was derived from the 5' GNACHE probe, and (2) the 3' 8A probe which includes the 1.5 kb fragment that was previously used to detect ACHE gene amplification phenomenon (39, 145). The two regional probes were d in blot hybridizations of FvuII digested peripheral blood cell DNA samples a two different patients with hematopoietic disorders and a control, apparently lthy individual; A controlled quantity of transferred plasmid DNA was employed examine whether intensified bands in the genomic blots reflected multiple copies the probed DNA fragments (Figure 4B).

ure 4. The ACHE-DNA-5'-attenuating domain and its in vivo amplification



Hypothetical secondary structure of ACHE mRNA and/or DNA

Stable RNA/DNA structures in the ACHE coding sequence were searched for, using FOLD program of the University of Wisconsin Sequence Analysis software package (GCG), which allows for G-C, A-T, and the irregular G-U pairing (170). The structure presented above (nucleotides 200-500 in the composite sequence initiating at residue 200 in Figure 2) is the most stable structure for this sequence and was found to have free energy of -117.0 kcal/mole.

5'- and 3'-ACHE DNA probes display distinct intensities and patterns in blot hybridization

Two plasmid ACHE DNA probes were employed to selectively detect 5'- and 3'-domains in the ACHE gene. The 5'-GNACHE probe (Figure 2A) contained a 3-kb insert initiated at its 3'-end, with the putative attenuation sequence displayed in Figure 4A. The 2-kb-long 3'-NBG8A probe (Figure 2A) was composed of sequences downstream from the attenuation region of the ACHE coding sequence. Ten μ g of purified DNA from the peripheral blood of a patient with a platelet disorder carrying approximately 100 copies of the 5'-domain of the ACHE gene (a), from another leukemic patient with approximately 20 copies of this sequence (b) or from a normal individual with an estimated 1 copy of this DNA (c), were digested to completion with PvuII (Boehringer Mannheim) and electrophoretically separated on a 1% horizontal agarose gel (1.2 mA/cm; 18 hr). One hundred μ g of 5.3 kb long genomic ACHE DNA sequence in its plasmid vector (p) were subjected to similar procedures. This genomic DNA includes a Sac I fragment, starting 1.5 kb upstream of the initiator AUG and extending into the first Sac I site at its 3'-region (Figure 2A), therefore its restriction pattern should resemble that of the native ACHE gene. DNA was transferred to a GeneScreen membrane (NEN, DuPont) and hybridized with the [^{32} P]-labelled 5'- and 3'- probes (marked below), as recently described (39). Exposure was overnight and development -1 min, except for lane a (probe), which was developed for 20 sec, to prevent blackening of the entire film. Note complex, non-overlapping signals and variable labelling intensities.

The 5' SphI-GNACHE probe labelled an approximately 3 kb fragment or a slightly shorter 2.5 kb fragment which were intensely labelled in the genomic DNA from both patients. In addition, several other fragments of variable lengths were observed in the genomic blots from these two patients, reflecting ACHE gene amplification which probably extends upstream from the previously demonstrated region (7). Only one of the labelled bands could also be seen in control DNA (Figure 4B), indicating different modifications in the PvuII sites included in the two DNA samples carrying amplified ACHEDNA. In contrast, the 3' NBG8A probe labelled, in plasmid and control genomic DNA samples, fragments of approximately 3.0, 1.20, and 0.5 kb in length, which were all labelled with rather similar efficiency in patients' DNA samples. In addition, one of the patients' DNA samples displayed 0.4 and 0.3 kb long fragment which was strongly intensified with the 3'-probe. The genomic DNA from each of these two patients as compared with control DNA recently includes amplified ACHEDNA with higher copy numbers of the 5' DNA region compared with the 3' domain, possibly reflecting variabilities in the 3'-initiation point of the ACHEDNA amplification unit. Further experiments will be required to determine the boundaries of the core amplification unit and the different modifications and probable attenuation in its 5'-domain in various cases of the ACHE gene amplifies.

1.2 Comparative analysis of acetyl- and butyrylcholinesterase coding sequences

A. Codon usage analysis

The nucleotide compositions of the coding regions of the human ACHE and BCHE genes are listed in Table IVA. The sequences analyzed begin in each case with the presumptive initiator AUG codon and end with the first "nonsense" codon in the cDNAs encoding BCHE (149) or ACHE (111). Both sequences encode an "asymmetric" CHE form.

The ACHE and BCHE coding sequences displayed almost mirror-image usage of nucleotides with ACHEcDNA being enriched in G,C-residues, and BCHEcDNA in A,T residues (Table IVA). A striking difference in the frequency of CG dinucleotides (5.9% in ACHEcDNA vs. 0.8% in BCHEcDNA) implies a particularly high potential for DNA methylation (173, 174) within the ACHE coding sequence, in comparison to BCHE (Table IVB). Therefore, the conspicuously high CG dinucleotide frequency in the human ACHE coding sequence may reflect a previously unforeseen mode of regulation, retarding the expression of this gene where the production of BCHE would be less restricted.

The distribution of codons in the open reading frames is clearly different for the two genes. This is most apparent for the amino acids with multiple codon choices. For example, 45 of the 68 leucine residues in ACHE are encoded by CTG, whereas in BCHE, only 6 of the 55 leucines translate from this codon (Table IVC). Moreover, certain codons are completely absent in the ACHE gene but not in the BCHE one, like ATT. The third nucleotide choices for the BCHEcDNA sequence are typical of tissue-specific genes (172). In contrast, the G,C-rich ACHE gene resembles the α -skeletal muscle actin and the c-mos oncogene both in its unusual nucleotide composition and in its tissue-specific expression (172). Altogether, the amino acid composition of ACHE also presents several differences as compared with BCHE (Table V).

TABLE IV: Nucleotide composition and frequencies in human cholinesterase genes

A. Human ACHE and BCHE: sequences encoding polypeptides

ACHE (2097 nucleotides)

BCHE (2325 nucleotides)

Nucleotide	No.	%	Nucleotide	No.	%
A	370	(17.6)	A	772	(33.2)
C	682	(32.5)	C	402	(17.3)
G	656	(31.3)	G	457	(19.7)
T	389	(18.6)	T	694	(29.8)
A + T	759	(36.2)	A + T	1466	(63.1)
C + G	1338	(63.8)	C + G	859	(36.9)

B. Dinucleotide frequency :

ACHE

AA	80	(3.8)	CA	127	(6.1)	GA	122	(5.8)	TA	40	(1.9)
AC	116	(5.5)	CC	266	(12.7)	GC	189	(9.0)	TC	111	(5.3)
AG	119	(5.7)	CG	124	(5.9)	GG	239	(11.4)	TG	174	(8.3)
AT	54	(2.6)	CT	165	(7.9)	GT	106	(5.1)	TT	64	(3.1)

BCHE

AA	310	(13.3)	CA	162	(7.0)	GA	157	(6.8)	TA	142	(6.1)
AC	112	(4.8)	CC	87	(3.7)	GC	77	(3.3)	TC	126	(5.4)
AG	148	(6.4)	CG	19	(0.8)	GG	114	(4.9)	TG	176	(7.6)
AT	201	(8.6)	CT	121	(5.2)	GT	100	(4.3)	TT	100	(4.3)

C. Distribution of codons in the open reading frame:

ACHE

TTT Phe	9 (1.5)	TCT Ser	1 (0.5)	TAT Tyr	3 (0.5)	TGT Cys	2 (0.3)
TTC Phe	20 (3.3)	TCC Ser	11 (1.8)	TAC Tyr	18 (2.9)	TGC Cys	6 (1.0)
TTA Leu	0 (0.0)	TCA Ser	3 (0.5)	TAA End	0 (0.0)	TGA End	0 (0.0)
TTG Leu	3 (0.5)	TCG Ser	2 (0.3)	TAG End	0 (0.0)	TGG Trp	17 (2.8)
CTT Leu	3 (0.5)	CCT Pro	8 (1.3)	CAT His	2 (0.3)	CGT Arg	5 (0.8)
CTC Leu	16 (2.6)	CCC Pro	24 (3.9)	CAC His	13 (2.1)	CGC Arg	11 (1.8)
CTA Leu	1 (0.2)	CCA Pro	10 (1.6)	CAA Gln	3 (0.5)	CGA Arg	7 (1.1)
CTG Leu	46 (7.5)	CCG Pro	9 (1.5)	CAG Gln	21 (3.4)	CGG Arg	14 (2.3)
ATT Ile	1 (0.2)	ACT Thr	2 (0.3)	AAT Asn	5 (0.8)	AGT Ser	5 (0.8)
ATC Ile	8 (1.3)	ACC Thr	7 (1.1)	AAC Asn	12 (2.0)	AGC Ser	12 (2.0)
ATA Ile	0 (0.0)	ACA Thr	8 (1.3)	AAA Lys	3 (0.5)	AGA Arg	0 (0.0)
ATG Met	9 (1.5)	ACG Thr	9 (1.5)	AAG Lys	7 (1.1)	AGG Arg	6 (1.0)
GTT Val	3 (0.5)	GCT Ala	9 (1.5)	GAT Asp	8 (1.3)	GGT Gly	9 (1.5)
GTC Val	11 (1.8)	GCC Ala	35 (5.7)	GAC Asp	21 (3.4)	GGC Gly	21 (3.4)
GTA Val	5 (0.8)	GCA Ala	5 (0.8)	GAA Glu	3 (0.5)	GGA Gly	9 (1.5)
GTG Val	35 (5.7)	GCG Ala	6 (1.0)	GAG Glu	31 (5.0)	GGG Gly	19 (3.1)

BCHE

TTT Phe	30 (5.0)	TCT Ser	7 (1.2)	TAT Tyr	14 (2.3)	TGT Cys	5 (0.8)
TTC Phe	12 (2.0)	TCC Ser	5 (0.8)	TAC Tyr	6 (1.0)	TGC Cys	5 (0.8)
TTA Leu	9 (1.5)	TCA Ser	10 (1.7)	TAA End	0 (0.0)	TGA End	0 (0.0)
TTG Leu	16 (2.7)	TCG Ser	0 (0.0)	TAG End	0 (0.0)	TGG Trp	19 (3.2)
CTT Leu	14 (2.3)	CCT Pro	12 (2.0)	CAT His	9 (1.5)	CGT Arg	2 (0.3)
CTC Leu	6 (1.0)	CCC Pro	3 (0.5)	CAC His	1 (0.2)	CGC Arg	1 (0.2)
CTA Leu	4 (0.7)	CCA Pro	13 (2.2)	CAA Gln	11 (1.8)	CGA Arg	3 (0.5)
CTG Leu	6 (1.0)	CCG Pro	2 (0.3)	CAG Gln	9 (1.5)	CGG Arg	2 (0.3)
ATT Ile	13 (2.2)	ACT Thr	13 (2.2)	AAT Asn	26 (4.3)	AGT Ser	9 (1.5)
ATC Ile	6 (1.0)	ACC Thr	6 (1.0)	AAC Asn	14 (2.3)	AGC Ser	8 (1.3)
ATA Ile	12 (2.0)	ACA Thr	15 (2.5)	AAA Lys	27 (4.5)	AGA Arg	14 (2.3)
ATG Met	13 (2.2)	ACG Thr	3 (0.5)	AAG Lys	10 (1.7)	AGG Arg	2 (0.3)
GTT Val	10 (1.7)	GCT Ala	11 (1.8)	GAT Asp	17 (2.8)	GGT Gly	17 (2.8)
GTC Val	7 (1.2)	GCC Ala	10 (1.7)	GAC Asp	7 (1.2)	GGC Gly	5 (0.8)
GTA Val	8 (1.3)	GCA Ala	12 (2.0)	GAA Glu	26 (4.3)	GGA Gly	18 (3.0)
GTG Val	8 (1.3)	GCG Ala	1 (0.2)	GAG Glu	11 (1.8)	GGG Gly	7 (1.2)

It is interesting to note that the proteins encoded by these two sequences have almost identical amino acid compositions (Table V). In particular, the content of acidic and hydrophobic residues was precisely retained to be almost equal in the two proteins. The somewhat lower content of basic amino acid residues in ACHE (51 as compared to 61 in BCHE) deviates from this rule, and results in a more acidic isoelectric point predicted for ACHE (5.42 vs. 7.38 for BCHE).

TABLE V: Amino acid composition in complete polypeptides

ACHE (614 a.a)			
Ala	55 (9.0)	Leu	69 (11.2)
Arg	43 (7.0)	Lys	10 (1.6)
Asn	17 (2.8)	Met	9 (1.5)
Asp	29 (4.7)	Phe	29 (4.7)
Cys	8 (1.3)	Pro	51 (8.3)
Gln	24 (3.9)	Ser	36 (5.9)
Glu	34 (5.5)	Thr	26 (4.2)
Gly	58 (9.4)	Trp	17 (2.8)
His	15 (2.4)	Tyr	21 (3.4)
Ile	9 (1.5)	Val	54 (8.8)
Acidic	(Asp + Glu)		63 (10.3)
Basic	(Arg + Lys)		53 (8.6)
Aromatic	(Phe + Trp + Tyr)		67 (10.9)
Hydrophobic			
(Aromatic + Ile + Leu + Met + Val)	208		(33.9)

Molecular Weight of naked polypeptide = 67803.

BCHE (602 a.a.)			
Ala	34 (5.6)	Leu	55 (9.1)
Arg	24 (4.0)	Lys	37 (6.1)
Asn	40 (6.6)	Met	13 (2.2)
Asp	24 (4.0)	Phe	42 (7.0)
Cys	10 (1.7)	Pro	30 (5.0)
Gln	20 (3.3)	Ser	39 (6.5)
Glu	37 (6.1)	Thr	37 (6.1)
Gly	47 (7.8)	Trp	19 (3.2)
His	10 (1.7)	Tyr	20 (3.3)
Ile	31 (5.1)	Val	33 (5.5)
Acidic	(Asp + Glu)		61 (10.1)
Basic	(Arg + Lys)		61 (10.1)
Aromatic	(Phe + Trp + Tyr)		81 (13.5)
Hydrophobic			
(Aromatic + Ile + Leu + Met + Val)	213		(35.4)

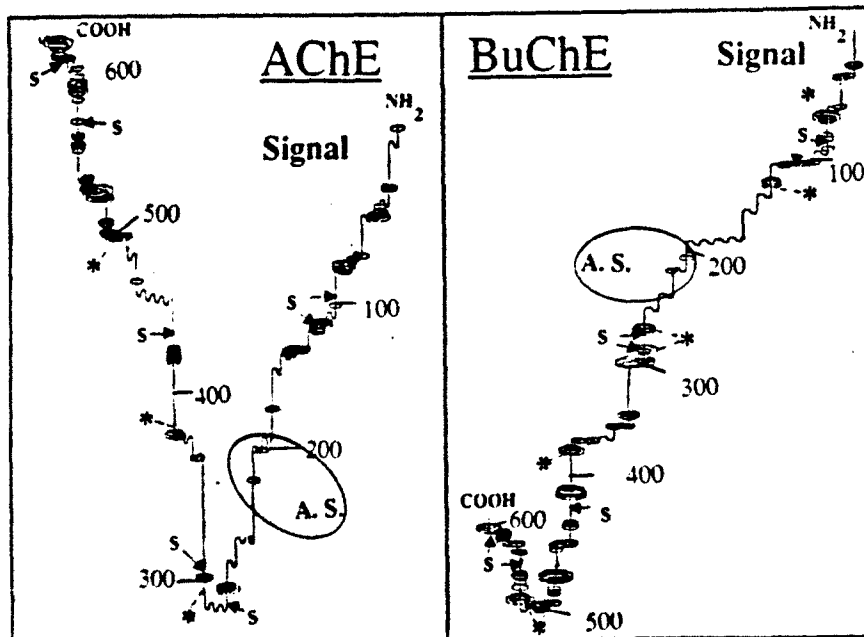
Molecular Weight of naked polypeptide = 68425.

B. Surface probability measurements

The polypeptide chains encoded by human ACHE and BCHE cDNAs were both subjected to computerized plot structure analysis according to the Chou and Fassman prediction (175). This analysis provides best-guess predictions regarding folding patterns within the analyzed proteins as well as evaluations on the probability of particular peptide domains to be located at the surface of the fully folded enzymes. Figure 5 presents these predictions for human ACHE and BCHE. As expected, the active-site domain in each protein is embedded within a part of the molecule with a particularly low surface probability. In contrast, regions with higher likelihood of being at the surface appear in both enzymes at the two ends of the active site. However, in spite of the conserved positions of the seven cysteine residues in these two proteins and their considerable amino acid sequence similarities, clear differences could be observed in the folding pattern predicted

for their polypeptide chains. Thus, the low-surface-probability domain in the ACHE protein extends over a region longer than 300 amino acids, whereas in BCHE it is limited to approximately 100 residues (Figure 5). This indicates a deeper groove for the ACHE active site, and may explain its high substrate specificity as compared with that of BCHE (13).

Figure 5. Surface probabilities for human ACHE and BCHE

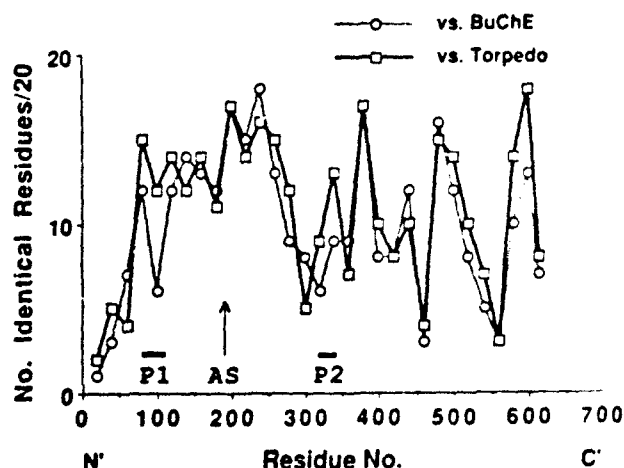


The polypeptide sequences encoded by ACHEcDNA and BCHEcDNA, designated AChE and BuChE, respectively, were subjected to plot-structure analysis by the Chou and Fasman prediction (175). Cysteine residues are noted by S- and potential glycosylation sites by stars. Peptide domains with high surface probabilities are encircled and active site regions are noted by oval signs.

C. Are there any ACHE-characteristic peptides?

Comparative analysis of the ACHE and BCHE polypeptides provided us, for the first time, with the opportunity to examine the sequence differences between the ACHE and BCHE proteins as expressed in a single species. To search for peptide domains that are characteristic of ACHE but not of BCHE, we divided both human sequences into 20-amino-acid-long units and counted the number of identically aligned residues in each (Figure 6). A similar comparison was then performed with *Torpedo* ACHE (156). The two comparisons revealed quite similar patterns, with particularly low levels of homology at the signal peptide region and a high level of homology in the vicinity of the active site (Figure 6). In addition, two specific peptides (P1, amino acid residue No. 82-112 and P2, No. 325-341) were revealed which displayed considerably higher homologies between the two evolutionarily remote ACHEs than between ACHE and BCHE from man. The possibility that these peptides may be implicated in the biochemical properties which distinguish ACHE from BCHE may now be examined by searching for *in vivo* variants of the ACHE gene, as well as by site-directed mutagenesis.

Figure 6. ACHE-specific peptides



Amino acid similarities are plotted for human ACHE as compared with human BCHE (BuChE) and with *Torpedo* ACHE. Peptide domains with considerably higher homologies between ACHEs are marked (P1 and P2). AS: Active site.

1.3 Preferential transcription of acetylcholinesterase over butyrylcholinesterase mRNAs in fetal human cholinergic neurons

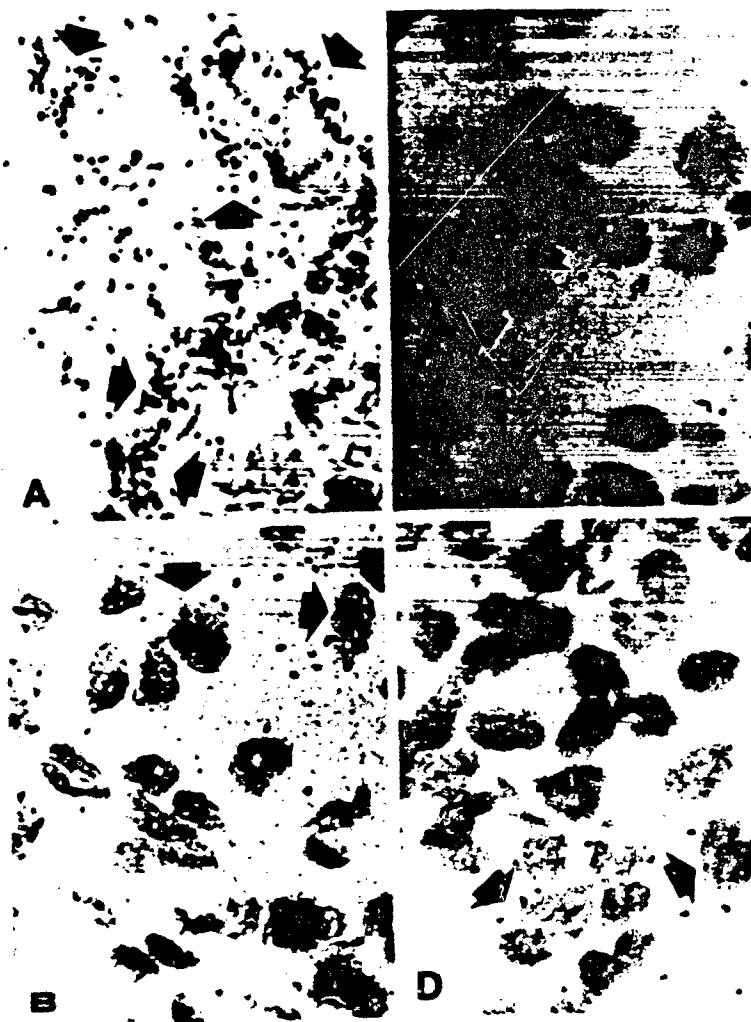
Cholinergic neurons in various regions of the primate brain acquire their biochemical and electrophysiological properties (176, 177) throughout fetal development in an intricate and complex pattern, which was studied in the human brain primarily by anatomical methods (178, 179). One of the characteristic properties displayed by such neurons is the expression of catalytically active ACHE (180, 181), which is generally accepted as a marker for their cholinergic nature (182). In the human brain, the basal nuclei present an example for a cholinergic region with intensive ACHE staining (183). However, ACHE activities may be observed in the developing fetal brain in many regions which are clearly not destined to become cholinergic (184). These include the developing human cerebellum (185, 186).

ACHE is transiently expressed in fetal noncholinergic neurons (186). Its level of expression and the developmental changes in its concentration are controlled independently from the production of muscarinic receptors (186). In contrast, ACHE production is accompanied by expression of the closely related enzyme BCHE (37). This early expression of CHEs has been assumed to reflect the involvement of these enzymes in differentiation processes (187) and to be unrelated to the acquirement of cholinergic functioning. In contrast, the staining of ACHE activities in the mature cholinergic brain regions rivals that of BCHE (188), indicating that the expression of each of these enzymes may be independently controlled. To examine whether this is the case, and to find out if the production of CHEs in fetal human brain is regulated at the transcription level, *in situ* hybridization was employed. Our findings demonstrate the preferential transcription of ACHEmRNA over BCHEmRNA in the cholinergic basal brain nuclei but not in cerebellar neurons, implicating the selective induction of ACHE biosynthesis in developmental processes of cholinergic neurons.

In situ hybridization was utilized to study regulation of synthesis of CHEs in developing human brain neurons. For this purpose, ACHEcDNA (111) and BCHEcDNA (149) were labelled with [³²S]; and hybridized with frozen sections (18-24 weeks gestation) from the cholinergic basal nuclei (189) as compared with the non-cholinergic cerebellum (185). Reproducible labelling was observed over the ACHE-secreting Golgi type I and II neurons (190) in fetal basal nuclei as well as in

fetal cerebellar external and internal granular neurons and Purkinje cells, i.e., in all of the cerebellar cell types characterized by intensive biosynthetic activities in the developmental period (191-193). Labelling was, however, absent in RNase-treated sections, confirming its dependence on mRNA presence (Figure 7).

Figure 7. Preferential labelling of fetal putamen neurons with ACHECDNA over [^{35} S] BCHCDNA

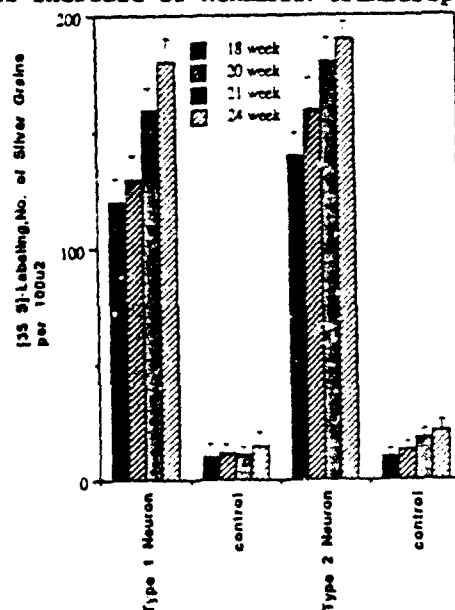


10 μ m frozen sections from the putamen region in fetal human brain (24 weeks gestation) were incubated for *in situ* hybridization with [^{35} S]-labeled ACHECDNA (A,B) or BCHCDNA probes (C,D) purified by agarose gel electrophoresis and electroelution. Pretreatment with RNase (B,D) was utilized to reveal specificity

of labelling. Hybridization and washing conditions were as previously described (35). Exposure for emulsion autoradiography was 14 days and counterstaining was performed with haematoxyline-eosine. Note the intensive labelling with ACHECDNA probe (A) as compared with the much weaker signals obtained with BCHECDNA probe (C), while negative RNase controls (B,D) were equally low for both cDNAs. Neuronal perikarya with variable labeling intensities are marked by arrows.

The intensity of labelling with the two distinct CHE probes, which were shown to display very low levels of cross-labelling (145), varied considerably between brain regions and in different developmental stages of particular regions. In basal nuclei neurons, labelling intensities with ACHECDNA increased from an average of 130 ± 25 grains/100 μ^2 at 18 weeks to 185 ± 28 grains at 24 weeks, reflecting a considerable enhancement in the levels of ACHEmRNA within the basal brain nuclei during this period of brain development. Similar patterns of developmental increases were found for Golgi type I and II neurons, and labelling was similarly sensitive to pretreatment with RNase (Figure 8).

Figure 8. Developmental increase of ACHEmRNA transcripts in basal nuclei neurons



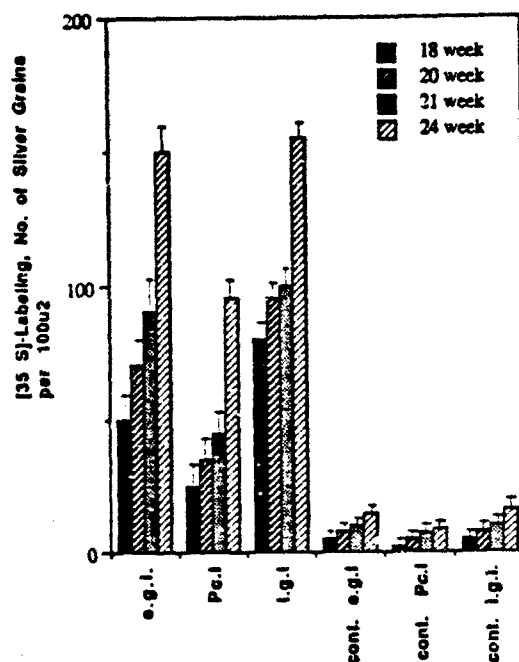
The intensity of labelling with [^{35} S]-ACHECDNA in Golgi type I and II neurons within basal brain nuclei was measured by *in situ* hybridization with frozen brain sections of 18-24 weeks gestation. Distinct types of neurons were microscopically detected by their size and general structure. Values were obtained by counting the number of silver grains in specific types of neurons within 25 fields of 100 μ^2 in size from at least five unrelated samples and by then calculating an average and standard deviation for each age group. Control sections were pre-treated with RNase to digest all mRNA and were equally analyzed. Note the significant developmental increase in labelling of both types of neurons.

In contrast with ACHEmRNA levels, there was no significant increase in the levels of BCHEmRNA in the basal nuclei. Labelling intensities with BCHECDNA were 8 ± 2 and 15 ± 3 grains/100 μ^2 at 18 and 24 weeks, almost as low as the levels measured for RNase treated control sections. Thus, Golgi type I and II neurons in the developing fetal brain synthesize ACHEmRNA with high preference over BCHEmRNA.

The different types of cerebellar layers were selected as a comparative model system for noncholinergic neurons. Labelling of cerebellar frozen sections resulted in generally lower average labelling signals with [^{35} S] ACHECDNA. These increased from 50 ± 20 grains/100 μ^2 at 18 weeks to 125 ± 18 grains/100 μ^2 at 24

weeks and were relatively more intense over granular neurons as compared with Purkinje cells. Interestingly, the levels of ACHEmRNA reflected by this labelling approach appeared to be essentially similar in the young, migrating, external granular neurons but were considerably lower throughout this period in the earlier developing Purkinje cells (Figure 9).

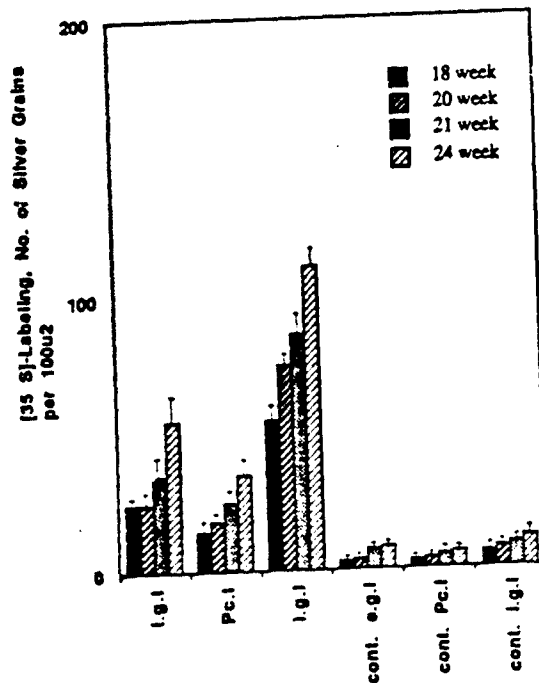
Figure 9. Developmental differences in ACHEmRNA levels within cerebellar cell layers



Frozen sections from fetal human cerebellum were hybridized with [³⁵S] ACHECDNA as described for Figures 8 and 9. The external granular layer (egl), Purkinje cell layers (Pcl), and internal granular layer (igl) were detected microscopically as detailed previously (194) and analyzed separately. The number of grains/100 μ^2 was determined for each layer by counting 25 fields, and average values and standard deviations are presented. Control sections were pretreated with RNase as detailed above. Note the developmental increases in ACHEmRNA levels, the apparently similar levels in egl and igl and the lower labelling intensities in the already developed Pcl.

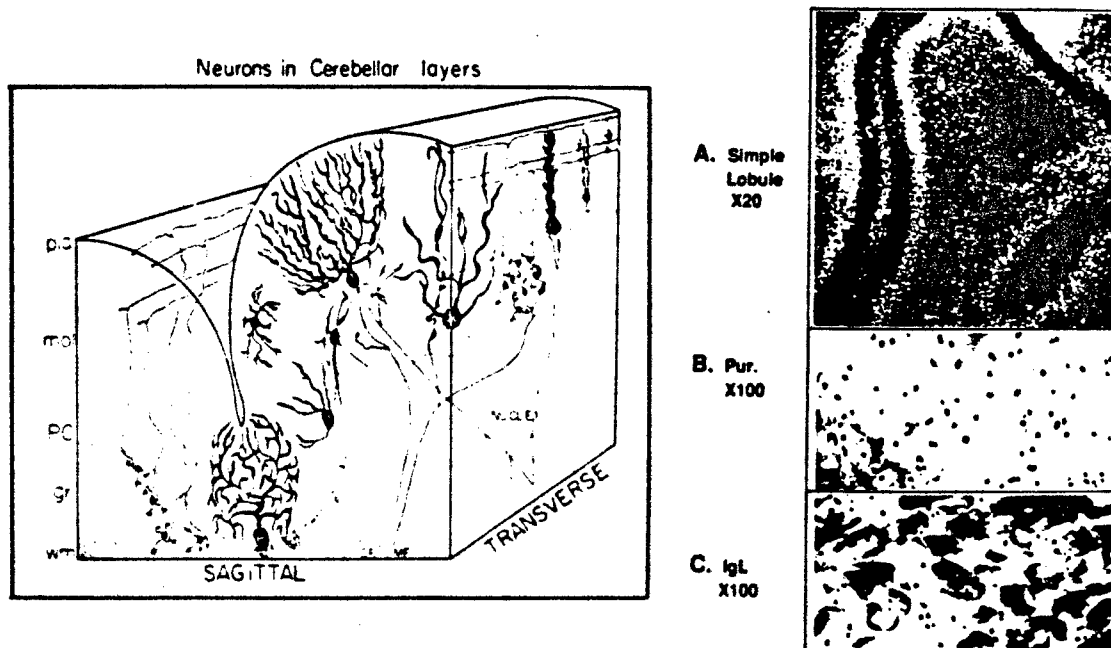
Labelling of the cerebellar sections with [³⁵S] BCHECDNA resulted in developmental patterns different from those obtained with [³⁵S] ACHECDNA. Average BCHECDNA signals increased from 25 $\pm$ 13 grains/100 μ^2 at 18 weeks to 55 $\pm$ 15 grains/100 μ^2 at 24 weeks. Particularly low signals were observed over Purkinje cells and relatively high signals over the mature, postmigration internal granular layer (Figures 10,11). These findings demonstrate a significant two-fold increase in the levels of BCHECDNA which takes place during the developmental migration of cerebellar granular neurons.

Figure 10. BCHEmRNA levels increase during the postmitotic migration of granular neurons in fetal human cerebellum



Experimental details were similar to those under Figure 9, except that [^{35} S] BCHECDNA was used as a probe. Note the low levels of BCHECDNA labelling over the Purkinje cell layer (Pcl) and the developmental increases in all of the cerebellar layers, with higher levels of labelling in the mature, postmigration internal granular layer (igl) as compared with the young, premigration external granular layer (egl).

Figure 11. AChE-mRNA labelling in pre- and postmitotic granular neurons in fetal human cerebellum



Experimental details were similar to those under Figure 10. A transverse section in the cerebellar simple lobule is displayed. Note the low levels of AChE-cDNA labelling over the Purkinje cell layer (B;Pur) and the higher levels of labelling in the granular layer (C;Igl).

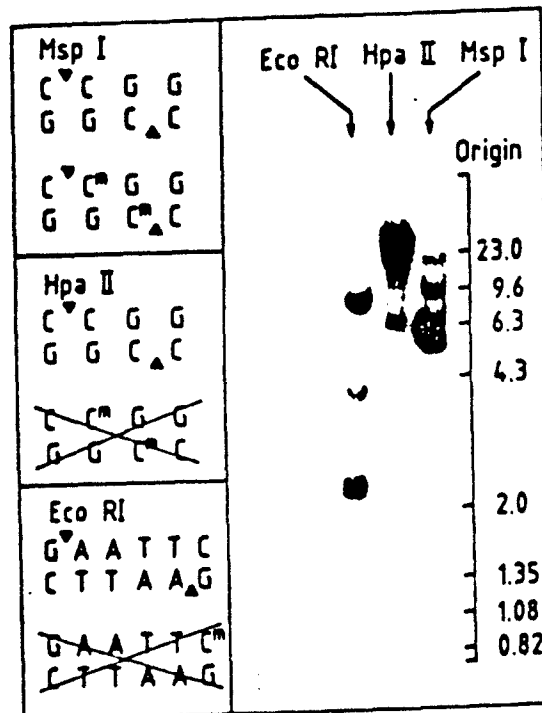
1.4 Coordinated expression of the cholinesterase genes in developing human oocytes

Ovarian DNA was first extracted and subjected to enzymatic restriction, agarose gel electrophoresis, and DNA blot hybridization with the ^{32}P -labelled full-length BCHE cDNA. The restriction pattern observed (Figure 12) was apparently similar to that determined for the BCHE genes in other tissues (149), suggesting similar organization for the BCHE gene in different tissues. The apparent difference between HpaII and MspI restriction patterns of the ovarian BCHE gene demonstrates the presence of methylated CpG dinucleotides (Figure 12).

To determine directly the efficiency of transcription from the BCHE gene in specific ovarian cell types, *in situ* hybridization with [^{35}S]-labelled BCHE-cDNA was employed. Reproducible hybridization signals, observed as densely distributed silver grains, were detected in single cells, identified by their form and positioning as oocytes, within follicular structures of different developmental stages in ovarian sections from several unrelated individuals. In several cases, the oocyte itself could not be seen, due to the density of silver grains covering it, and was identified mainly by the surrounding follicular structure. Figure 13 presents an autoradiograph of part of such a section, counterstained by hematoxylin-eosin, and accompanied by a camera lucida drawing of the various

follicles within this field. It is clear from this picture that most, but not all, follicles displayed positively labelled oocytes producing BCHE mRNA. The unlabelled follicles were most probably sectioned at levels in which the oocyte was not included.

Figure 12. Ovarian DNA blot hybridization reveals methylated CpG dinucleotides in gene

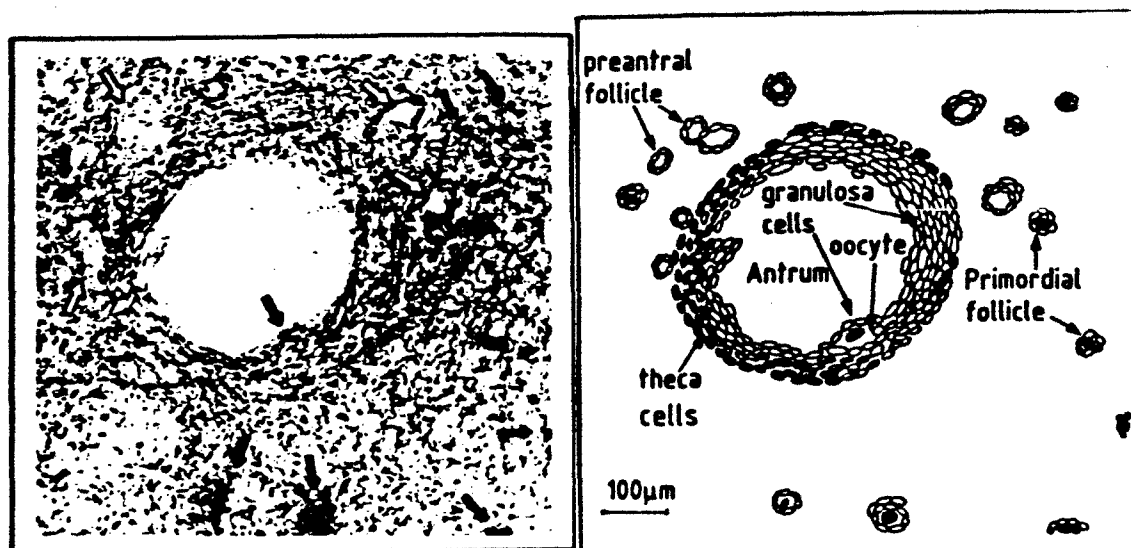


Ovarian genomic DNA (20 µg) was restricted separately with the enzymes *EcoRI*, *HpaII*, and *MspI*; separated by agarose gel electrophoresis; blotted onto nitrocellulose paper; and hybridized with the full-length human BCHE cDNA probe, labelled with ³²P by nick translation. Autoradiography was at -70°C for three days, with an intensifying screen. Note the difference between the methylation-sensitive *HpaII* and *MspI* lanes, suggesting the presence of CpG dinucleotides within the DNA fragments hybridizing with BCHE cDNA. The sequences restricted by each of these enzymes are noted at left.

Alternatively, it is possible that some oocytes do not contain BCHE mRNA, perhaps due to chromosomal defects (195). In contrast, atretic follicles were always devoid of hybridization signals (not shown).

Several control experiments were performed to exclude the possibility of nonspecific hybridization to other mRNAs and to verify the validity of the results. Thus, an irrelevant DNA fragment from a nonexpressed intron of the human superoxide dismutase gene (196) was similarly labelled and hybridized in parallel to several ovarian sections, with completely negative results. Other sections were pretreated with pancreatic RNase prior to hybridization with the [³²S]BCHE cDNA probe. These sections also remained totally unlabelled. The control experiments, as well as the selectivity of the hybridization signals, demonstrated that the *in situ* hybridization reactions with the [³²S]BCHE cDNA probe were highly specific and depended on the presence of intact mRNA within the ovarian sections.

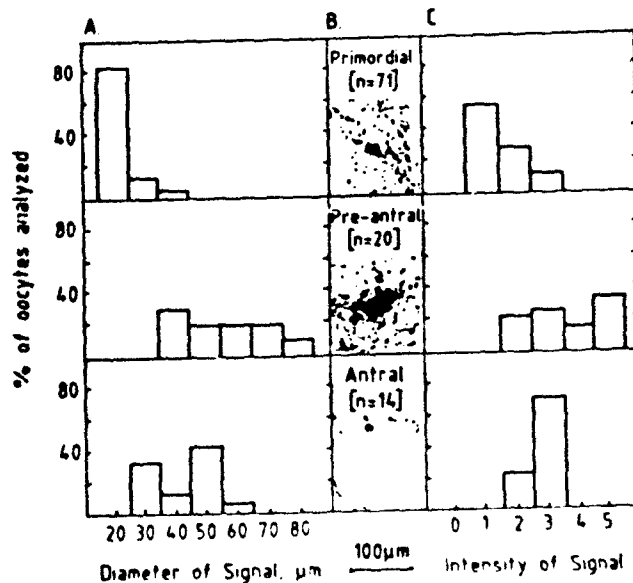
Figure 13. In situ hybridization of a frozen ovarian section with [^{35} S]BCHEcDNA .



An ovarian section was hybridized with full-length BCHEcDNA and labelled with ^{35} S by nick-translation. The washed slice was covered with photographic emulsion and exposed for 3 weeks. Counterstaining was with hematoxylin-eosin. The photomicrograph (left) and camera lucida drawing (right) display a representative area of the ovary. Strong signal intensity is depicted in oocytes at different stages of development (black arrows). Other follicles, probably those that did not include oocytes at the level of sectioning, did not show such signals (white arrows). Note partial loss of granulosa cells during the prehybridization processing steps of the ovarian section.

To evaluate the significance of these hybridization results, a semiquantitative analysis was performed on oocytes in structurally characterized primordial, preantral, and antral follicles. The hybridization signals could be characterized either by the density of silver grains or by the dimension of the labelled area, both reflecting the level of BCHEmRNA in the examined oocyte. Therefore, the density of grains on an arbitrary scale of 1-5 and the dimension of the hybridization signals were determined separately for each of the 105 photomicrographed follicles (Figure 14).

Figure 14. Transient enhancement of BCHE mRNA levels in preantral human oocytes



One-hundred-fifty primordial, preantral, and antral oocytes with positive hybridization signals were visually analyzed for signal diameter (A) and grain density (C), both estimated according to arbitrary scales of 1-5 and presented as percentages of the examined oocytes. Examples from hybridization signals observed at the three different stages of oocyte development are presented in (B). Note that preantral oocytes appear to display signals that are reproducibly bigger in diameter and higher in density than those observed in primordial and in antral oocytes.

Preantral oocytes were found to display significantly higher signals by both criteria, as compared with primordial and antral oocytes, suggesting that the level of BCHE mRNA is transiently high in the preantral stage of oocyte development.

The biochemical properties of ovarian CHEs were determined by [^{3} H]ACH hydrolysis measurements, inhibition studies, and sucrose-gradient centrifugations performed on ovarian extracts prepared from several specimens. The specific activity of total CHEs in the ovary was found to be ca. 535 nmoles ACH hydrolyzed/g/hr, comparable to the activities observed previously in extracts from human brain tissues (186). This implies that CHE represents ca. 1.10^{-5} M of total ovarian protein. Ovarian CHE activity was predominantly (75%) soluble in buffer. Minor CHE activities remained, however, associated with the insoluble fraction, from which it could be extracted with Triton X-100 alone or with Triton X-100 and salt. A major part (61%) of the ovarian CHE activity could be blocked with the selective anti-ACHE inhibitor BW284C51, suggesting that it represented ACHE activity. Sucrose-gradient centrifugation in the presence of known sedimentation markers (184) revealed the presence of light ACHE forms, probably dimers, sedimenting as 5.5-6.5S in the buffer-soluble fraction of the ovarian extracts. Additional, yet lighter and apparently monomeric, molecular forms (4.0-5.0S) were observed in the Triton-extractable fraction, which also included a small but reproducible peak of heavy (16S) ACHE activity. In some, but not all, samples, a soluble 11-12S peak of BCHE activity resistant to BW284C51 inhibition but sensitive to 1.10^{-6} M of the OP inhibitor iso-OMPA could be detected. This activity most probably represents plasma BCHE tetramers reflecting blood contamination (197).

2. Search for the molecular origins of BCHE polymorphism by cDNA screening, deletion mutagenesis, and Xenopus oocyte coinjections

BCHE displays tissue-specific polymorphism with respect to subunit assembly, hydrodynamic properties, and subcellular compartmentalization. To search for the molecular origin of this polymorphism, screening of various human cDNA libraries was carried out using both specific oligonucleotide probes (152) or a full-length cDNA (149). To produce recombinant BCHE, the full length cDNA-encoding human BCHE (149) was subcloned into the pSP64 transcription vector (161) and was used as a template. Xenopus oocytes were microinjected with the transcription products using 50 nl of 0.1 mg/ml mRNA/oocyte (148), and were incubated and homogenized as described (111). Enzymatic activity was measured according to Ellman et al. (198). Immunofluorescence detection was carried out as described (199), using rabbit polyclonal antibodies prepared against Torpedo ACHE, generously provided by Dr. S. Camp and P. Taylor.

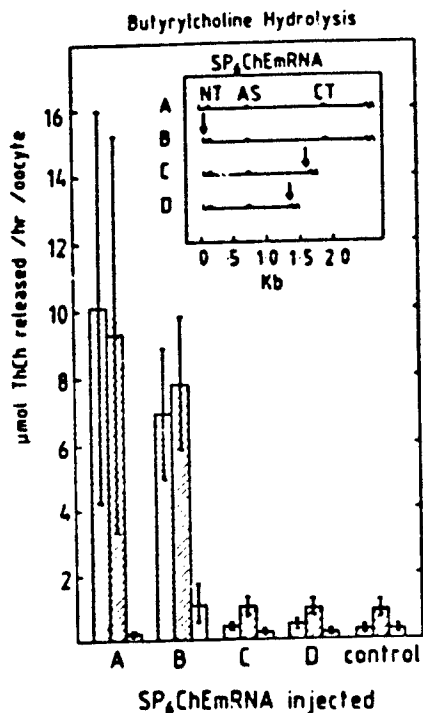
2.1 Evidence for a single transcript

Screening of several cDNA libraries from various tissue origins (human adult liver and lymphocytes and fetal brain, muscle, and liver) with the oligonucleotide and cDNA probes indicated above resulted in the isolation of several identical cDNA clones, all coding for BCHE, suggesting that the human BCHE gene encoding this enzyme is most probably transcribed into a single transcription product in all tissues. These findings imply that alternative splicing is not responsible for the heterogeneity observed in liver, muscle, and brain BCHE forms. In the absence of indications for the involvement of transcriptional control, posttranscriptional mechanisms may well be at the origin of BCHE heterogeneity. Therefore, oocyte microinjection experiments were initiated with clone-produced synthetic BCHEmRNA.

2.2 Partial deletion constructs

When injected into Xenopus oocytes, synthetic mRNA transcribed off a cDNA-encoding human BCHE induced the production of a protein displaying the substrate specificity and sensitivity to selective inhibitors characteristic of native BCHE, and which clearly distinguish it from ACHE (87,199). Enzymatic restriction of the complete SP64-BCHEcDNA construct was then performed to produce a series of partially deleted clones which were then similarly transcribed and expressed in oocytes (Figure 15). Thus, HinfI restriction of this 2450-bp-long clone (Figure 15A) removed the 5'-end of BCHEcDNA to produce a 2380-bp-long insert with only one AUG codon (B); HincII digestion produced a 1615-bp-long BCHEcDNA shortened at its 3'-end which encodes a 507-amino-acid-long protein (C) and StuI restriction produced a yet shorter 1257-bp-long insert encoding a 367-residue protein (D, see Figure 15). The 5'-end deletion did not significantly affect BCHE synthesis in oocytes microinjected with its transcription product. In contrast, the 3'-end deletions completely abolished the production of catalytically active BCHE (Figure 15). These experiments demonstrated that the entire sequence (574 amino acid residues) of the mature protein is essential for either production or stabilization of the catalytically active enzyme.

Figure 15: The entire C'-terminus of the polypeptide sequence is essential for production of a catalytically active enzyme



Hydrolytic activities of BCHE species produced in microinjected *Xenopus* oocytes from gradually shortened BCHEmRNAs (*Sp₆ChEmRNA*) are displayed in μmole thiocholine (ThCh) released/hr/oocyte in columns ± standard evaluation of the mean (3 separate experiments). The length of each BCHEmRNA type which was injected is displayed in kilobase (Kb; inset). NT: N'-terminus. AS: Active site. CT: C'-terminus. Control: parallel activities induced by homogenates of oocytes injected with Barth medium (frog saline). Dotted columns represent total hydrolytic activities, hatched columns-activities in the presence of 1x10⁻³ M BW284C51 and empty columns-activities in the presence of 1x10⁻³ M iso-OMPA.

2.3 Molecular form polymorphism

Microinjected alone, synthetic BCHEmRNA induced the formation of primarily dimeric BCHE. This first level of oligomeric assembly may therefore be spontaneous, or may require a catalytic mechanism that is already available in the oocyte (Table VI).

Table VI Molecular forms of recombinant BCHE produced in microinjected Xenopus oocytes

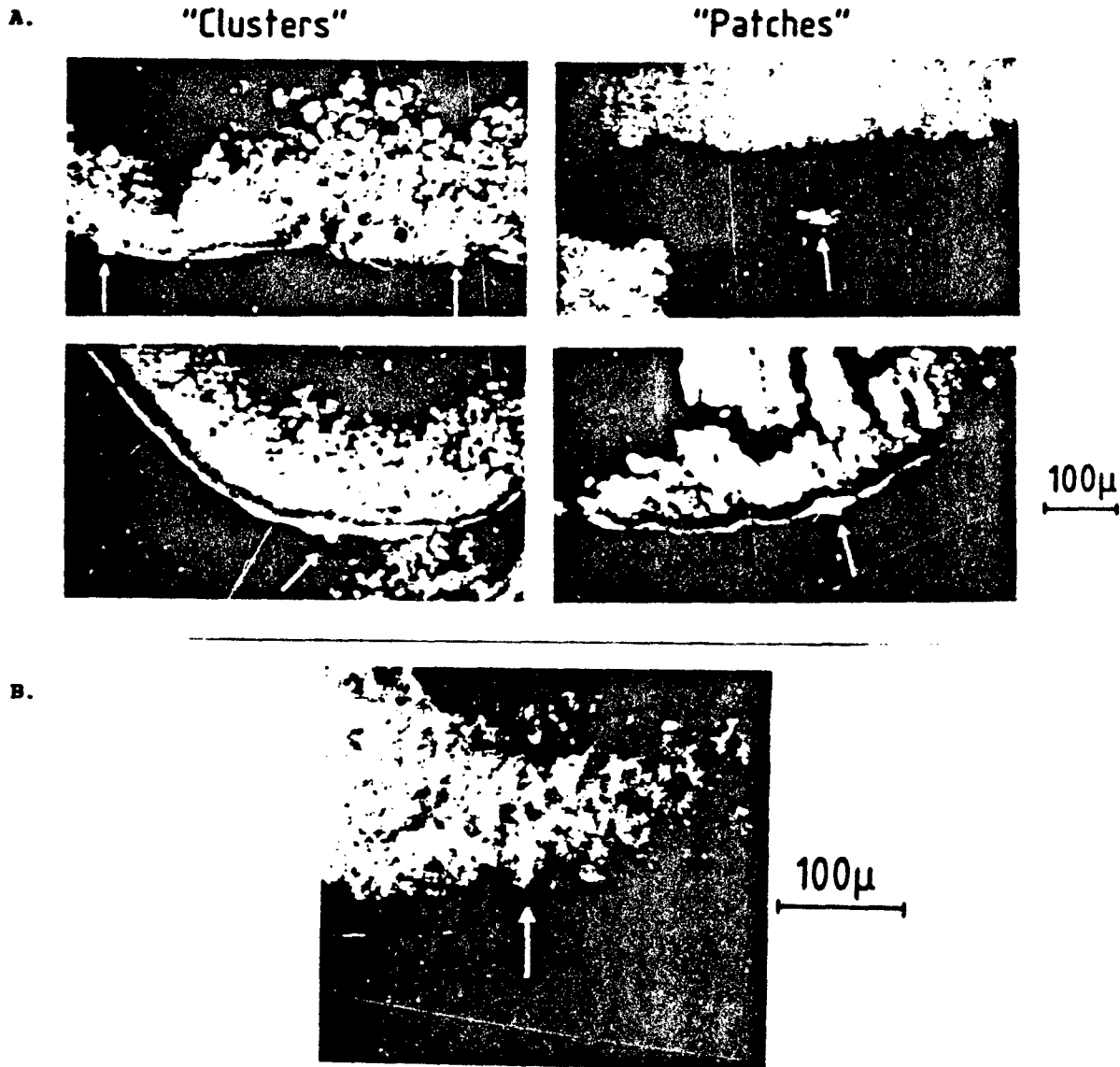
Co-injected mRNA	Molecular Forms
None	Dimer
Fetal Brain	Dimer + Tetramer
Fetal Muscle	Dimer + Tetramer + Tailed 16S
Fetal Liver	Monomer + Dimer

It should be noted in this respect that the detection of endogenous CHE activities in Xenopus oocytes (58) and high levels of BCHEmRNA in human oocytes (197) indicate that BCHE represents a natural endogenous oocyte protein. The co-injection of tissue-extracted poly(A)⁺mRNAs induced higher levels of multi-subunit assembly (Table VI)--likely including the incorporation of noncatalytic subunits. It indicates that additional protein species, not available in the oocytes, are required to direct the biosynthesis of more complex molecular forms. Given the high degree of tissue-specific polymorphism of CHE molecular forms, it is not surprising to find these additional factors expressed in a tissue-specific manner.

2.4 Transport and extracellular surface association

Immunocytochemical analysis of the oocyte-produced enzyme showed its aggregation on the oocyte surface into cluster (1-10 μm^2) and patch (10-100 μm^2) structures (Figure 16). About 30 min after injection, the immunoreactive BCHE accumulation was detectable at the external oocyte surface. Two and a half hours after injection, fluorescent BCHE clusters were bigger, more frequent, and more intense than those seen at the earlier time points (199). The newly expressed enzyme was detectable as intracellular immunoreactive structures (Figure 16). Tunicamycin-pretreated oocytes were capable of expressing active BCHE, but not of transporting it to the cell surface (199). Supplementation with tissue-specific mRNAs induced alterations in the features of the membrane-bound aggregates (Figure 17C). The relative distribution of each type of formation varied between oocytes co-injected with mRNA from liver, brain, and muscle tissues in a manner consistent with the organization of CHE in the native tissues (200, 201). Enhanced aggregation appeared to be correlated to the appearance of molecular forms containing 4 or more catalytic subunits (87). Both brain and muscle mRNAs increased the number of patches and clusters as compared to BCHEmRNA alone (Figure 16A). Co-injection with tissue mRNAs, therefore, increased both the intensity and the surface area occupied by patches and clusters indicating the induction, by tissue-specific factors, of enhanced BCHE aggregation at the external surface of the oocyte (Figure 16E). The relative staining intensity of clusters and patches obtained with muscle mRNA qualitatively exceeded that obtained with brain mRNA. Together, these observations imply a qualitative and/or quantitative difference between CHE-related mRNAs in different tissues and suggests that these mRNA pools are capable of modulating tissue-specific usage of a single BCHEmRNA species.

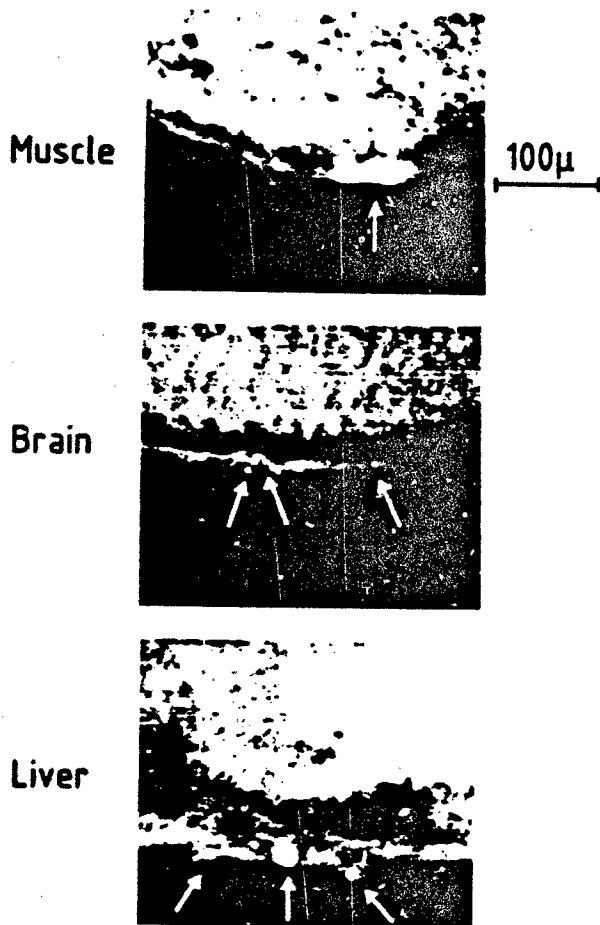
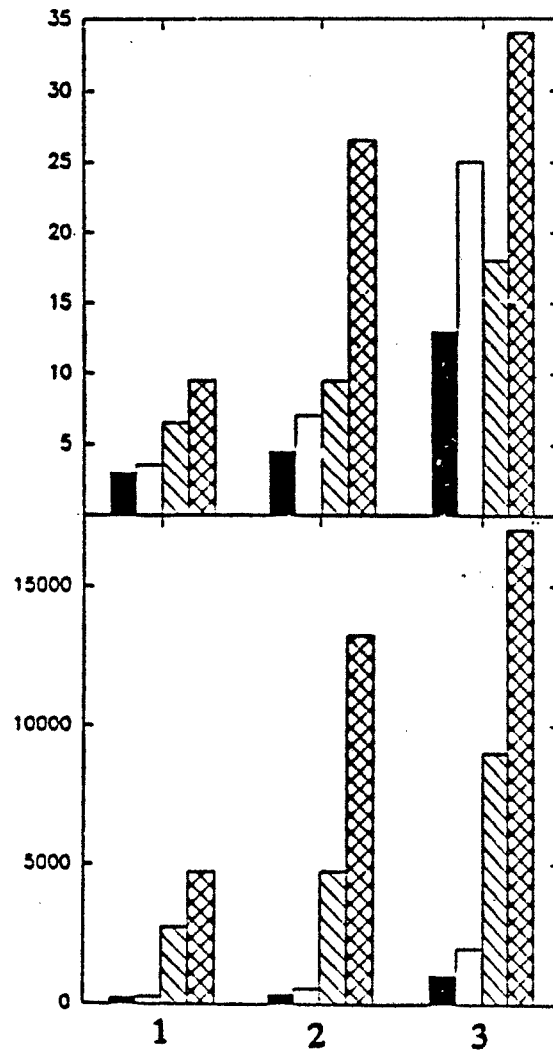
Figure 16. Transport and extracellular association of recombinant BCHE in microinjected oocytes



16A. Recombinant BCHE aggregates on the oocyte surface into cluster and patch structures.

16B. Intracellular transport of recombinant newly expressed BCHE in a brain poly (A⁺) mRNA co-injected oocyte; the arrow shows immunoreactive BCHE "en route" for the cell surface.

16C. Tissue-specific potentiation of BCHE assembly patterns on oocyte surface. Muscle mRNAs induced patch formation, brain mRNAs induced numerous clusters, but fuzzy aggregates were visible with liver mRNAs.

Figure 16C.**Figure 16D.**

16D Immunocytochemical quantification of BCHE distribution on individual oocytes.

Black column: clusters on vegetal pole.
 Open column: patches on vegetal pole.
 Oblique lines: clusters on animal pole.
 Crosshatched column: patches on animal pole.

1: BCHE mRNA injected alone.
 2: BCHE mRNA co-injected with brain mRNAs.
 3: BCHE mRNA co-injected with muscle mRNAs.

Top: number of clusters and patches
 Bottom B: Total immunoreactive area in μ m².

These findings demonstrate that liver, muscle, and brain express mRNAs encoding peptides which: i) are required for the biosynthesis of BCHE molecular forms consisting of multiple dimeric units, ii) may specify the incorporation of noncatalytic subunits, iii) direct the formation of a tissue-specific array of BCHE molecular forms, and iv) direct a tissue-specific organizational pattern of BCHE at the external surface of the injected oocytes.

3. Expression of alternatively terminated unusual cholinesterase messenger RNA transcripts, mapping to chromosome 3q26-ter, in nervous system tumors

3.1. Characterization of an unusual tumor-specific BCHEcDNA

Variable BCHEcDNA clones were searched for in cDNA libraries from several human tissue origins. These included fetal brain, liver, and muscle (17, 24, and 21 weeks gestation, respectively), neonate brain, and adult liver and lymphocytes. The number of BCHEcDNA clones isolated varied from 3 in the fetal brain library screen up to 40 in the fetal liver one. These were all, apparently, derived from the same BCHEmRNA transcript, identical to the 2.5-kb clones previously reported (149), as determined by restriction pattern analyses and partial DNA sequencing experiments (202).

In contrast with the invariant nature of BCHEcDNA clones from multiple normal tissue origins, libraries from both primary glioblastoma and neuroblastoma cells appeared to include two types of BCHEcDNA sequences. Nine of the 10 clones characterized in the glioblastoma library and six of the nine characterized in the neuroblastoma one were identical with the clones isolated from normal tissue sources. However, the remaining clones from both libraries, termed Gb5, displayed several unique features, detailed in Figure 17 and schematically presented in Figure 18A.

Figure 17. Alternative coding sequences of human BCHES

120	AAA TCG CAA ATC AAT ATG CAT ACC AAA GTC ACA ATC ATA TGC ATC AGA TTT CTC TTT TCG	1260	Ala Phe Leu Val Tyr Gly Ala Pro Gly His Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg
150	Met His Ser Lys Val Thr Ile Ile Cys Ile Arg Phe Leu Phe Thr	1320	AAA GAA TTT CAG GAA GGT TTA AAA ATA TTT TTT CCA GGA GTG ACT GAG TTT GGA AAG GAA
180	TTT CTT TTG CTC TGC ATG ATT GGG AAG TCA CAT ACT CAA GAT GAC ATC ATA ATT CCA	1380	Lys Glu Phe Gln Glu Gly Leu Lys Ile Phe Pro Gly Val Ser Glu Phe Gly Lys Glu
210	the Leu Leu Leu Cys Met Leu Ile Gly Lys Ser His Thr Glu Asp Asp Ile Ile Ile Ala	1440	TCC ATC CTT TTT CAT TAC ACA GAC TGG GTA CAT CAT CAG ACA CCT GAA AAC TAC CCG GAG
240	CA AAG AAT GGA AAA GTC ACA GGG ATG AAT TTT GGT GGC ACG GTA ACA GGC	1500	Ser Ile Leu Phe His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu
270	Thr Lys Asn Gly Lys Val Arg Gly Met Asn Leu Thr Val Phe Gly Gly Thr Val Thr Ala	1560	GCC TTG GGT CAT GTT GGT GAT TAT ATC TTA TGC CCT GCT GAG TTT GAG TTT ACC AAG
300	TT CTT GCA ATT CCC TAT GCA CAG CCA CCT CTT GGT AGA CTT GCA TTC AAA AAG CCA CAG	1620	Ala Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu Glu Phe Thr Lys
330	the Leu Gly Ile Pro Tyr Ala Gln Pro Leu Gly Arg Leu Arg Phe Lys Lys Pro Gln	1680	AAG TTC TCA GAA TGG GGA AAT AAT GCC TTT TTC TAC TAT TTT GAA CAC GCA TCC TCC AAA
360	CT CTC ACC AAG TGG TCT GAT ATT TGG AAT GCC ACA AAA TAT GCA AAT TCT TCG TGT CAG	1740	Lys Phe Ser Glu Trp Gly Asp Phe Phe Tyr Tyr Phe Glu His Arg Ser Ser Lys
390	er Leu Thr Lys Trp Ser Asp Ile Trp Asn Ala Thr Lys Tyr Ala Asn Ser Cys Cys Gln	1800	CTT CCG TCG CCA GAA TGG ATG GGA GTG CAT GCG TAT GAA ATT GAA TTT GTC TTT GGT
420	AC ATA CAT CAA AGT TTT CCA GGC TTC CAT GGA TCA GAG ATG TUG AAC CCA AAC ACT GAC	1860	Leu Pro Trp Trp Pro Glu Trp Met Gly Val Met His Gly Tyr Glu Ile Glu Phe Val Phe Gly
450	an Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn Pro Asn Thr Asp	1920	TTA CCT CTG GAA ACA GAT AAT TAC ACA AAA GCG GAG GAA ATT TTG AGT AGA TCC ATA
480	TC ACT GAA GAC TGT TTA TAT CTA AAT GTA TGG ATT CCA GCA CCT AAA CCA AAA AAT GCC	1980	Leu Pro Leu Glu Arg Arg Asp Asn Tyr Thr Lys Ala Glu Ile Leu Ser Arg Ser Ile
510	eu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp Ile Pro Ala Pro Lys Pro Lys Asn Ala	2040	GTG AAA CCG TGG GCA AAT TTT GCA AAA TAT GCG AAT CCA AAT GAG ACT CAG AAC AAT AGC
540	CT GTA TTG ATA TGG ATT TAT GGT GGT TTT CAA ACT GGA ACA TCA TCT TTA CAT GTT	2100	Val Lys Arg Trp Ala Asn Phe Ala Lys Tyr Gly Asn Pro Asn Glu Thr Gln Asn Asn Ser
570	hr Val Leu Ile Trp Ile Tyr Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val	2160	ACA AGC TGG CCT GTG TTC AAA AGC ACT GAA CAA AAA TAT CTA ACC TTG AAT ACA GAG TCA
600	AT CAT GCC AAG TTT CTC GGT CCG GTT AAG ACA GAT ATT GTA TGC TCA ATG AAC TAT AGG	2220	Thr Ser Trp Pro Val Phe Lys Ser Thr Glu Gln Lys Tyr Leu Thr Leu Asn Thr Glu Ser
630	Yr Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Ser Met Asn Tyr Arg	2280	AAA GTC TTG CAA ATG ACA GCA AAT ATT CAT GAA CCA TCG CAG TCG AAA CCA GCA TTC
660	TC GGT GCC CTA GCA TTC TTA TAT TGC CCA GCA AAT CCT GAG GCT CCA GCG AAC ATG GGT	2340	Lys Val Leu Glu Met Thr Gly Asn Ile Asp Glu Ala Glu Trp Glu Trp Lys Ala Gly Phe
690	al Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro Gly Asn Met Gly	2400	CAT CCG TCG AAC AAT TAC ATC ATG GAC TGG AAA AAT CAA TTT AAC GAT TAC ACT AGC AAG
720	TA TTT CAT CAA CAG TTG GCT CTT CAG TGC GTT CAA AAA AAT ATA GCA GCC TTT GGT GCA	2460	His Arg Trp Asn Asn Tyr Met Met Asp Trp Lys Asn Gln Phe Asn Asp Tyr Thr Ser Lys
750	eu Phe Asp Gln Gln Leu Ala Leu Glu Trp Val Gln Lys Asn Ile Ala Phe Gly Gly	2520	AAA GAA AGT TGT GTG GGT CTC TAA TTA ATA CAT TTA CCC TTT ATA GAA CAT ATT TTC CTT
780	AT CCT AAA AGT GTA ACT CTC TTT GGA GAA AGT GCA GCA GCT TCA GTT AGC CTG CAT	2580	Lys Glu Ser Cys Val Gly Leu
810	sn Pro Lys Ser Val Thr Leu Phe Gly Glu Ser Ala Gly Ala Ala Ser Val Ser Leu His	2640	TAG ATC AAG GCA AAA ATA TCA GCA GCT TTT TTA CAC ACC TAC TAA AAA AGT TAT TAT GTA
840	TG CTT TCT CCT GCA AGC CAT TCA TTG TTC ACC ACA GCC ATT CTG CAA AGT GCA TCC TTT	2700	GCT GAA ACA AAA ATG CCA GAA GCA TAA TAT TGA TTC CTC ACA TCT TTA ACT TAG TAT TTT
870	eu Leu Ser Pro Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Phe	2760	ACC TAG CAT TTC AAA ACC CAA ATG GCT ACA ACA TGT TTA ATT AAA TTT CAC AAT ATA AAG
900	AT GCT CCT TGC CCG GTA ACA TCT TAT GAA GCT AGC AAC ACA ACG TTG AAC TTA GCT	2820	TTC TAC AGT TAA TTA TGT GCA TAT TAA AAC AAT GCG CTG GTT CAA TTT CTT TCT TTC CTT
930	sn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr Leu Asn Leu Ala	2880	AAT AAA TTT AAG TTT TTT CCC CCC AAA ATT ATC AGT GCT CTG CTT TTA GTC ACG TGT ATT
960	AA TTG ACT GGT TCG TCT A A GAG AAT GAG ACT GAA ATA ATC AAG TGT CTT ACA AAT AAA	2940	TTC ATT ACC ACT CGT AAA AAG GTA TCT TTT TTA AAT GAA TTT TTA TGA AAC ACT GTA
990	ys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys	3000	CAC CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
1020	AT CCC CAA GAA ATT CTT CTG AAT GAA GCA TTT GTT GTC CCC TAT GCG ACT CCT TTG TCA	3060	GAG CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
1050	sp Pro Gln Glu Ile Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Ser	3120	CAC CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
1080	TA AAC TTT GGT CCG ACC GTG CAT GGT CAT TTT CTC ACT GAC ATG CCA GAC ATA TTA CTT	3180	GAG CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
1110	al Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu	3240	CAC CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
1140	AA CTT GCA CAA TTT AAA AAA ACC CAG ATT TTG GTG GGT GTT AAT AAA CAT GAA CCG ACA	3300	GAG CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
1170	lu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys Asp Glu Gly Thr	3360	CAC CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
1200		3420	GAG CAT AGT TTA CAA TTA TTA TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT

The nucleotide sequence of full-length "usual" BCHEcDNA and its inferred translation product are displayed (149, 157). Positions at which alternative nucleotides were found in the course of this work are ovally circled, and alternative nucleotides with their predicted amino acid products are shown above or below this sequence. Deleted nucleotides are marked by stars. The changes displayed below represent the Gb5cDNA clones. Altered nucleotides above are derived from another Lambda Gt10 library. The exclamation points mark the end of the alternative Lambda Gt10 or Gb5 DNA clones. See following sections for details of nucleotide-specific alterations.

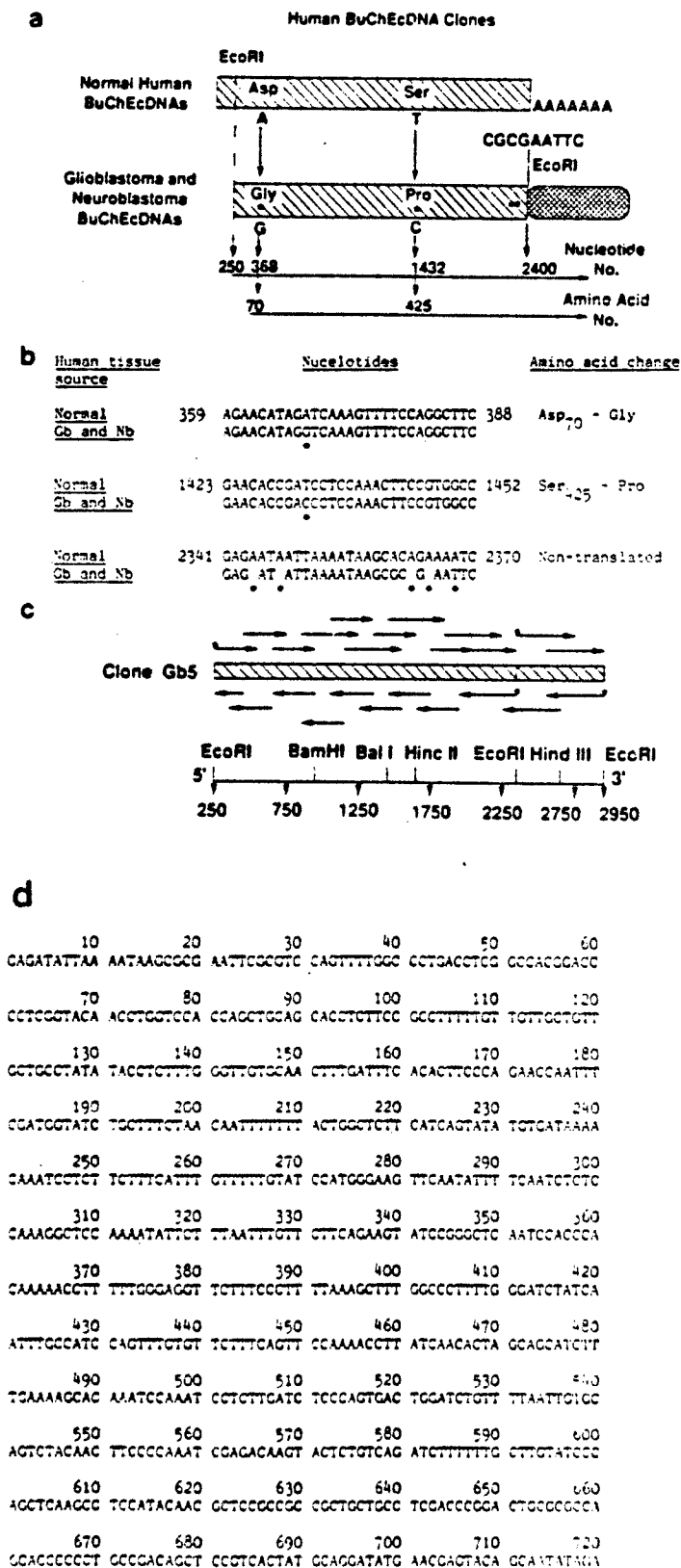
3.2. Unusual BCHEcDNA characterized by alternative termination and point mutations in the coding region

All of the Gb5 clones were characterized by a 0.7-kb EcoRI-excisable 3'-extension in the position where "normal" BCHEcDNA contains a poly(A) tail. Four adenosine residues in the consensus polyadenylation site were found to be deleted and a fifth modified into thymidine (Figure 17B). In the coding region, two point mutations ($A_{388} \rightarrow G$ and $T_{432} \rightarrow C$) appeared in the Gb5 clones. These implied changes in two amino acid residues, Asp70 into Gly and Ser425 into Pro (Figure 18B).

Interestingly, the Asp70 $\rightarrow$ Gly70 change has recently been found to repetitively occur in the BCHE gene of individuals with the "atypical" BCHE phenotype (16). This implicated the Asp $\rightarrow$ Gly substitution in BCHE with alteration(s) in the biochemical properties of the enzyme, with the following precautions:

- (a) It remained unclear whether the existence of Gly70 in human BCHE is a necessary and sufficient requirement to create the "atypical" phenotype or whether other mutations may cause similar changes in BCHE properties.
- (b) The description of the "atypical" BCHE phenotype in the clinical and pharmacological literature is not always consistent, and relates to several distinct alterations in BCHE properties as they relate to characteristics of the normal enzyme. For example, it appears that BCHE from various individuals having the "atypical" phenotype may display variable sensitivities to both dibucaine and succinylcholine. This raised the question of whether a single point mutation may alter the affinity of BCHE to both these inhibitors; our approach to resolve these issues is presented in section III,4.

Figure 18. Molecular properties of the Gb5 cDNA clones



A. Schematic characterization of the Gb5 cDNA clones

The originally isolated BCHEcDNA clones (top line) were found to be expressed in fetal and neonatal brain, in fetal and adult liver, in fetal muscle, and in adult lymphocytes (normal tissues). These were all 2400 nucleotides long and identical in all details and included A and T residues in positions 368 and 1432 (where nucleotide 1 corresponds to the first residue in the original BCHEcDNA clone). The Gb5 cDNA clones (bottom line) were found only in libraries from glioblastoma (Gb) and neuroblastoma (Nb) origins. Six such clones were fully sequenced and their coding regions were found to be similar to those of the original BCHEcDNA clones in most details, except that they included G and C residues in positions 368 and 1432. These alterations conferred two changes in amino acid residues: Asp70-Gly and Ser425-Pro, where residue 1 is the first one in the mature protein (157). Additional differences included several altered nucleotides at the 3'-nontranslated region (*) and a 3', 0.7-kb extension linked by a CGCGAATTC oligonucleotide.

B. Detailed nucleotide differences in Gb5 clones

Nucleotide differences (*) between usual BCHEcDNA from brain, liver, and lymphocytes (normal) and the mutated ones found in tumorous tissues, such as Gb and Nb, are represented. Nucleotides are numbered according to the published BCHEcDNA sequence and amino acids are marked according to the mature protein sequence. The changes in the nontranslated region appeared approximately 20 nucleotides upstream of the consensus region for polyadenylation and might explain the shift in the poly(A) site of the Gb5cDNA clones.

C. Sequencing strategy

The variable cDNA inserts encoding BCHE were isolated from the glioblastoma and neuroblastoma libraries and their restriction endonuclease EcoRI fragments were subcloned in the sequencing vectors M13mp18 and M13mp19 (Amersham, see section II). DNA sequencing was performed with the universal 17-mer primer (Amersham, no. 4511, * at the beginning of an arrow) or with unique 17-mer primers chemically synthesized from a previously confirmed BCHEcDNA sequence (149) arrows without *). Confirmed sequences were obtained from both strands of the cDNA as indicated by arrow length and direction for a representative Gb5cDNA clone of glioblastoma origin. The 5'-end of clone Gb5 was localized at nucleotide 250 of the originally isolated brain FBCHE12 clone (149).

D. Primary structure of the 3', 0.7-kb fragment in Gb5cDNA clones

The 0.7-kb sequence of the Gb5cDNA clones was numbered in the 5'-3' direction, with the EcoRI site presented in A and B at nucleotide 20. The sequence does not include any long open reading frames and is not homologous to available sequences in the EMBL databank.

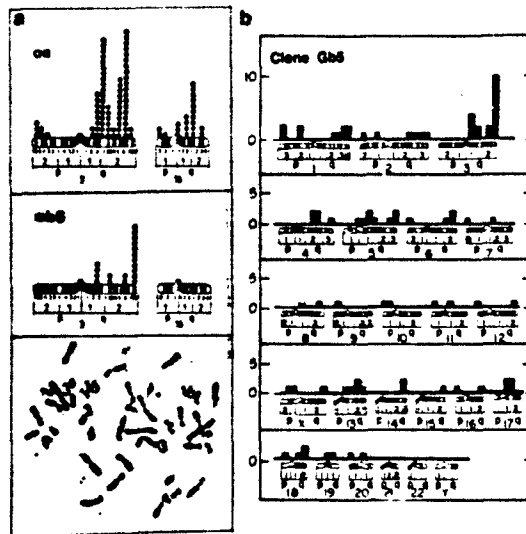
3.3 3'-extended BCHEcDNA maps to chromosome 3q26-ter

The unique sequence of the 3'-extended 0.7-kb DNA fragment characteristic of the Gb5 BCHEcDNA clones made it an appropriate probe for chromosomal mapping by *in situ* hybridization. When labelled with [³²S], it hybridized preferentially to a unique site on the long arm of chromosome No. 3, with most of the silver grains concentrated at the 3q26-ter position. This pattern of binding, presented in Figure 19A, differed significantly from the distribution of grains obtained with the full-length BCHEcDNA probe, which equally labelled two sites, termed CHEL1 and CHEL2, on

the long arm of Chr. 3 (120). These peaked at positions 3q21 and 3q26, respectively. In addition, "normal" BCHEcDNA labelled significantly the long arm of Chr. 16 at a third site termed CHEL3 (121). In contrast, the hybridization signal obtained with the Gb5 probe on Chr. 16 was negligible. An example of autoradiographed R-banded chromosomes after hybridization with the 0.7-kb fragment from Gb5cDNA is also displayed in Figure 19A.

The cumulative distribution of autoradiographic silver grains observed over photographed chromosome spreads hybridized with the Gb5-specific probe was plotted on a histogram representing the haploid human genome and divided into equal units scaled to the average diameter of a silver grain ($0.35 \mu\text{m}$; Figure 19B). This analysis revealed a unique localization of the Gb5-specific probe, which shares no homologies with other known genes in our data bank, onto the 3q26-ter position, where an amplified defective CHE allele was previously localized (146).

Figure 19. Chromosomal mapping of the Gb5 Clones by in situ hybridization



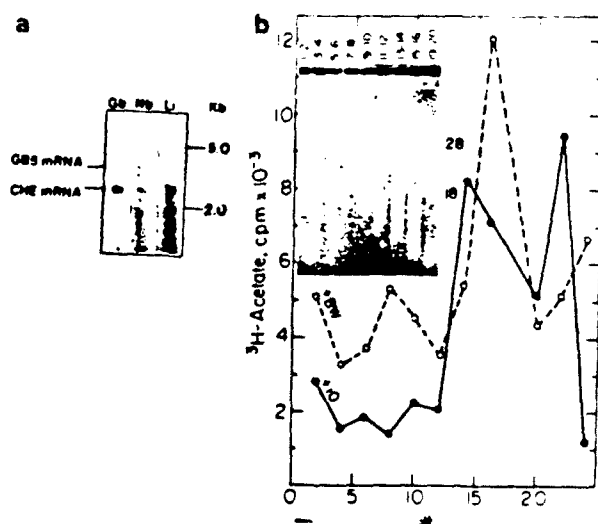
A: Gb5 cDNA hybridizes with chromosome 3 but not 16. Distribution of silver grains scored over chromosomes 3 and 16 from 52 Q- or R-banded metaphase spreads hybridized with the usual BCHEcDNA (CHE) or from 28 G- or R-banded metaphase spreads hybridized with the 0.7-kb fragment from Gb5cDNA are presented, together with a representative R-banded chromosome spread after hybridization with [^{35}S]Gb5cDNA and autoradiography (exposure, 7 days). Note that the [^{35}S]Gb5 probe labels both chromosomes 3 but not 16 in this representative chromosome spread (19a, bottom), whereas the CHE probe labels both chromosomes 3 and 16 (19a, top).

B: Unique position of Gb5 sequences over the human genome. Cumulative scores of silver grains are presented for G- or R-banded metaphase spreads hybridized with [³⁵S]Gb5cDNA. In a total of 28 cells from three unrelated volunteers having normal karyotypes which were scored, 21 copies of chromosome 3 in 19 cells (37% of no. 3 chromosomes) gave positive hybridization signals from a total of 97 grains which were associated with chromosomes, while labelling on all other chromosomes was insignificant. Sixty-six percent of the grains on chromosome 3 were concentrated within the region 3q26-ter. On chromosome 16, radioactive labelling remained at the background level. Statistical evaluation of the number of silver grains/unit chromosome length, assuming a Poisson distribution, indicated that the localization on chromosome 3q26-ter was the only one to be significant ($P < 0.025$).

3.4. 3'-Extended tumor BCHE mRNA transcripts are functional

Previous RNA blot-hybridization experiments, using the full-length BCHEcDNA as a probe, demonstrated a single 2.4- to 2.6-kb-long transcript in both brain and liver (149). When poly(A)⁺RNA from the same primary glioblastoma tumor which served to make the library and from IMR32 neuroblastoma cells was similarly examined, an additional faint band, 3.6 kb in length, was observed (Figure 20A). Its abundance in glioblastoma mRNA, relative to the 2.6-kb "normal" transcript, termed CHE, was in good agreement with the 1:10 Gb5:CHE ratio observed among cDNA clones. The total intensity of labelling in lanes loaded with glioblastoma mRNA was considerably higher than in neuroblastoma, in agreement with previous biochemical findings of BCHE overexpression in glioblastomas (41) and with the relatively low levels of BCHE in neuroblastoma cells (203).

Figure 20. Blot hybridization and *in ovo* translation of butyrylcholinesterase-encoding mRNAs from tumor and normal tissues



A: Blot hybridization with the usual BCHEcDNA was performed as previously detailed (149). mRNAs are represented by: Li, fetal liver; Nb, IMR32 neuroblastoma cells; and Gb, primary glioblastoma cells. Electrophoretic migration of RNA markers (5.0 and 2.0 kb) is marked.

B: Two size classes of mRNAs from glioblastoma induce the biosynthesis of BCHE activity in microinjected *Xenopus* oocytes. Fractionated RNA was injected into oocytes, and acetylcholine-hydrolyzing activities were separately determined radiometrically in the presence of 0.1 mM iso-OMPA (IO) or BW284C51 (BW) to account for the biosynthesis of AChE or BCHE, respectively. Spontaneous release of [³H]acetate and activity in control oocytes were subtracted. Arrow, sedimentation direction. Note the appearance of a heavy, rapidly migrating peak of BCHE-inducing mRNA in addition to the major 20S one.

Because of the difficulties to associate specific bands in tumor RNA blots with defined cDNAs in libraries, particularly when rare species of mRNA are pursued, we searched for unrelated experimental approaches to examine whether Gb5mRNA transcripts in glioblastoma tissue are functional. For this purpose poly(A)⁺RNA from such tumors was size-fractionated by sucrose-gradient centrifugation and microinjected into *Xenopus* oocytes. The acetylcholine-hydrolyzing activities produced in the oocytes were measured in the presence of selective inhibitors, which distinguish between BCHE and the related enzyme AChE. Induced BCHE activities, measured in the presence of 0.1 mM of the AChE-specific inhibitor BW284C51, were associated with two distinct mRNA size fractions.

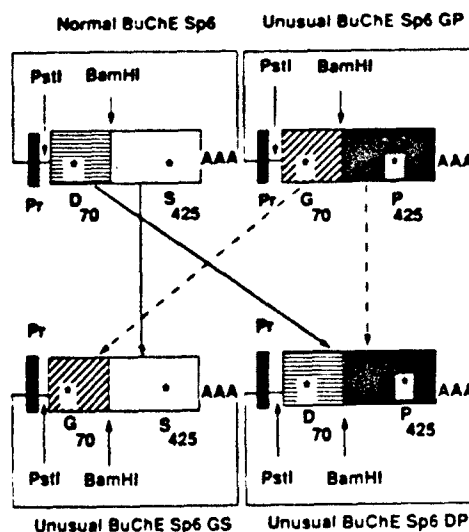
These sedimented as 20S and 24S, (approximately 2.5 and 4.0 kb in size). Activity associated with the faster-sedimenting mRNA peak was considerably smaller in height (Figure 20B). Both peaks associated with BCHE activities could clearly be distinguished from the AChE-encoding ones, which were measured in the presence of the BCHE-specific inhibitor iso-OMPA. The mRNAs inducing AChE displayed peaks of 20S and 9S, approximately 2.5 and 0.3 kb in length. It should be noted in this respect that parallel analyses with fetal brain mRNA detected only a single BCHEmRNA peak (148), suggesting that the heavy functional BCHEmRNA species was specific to the nervous system tumor.

4. Structure-function relationships in human butyrylcholinesterase variants

4.1 Biochemical properties of the unusual BCHE mutants

Synthetic mRNA transcribed from cloned human BCHEcDNA (149) directs the production, in microinjected *Xenopus* oocytes, of high levels of a catalytically active BCHE, demonstrating the substrate specificities and sensitivities to selective inhibitors characteristic of native serum BCHE (87). Transcription constructs encoding the double Asp70-->Gly70, Ser 425--> Pro425 mutation (GP) and the Asp70-->Gly70 (GS) or the Ser425-->Pro425 (DP) mutations alone were prepared and similarly employed to express synthetic, mutant BChEs readily available for biochemical analysis (Figure 21). Interestingly, microinjection experiments using synthetic mRNA encoding the Gly70 substitution yielded, on average, 4 to 6-fold lower BCHE activities per oocyte than those employing the native Asp70-encoding mRNAs, yet relatively normal Michaelis-Menten constants (K_m) towards BuTCh (Table II). These data are consistent with lower protein content (204), yet identical turnover numbers reported for human "atypical" serum BCHE (205), reflecting an intrinsic instability of either the mRNAs encoding these BCHE variants or the proteins themselves. The underrepresentation of "unusual" GP-BCHEcDNA clones in heterozygote-derived cDNA libraries together with the 1:10 ratio (GP:DS, DS being the "usual" BCHE allele containing Asp70 and Ser425), of translatable BCHEmRNA observed in the tissues from which those libraries were prepared substantiates our view that RNA instability is indeed the operative element in this phenomenon (155).

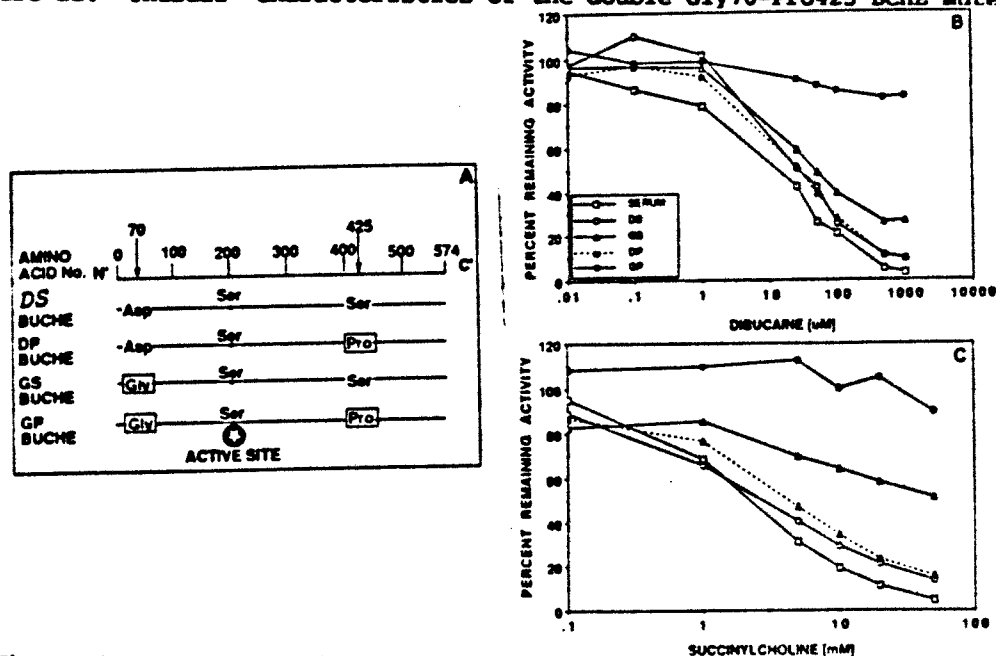
Figure 21. Construction of mutant human BCHE SP6 driven vectors



Variant sequences were constructed by replacing fragments in the "usual" human BCHEcDNA, which has previously been subcloned into the pSP64 transcription vector (Amersham International) carrying the RNA polymerase binding site of the salmonella phage SP6 (87) and is defined in the scheme as "Normal BCHE SP6." "Unusual" BCHEcDNA carrying the two mutations which confer the substitution of aspartate70 into glycine (D→G) and serine425 to proline (S→P), initially isolated from a primary glioblastoma library (155), was similarly constructed into the pSP64 vector and is defined in this scheme as "Unusual BCHE SP6 GP." Double-enzymatic restriction with PstI and BamHI removed a DNA fragment containing the Gly70 or Asp70 domain from each of these constructs. Agarose gel electrophoresis (0.8% in TBE buffer) and electroelution was employed to isolate each of the four DNA fragments created by this digestion, and reciprocal ligation with T4 DNA ligase created two new recombinant variants, carrying a single mutation each and defined as "Unusual BCHE SP6 GS" and "Unusual BCHE SP6 DP" in the scheme. Each variant was fully sequenced to confirm the validity of these engineering experiments.

The "atypical" BCHE phenotype is clinically characterized by the resistance of the variant enzyme to succinylcholine (SuCh) and dibucaine (15). Using the separate Gly70 and Pro425 mutations included in distinct transcription constructs, the contribution of each mutation towards this defective phenotype was assessed. For this purpose, IC₅₀ values with dibucaine or SuCh were determined for each of the recombinant mutant proteins (muteins) as compared with the native enzyme present in normal human serum (Figure 22). The single Gly70 mutation was found in these measurements to increase the IC₅₀ with SuCh by more than an order of magnitude, in agreement with the recent finding of this mutation in patients with records of postanaesthetic apnea (16).

Figure 22. "Unusual" characteristics of the double Gly70-Pro425 BCHE mutein



A: The variant oocyte-produced BCHE muteins which were examined included the normal human serum Asp70-Ser425 (DS) BCHE (149) and the Gly70-Pro425 (GP) "unusual" BCHE encoded by BCHEcDNA isolated from glioblastoma and neuroblastoma sources (155). To analyze the independent effects of each of the point mutations included in GP-BCHE, DP and GS BCHEcDNA constructs were prepared from the DS and GP BCHEcDNA, as described in the legend to Figure 21. The SP6 vectors served to prepare the synthetic mRNA which, in turn, was injected into *Xenopus* oocytes to yield the various functional BCHE muteins.

B: For inhibitor measurements, clear oocyte supernatants were employed for spectrophotometric assessment of BCHE activities in multiwell plates (see section II). Curves present percent remaining activities vs. dibucaine concentration for the various synthetic BCHEs.

C: Succinylcholine inhibition was measured for each of the variant BCHE muteins as detailed under B, except that BuTCh concentration was 1 mM. Note that the double GP mutein was resistant to both dibucaine and succinylcholine, whereas the single GS mutein was insensitive to succinylcholine inhibition only.

An apparently absolute resistance to SuCh was noted for the double mutant GP (IC₅₀ > 1000 mM, Figure 22), demonstrating a synergistic contribution of the Pro425 substitution towards an exceptionally pronounced "atypical" phenotype. Moreover, the IC₅₀ for dibucaine was only increased by 2-fold in the GS mutein as compared with DS or DP (Figure 22). In contrast, the double GP mutant became completely resistant to dibucaine as well. Thus, the combination of Gly70 and Pro425 substitutions makes human BCHE fully resistant to SuCh and dibucaine inhibition.

In Table VII, the biochemical characteristics of BCHE muteins reveal disruption of anionic site interactions. Hydrolytic activities of the various BCHE muteins measured at room temperature as detailed under Figure 21 are expressed in nmoles/oocyte/hr following injection of 2 ng per oocyte of each of the relevant synthetic BCHEmRNA transcripts and 18 hr incubation at 16°C.

TABLE VII. Biochemical characteristics of BCHE muteins reveal disruption of anionic site interactions

A. Hydrolytic Activities

<u>Normal Serum</u>	<u>DS</u>	<u>DP</u>	<u>GS</u>	<u>GP</u>
211.8 (per ul)	34.7	28.9	8.7	5.8

B. Apparent Substrate Affinity/Association Constants (mM)

ButCh (Km)	2.3	1.9	3.8	2.4	3.7
SuCh (Ki)	1.6	1.8	3.4	42.1	>1000
BenzCh (Ki)	0.6	0.5	1	0.3	0.3
PropCh (Ki)	8.7	8.2	8.9	9.9	9.8
ACh (Ki)	44	30	30	113.3	145.4

C. IC50 values (uM)

Dibucaine	12	25	25	50	>1000
SuCh	2300	2800	4300	60000	>10 ⁶
DFP	0.3	0.3	0.4	0.3	0.2
Paraoxon	0.3	0.3	0.3	0.2	0.3
iso-OMPA	2	2.3	5.3	>1000	14
Ecothiophate	0.2	0.2	0.2	3.5	17
Bambuterol	0.2	0.3	0.3	70	12
Physostigmine	0.5	0.7	0.1	7	0.7

A: Hydrolytic activities of the various BCHE muteins measured at room temperature as detailed under Figure 21 are expressed in nmoles/oocyte/hr following injection of 2 ng per oocyte of each of the relevant synthetic BCHEmRNA transcripts and 18 hr incubation at 16°C. One- μ l samples of an apparently normal, undiluted serum served as control for native BCHE properties. Spontaneous hydrolysis of butyrylthiocholine was subtracted. Data represent the average of 27 experiments from 15 independent microinjections and six unrelated transcriptions. About 80% of the microinjection experiments yielded activities in the noted range.

B: Apparent Michaelis-Menten constant (Km, ButCh) and inhibition coefficients (Ki) for succinylcholine (SuCh), benzoylcholine (BenzCh), propionylcholine (PropCh), and acetylcholine (ACh), all from Sigma (St. Louis, Ma.) were calculated, in mM from activity assays performed as detailed in section II (three separate experiments from two unrelated transcriptions with S.E.M. <10%).

C: Fifty percent inhibition constants (IC50) in μ M were determined from inhibition curves representing three experiments and two microinjections for each inhibitor.

The examined mutations conferred marked reductions in the ability of some ligands to interact with the mutein BCHEs. Most striking was the effect of the double Gly70-Pro425 mutation on SuCh and dibucaine binding--essentially abolishing measureable interaction (Figure 22B,C; Table VII). Alone, the Gly70 mutation also induced a significant, albeit smaller, decrease in SuCh interaction, but only a modest 2-fold decrease in its dibucaine IC50 in comparison with DS BCHE (Table VII). In contrast, the Pro425 mutation alone failed to change the ability of

display the "atypical" BCHE phenotype with respect to SuCh sensitivity, only those with the GP mutein would also display the abnormal "dibucaine numbers" characteristic of certain BCHE variants (126). Several choline substrates, such as benzoylcholine and propionylcholine, displayed no detectable change in binding to any of the recombinantly produced proteins (Table VII). Other substrates, such as ACh or SuCh, bound preferentially to the Asp70-containing BCHEs but not to the Gly70-containing ones. This demonstrates that Asp70 exerts a critical role in the anionic binding of only certain ligands, and suggests that other negatively charged amino acid residues within BCHE must play roles in anionic site binding in accord with previous postulates (206, 207).

The oocyte-produced BCHE muteins were further examined for their abilities to bind anti-CHE OP agents and carbamate drugs. There was no change in the IC₅₀ values observed for any of the recombinant BCHEs with diisopropylphosphorofluoridate (DFP) or O,O'-diethyl O" para-nitrophenylphosphate (paraoxon), the toxic metabolite of parathion (Table VII).

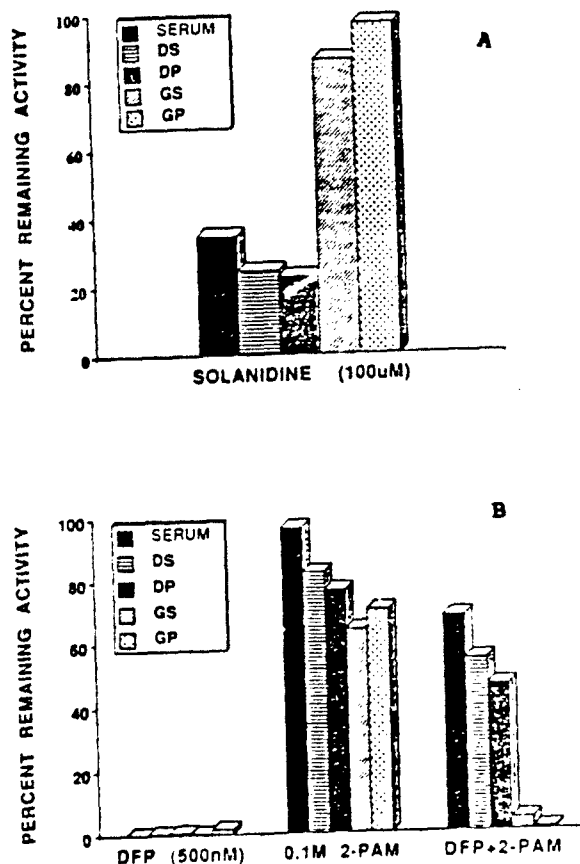
4.2 Resistance of glycine70 BCHE muteins to solanidine

Recombinant BCHE variants were tested for their resistance to solanidine, a naturally occurring steroidal alkaloid with previous reports of acute poisonings due to its dietary consumption (115). At 100 μ M concentration, solanidine blocked 65% of the normal serum enzyme and 75% of its oocyte-produced recombinant BCHE muteins, while BCHEs possessing the Gly70 mutation (GS and GP) remained fully active in the presence of 100 μ M solanidine (86 and 98% remaining activities, respectively; see Figure 23), demonstrating that the aspartate70-to-glycine substitution is sufficient to render BCHE resistant to physiologically toxic concentrations of this common solanoid compound.

4.3 Inhibited BCHEs carrying the glycine70 mutation resist oxime reactivation

Oxime reactivation is employed in research and therapy to reverse the inhibition of CHEs by poisonous OP compounds (208). Estimations are that up to 1 million poisoning cases occur per year, worldwide, due to agricultural uses of OP insecticides leading to inhibition of AChE and BCHE (reviewed in 209). To examine whether BCHE in individuals carrying the Gly70 and/or the Pro425 mutations in their CHE genes would potentially respond to oxime therapy, recombinant BCHE muteins were first subjected to complete inhibition by 500 nM of the OP compound DFP and then incubated with 10 mM of the commonly used oxime 2-PAM (Figure 23). When administered alone, 2-PAM caused a limited reduction in the activities of all four recombinant BCHEs.

Figure 23. Solanidine resistance and 2-PAM reactivation of BCHE mutants



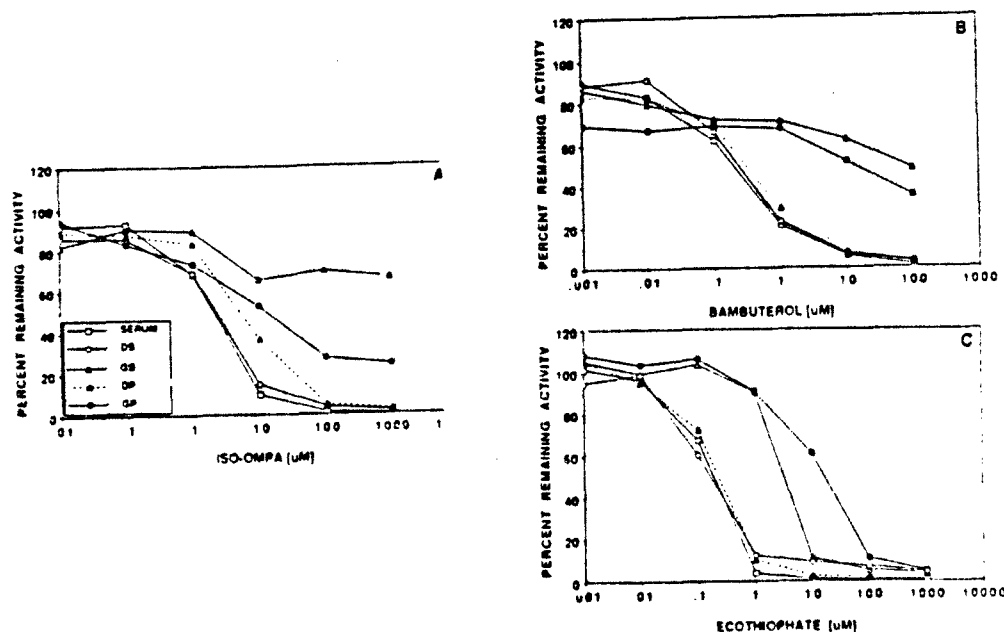
A. The single aspartate70-to-glycine substitution fully confers solanidine resistance. Recombinant mRNA was synthesized *in vitro* from each of the four BCHE SP6 variants depicted in Figure 20 and was microinjected into *Xenopus* oocytes as detailed under section II. BCHE activities in oocyte homogenates were tested for their resistance to inhibition by 100 μ M solanidine as compared with native BCHE in normal serum. Note that percent remaining activities were similarly low in the serum, DS, and DP enzymes, whereas the two variants carrying the glycine70 substitution, GS and GP, displayed complete resistance to this naturally occurring steroidal alkaloid.

B. Glycine70 substitution prevents reactivation of DFP-intoxicated BCHE variants. Serum and oocyte homogenates displaying the four recombinant BCHE variants were incubated with 500 nM DFP for 30 min, after which 2-PAM was added to the final concentration of 10 mM for a further incubation of 2 hr at room temperature. Ten- μ l aliquots were subsequently taken for BCHE assay. Percent remaining activities are shown following DFP treatment alone, 2-PAM alone, or DFP followed by 2-PAM. Note that both the GS and the GP variants remained fully inhibited under conditions in which the DS and DP variants were reactivated by 2-PAM.

Following complete DFP inhibition, 2-PAM restored 65% and 55% of normal serum BCHE as well as of the oocyte-produced normal DS counterpart. The DP enzyme, carrying the Pro425 mutation was also reactivated, although to a somewhat lower level, 45%. In contrast, both of the recombinant muteins carrying the Gly70 mutation (GP and GS) completely failed to be reactivated by 2-PAM (Figure 23). Furthermore, 20 mM of the nonspecific nucleophile hydroxylamine effectively reversed DFP intoxication in all of the recombinant BCHE muteins, including GS and GP (not shown), demonstrating that the resistance of these muteins to 2-PAM reactivation was not due to augmentation of covalent "ageing" BCHE modifications by the glycine70 residue. In view of the generally accepted notion that 2-PAM interacts with charged amino acid residues at the anionic site of CHEs (13), this finding further demonstrates the involvement of aspartate70 in anionic site interactions. This is in line with the increased resistance of the Gly70-containing BCHEs to ecothiophate (Table VII), since both compounds are known to bind the anionic site (5, 210).

It is interesting to note that the effects of Gly70 and Pro425 are not always additive. Physostigmine displayed an abnormally high IC₅₀ value only towards the singly mutated GS-BCHE (Table VII). Furthermore, this mutein displayed a significantly greater reduction in affinity toward iso-OMPA, physostigmine and bambuterol (1-3, 5-bis-(N,N-dimethylcarbamoyloxy phenyl)-2-t-butylaminoethanol-hydrochloride), a long-duration anti-asthmatic carbamate prodrug (206), than the double-mutant GP did, while the opposite phenomenon was observed with ecothiophate, dibucaine, SuCh, and ACh (Figure 24 and Table VII).

Figure 24. Asp70 mutagenesis confers variable resistance on BCHE towards OPs and carbamate compounds



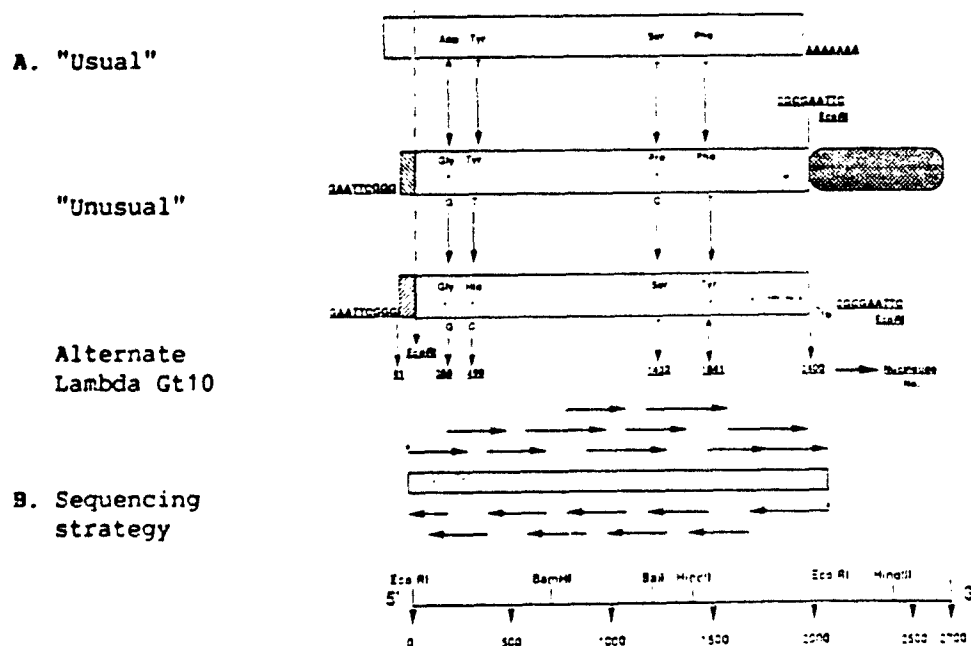
Inhibitor assays are described in section II. A 45 min pre-incubation period was employed with each inhibitor. Values represent means of three separate experiments, each repeated in duplicate from two separate microinjection experiments with S.E.M. < 10%. Iso-OMPA was from Sigma (St. Louis, Mo.), bambuterol was gratefully received from Draco (Sweden) and ecothiophate was from Ayerst laboratories (Montreal, Canada). Note that bambuterol inhibition is plotted on the same scale of concentrations as ecothiophate; iso-OMPA is plotted on a 10-fold higher scale than the other two inhibitors.

4.4 Additional nucleotide substitutions characterized in a Lambda Gt10 BCHE cDNA clone

Other BCHEcDNA clones prepared by EcoRI digestion from Lambda Gt10 phages which were hybridized with [³²P]BCHEcDNA were fully sequenced to investigate the possibility that they carry additional, yet-unknown nucleotide substitutions in the BCHE coding sequence.

Several positive clones were isolated and further analyzed using two regional probes. These were an end-labelled oligonucleotide probe complementary to nucleotides 160 through 183 in the BCHEcDNA sequence (149) and a random-primer-labelled, 1-kb-long cDNA fragment isolated by RsaI digestion from the 3'-end of BCHEcDNA. One of the Lambda Gt10 clones which was picked was 2136 bp long and was termed 16L2. Enzymatic restriction and DNA sequencing demonstrated that it contained most of the coding region for BCHE. However, the 16L2 clone was found to include several nucleotide differences, some of which confer altered amino acid residues in the encoded protein and which represent a yet-undescribed variant of human BCHEcDNA. Figure 25 presents in detail the characteristic properties of these cDNA sequences as compared with the previously described "usual" and "unusual" BCHEcDNAs and displays the sequencing strategy employed to characterize this clone. Substituted nucleotides and amino acids in this sequence are also included in Figure 25.

Figure 25. Novel variant butyrylcholinesterase cDNA clones



A. The clone isolated by Lambda Gt10 screening is schematically represented in comparison to the "usual" BCHEcDNA (149) and to the "unusual" one (155). Alternate amino acids and nucleotides are shown (*).

9. A Lambda Gt10 BCHEcDNA clone isolated by screening with [³²P]BCHEcDNA was sequenced from both strands, using the universal M13 primer (*) or other oligodeoxynucleotides (with unlabelled arrows). Fragment size and restriction sites are shown below.

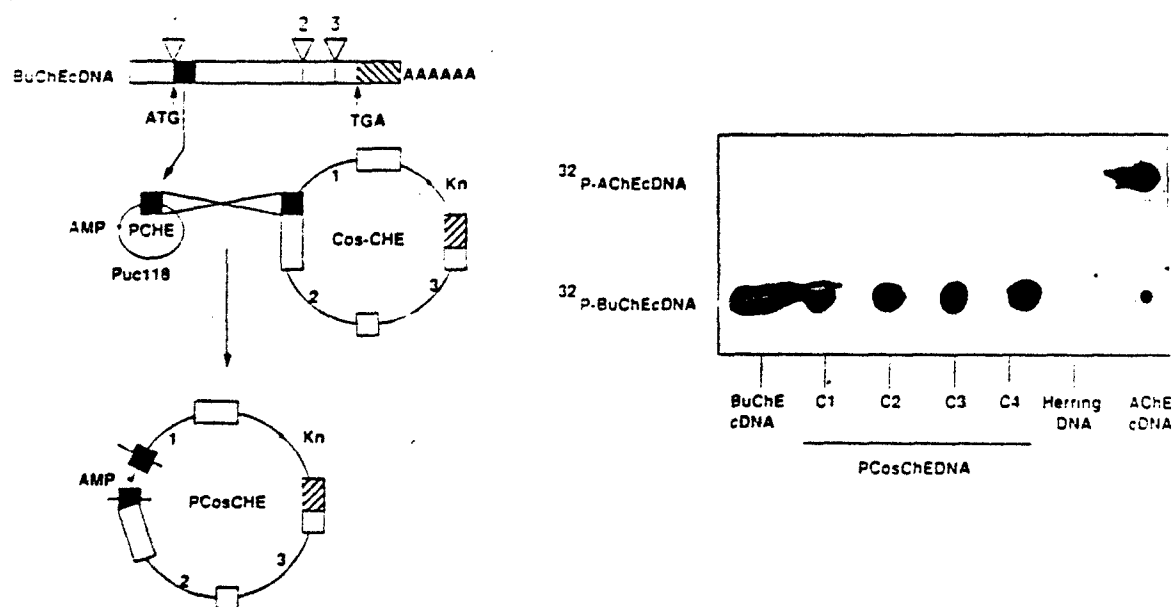
Interestingly, the 16L2-isolated BCHEcDNA sequence encoded two novel variations in its conferred protein product, in addition to one previously shown alteration: (1) Asp70 was changed into Gly, similar to the alteration in the "unusual" BCHEcDNA; (2) Tyr114 was changed into His; (3) Phe563 was changed into Tyr. It should be noted that Ser425 remained unchanged in this variant sequence and that several novel alterations were also found in the 3'-nontranslated sequence in this clone. Altogether, this BCHEcDNA therefore represents a yet-unknown combination of point mutations in the human BCHE coding sequence and awaits characterization in the oocyte system.

5. Human acetylcholinesterase and butyrylcholinesterase are encoded by separate genes

5.1. Recombinant screening of cosmid CHEDNA clones

The presence of both BCHE and AChE coding sequences in the cosmid library selected for the experiments was first verified by DNA blot hybridization. For this purpose, the cosmid library was amplified and total DNA extracted from it was digested to completion with EcoRI and was electrophoretically separated in parallel to human genomic DNA. Blot hybridization with BCHEcDNA and AChEcDNA probes confirmed that both sequences are represented in this library (not shown). Homologous recombination screening, using a 5'-190-bp-long fragment from BCHEcDNA cloned in puc118 (87), resulted in the isolation of four different cosmid pCosCHEDNA clones designated C1-4. Figure 26 schematically presents the recombination experiment.

Figure 26. Recombinant screening and isolation of CHE cosmid clones



A. A 190 bp fragment from the 5'-domain of BCHEcDNA (159) was inserted into the ampicillin (Amp)-resistant vector puc118 (PCHE) and transfected into the rec⁺ *E. coli* strain BHB3169, which permits natural recombination. PCHE-carrying bacteria were, in turn, infected by the genomic kanamycin-resistant cosmid phage library and recombination between PCHE and CHE cosmid clones (CosCHE) took place. Dual

antibiotic-resistant recombinant clones (PCosCHE) were purified for analysis.

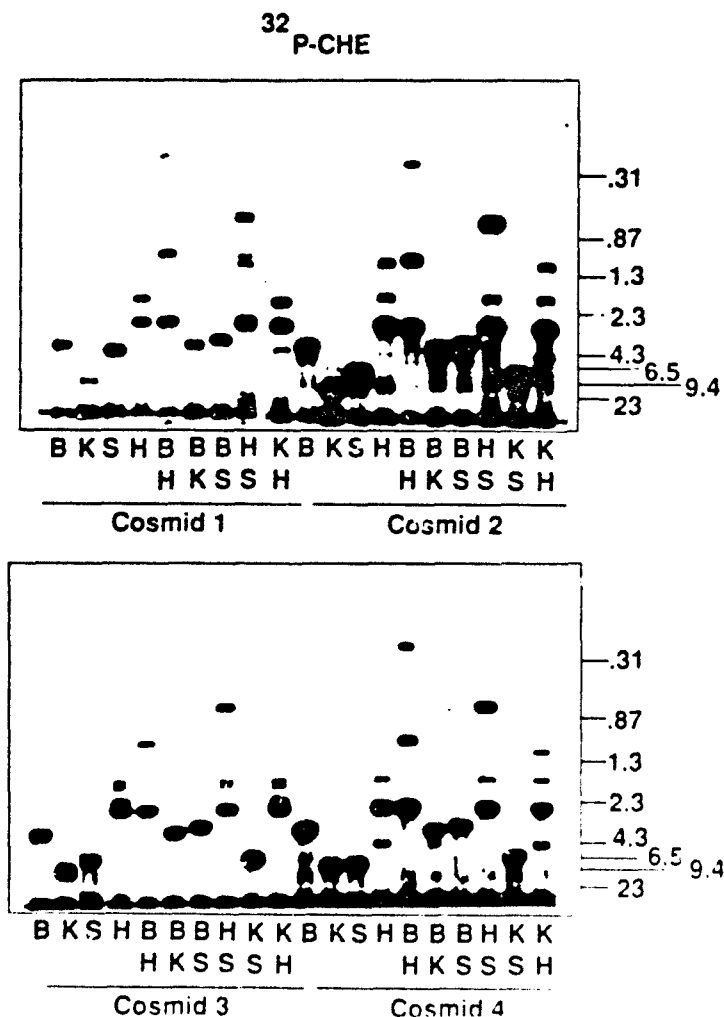
B. One μ g of DNA from four such clones (PCosCHEDNA clones C1-C4) and 60 ng of either BCHE or ACHEcDNA were blotted onto nitrocellulose filters, with herring DNA used as a negative control. Probes employed were labelled with [^{32} P] as detailed in section II. Note that all cosmid clone DNAs hybridized with BCHEcDNA but not with ACHEcDNA.

5.2. Blot-hybridization analysis of pCosCHEDNA fragments

DNA extracted from each of the pCosCHEDNA clones was tested for its ability to hybridize with BCHEcDNA and ACHEcDNA. All four clones were clearly positive with the first probe but negative with the latter, demonstrating that they all included BCHE-coding sequences but not ACHE-coding ones (Figure 26).

Enzymatic restriction, agarose gel electrophoresis, and ethidium bromide staining of these pCosCHEDNA preparations revealed that the four isolated cosmid DNA fragments were all approximately 50 kb in size and displayed highly similar, although not identical, restriction patterns with four different enzymes. Blot hybridization with BCHEcDNA again demonstrated similar restriction patterns, suggesting that all four cosmid clones were derived from the same gene and encompassed overlapping DNA fragments (Figure 27).

Figure 27. Blot hybridization of human cosmid CHEDNAs



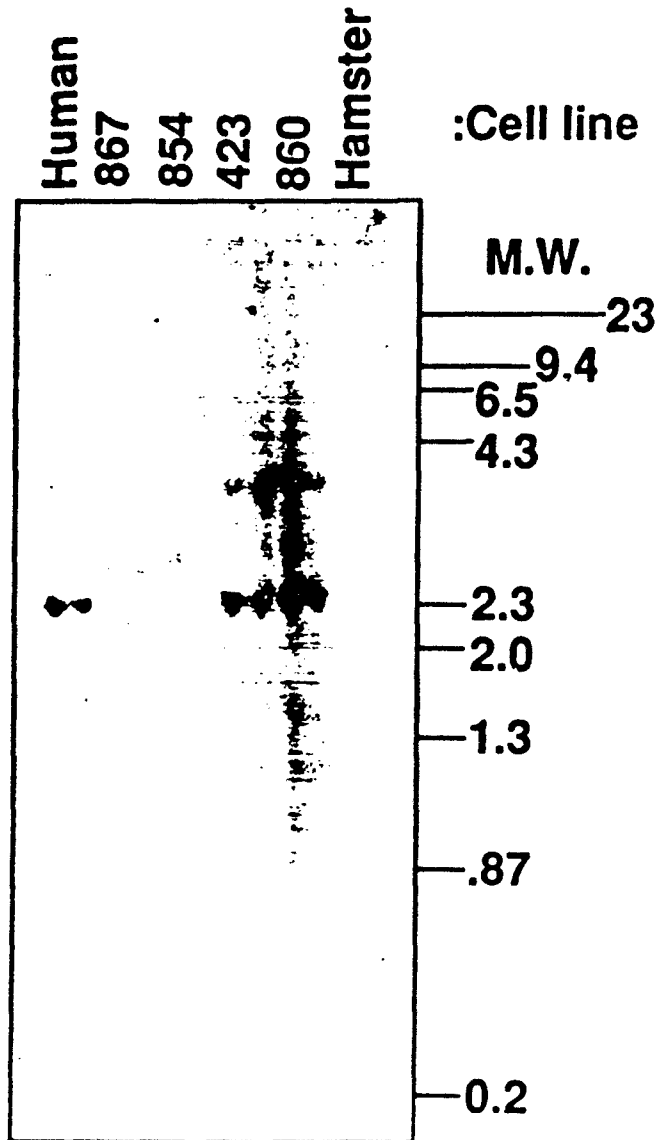
DNA from cosmid clones was digested to completion by one or more of the restriction enzymes *SacI* (S), *HindII* (H), *BamHI* (B), and *KpnI* (K) and electrophoresed in a 0.8% TBE agarose gel (TBE = Tris-borate 0.089 M, boric acid 0.089 M and 0.002 M EDTA). [³²P]BCHEcDNA probe was employed in hybridization. Size markers were Lambda-HindIII- and *PhiX*-HaeIII-digested fragments; the migration positions of some are illustrated. Highly similar restriction patterns can be observed in all of the cosmid DNA preparations in both single- and double-enzymatic restrictions. Note that KH double restrictions reveal a closer restriction pattern between Cosmid 1 and 3 than between cosmid 2 and 4.

All four cosmid clones originally had to include the 5'-190-bp-sequence to recombine with the puc118 plasmids which contributed their ampicillin resistance. Moreover, they all hybridized with the 0.7-kb 3'-extension of BCHEcDNA, designated Gb5, that we previously found in cDNA libraries from nervous system tumors. This demonstrated that all four cosmids included the entire sequence encoding BCHE, plus adjacent sequences. In addition, the cosmid DNAs appeared to contain several intron sequences. For example, the enzyme *HincII*, having a unique restriction site in BCHEcDNA (149, 157), restricted the various cosmid DNAs into at least three BCHEcDNA hybridizing fragments (Figure 27). This suggested the existence of more than one intron in these DNA fragments, in complete agreement with observations of others (167). It should be mentioned in this respect that the 190-bp fragment that was used to recombine with the CHEDNA cosmids had to be actively integrated into the isolated cloned sequences. Therefore, the organization of these DNA fragments should not necessarily be identical to that of the native gene, and these blots were, hence, not used to construct detailed restriction maps for the BCHE gene. Since the Gb5 3'-extended fragment was mapped to the unique 3q26-ter position by *in situ* hybridization (155), these findings also indicated that all of the isolated pCosCHEDNA clones were derived from a single BCHE-encoding gene, localized on the long arm of chromosome on no. 3.

5.3. Chromosome blot panel hybridization

The assignment of the BCHE gene to a unique position on chromosome 3 was further confirmed by DNA hybridization employing chromosome blots (Bios. Incorp.). These included DNA from different human/hamster hybrid cell lines carrying one or more human chromosomes (211). Only DNA from cell lines including human chromosome 3 contained the informative 2.4-kb *Hind III* restriction fragment hybridizing with BCHEcDNA (16, 149). Furthermore, the pCosCHEDNA fragments were identical with those observed in parallel lanes loaded with DNA from human lymphocytes. In contrast, hamster genomic DNA or hamster/human cell hybrids carrying human chromosomes other than no. 3 displayed no positive bands when hybridized with [³²P]BCHEcDNA. Figure 28 presents representative examples for these hybridization results and Table VIII summarizes the somatic cell hybrid mapping panel, clearly demonstrating full concordance with the chromosome no. 3 assignment in all of the five examined cell lines which carried this chromosome.

Figure 28. Human/hamster chromosomal blot hybridization



DNA from human/hamster somatic cell hybrids containing one or more human chromosome/s on a background of hamster chromosomes was enzymatically restricted with HindIII. Blots were prepared by Bios. Corp. and hybridization performed with [³²P]BCHEcDNA. For chromosomal contents, see Table VIII. A 2.3-kb HindIII fragment characteristic of the human BCHE gene appeared only in lanes loaded with DNA from cell hybrids containing human chromosome 3 (cell lines 423, 860) or with total human DNA, but not in lanes loaded with hamster DNA or with DNA from hybrid cell lines that do not carry the human chromosome 3 (cell lines 867, 854).

Table VIII. Human/hamster somatic cell hybrid panel: hybridization with BCHEcDNA

cell line	CH1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
867	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-
854	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
423	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
868	+	-	-	+	-	+	+	-	-	-	+	100	-	-	-	-	-	-	-	-	+	+	-	-	-
883	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
909	-	-	-	-	-	D	+	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-
151	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
811	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
967	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
734	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
968	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-
683	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	+	+	+	-	-	+
587	+	-	-	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+	-	-	+
750	-	-	-	-	-	D	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	+	+	-	-
1099	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
940	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
937	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-	-
861	+	-	-	+	-	+	-	-	-	-	-	75	-	-	-	-	-	-	-	-	-	-	-	-	-
1079	+	-	-	+	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
1006	-	-	-	-	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	+
750	-	-	-	-	D	+	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+	+	-	-	+
904	-	-	-	-	D	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-	+
909	-	-	-	-	D	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
862	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1049	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
212	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Discordance		26	22	0	26	74	30	22	33	33	7	15	33	37	41	26	22	26	30	30	26	37	26	30	22

Twenty-seven different hybrid cell lines containing one or more human chromosomes were examined in each consecutive lane. Chromosomes present in each hybrid cell line are denoted by + signs. The percentage of hybrids that were discordant with the human BCHEcDNA sequence is given for each chromosome. Notice that only DNA from hybrid cell lines containing chromosome 3 were found to positively hybridize with BCHEcDNA. D-deleted.

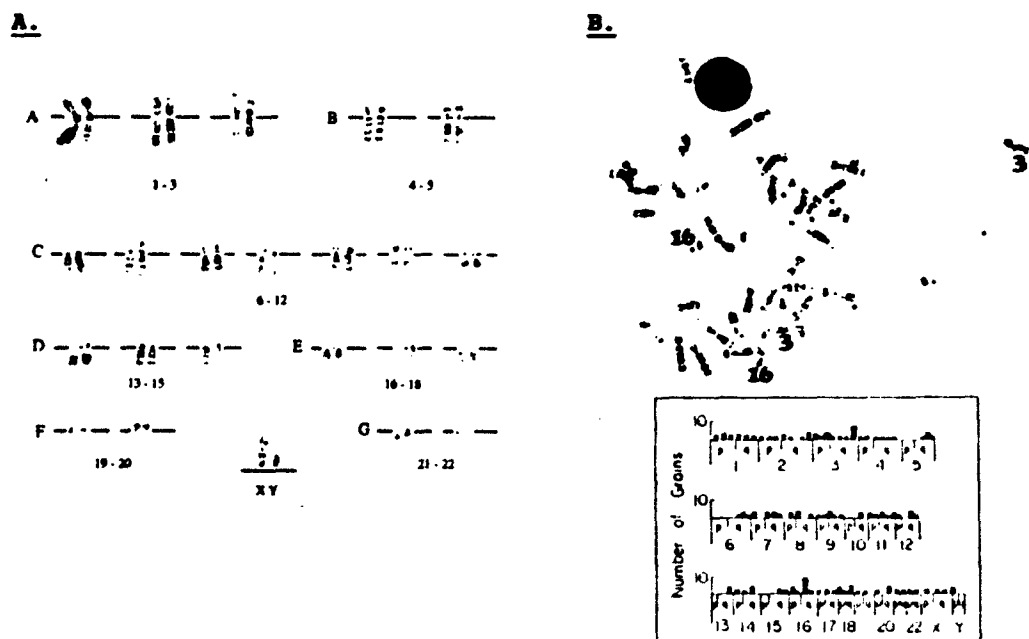
In contrast with chromosome 3, all other chromosomes, including chromosome 16, appeared not to carry BCHEcDNA-positive sequences. Exclusion of chromosome 16 was shown by two independently derived discordant hybrids, and one hybrid cell line with chromosome 3 as the only human chromosome was clearly positive for the human-specific fragment.

5.4. Gene-mapping by in situ hybridization

Cosmid recombination is an efficient and highly selective process, which takes place only with fully complementary sequences (150). The chromosome blots were hybridized under denaturing conditions and washed at high temperature and low salt, ensuring the stringency of the hybridization reaction. In contrast, in situ hybridization with spread chromosomes is limited in that it is performed in the presence of chromatin and washing conditions are generally rather mild (212). To

re-examine the validity of previous in situ hybridization with BCHEcDNA, which demonstrated positive labelling of chromosome 16 in addition to 3 (120), we performed these mapping experiments under highly stringent washing conditions. Significant labelling again appeared on chromosomes 3 and 16 (Figure 29), reconfirming the presence of Chr.-16-BCHEcDNA-like sequences, in at least some individuals.

Figure 29. In situ hybridization of [^{35} S]BCHEcDNA to human lymphocyte metaphase chromosomes



A. A representative chromosomal R-banding karyotype and spread. Silver grains appear to be associated with both chromosome 3 and chromosome 16.

B. Cumulative distribution of grains on human chromosomes from 14 metaphase spreads. Equally significant accumulation of grains was observed on chromosomes 3 and 16.

6. In vivo amplification of the human CHE genes

6.1 In individuals exposed to organophosphorous poisons

The oocyte expression studies demonstrated that BCHE can serve as a potential scavenger of cholinesterase inhibitors, for example, OP poisons. This may become crucial under conditions in which BCHE and/or ACHE activities are essential for yet-undefined developmental processes: One may postulate a situation in which a developing cell producing sufficient quantities of active CHEs will survive under exposure to such poisons, while cells or organisms with defects in the expression of CHEs may not.

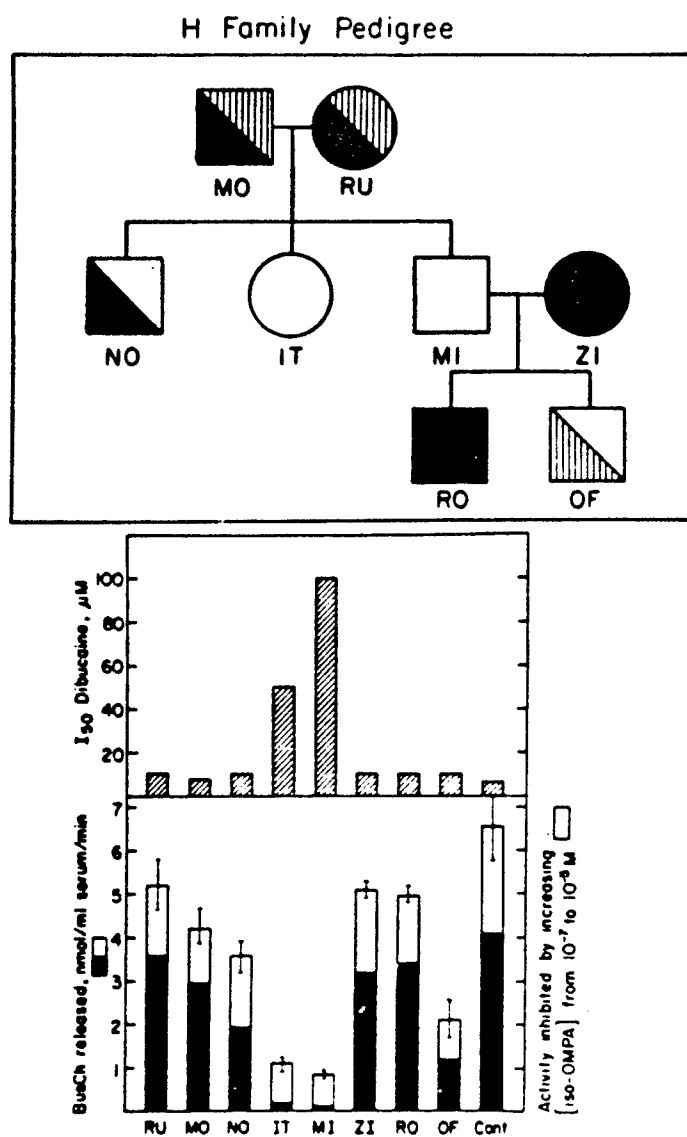
Exposure to metabolically poisonous materials which are primarily aimed to destroy developing or rapidly dividing cells is a common paradigm in chemotherapy. A well-known example is the use of methotrexate, an inhibitor of the enzyme dihydrofolate reductase, in the treatment of leukemia. The cytotoxic effect of methotrexate depends, however, on the ability of the drug to block DHFR activity completely. Under conditions of overproduction of DHFR, cells may, therefore, divide even in the presence of high methotrexate concentrations. One way by which cells become overproducers of DHFR and resistant to methotrexate cytotoxicity is by

The discovery of DHFR gene amplification led to many further studies of controlled exposure to cytotoxic inhibitors, and it was found that in many such cases the genes encoding the target proteins to these inhibitors amplified, conferring resistance to the toxic effects. It is generally assumed that gene amplification occurs spontaneously, with higher frequency in transcriptionally active genes and dividing cells. Under conditions in which the amplification of a particular gene will provide the cell in which it occurred with a survival advantage over other cells, the amplified DNA will become inheritable. Thus, drug-resistant cell lines were developed with progressive increases in DHFR (or other genes) copy numbers (214, reviewed in 88).

Anti-sense oligonucleotide experiments (see section III.7) demonstrate that CHE activities are essential for the well-being of various developing cells. Under exposure to CHE inhibitors, such cells may become analogous to leukemic cells treated with methotrexate, and it would be conceivable to expect that when CHE genes amplify in these cells, they provide them with a survival advantage over cells with non-amplified CHE genes.

The commonly used OP agricultural insecticides are effective CHE inhibitors, which are toxic to the human enzymes as well (215). Particularly vulnerable to these poisons are individuals with genetic BCHE defects, a relatively common trait in Israel (132). The serum BCHE in these individuals displays low or abnormal catalytic activities and insensitivity to specific BCHE inhibitors, for example, bucarine (15). When exposed to OP poisons, such individuals would be subjected to acute inhibition of BCHE activities, which may result in interference with growth of certain cell types in their bodies. This situation fulfills the two prerequisites for in vivo amplification of the BCHE gene, as it provides a selection pressure for the survival of cells in which BCHE gene amplification occurred. If BCHE activity is necessary for germ cells development, BCHE gene amplification will be observed in all cells of the descendent progeny. Figure 30, presents an example for a family pedigree of such individuals and the BCHE activities measured in them (see also, 146). Two members in this family presented BCHE gene amplifications: one of the sons, M.I., and one of his own sons, O.F. In contrast, the parents (M.O. and R.U.) had normal copy numbers of BCHE genes. Gene-mapping by in situ hybridization has further demonstrated that the amplified CHE sequences were localized in the 3q26-ter position, either indicating that the amplification occurred in loco, where we have previously mapped the original site of this gene, (120, 121) or suggesting that it was inserted therein. Figure 31 presents a schematic distribution of the gene-mapping results.

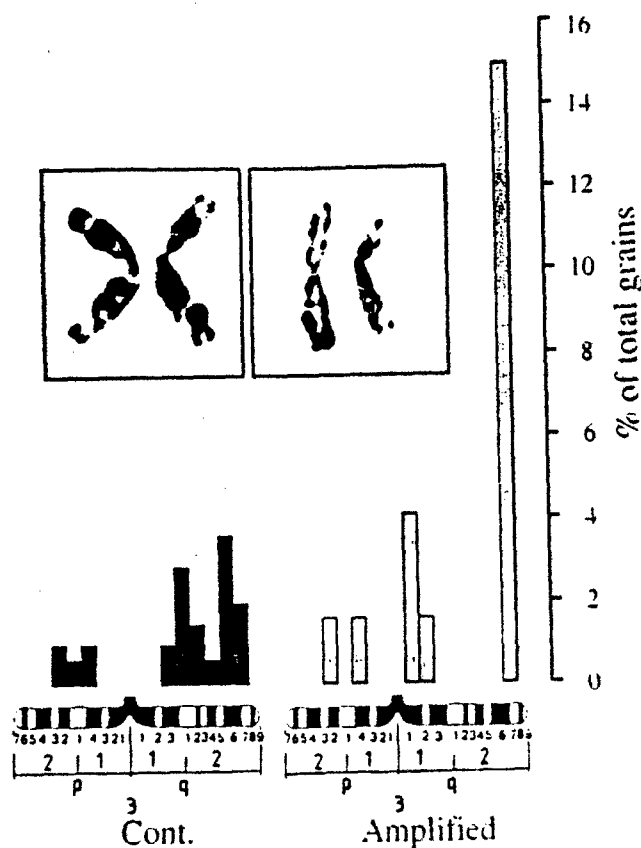
Figure 30. The H family pedigree and BCHE activities



A. Family pedigree and phenotypic BCHE properties.

B. BCHE catalytic activities were measured spectrophotometrically by following butyrylthiocholine (BuSCh) hydrolysis as described (146), in the absence or presence of iso-OMPA (tetraisopropyl pyrophosphoramide) or dibucaine (2-butyryl-5-methyl-6-phenyl-1,2,4-triazine-3-carboxamide).

Figure 31. Chromosomal mapping of amplified BCHE genes

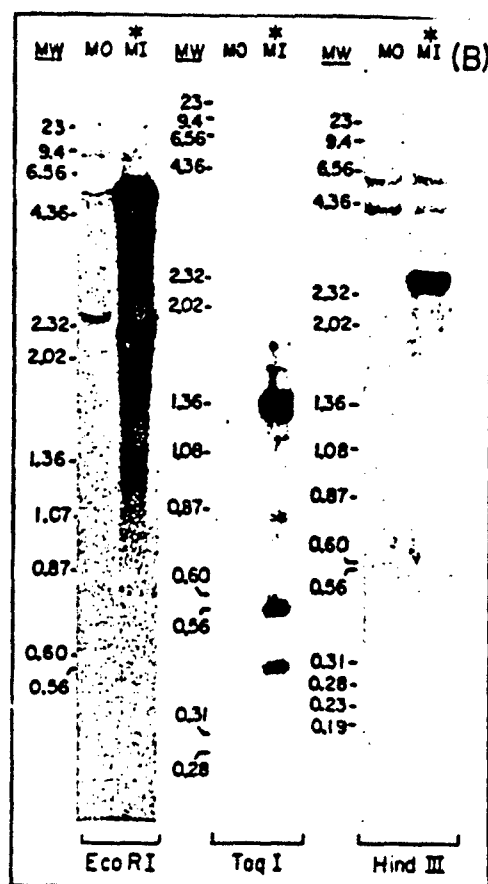
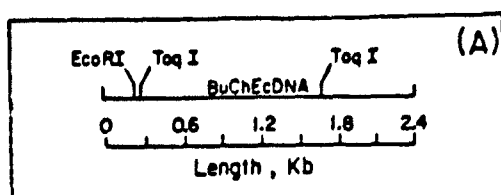


In situ hybridization with M.I. and control chromosomes was done as described (120). Cumulative number of silver grains for 21-chromosome spreads from each sample is presented.

To initiate structural analysis of the amplified BCHE gene, DNA blot hybridization was employed. Figure 32 presents a representative DNA blot with M.I. and M.O.'s DNAs. Enzymatic restriction followed by blot hybridization reproducibly revealed irregular positions for EcoRI sites spanning the amplified fragment in M.I.'s DNA, resulting in hybridizable bands of variable sizes. In contrast, Hind II restriction yielded a single 2.7-kb-long amplified band. Finally, a 1.4-kb *aql* fragment from the central part of the coding region was considerably more amplified than the external, smaller *TaqI* fragments. Altogether, this analysis indicated an initial "onion-skin" amplification event (213), creating a tandemly repeated core amplification unit which contains HindIII and *TaqI* sites and with *coRI* sites being external to this core unit.

Figure 32. DNA blot hybridization reveals the amplified BCHE genes B.

A.



A. BCHEcDNA contains one and two EcoRI and TaqI restriction sites, respectively
 B. DNA blot hybridization was performed as described (148). Exposure was for 1 wk.

5.2 In blood cell disorders

To become inheritable, the amplification of CHE genes should occur in germline cells. Logical candidates are developing sperm cells, in which CHE activities are known to be expressed (55). However, one can only speculate on the origin of this unprecedented phenomenon in humans, and it remains to be proven whether OP exposure can be implicated with it. In addition, the first finding of amplified BCHE genes in humans raised the question of whether this is a unique, exceptional event or whether it reflects a recurrent phenomenon. Also, we postulated that the ACHE genes should be subject to the same survival pressures under OP exposure, and we wished to find out whether these genes amplify as well. Finally, we knew that both genes are transiently expressed in embryonic development and display particularly high levels of expression in various malignant tumors.

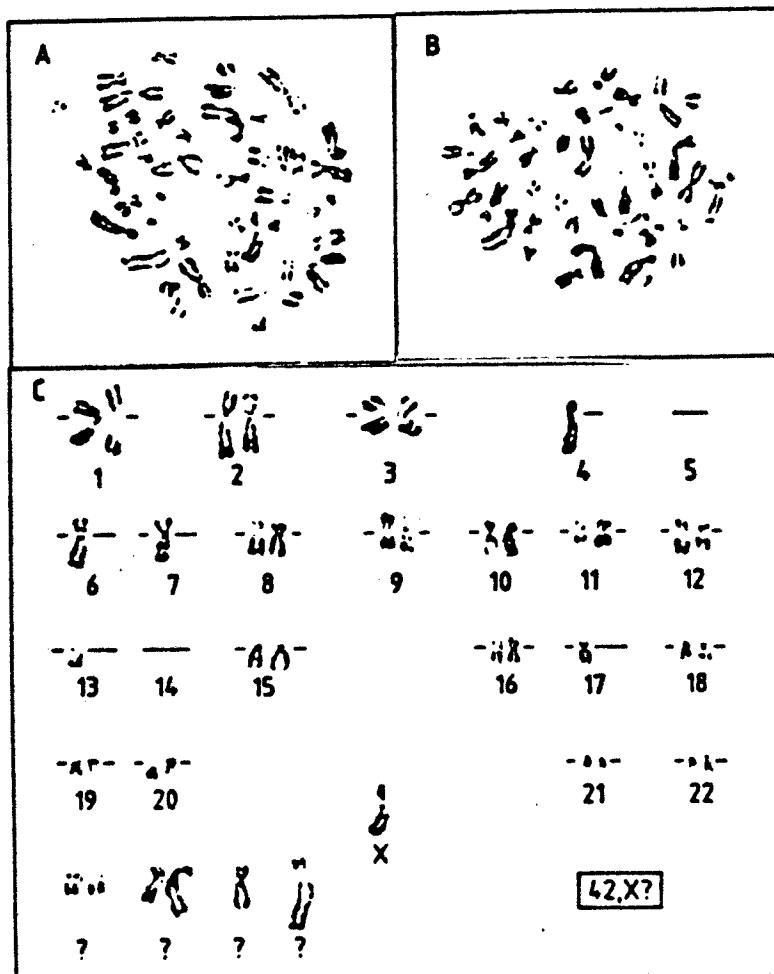
Therefore, further amplification cases were pursued in tumors in which the Chr. 3q26-ter position, where the BCHE gene resides, tends to break frequently. This survey was based on the assumption that gene amplification could be a putative origin for such breakage (see, for example, reports on breaks in Chr. 7 at the position of the amplified EGFR-erb2 oncogene in glioblastomas, 216).

Breaks in the long arm of chromosome no. 3 appear repeatedly in leukemias (217). This happens in patients with rapid progress of the disease and abnormal platelet counts, which implies defects in promegakaryocytopoiesis (218). Because of the previous publications implicating CHEs with this hemocytopoietic process (219, 220), we examined DNA from patients with leukemias and/or platelet disorders for BCHE and ACHE gene amplification. Four of sixteen samples of leukemic DNAs and three of five from patients with platelet disorders were found to have 10-200 copies of both of these genes (145). This raised the question of whether the co-amplification of CHE genes in hemopoietic cells could be linked with enhanced production of their protein products and involved in the etiology of hemocytopoietic disorders (88). This question became yet more pertinent in view of our other observation that the 5'-attenuating domain in the BCHE gene is

6.3. In ovarian adenocarcinomas

In order to further pursue these putative correlations between CHE gene amplification and overexpression, we searched for parallel phenomena in ovarian carcinomas. It has long been known that CHEs are intensively expressed in these tumors (37). Using *in situ* and blot hybridization, we could show that co-amplification of the BCHE and ACHE genes was correlated with their overexpression in ovarian carcinomas (39).

Figure 33. Chromosome spreads and a representative partial karyotype from an ovarian papillary adenocarcinoma tumor

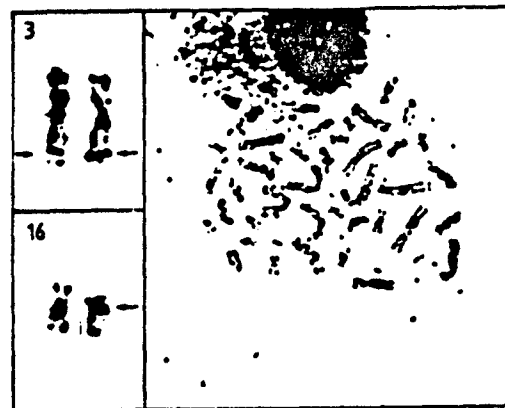


Chromosomes were prepared from a papillary adenocarcinoma tumor essentially as described elsewhere (121). Briefly, tissue samples were treated immediately following operation with 10 ml/50 mg tissue of RPMI 1640 medium (Gibco) containing 10 I.U./ml of heparin as well as penicillin, streptomycin and neomycin (Biolabs, 1:1000). Minced tissue was transferred into 3 ml of fresh RPMI medium containing 20% fetal calf serum and 4 mg/ml colcemid for 60 min at 37°C with 5% CO₂. Medium was replaced by 0.8% Na-Citrate for 15 min at 37°C for hypotonic treatment. Chromosomes were fixed by two successive 10-min incubations in methanol: acetic acid mixture (3:1, V/V). Cells were dissociated in 60% glacial acetic acid and spreads were manually dropped onto glass slides. Chromosomes were then G-banded,

one or two of the chromosomes 4-7, 13, 14, X and 17 are missing, whereas 6 unidentifiable chromosomes appeared, probably the results of translocations and deletions.

Localization of specific genes in chromosomes from malignant tumors is generally rather difficult due to the appearance of multiple chromosomal aberrations in tumor cells. When cells from ovarian tumors were grown in culture, we repeatedly detected missing chromosomes as well as abnormally sized and banded tumor-specific chromosomes (Figure 33). However, the use of *in situ* hybridization with BCHEcDNA permitted the direct localization of the BCHE genes in chromosomes 3 and 16 in tumor chromosomes (Figure 34), in agreement with our previous gene-mapping studies in adult (120) and fetal chromosomes (121).

Figure 34. BCHE gene-mapping by *in situ* hybridization onto ovarian carcinoma chromosomes



Chromosome spreads were prepared as in Figure 33 and subjected to *in situ* hybridization with [³²S]BCHEcDNA as detailed previously (120, 121). Exposure was for 12 days. Note labelling of chromosomes no. 3 and one chromosome no. 16 (left) and abnormalities in karyotype.

BCHE/ACHE gene amplification was observed in each ovarian tumor sample in which the *RAF1* or *SIS* oncogenes were amplified. Moreover, several of these primary tumors displayed amplified BCHE/ACHE genes, although none of the examined oncogene probes detected an amplified signal (39). Further experiments will be required to determine whether CHE gene amplification occurs earlier than that of oncogenes in the course of tumor progression.

7. Manipulations of BCHE gene expression modulate murine megakaryocytopoiesis *in vitro*

7.1 Anti-sense oligonucleotide to BCHEcDNA blocks megakaryocytopoiesis in culture

Bone marrow cultures were employed to assess the etiological involvement of BCHE in megakaryocytopoiesis. Cells from the marrow of femur and tibia of 8 to 12-week-old endotoxin-resistant C3H/HeJ mice were cultured in LPM synthetic medium (Biological Industries, Beit haemek, Israel) containing 10% conditioned medium from WEHI-3 cells, 1% BSA, 10⁻⁴ M thioglycerol, and 1% methylcellulose. In 35-mm Petri dishes (Falcon 1008), or 24-well tissue-culture Costar plates, 0.5-1.0 X

with high humidity. The cultured murine bone marrow cells were then incubated with an anti-sense oligodeoxynucleotide (AS-CHE ϕ , Figure 35A) targeted to human BCHEmRNA (149), which is almost identical to mouse BCHEmRNA in its nucleotide sequence (Soreq et al., unpublished observations). AS-CHE ϕ was found to block translation of recombinant BCHEmRNA in microinjected *Xenopus* oocytes up to 80% and to hybridize *in situ* with mouse cell suspensions (not shown).

To permit optimal uptake of the oligonucleotide into the cells, they were incubated in methylcellulose supplemented with the LPM synthetic serum substitute and conditioned medium from WEHI-3 cells (WEHI), a rich source of the multilineage hematopoietic growth factor interleukin 3 (IL-3) (221). Following 4 days in culture, AS-CHE ϕ was found to reduce the total number of colonies (Figure 35B, inset) and the relative proportion of megakaryocytes in the culture population (Figure 35B).

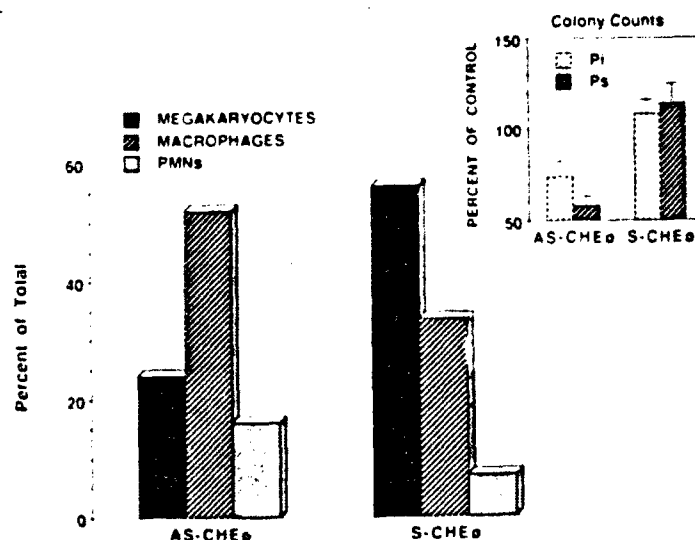
In addition, megakaryocyte colonies grown in the presence of AS-CHE ϕ appeared considerably smaller than controls (not shown). Pulse-labelling with 50 μ Ci of [^{35} S]-methionine (1000 Ci/mole) per culture demonstrated that gross rates of nascent protein synthesis at day 4 remained unaffected by either of these oligonucleotides, at a level of $(7\pm 3)\times 10^4$ cpm/culture/24 hr. This implied that the AS-CHE ϕ effect reflected a specific suppression of a gene product required for development and not a general toxicity. A stable phosphorothioate (222) analog of AS-CHE ϕ elicited a similar depression of colony formation in the cultures (Figure 35B, inset), while both phosphate and phosphorothioate complementary sense oligodeoxynucleotides (S-CHE ϕ , Figure 35A) had no effect on either colony counts or their cellular composition (Figure 35B).

Figure 35. Anti-sense BCHE oligonucleotide inhibition of megakaryocytopoiesis

A.

AS-CHE ϕ	3' TAC GTA TCG TTT CAG 5'
BuCHEmRNA 5'...	AUC AAU AUG CAU AGC AAA GUC ACA...3'
	met his ser lys val thr
S-CHE ϕ	5' ATG CAT AGC AAA GTC 3'

B.



A. Oligonucleotides: Phosphorothioate (P_s)- and phosphate (P)-containing sense (S-CHE ϕ) and anti-sense (AS-CHE ϕ) 15-mer oligodeoxynucleotides were synthesized using an Applied Biosystem 380B DNA synthesizer. The phosphate oligonucleotides were synthesized by the phosphoramidite method, the phosphorothioates by the H-phosphonate method. All were purified by reversed phase

high-performance liquid chromatography (HPLC), the phosphorothioate oligonucleotide before and after removal of the high-performance liquid chromatography dimethoxytrityl group. The region spanning the initiator AUG in BCHEmRNA (149) and the concentration of the oligonucleotides used were selected in view of the experience of others (230), and AS-CHE ϕ was confirmed to be potentially effective in the examined final concentration of 5 μ M (expressed as oligomer concentration) on the basis of its ability to block translation of synthetic BCHEmRNA when co-injected in this concentration into *Xenopus* oocytes. Also, when tested in culture, 2.5 μ M of AS-CHE ϕ was not effective at all, while 10- and 15- μ M concentrations of both AS-CHE ϕ and S-CHE ϕ were toxic to the cultured cells.

B. Differential cell analysis of anti-sense-oligonucleotide-treated, semisolid bone marrow cultures: Colonies grown in serum-free methylcellulose cultures containing sense or anti-sense CHE oligonucleotide (P_s) were picked with drawn-out Pasteur pipettes, concentrated (5 min at 500Xg) by Cytospin (Shandon; 2) centrifugation in phosphate-buffered saline (PBS), stained with May-Grunwald Giemsa, and analyzed microscopically. Megakaryocyte colonies contained at least four cells, macrophage or granulocyte colonies consisted of 50 or more cells. The relative fraction of each cell type represented among the total cells recovered from two independent experiments are shown. Almost all colonies were pooled from each plate and at least 500 cells were counted for each data set. The distribution obtained with S-CHE ϕ was essentially identical to that observed in control (no oligo) cultures.

Inset. Colony counts. Total number of all types of colonies, including megakaryocytes, granulocytes, and macrophages, were scored using a Zeiss Stereozoom binocular after 4 days' growth in the presence of P_s or P oligonucleotides. Plots present percent of control (no oligo) culture counts. Data represent average of two (P_s) or 3 (P) independent experiments $\pm$ (S.E.M.).

7.2. Conditioned medium from BCHE-producing *Xenopus* oocytes promotes murine megakaryocytopoiesis

In order to further investigate the role of BCHE as a regulatory element in the megakaryocytopoietic pathway, incubation medium from *Xenopus* oocytes microinjected with recombinant BCHEmRNA (87, 223) (BCHE-OCM) was added to bone marrow cultures. BCHE-OCM significantly augmented colony formation in a variety of semisolid and liquid culture conditions (Table IX). A 2-fold dilution of the BCHE-OCM reduced the effect to 33% of the initial level, whereas a 10-fold dilution completely abolished it (not shown). Conditioned medium from control oocytes injected with Barth's saline solution (Brt-OCM) also elicited an enhancement of colony formation, in concurrence with reports on the presence of platelet-derived growth factor (PDGF) (224) and other growth-stimulating factors (225) in oocyte-conditioned media. However, the Brt-OCM effect was significantly smaller than that observed with BCHE-OCM ($P < 0.025-0.001$) (Table IX). Furthermore, antibodies elicited against purified human PDGF (226) completely prevented the Brt-OCM-augmentation effect, while BCHE-OCM enhancement was only partially blocked (Table IX).

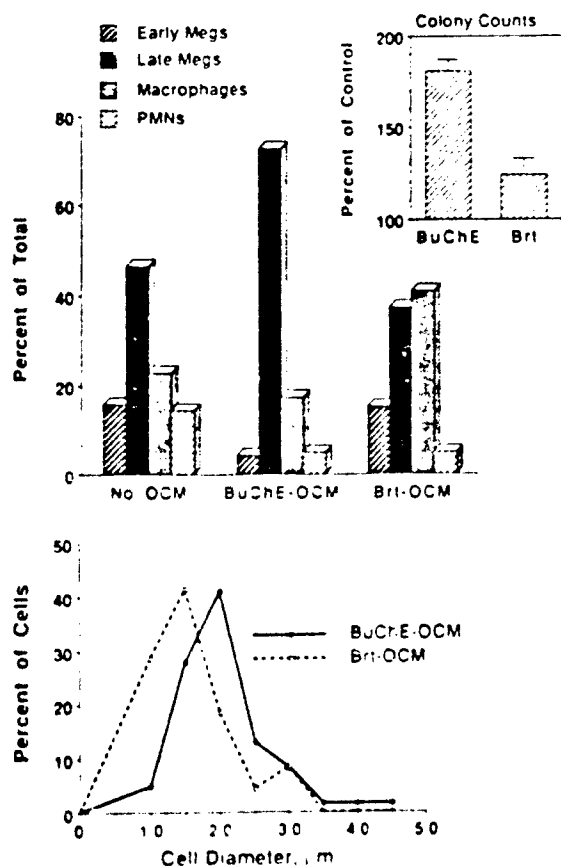
Table IX. Effect of oocyte conditioned media on hematopoietic colony formation

Medium	DAY	No. Expts.	No OCM	+BCHE-OCM	+Brt-OCM
HS/WEHI	3	8	1.00+/-0.03 (59-276)	1.81+/-0.06 P<0.001	1.24+/-0.09 P<0.001
	4	11	1.16+/-0.07 (72-252)	2.03+/-0.12 P<0.001	1.50+/-0.08 P<0.001
HS/WEHI + α PDGF	4	1	1.14+/-0.08 (417-646)	1.26+/-0.05 n.d.	1.01+/-0.09 n.d.
HS/IL-3	3	1	1.00+/-0.10 (61-277)	2.02+/-0.06 P<0.008	1.25+/-0.05 P<0.085
	4	1	1.69+/-0.05 (122-276)	2.34+/-0.17 P<0.01	1.90+/-0.30 P<0.055
LPM/WEHI	4	4	1.00 +/-0.07 (221-386)	1.43+/-0.04 P<0.007	1.28+/-0.03 P<0.02
LPM/WEHI + α PDGF	4	2	n.d.	n.d.	0.60+/-0.04 P<0.001 (466)

Average total number of colonies per culture dish +/- S.E.M. (as a fraction of control culture on first day counted) is shown for various culture media with and without the addition upon initiation of culture of conditioned medium (0.5%, V:V) from *Xenopus* oocytes injected with synthetic BCHE-mRNA (BCHE-OCM), or Barth's saline solution (Brt-OCM). Oocyte microinjections were as described (87): HS/WEHI - 15% horse serum (Gibco), 10% conditioned medium from WEHI-3 cells, 10^{-4} M thioglycerol (Sigma), and 1% methylcellulose (DOW, A4M Premium) made up in Iscove's modification of Dulbecco's medium (IMDM) (Gibco); HS/WEHI- α PDGF - as above, with 1:40 dilution of rabbit antihuman PDGF antiserum; HS/IL-3 - as above with 20 U/ml purified recombinant murine IL-3 replacing WEHI-conditioned medium; LPM/WEHI - LPM synthetic serum substitute containing 10% conditioned medium from WEHI-3 cells, 1% defibrinated bovine serum albumin (BSA) (Sigma, fraction V), 10^{-4} M thioglycerol, and 1% methylcellulose; LPM/WEHI- α PDGF - as above with 1:40 α PDGF. Statistical significance (P) of deviations was calculated by Student's t-test vs. no OCM controls. Numbers in parentheses represent range of colonies counted. n.d. - not determined.

Differential cell counts attributed the increase in colony-forming units in BCHE-OCM-treated cultures to enhanced megakaryocyte proliferation and maturation, while the increase in Brt-OCM-treated cultures appeared in the macrophage population (Figure 36). A substantial increase in the number of late as opposed to early megakaryocytes was also observed in BCHE-OCM-treated cultures as compared with both control and Brt-OCM-treated cultures. This shift was accompanied by a significant increase in the average diameter of megakaryocytes (Figure 36), indicating that BCHE-OCM played a specific role in megakaryocyte maturation. Colonies cultured in horse-serum-containing media were larger, developed faster, and contained a higher proportion of megakaryocytes than those grown on LPM. Therefore, each type of experiment was evaluated separately. In both horse serum (HS)-containing and HS-free media, cultures deprived of WEHI-3-conditioned medium gave rise to colonies which were small, and poorly developed, and which disintegrated after day 3, indicating that the oocyte-conditioned medium (OCM) affects megakaryocyte colony formation by synergizing with the IL-3 in WEHI. Substitution of recombinant-murine-purified IL-3 for WEHI confirmed this conclusion (Table IX).

Figure 36. BCHE-OCM augmentation of megakaryocytopoiesis



A representative differential cell analysis (one of three experiments) of colonies picked from serum-fortified methylcellulose cultures. Cultures were grown with or without addition of 0.5% incubation medium from *Xenopus* oocytes injected with synthetic BCHEmRNA (BCHE-OCM), or Barth's saline solution (Brt-OCM). Early megakaryocytes, defined as immature forms with one or two nuclei, were distinguished from late ones, characterized by their larger size and tendency to shed cytoplasmic fragments. Note relative increase in late-stage megakaryocytes upon addition of BCHE-OCM, and increase in macrophages in Brt-OCM-containing cultures.

Inset. Colony counts. Total number of colonies observed after 3 days incubation is plotted as percentage of control (no OCM) cultures $\pm$ S.E.M. (n =eight independent experiments; triplicate cultures per experiment).

(bottom) - BCHE-OCM stimulates megakaryocyte growth. Percent of cells in each size range (5 μ m intervals) are plotted for BCHE-OCM- and Brt-OCM-treated cultures. For each culture type, 60-70 cells were measured. Average diameter of megakaryocytes grown in the presence of BCHE-OCM ($18.8 \mu\text{m} \pm 0.63$ S.E.M.) was found to be significantly larger ($F < 0.025$) than those grown in the presence of Brt-OCM ($14.4 \mu\text{m} \pm 0.85$ S.E.M.), an increase comparable to that observed in the *in vivo* response to the stimulus of thrombocytopenia on megakaryocyte growth.

BCHE-OCM exerted a more pronounced enhancement effect in methylcellulose cultures supplemented with 15% horse serum, a rich source of tetrameric BCHE (15), than that observed under serum-free conditions (Table IX). Given the negligible

BCHE enzymatic activity contributed by the oocyte-conditioned medium in these cultures (5.01% of serum contribution; 87), it appears unlikely that the stimulating factor in BCHE-OCM is catalytically active BCHE. Moreover, 5-min heating to 45°C resulted in a 40% reduction in the BCHE-OCM effect, while BCHE activity remained 95% intact. Therefore, these results suggest the presence of an as-yet-unknown heat-sensitive amphibian factor secreted by the oocytes in response to the intracellular accumulation of heterologous heat-stable, human BCHE--an enzyme not normally synthesized in *Xenopus* oocytes (58).

Although cholinergic mechanisms have been implicated in megakaryocytopoiesis (145, 127) and AChE is recognized as a specific marker of murine megakaryocytes (228), these data provide the first evidence for BCHE involvement in megakaryocyte development. In order to directly examine whether BCHE is expressed in these cells, we performed cytochemical CHE staining (229) of megakaryocytes grown in LPM liquid cultures. Activity staining was pronounced, and appeared sensitive to inhibition by both the AChE-specific inhibitor BW284C51 and the BCHE-specific inhibitor iso-OMPA (Figure 37, panel C), indicating the presence of both enzymes - and directly revealing the previously undetected presence of BCHE in these cells.

Figure 37. BCHE-OCM influences the morphology of hematopoietic cells



Liquid cultures were grown in LPM +/- BCHE-OCM and cells were picked and stained as in the legend to Figure 34 and photographed in a Zeiss AvioPlan microscope equipped with an HC100 camera.

A,B. Representative microscope fields. A Control cells. Note heterogeneity of cellular morphologies. B - Megakaryocyte-enriched BCHE-OCM treated culture. Note the presence of multicell megakaryocyte colony (bottom) and mature megakaryocytes (top).

C. Cytochemical staining of megakaryocytes for CHE activities

Fixed cells were stained for CHE activity by indolyl acetate (229) in the absence of inhibitors (top), in the presence of 10^{-5} M of 1,5-bis (4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51), a specific AChE inhibitor (second), or tetraisopropylpyrophosphoramidate (iso-OMPA), a selective BCHE inhibitor (third), or both inhibitors (bottom). Note the partial sensitivity of staining to both inhibitors, indicating the presence of both AChE and BCHE in murine megakaryocytes.

IV. Scientific and Environmental Implications

1. The human ACHE gene: Molecular cloning and comparison to the BCHE gene

1.1 Differential codon usage in the human ACHE and BCHE genes

Amino acid sequence homologies to other CHes, along with the acetylthiocholine-hydrolyzing activity of the recombinant enzyme and its inhibition by ACHE-specific but not by BCHE-specific inhibitors, clearly identify our composite nucleotide sequence as a DNA encoding human ACHE. This makes Homo sapiens the first species in which ACHE and BCHE sequences may be compared. The overall sequence conservation between these two proteins implies some common physiological role(s) and is compatible with the cross-homologies displayed by antibodies directed against recombinant, nonglycosylated BCHE peptides (163). In contrast, the considerable differences between these primary amino acid sequences and their dissimilar glycosylation levels may explain their previously reported lack of cross-immunoreactivity (139).

ACHEDNA presents a G,C-rich pattern of codon usage, characteristic of genes with tendencies to replicate and be transcribed late in the cell cycle (172). In contrast, BCHEDNA is A,T-rich (149,157), as expected of a gene with transiently high levels of activity in dividing cells (38). This clear distinction indicates that although these two genes apparently diverged long ago in evolutionary history, selection pressures kept their encoded polypeptides from extreme evolutionary drift. Consequently, there is essentially no cross-hybridization between ACHE- and BCHEDNA (145).

The susceptibility of the ACHE gene for recurrent amplification in leukemias (145) and ovarian tumors (39) may be implicated with its tumorigenic expression (88). The different signal intensities which were observed when 5' and 3' regional ACHEDNA probes were hybridized with patients' DNA samples (111) indicate that the predicted G,C-rich structure at the 5'-end of the ACHE-coding sequence may, in certain cases, terminate the amplification process in vivo. Moreover, the considerable variability in the PvuII restriction patterns, between DNA samples reflecting independent amplification events, as shown with the 5'-probe, may be due to rearrangements in this DNA region more frequently than in the 3'-domain.

The findings presented in this report demonstrate amplified and structurally modified ACHE genes in the tested primary DNA samples, in extension of previous observations (39, 145). This further suggests that earlier biochemical studies of ACHE activities (141) may have reflected underestimates of the extent of pathological changes which occur in the ACHE gene in tumors. The G,C-rich attenuator sequence in ACHEDNA may also serve to control the transcriptional activity of this gene, a phenomenon similar to the attenuating function of structures with similarly high free energy which have been observed in viral sequences (171). Our present observations, therefore, predict previously unexpected mechanisms to regulate the production of ACHE in healthy and neoplastic cells that may now be investigated using cloned human ACHEDNA sequences.

1.2 Differential modes of expression for the ACHE and BCHE genes in developing neurons

Comparison of labelling efficiencies with the two CHE probes reveals that the production of their corresponding mRNAs increased consistently throughout this period in fetal brain development in both of the regions that were studied, with, however, very different rates and developmental patterns. The levels of ACHEmRNA were found to be considerably higher than those of BCHEmRNA in basal nuclei neurons, while BCHEmRNA production was relatively more intensive in the cerebellum. Moreover, the ratio between ACHEmRNA and BCHEmRNA remained close to 2 in the developing fetal cerebellum during this period in human brain development, while the much higher ratio in basal nuclei was depressed from 15- to 10-fold. These

differentiating noncholinergic neurons. This would support the notion that specific CHEs may exert pivotal growth-related roles in neuronal commitment and differentiation.

Transient histochemical staining of CHE activities was also noted in several neocortical areas (139, 231), most of which display divergent mechanisms of regulation for muscarinic receptors (186). It will be interesting to examine whether the enzymatic staining in these areas reflects as well the coordinated production of AChE and BCHE. Similarly, one would like to know which transcripts are produced in the visual cortex, where the transient patterns of AChE activity depend on the normal neonatal development of functioning neurons (187).

The use of molecular biology approaches in general, and particularly in situ hybridization, adds a new dimension to the anatomical and histochemical studies of nervous system development. In our case, it enabled the distinction between types of developing neurons in brain areas that were apparently similar in their positive cholinergic histochemical staining. This was done by demonstrating the selective induction of transcriptional processes leading to the accumulation of the RNA species encoding one particular enzyme, AChE, in fetal cholinergic neurons but not in noncholinergic ones. In addition, these findings could indicate when during fetal development the basal nuclei neurons become capable of receiving cholinergic inputs.

1.3 Coordinated regulation of the BCHE/AChE genes in human oocytes

The aim of this part in our study was to reveal whether the CHE genes are expressed in human oocytes and, if so, at what level of transcription and in which developmental stage. The high resolution, specificity, and sensitivity of the in situ hybridization reaction enabled us to resolve this question. When combined with biochemical analysis, our findings demonstrate that within the mature human ovary, oocytes produce catalytically active AChE monomers and dimers that are predominantly soluble in buffer. In contrast, no other cell types in the ovary produce CHEs, indicating that the switching on of CHE genes occurs in the human ovary by selective regulation processes taking place in the nuclei of developing oocytes arrested at their mitotic division.

The conservative cytochemical staining of CHE activities displayed an intricate pattern of cholinceptive parasympathetic nerve fibers in the mammalian ovary. The innervation density was found to be higher in the intraovarian part of the follicle, where the oocyte is localized (232). Thus, it is difficult to employ the cytochemical staining technique to demonstrate CHE activity within the oocyte itself. In contrast, the nerve fibers do not contain any mRNA and would, therefore, not be detected in the in situ hybridization technique. In Xenopus oocytes, AChE dimers are predominantly associated with the oocyte membrane (233). However, it is possible that oocyte proteases (234) release the oocyte AChE from the membrane fraction. Further experiments will be required to resolve this issue.

The DNA blot hybridization implies that apparent differences in the levels of CHEs or of CHEmRNAs in major ovarian cell types should be attributed to alterations at the transcriptional and/or posttranscriptional levels, rather than to changes in the organization of CHE genes or in the extent of DNA methylation in them. However, it should be noted that selective changes in oocyte DNA methylation would be difficult to detect by DNA blot hybridization. Thus, our experiments cannot exclude this possibility.

The pronounced level of transcription of CHE genes in the oocytes is of a particular interest. Over 50 grains could be counted in single oocytes exposed for 15 days, indicating a high content of CHEmRNA within the oocyte. In a quantitative analysis of in situ hybridization using [³²S]pCO2 cDNA encoding the H₂ histone protein and two-cell sea urchin embryos, Cox *et al.* (235) counted a similar number of grains and estimated that the pCO2 mRNA represents 15% of the total mRNA content in the developing embryos. Considering that the mature human ovary contains ca. 10⁶ oocytes only, this high intensity of labelling is in complete agreement with the relatively low specific activities of AChE as determined by biochemical methods.

The selectivity and the intensity of BCHEmRNA production throughout oocyte development suggests that BCHE may be required for oocyte growth and maturation processes. This conclusion is in agreement with the observation that muscarinic ACHR responses (59), which may be mimicked in Xenopus oocytes by inositol 1,4,5-triphosphate (236), trigger the activation of amphibian and starfish oocytes by progesterone (237). Furthermore, both ACHE and muscarinic receptors appear to be seasonally regulated in Xenopus oocytes inversely to the reproductive cycle (57, 58, 238). Altogether, this may indicate an involvement of cholinergic responses in the differentiation process, leading to meiotic maturation of oocytes in a selected group of antral follicles (239), and suggests that these cholinergic responses could function through changes in the metabolism of phosphoinositides. The particularly high levels of ACHEmRNA at the preantral stage may reflect a transient enhancement in these processes at this stage.

According to an alternative theory, the induced transcription of CHE genes in the oocytes could indicate that ACHEmRNA is accumulated in the oocyte throughout its development for later use during and after fertilization processes. An example of a parallel phenomenon is the accumulation of excess histone mRNAs in developing sea-urchin oocytes (240).

This suggestion is in accordance with observations that the cholinergic antagonist quinuclidinylbenzoate (QNB) interferes with fertilization in the mouse (241), that the muscarinic cholinergic responses in oocytes of Xenopus disappear after fertilization (242), and that ACH induces polyspermy in sea-urchin oocytes (243). The intensive production of CHEs may account for the high catalytic activity of BCHE in embryonic tissues, where it appears to accompany periods of high proliferation rates (61). Further experiments using CHEcDNA probes should be performed in order to determine whether any of the theories suggested above are correct. In addition, the *in situ* hybridization approach may be employed to examine the transcriptional activity of other genes assumed to be involved in oocyte development and maturation.

2. Expression of cloned ACHE and BCHE in microinjected Xenopus oocytes

2.1 Biochemical characteristics

When microinjected into Xenopus laevis oocytes, synthetic mRNA transcribed from a cDNA-template-encoding human serum BCHE efficiently induced the biosynthesis of a protein displaying the catalytic activity, substrate specificity, and sensitivity to selective inhibitors characteristic of native serum BCHE. These results indicate that the ligand-binding specificities which characterize BCHE and distinguish it from the homologous ACHE reflect properties which are inherent to the primary amino acid sequence of the molecule. Furthermore, the level of BCHE activity induced by the synthetic message facilitated biochemical characterizations of the oocyte-produced enzyme not previously accessible using tissue-RNA-injected oocytes. Yet, the 10^2 - to 10^3 - fold enhancement of specific activity (induced activity/ng injected RNA) achieved in these studies over that previously achieved with total poly (A⁺) RNA (148) does not correlate directly with the 10^3 -fold enrichment of BCHEmRNA over that observed in both brain and muscle mRNA preparations by RNA dot-blot hybridization (35). This lack of correlation between the level of induced CHE activity and the level of specific CHE message implies that restrictions on the diffusion of injected RNA and/or competition for translational elements may be limiting factors in BCHE biogenesis in the oocytes.

Although 60-70% of the oocyte-produced BCHE fractionates into a detergent-extractable pool, we have as yet no information regarding the mode of association of the enzyme with the oocyte membranous fraction from which it is detached by detergent. The enzyme present in this fraction appears catalytically identical to that recovered from the low-salt-soluble fractions. Furthermore, the observed lack of aggregation in the absence of detergent suggests that these molecules do not possess the strongly hydrophobic regions characteristic of a true membrane-bound CHE. Nonetheless, subcellular oocyte fractionation does generate a reproducible distribution of synthetic CHE into membrane- and non-membrane-associated pools.

a factor here. However, immunocytochemical analysis of frozen oocyte sections demonstrated the extracellular accumulation of BCHE molecules in these co-injected oocytes (88) and supported the notion that the synthetic BCHE does, in fact, stably associate with the oocyte plasma membrane and/or its associated extracellular components.

One approach to account for the apparent discrepancy in the subcellular distribution of the enzyme is to assume that the open reading frame at the 5'-region of the BCHEcDNA employed (149) is indeed translated into protein and is maintained in a given population of nascent BCHE. This relatively hydrophobic sequence of amino acids could act as an internal signal sequence capable of anchoring the enzyme to the membrane in a manner similar to that described for the asialoglycoprotein receptor membrane anchor (244). This approach might imply that the mechanisms required to remove the BCHE leader sequence from the protein encoded by this cDNA are deficient. Thus, these results may offer an explanation for the observation of multiple ATG start signals in the longer BCHEcDNA clone isolated from a different brain library (157).

Microinjected alone, synthetic BCHEmRNA induced the formation of primarily dimeric BCHE. This first level of oligomeric assembly may, therefore, be spontaneous, or, it may require a catalytic mechanism that is endogenously available in the oocyte. The observation that tissue-extracted mRNAs induced higher levels of multi-subunit assembly--possibly including the incorporation of noncatalytic subunits--indicates that additional protein species are required to direct the biosynthesis of more complex molecular forms. Given the high degree of tissue-specific polymorphism which has been described for CHE molecular forms, it is not surprising to find that these additional factors appeared to be expressed in a tissue-specific manner. Nonetheless, the nature and number of these factors remain to be elucidated. It is interesting to note that in oocytes co-injected with synthetic BCHEmRNA and muscle poly (A)⁺ RNA, the pattern of BCHE forms resembles that of muscle ACHE. This could imply that *in vivo* ACHE and BCHE compete for the available noncatalytic subunits, whereas in the injected oocytes, the excess of BCHE binds these components without hindrance.

For ACHE, a variety of posttranslational modifications, including glycosylation, oligomeric assembly, association with noncatalytic subunits, and the attachment of phospholipids, have been extensively described and implicated in defining the enzyme's quaternary structure and/or subcellular localization in various tissues (8). However, the mechanisms by which these modifications are targeted and regulated have not been characterized. The discovery of a C-terminal variant of *Torpedo* AChE (245) which correlates specifically with the dimeric membrane-bound form of the enzyme led to the demonstration (85) that alternative mRNA-splicing plays a role in specifying the mature character and subcellular destination of AChE in that system. In microinjected oocytes, where the multiple BCHE forms presumably diverge from a pool of identical nascent translation products, the regulation of BCHE polymorphism must rely exclusively on post-translational factor(s). However, these experiments (and the failure of other researchers to find alternative BCHEcDNA clones) do not exclude the possibility that alternative mRNA splicing may contribute to BCHE heterogeneity *in vivo*.

2.2 Immunohistochemical approach

When injected into *Xenopus* oocytes, synthetic mRNA transcribed off of a cDNA-encoding human serum BCHE induced the production of a protein displaying the catalytic activity, substrate specificity, and sensitivity to selective inhibitors characteristic of native BCHE, and clearly distinct from ACHE. However, its mode of interaction with the oocyte surface resembled that of ACHE.

Taking the average membrane-associated activity as 4.8 nmole butyrylthiocholine hydrolyzed/hr/oocyte implies 8.0×10^{11} molecules substrate hydrolyzed/sec/oocyte. Assuming a turnover rate of 1×10^4 molecules/active site/sec (197) implies 8.0×10^7 catalytic sites/oocyte in the membrane-associated fraction, or 4×10^7 dimeric BCHE molecules/oocyte. This very small amount of protein would not be sufficient for protein labelling or immunoblot analysis. but

could be studied by catalytic activity measurements and by immunofluorescence microscopy.

The conspicuous intracellular accumulation of enzyme induced by tunicamycin indicates that posttranslational glycosylation plays an essential role in the transport of BCHE to the external surface. The unequal animal-vegetal pole distribution of induced BCHE in the oocytes indicates an active, polar asymmetry, which has been described previously for morphological characteristics, such as the yolk platelets or cytoskeletal elements (246-249) and also for a maternal mRNA localized in the vegetal hemisphere (250). Our present findings suggest that the polar build-up of the oocyte cytoskeletal elements takes an active part in directing newly synthesized proteins to their ultimate site of association at pre-defined extracellular positions in a manner similar to other polarized cell types (251) and that the animal pole of the *Xenopus* oocyte is preferentially designated for deposition of nascent surface-associated proteins. Another possibility is that the injected mRNA itself is transported preferentially to the animal pole prior to its translation. However, it should be noted that there is no apparent region in the noncoding sequences of BCHEcDNA (149) that could indicate such a special mechanism of intracellular transport.

In an oocyte injected with synthetic BCHEmRNA alone, or together with liver mRNA, approximately 8000 sq. microns or 0.1% of the oocyte surface was occupied by high-density immunoreactive ChE molecules. Therefore, the density of ChE molecules in a cluster or patch can be estimated to be about 5000 molecules per sq. micron. It is interesting to note that both the nicotinic acetylcholine receptor (252, 253) and AChE in neuromuscular junctions and along neuronal dendrites (104, 254, 255) were estimated to be aggregated at molecular densities within the same order of magnitude. This observation could indicate that the organization of membrane-bound molecules within the extracellular surface reflects a precisely regulated physiological property of the involved subcellular structures which is conserved through evolution.

Supplementation with brain or muscle mRNAs increased the number and intensity of patches and clusters indicating the induction, by tissue-specific factors, of enhanced BCHE aggregation at the external surface of the oocyte. Such elements were found to be required for the aggregation of both the nicotinic AChE receptor and acetylcholinesterase at the neuromuscular junction (200, 201, 256), and it would be intriguing to reveal whether the situation here is parallel.

Although both brain and muscle mRNAs induced both patches and clusters, the relative distribution of each type of formation varied between oocytes co-injected with mRNA from the two tissue types in a manner consistent with the organization of ChEs in the native tissues (193, 200, 201). Furthermore, the relative staining intensity of clusters and patches obtained with muscle mRNA qualitatively exceeded that obtained with brain mRNA. Together, these observations imply a qualitative and/or quantitative difference among mRNAs involved in organizing ChEs at the cell surface in different tissues. Furthermore, this information suggests that these mRNA pools are capable of modulating tissue-specific usage of a single BCHEmRNA species.

Several classes of membrane-anchoring elements have been considered in conjunction with AChE. Considerable evidence implicates covalently linked glycolipids--most prominently, phosphatidylinositol--as a principal mechanism for the anchorage of globular dimeric AChEs in plasma membranes (81, 257). In addition, noncatalytic subunits and proteoglycans have been implicated in the attachment of ChEs to membranes in the brain (258) and to the basal lamina at the neuromuscular junction (259, 260). We do not yet know which of these mechanisms, if any, is operative for BCHE in our system. It has recently been demonstrated that alternative mRNA processing is involved in specifying mRNAs encoding hydrophobic membrane-bound AChE in *Torpedo* (27, 85). However, it is still not known whether this model is applicable in BCHE biogenesis since the BCHEmRNA we injected represents a unique transcript and there are, so far, no indications for alternative splicing in this gene.

Taken together, these findings demonstrate that both muscle and brain, but not liver, express mRNAs encoding peptides which direct a tissue-specific

3. Unusual BCHEmRNA transcripts

Functional, 3.6-kb-long unusual Gb5 BCHEmRNA transcripts, including the mutation characteristic of the "atypical" BCHE allele, were detected by cDNA screening, RNA blot hybridization, and *Xenopus* oocyte translation in glioblastoma and neuroblastoma cells, but not in fetal or adult normal tissues. The use of alternative polyadenylation sites and production of long, 3'-extended mRNA transcripts in tumor cells has been previously noted for other genes, such as the DHFR gene coding for dihydrofolate reductase (261), and was generally assumed to reflect enhanced transcription rates and "readthrough" mechanisms for these genes in cells subject to rapid proliferation and growth (262). In contrast, the extended BCHEcDNA clones include modified polyadenylation signals that may account for the "readthrough" and alternative termination of the corresponding transcription. It should be noted that some of the modifications observed in the Gb5 clones appeared in a BCHEcDNA sequenced by others (157), perhaps indicating that gradual alterations occur in this gene by a genetic drift mechanism. Furthermore, the BCHE gene does not include any introns at the 3'-sites where sequence alterations were detected in the Gb5 clones (16, 167). Also, no evidence for any alternative splicing of BCHEmRNAs has been demonstrated as yet. Therefore, we conclude that the Gb5 transcripts are most probably the result of several point mutations within the non-translated 3'-region of a novel type of the "atypical" BCHE gene, creating the correct conditions for alternative termination. It is highly unlikely that the Gb5 clones resulted from artifactual recombination during libraries' construction, since (i) the same artifact would not have occurred twice in two unrelated libraries; (ii) the Gb5 fragment would not have mapped to the same chromosomal position as the BCHE gene if it had originated from another gene, and (iii) the Gb5 fragment was found to be included in all of the cosmid CHEDNA clones.

3.1. Chromosomal localization of the mutated Gb5-BCHE gene

When used as a probe for chromosomal mapping by *in situ* hybridization, the Gb5 3'-0.7-kb fragment bound exclusively to the unique 3q26-ter position and did not significantly label the additional 3q21 or 16q12-24 sites that are also labelled with full-length BCHEcDNA (120). The unique mapping site suggests that the functional Gb5 transcripts originate from the 3q26-ter site, termed CHEL2 (121). This confirms and refines previous genetic-linkage mapping which localized the CHE locus to 3q25-ter (112, 263) but does not exclude the possibility that other BCHEmRNA transcripts might be produced, perhaps in other cell types, from the 3q21 (CHEL1) or 16q12-24 (CHEL3) site (264).

3.2 Functional characteristics of extended BCHEmRNA

Interestingly, we found low numbers of 3'-extended Gb5 BCHEcDNA clones as compared with the "normally" terminated ones, consistent with the low intensities of their corresponding bands in the glioblastoma RNA blots. The fraction of translatable BCHEmRNA observed in the oocytes injected with slowly sedimenting mRNA was also relatively small. Altogether, this indicates that the 3'-extended BCHEmRNA transcripts are functional, but does not prove whether their protein product is as active as the normal ones. The finding of mutations characteristic of the "atypical" BCHE gene in these unusual transcripts may indicate that the low levels of the atypical BCHE results from unstable unusual BCHEmRNAs. This is also in agreement with the low levels of active BCHE produced by these unusual BCHEmRNA in microinjected oocytes. However, neither Gb5 mRNA stability nor the amino acid modifications can yet explain BCHE overexpression in nervous system tumors, a question which should be further examined. Possibilities are that other tumor-

specific mRNAs affect the efficiency and mode of expression of Gb5 BCHE mRNA transcripts, contributing to the altered levels of the enzyme in nervous system tumors (41, 265, 266) and to the production of BW- and IO-sensitive BCHE dimers in the patients' serum (141). Other options include control-element mutations, affecting the rate of transcription.

3.3 Induction of ACHE expression by a 9S glioblastoma mRNA fraction

Tissue-specific mRNAs do affect the expression and mode of assembly of nascent, clone-produced BCHE polypeptides in microinjected *Xenopus* oocytes (87, 88). The 9S-sized fraction of glioblastoma mRNA, which induces ACHE expression in the oocytes but is clearly too small to encode the catalytic subunit, could be an example for such processes. Further studies will be required to prove whether similar mechanisms control the overexpression of BCHE during embryogenesis (37, 193).

3.4 Heterozygosity of mutated BCHE cDNAs

Normal, nonextended BCHE cDNA clones in the tumor libraries were found to either include the wild-type nucleotide sequence or the "atypical" one, implying heterozygosity for the individuals having these tumors. In contrast, all six Gb5 clones that were sequenced displayed the "atypical" Asp→Gly mutation as well as the alteration Ser425 →Pro, an additional and yet-unreported polymorphism in the coding region. The effects of these mutations on the biochemical properties of BCHE produced from the Gb5 sequence were further examined by expressing Gb5 cDNA in *Xenopus* oocytes (See Experimental Observations). In particular, properties such as substrate specificity and sensitivity to selective inhibitors (e.g., succinylcholine and organophosphorous poisons) were examined.

An additional possibility which should be considered is that the Gb5 transcripts originate from amplified BCHE genes, and that the altered expression of BCHE in tumors reflects this amplification. A family of farmers with a defective CHE phenotype who have long been exposed to the organophosphorous insecticide parathion was recently found to have an inheritable *de novo* amplification of the CHE gene at position 3q26-ter (146). More recently, the BCHE gene was found to be frequently amplified in leukemias and platelet disorders (145), as well as in ovarian tumors (39). The finding of a modified transcription pattern from another defective allele of the same gene in brain tumors raises the question of whether this also reflects gene amplification events. Experiments aimed to reveal whether the "atypical" allele of the CHE gene is amplified in brain tumors should be performed, and the effects of exposure to organophosphorous poisons on the normal and "atypical" human CHE genes need to be examined in various expression systems or in transgenic mice.

4. In ovo expression of human BCHE muteins reveals structure-function relationships

The analysis of recombinantly produced BCHE muteins is a novel approach in analysis of naturally occurring allelic differences in ChEs and was employed to discover functionally important regions and amino acid residues responsible for "atypical" characteristics. When analyzing the Km's and inhibition patterns of these oocyte-produced BCHEs, the question arises as to their similarity to or dissimilarity from the naturally occurring human serum BChE. Microinjection of the usual SP6-produced RNA, which encodes the most common allele, into *Xenopus* oocytes, did, in fact, produce a protein that is nearly identical to the human-produced protein in its inhibitory and kinetic properties. Therefore, this rapid method of

expression is most efficient for studying structure-function relationships.

Full log dose-response inhibition curves with dibucaine and, more importantly, SuCh to calculate IC50 values provided a complete characterization of the differences caused by each of the mutations alone and by the two of them together. Such inhibition studies were performed for two reasons. First, phenotyping "atypical" individuals on the basis of dibucaine numbers (126) required the use of a different and specific assay condition. Second, it allowed us to overcome anomalies in which BCHE may be observed as "normal" from its apparent interaction with dibucaine, yet is seen to behave in a completely contrasting manner with SuCh and fails to bind the muscle relaxant. Such surprising patterns of inhibition have been observed following the phenotyping of a number of different human serum BCHE samples from individuals displaying clinical SuCh sensitivity (267). The double GP mutein appeared in our experiments to be far more resistant to both dibucaine and SuCh than the single GS mutein, whereas the proline425 substitution alone was ineffective with both ligands. It is possible that such differences have so far been overlooked by the standard clinical protocol, in which single-point reference numbers are employed as indications for dibucaine or SuCh resistance (see ref. 16 for an example).

4.1. Distinct effects of the single Gly70 and Pro425 mutations

The single mutation Asp70 to Gly70 has an effect on the binding of inhibitors; binding of inhibitors and some choline-esters to the anionic site is markedly decreased. This is clearly observed in the inhibition profiles. The strongest evidence is observed when 2-PAM, an anionic-site oxime which dephosphorylates the esteratic site via nucleophilic attack following OP intoxication, is employed. This compound was unable to reactivate Gly70-containing muteins but was effective with Asp70-containing CHEs. The conclusion drawn is that Asp70 plays an important and pivotal role in the anionic site, especially with ACh, BCh and positively charged inhibitors. This does not prove, however, that it is the only key charged residue in the anionic site. Some choline-esters bind efficiently even in the presence of the Gly70 mutation. Other charged residues might, therefore, have similar importance (268). Others have indeed suggested that six to nine is the effective number of charges on the Torpedo AChE active site (25).

In contrast to Asp70, Ser425 appears not to be directly involved in ligand-binding, since its replacement by proline in DP-BCHE displays completely normal substrate- and inhibitor-binding profiles. Rather, the proline mutation may cause conformational changes, such as displacement or distortion of a nearby peripheral or secondary electronegative anionic site, as observed by its small shift in K_m to BuTCh. A highly conserved and negatively charged Glu-Ile-Glu tripeptide appears upstream from Ser425 in human AChE (11, 111) and BCHE (11), as well as in bovine (169) and Torpedo AChE (156), and may be a candidate for such an anionic site component. The apparent crucial role of Asp70 within BCHE in binding anionic-site-directed ligands was further probed by reactivation experiments with the oxime 2-PAM, previously shown to reverse organophosphorous intoxication of the esteratic-site serine in CHEs (208) by an action specifically mediated by anionic-site binding (210). Replacement of aspartate70 by glycine completely prevented this reactivation, regardless of the additional substitution of serine425 to proline. Aspartate70 is, therefore, an essential requirement to the interaction of 2-PAM with the anionic site in human BCHE.

4.2 Synergistic contributions to biochemical changes in doubly mutated CHE variants

The doubly mutated-oocyte-produced BCHE mutein GP displayed different inhibition patterns than either single mutein GS or DP did. Thus, alteration of the protein structure is strongly implicated to be a result of the specific changes in the Asp70 region and the Ser425 domain. Synergistic effects of the Gly70 and Pro425 mutations further suggest that Asp70 and Ser425 may be sterically proximal to each other in mature human BCHE, implying interactions between cysteine loops A and C in CHEs in general (72).

The general nature of these considerations is clearly indicated by the large number of identically aligned amino acid residues in the peptide regions harboring Asp70 and Ser425 throughout the entire family of CHEs. In particular, the highly conserved glutamate-isoleucine-glutamate (EIE) domain upstream from serine425 should be noted. This electronegative domain may be of crucial importance in the presumed anionic-site binding of certain non-thio containing choline substrates by Gly70-containing BCHE muteins (269). The importance of such domains in CHEs which serve to bind positively charged choline substrates should be further examined either by pursuing additional and yet-unknown variants in the CHE genes from humans or other species, or by employing site-directed mutagenesis in conjunction with the efficient *Xenopus* oocyte microinjection assay to examine substitutions in pre-defined peptide domains.

5. Isolation of alternative BCHEcDNA sequences from cDNA libraries

Several recently accumulated publications describe natural variants of the human BCHE-coding sequence. These include the "silent" variant (270) and the "atypical" one (271), in addition to the H, K, and J variants (17). Interestingly, most of these *in vivo* variants include one or two point mutations, which tend to cluster in the N-terminal and C-terminal domains of the BCHE protein, from both sides of the active-site serine. The "unusual" BCHE-coding sequence which has been isolated in the course of this work also fits into this group of naturally occurring BCHE variants, which prompted us to rescreen for yet more variants within our previously isolated BCHEcDNA clones. Careful sequencing of Lambda Gt10 clones screened with [³²P]BCHEcDNA then revealed the "alternative" BCHE-coding sequence, in which the previously characterized Asp-->Gly substitution appeared together with two more changes, which were both previously unknown. These were the His114 and the Tyr mutations, which again belong to the N'-terminal and C'-terminal clusters of amino acid substitutions in the BCHE-coding sequence. Further experiments will be required to reveal the biochemical effect(s) asserted by these new mutations on the BCHE protein. When added to the long list of other mutations, these apparently permissive alterations in the BCHE coding sequence may shed new light on the peptide domains which are necessary prerequisites to the catalytic activity of this evolutionarily conserved enzyme, versus amino acid residues which may be substituted by others.

5.1 Does chromosome 16 contain a CHE gene?

Cosmid recombination offers a valuable selectivity not obtainable by conventional hybridization screening. The efficiency of homologous recombination in this method was estimated to be 1:10³ (158), which implies a frequency of 1:10⁶ for CHEDNA sequences in the human genome (a single-copy gene). In addition, the cosmid recombination approach ensures high specificity of the screening procedure. Sequences bearing a 10% divergent target homology were shown to recombine with a frequency about two orders of magnitude lower than the rate for

the perfectly homologous target, which would be well below our limit of detection (158). This implies that if a moderately modified CHE gene resides on chromosome 16, it might not have been selected by the cosmid recombination approach, although it could potentially yield a positive signal in the in situ hybridization experiments. However, the hybrid cell lines also displayed a single chromosomal location, reinforcing the question whether BCHEcDNA-hybridizing sequences truly reside on chromosome 16.

DNA blot hybridizations suggested that the genomic DNAs which were used to construct both the cosmid library and the hybrid cell lines carried a single copy of the CHE gene containing the 0.7 kb Gb5 3'-extension fragment that was recently mapped to the 3q26-ter position. This, in turn, indicated that the same BCHE gene may be expressed in different tissues, including liver, brain, and various tumor and embryonic cells. In contrast, gene-mapping by in situ hybridization demonstrated significant labelling of two sites on chromosome 3 and an additional one on chromosome no. 16, even under highly stringent washing conditions. While the two apparent sites for BCHEcDNA hybridization on the long arm of chromosome no. 3 might be explained by variations in chromosome condensation between preparations, the chromosome 16 hybridization was certainly due to a separate binding reaction. Dual labelling of human chromosomes by cDNA probes encoding a single enzyme is not exceptional. For example, the cDNA encoding the trifunctional human enzyme methylenetetrahydrofolate dehydrogenase - methenyltetrahydrofolate cyclohydrolase - formyltetrahydrofolate synthetase was recently found to label both chromosome X and chromosome 14 (272). However, in that particular case, two distinct DNA fragments were associated with the two labelled sites and appeared on DNA blots as well, whereas our findings present a contradistinction between the hybridization to blots and chromosomes.

There are two possible explanations to account for the difference between our observations in the in situ hybridization experiments and those of the other methods: (i) The in situ hybridization approach might be principally less selective than blot hybridization and cosmid recombination, permitting binding of probe DNA to chromosomal sites harboring similar, but not identical sequences. It is quite probable that such sequences do exist, in light of the finding of several CHE-related genes in the mammalian genome. However, although these encode various homologous proteins with unique physiological substrates other than ACH, their sequence homologies at the DNA level are not particularly high. Moreover, the in situ hybridization experiments were performed using probe concentration, ionic strength, and hybridization time conditions that fully favor specific hybridization but do not allow nonspecific interactions (273). (ii) Alternatively, one may postulate that some of the individuals examined do carry a BCHEcDNA-like sequence on their chromosome no. 16, while the DNA used to construct the cosmid library and the hybrid cell lines was prepared from individuals who do not carry this chromosome 16 gene. Recent findings demonstrate genomic differences between specific DNA sequences in particular individuals. For example, the number of ubiquitin-coding sequences tends to change by unequal crossing over, generating variation among individuals in ubiquitin-coding unit number at the human u_{bc} polyubiquitin locus (274). Furthermore, inheritably high levels of serum BCHE activities were detected in Japanese families (275). It is, therefore, possible that the BCHE gene was subjected to recent evolutionary pressures, expressed in genetic differences within the human population.

Whittaker (15) has summarized the frequency of various BCHE genes and their alleles in various populations. This analysis again revealed considerable variations among human populations in the phenotypic expression of serum BCHE. Thus, it appears that populations display no CHE2 phenotype, whereas others demonstrate high levels of expression of this C5 variant of serum BCHE. In view of the recent genetic-linkage analysis, this evidence does not necessarily suggest

that the chromosome 16 BCHEcDNA hybridizing locus is the same as that regulating the C5 phenotype. In different studies, the C5 has been weakly linked to chromosome 16 (118, 122) or to the alpha crystalline gene cluster on chromosome no. 2 (123), the latter linkage having a convincing Lod score of 4.21. Furthermore, a family of 15 members was found to have no crossovers between the alpha crystalline probe and the C5 phenotype, suggesting that the two genes are in close proximity (123). This is strong evidence that the C5 locus, at least in the examined individuals, lies on chromosome 2. Further experiments will be required to determine the yet unclear relationships between the chromosome 16 genetic linkage to the C5 phenotype and the in situ hybridization labelling on this chromosome.

5.2 ACHE and BCHE are encoded by distinct genes

The findings presented in this report imply that distinct genes encode for BCHE and AChE in man. Although they encode for highly similar proteins, (>50%;11) these two genes do not display sequence homology at the level of DNA, suggesting that they diverged very early in evolution. While the CHE gene remained rather primordial in its A,T-rich base composition, resembling many tissue-specific genes, the ACHE gene continued to evolve and gradually acquired the G,C-rich composition characteristic of certain other gene families (172). It is not yet clear whether the BCHE and the ACHE genes are co-localized on the same chromosomal position; ACHE-gene-mapping experiments by in situ hybridization, pulse-field gel electrophoresis, and RFLP analyses will be required to resolve this issue and to determine whether the BCHE and ACHE genes co-amplify in various biosystems because of their geographically close positions or due to similar environmental signals affecting the genes in the same manner. It should be noted in this respect that the promoter and other regulatory sequences controlling the expression of the BCHE and the ACHE genes have not been isolated, and that sequence homologies between these regions may yet be revealed.

6. Putative involvement of BCHE in development

BCHE overexpression in brain tumors (41), its altered properties in carcinoma patients (141), its early production during embryogenesis and cell proliferation (37,38,276), and the amplification of the BCHE gene in hemopoietic disorders (145) and in ovarian tumors (39) are all suggestive of a developmental role for this protein. In view of this, it would be interesting to examine the possibility that mutation(s) and/or amplification of this gene are causally involved with the development of nervous system tumors and with their rapid rate of progression. Furthermore, it would be interesting to determine if there is a correlation between various developmental abnormalities or infertility and the appearance of "atypical" BCHEs. In addition, there might exist a physiologically important substrate other than ACh which BCHE can bind to and degrade. All of these topics may be approached with the advent of the new probes and expression vectors; rapid development in this field, accordingly, may be expected.

6.1. Evolutionary implications

Clearly, the CHEs are a distinct gene family, as shown by the high levels of identically aligned residues in the DNA and protein sequences of various members. To demonstrate the consensus amino acid residues characterizing the CHE gene family, several CHE-like proteins from different species were subjected to profile analysis. These included human BCHE, Torpedo AChE, Drosophila Esterase-6, bovine

thyroglobulin, rat lysophospholipase, and rabbit microsomal esterase. Table X presents these sequences and the alignment results, clearly indicating that these proteins all belong to the same superfamily and should have originated from a single gene.

The predicted primordial gene for the CHE superfamily predates thyroid development, since the presence of CHE sequences at the 3'-terminus of the thyroglobulin protein is a prerequisite for thyroxine production (277). This implies that the primordial CHE gene would have had to duplicate, and fuse to, thyroglobulin sequences prior to the appearance of thyroid glands. Thyroid organs developed in fish about 400-450 million years ago. Therefore, the fusion of CHE-coding sequences with the thyroglobulin gene should have occurred during the 350-million-year-long Precambrian period, before the emergence of fish, yet after the separation of vertebrates from invertebrates (277).

Furthermore, CHE-like proteins existed long before the Precambrian period. The wide evolutionary range of species distribution for CHEs implies that their original role was not in neuronal transmission, since CHE expression in bacteria, plants, and unicellular organisms clearly preceded nervous system evolution. This reopens the question, "What could have been the initial role of CHE-like proteins?" A putative function in metabolism has been suggested for CHEs in unicellular organisms. For one strain of *Pseudomonas*, at least, a feedback response of enhanced CHE production was demonstrated by growth in the presence of acetylcholine (278). This indicates a metabolic role for the ancestral enzyme, which could explain CHE existence in single-cell organisms. With the evolution of other, more efficient metabolic proteins (such as serine proteases), novel environments, and more complex metabolic pathways, the use of CHEs for such roles might have become relatively inefficient and, possibly, obsolete. At the same time, it is likely that CHEs acquired a variety of novel functions in newly developing physiological systems. Regulation of movement has also been repeatedly proposed as a role for CHEs. This hypothesis is based on the demonstration of CHEs in cilia-containing cells, where they principally could have been initially utilized to control motility. Subsequently, these cells could eventually have evolved into nervous systems. A combination of these two hypotheses provides reasonable explanations for the various roles of CHE-related proteins as involved with pheromone metabolism in insects (Esterase 6; 279), as a lysophospholipase, or even as the thyroxine precursor. In parallel with the organisms becoming more complex, so did the acquired CHE functions.

It is interesting to note that the various CHE-related proteins, such as lysophospholipase and microsomal esterase, are clearly homologous to human CHEs, yet they are unable to degrade acetyl- or butyrylcholine. Instead, they have their specific substrates that are sterically similar to choline esters. Because this is the case, it is possible to suggest that BCHE, which has a wide variety of substrates, might have a physiological substrate other than choline esters. One possibility, which was raised in 1952 by Whittaker and Harris (reviewed in 280) is that BCHE acts as a scavenger, and is responsible for the degradation of naturally occurring CHE inhibitors, such as solanine, and for their removal from the circulation, as well as for protecting other members of this protein family. An alternative possibility is that the "native" physiological substrate of BCHE has not yet been found.

As shown above, human ACHE and BCHE are encoded by separate and distinct genes. It also appears that most individuals should contain one copy of each, as suggested by linkage analysis and DNA isolation, though some individuals might contain additional copy(ies) of the BCHE gene that could also account for the overexpression of BCHE, as observed in a Japanese family (275). Interestingly, lysophospholipase, which was isolated in rat, should have a human homologue capable of degrading lysolecithin. A profile search (see Methods) allowed for the

identification of rabbit microsomal esterase as a related protein that once again, should have a human homologue. Human ACHEDNA and BCHEDNA do not cross-hybridize; however, the present finding of hybridization on chromosome 16 might reflect the existence of a BCHE homologue encoded by chromosome 16 sequences.

DNA mutagenesis (281, 282) and amplification (283, 284) have been observed in insect esterase genes and have been correlated to insecticide exposure. The recently discovered de novo amplification of an aberrant CHE gene in individuals subjected to chronic exposure to the agricultural OP insecticide parathion (146) suggests that such hereditary alterations in human DNA may reflect the adjustment of our genetic repertoire to a changing environment. In view of the currently increasing levels of ecological and pharmacological exposure to CHE inhibitors, it should be interesting to search for yet-unknown human CHE variants which might emerge in response to such exposure and to use the microinjection approach to study these mutants.

6.2 Does the mutability in the BCHE gene reflect a selection advantage?

As mentioned above, BCHE is generally assumed to act as a scavenger, removing compounds which are toxic to acetylcholine-binding proteins from the circulation (15). Biochemical, genetic, and molecular biology approaches have gradually revealed that the BCHE gene appears in several differently mutagenized allelic variants (15,17,155). Therefore, naturally occurring toxic compounds that normally interact with CHEs, and whose binding to mutated BCHEs is reduced, have been sought. Assuming that BCHE activity is essential for certain vital processes, either directly or as a replacement for inhibited ACHE, such mutations may provide the individuals carrying them with a selection advantage. This would explain both the abundance of CHE alleles and their differing incidence among populations, which may be susceptible to distinct toxic compounds (15).

The steroidal alkaloid solanidine apparently interacts selectively with mammalian BCHE (64) but not ACHE (285) and inhibits their catalytic activities. This aglycone, as well as its parent glycoalkaloid compound solanine, are naturally present in all members of the solanum plant family, including Solanum tuberosum (potatoes) and are likely to be present in extremely high and toxic concentrations in blighted potatoes.

Table X. Consensus sequence alignment in various members of the CHE gene superfamily

[illegible]

In addition to these recorded acute toxicities, solanidine was also found to be teratogenic in mammals (286). Therefore, the reduced ability of "atypical" BCHE to bind the solanoid poisons can clearly have a survival advantage, particularly in heterozygotes to this mutation where scavenging and catalytic activities may be retained under intoxication. Findings herein indicate that the substitution of aspartate70 into glycine is by itself sufficient to provide individuals with such advantage by modification of the BCHE anionic site.

The molecular genetics approach has demonstrated that the N-terminal Gly70 mutation is frequently linked with additional substitutions, altering single-amino-acid residues in the C-terminal part of the BCHE molecule (167). The Ser425-to-pro mutation which co-appeared with Gly70 in glioblastoma and neuroblastoma tumors (155) demonstrates yet another case of such linkage. Figure 38 summarizes the currently available data on BCHE gene mutability in man. According to our line of thought, the combination of two amino acid substitutions should provide the enzyme with yet higher resistance, at least towards some anionic site ligands, to create the selection advantage leading to the observed genetic linkage. The apparent synergistic resistance of the double GP mutant to inhibition by SuCh and dibucaine demonstrates that this may be true. Further experiments will be required to determine the abundance of the double GP substitution and whether other C'-terminal modifications linked to the Gly70 substitution are similarly effective with anionic-site ligands. Also, other naturally occurring inhibitors (see, for example, ref. 287), which may explain the biological background to these mutations should be pursued.

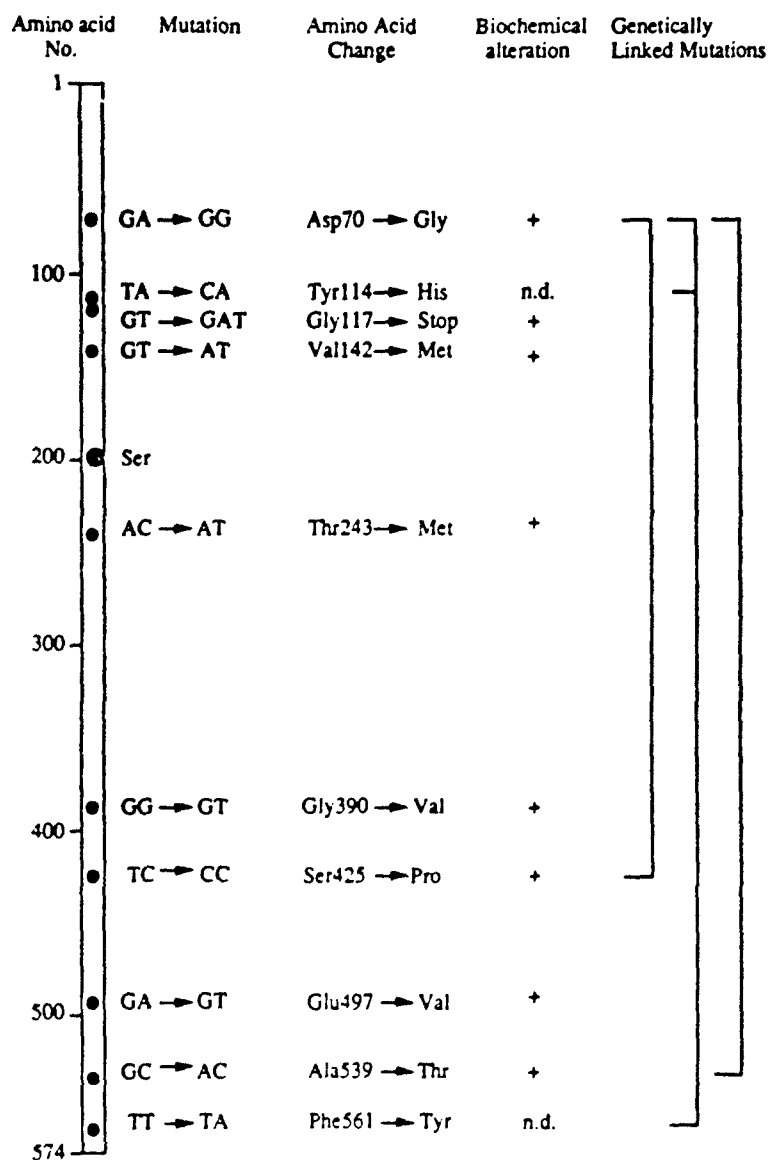
To resume the above-predicted evolutionary selection pressure, BCHE should have a developmentally essential role either in germ cells or early in embryogenesis. The intensive expression of the BCHE gene in developing human tissues (37) and oocytes (35) and the transiently elevated levels of BCHE in embryonic human brain (184) are all indications that BCHE might have a developmental role in man. The recent observation that a synthetic phosphorothioate "anti-sense" oligonucleotide spanning the initiator AUG sequence in human BCHEmRNA inhibits bone marrow cell development in culture (288) directly proves an essential role for BCHE in mammalian hemopoiesis, whereas the apparently inheritable germline amplification of the BCHE gene in two generations from a family of farmers exposed to the agricultural anticholinesterase insecticide parathion (146) points towards a function in germ cells. Altogether, it therefore appears that BCHE activity is indeed developmentally essential, which creates an evolutionary pressure for genetic modifications in the BCHE gene under conditions in which its protein product is blocked by inhibition.

7. In vivo amplification of the human CHE genes

7.1. Inheritable amplification of defective BCHE genes in individuals exposed to organophosphorous insecticides

The first case of a CHE gene amplification was found in a family of farmers with a defective BCHE phenotype who were under repetitive exposure to the agricultural OP insecticide parathion (p-nitro phenyl diethyl thionophosphate). The H. family parents were employed in agricultural work and were exposed to parathion since 1959. Several mild incidents of organophosphorous intoxication in the mother were symptomatically treated. Routine BCHE tests, run in later years by the health authorities, indicated particularly low BCHE activities in the serum of one of the sons.

Figure 38. Currently available data on mutability in the human BCHE gene



The 10 different point mutations which were recently discovered in the human BCHE gene (ref. 17,155, and unpublished observations) are presented schematically on a scale of the primary amino acid sequence in the BCHE protein. Active-site serine 200 is noted by a circle. The nucleotide substitutions and the amino acid changes conferred in each mutation are displayed. Evidence for alterations in the biochemical properties of the resultant variant proteins is noted by +. n.d.: not determined. Vertical lines note cases in which more than one mutation appeared in a single gene.

When, in spite of instructions, this son took part in parathion spraying, he displayed acute characteristic apnea (289) and was hospitalized and artificially respiration. A daughter of this family suffered from infertility problems and postanesthetic apnea following succinylcholine administration during the course of a laparoscopy. Serum BCHE measurements demonstrated low butyrylthiocholine hydrolytic activities, high sensitivity to OP BCHE inhibitors, and resistance to dibucaine for the serum butyrylcholine hydrolytic activities from both daughter and son, as expected from "silent" BCHE (15). Relatively low activities were also found in the serum of one of the son's children. Upon DNA blot hybridization, peripheral blood DNA from this son and his child was found to contain about 100 copies (quantified by dot blots of 6 dilutions from each DNA) of a genomic DNA fragment hybridizing with BCHEcDNA. All of the other members of the family (the parents and the daughter) displayed completely normal hybridization patterns and intensities.

Detailed karyotype analysis of lymphocyte chromosomes from these family members revealed apparently normal G- and Q- banding patterns. There were no abnormal extrachromosomal structures, which are characteristic of unstable amplified genetic material, and none of the homogeneously stained regions commonly observed in chromosomes containing large amplification units (262). In situ hybridization of lymphocyte chromosomes from these individuals with ^(35S)-labelled BCHEcDNA revealed intensive labelling in the 3q26-ter region in chromosomes from the individual having the amplified CHE gene, whereas the labelling on chromosomes from other family members was indistinguishable from that of controls (146). Thus, the amplified CHE gene appears to have been stably integrated at, or close to, its original position on chromosome no. 3 (120).

The absence of BCHE gene amplification in the parents and its presence in one of their sons and a grandson, demonstrated a de novo appearance of an inheritable gene amplification, or the predisposition for such an event (290). There is no recorded precedence for such a phenomenon in humans, although gene amplification was found, as detailed above, in many genes of cultured cells and primary tumors (214,261).

7.2 Co-amplification of CHE genes in leukemias and platelet disorders

The finding of a de novo inheritable amplification of the BCHE gene in individuals exposed to commonly used CHE inhibitors raised several questions. First, "Was this an incidental unrepresentative exception, or did it reflect a significant phenomenon and occurred elsewhere too?" Second, "If BCHE genes do amplify nonrandomly, would ACHE genes amplify as well, as expected from their functional relatedness?" Third, "Could such amplification events take place in somatic or tumor cells in a non-inheritable manner and, if so, would this have specific clinical implications?"

To answer these questions, we searched for evidence on disease in which non-random aberrations occur in the 3q26-ter site, where we mapped the BCHE gene, since gene amplifications and chromosome breakage appear to be associated phenomena in multiple tumor types. We found that the 3q26-ter region is frequently subjected to deletions, inversions, and translocations in acute myelodysplastic leukemias (217). Moreover, all patients having such breakages featured enhanced megakaryocytopoiesis, altered platelet counts, and rapid progress of the disease (218). That was particularly intriguing, since the administration of acetylcholine analogs and cholinesterase inhibitors has been shown to induce promegakaryocytopoiesis and to enhance platelet formation in the mouse, both in vivo (220) and in bone marrow cultures (36).

Based on this indicative correlation, we tested the cDNA probes for the BCHE and the ACHE genes in blot hybridization with peripheral blood DNA from various

leukemic patients. One-hundred- to two hundred-old intensified hybridization signals and modified restriction patterns were observed with both cDNA probes in four of the 16 leukemia DNA preparations examined (145). These reflected the amplification of the corresponding ACHE and BCHE genes and alterations in their structure. Parallel analysis of 30 control samples revealed nonpolymorphic, much weaker hybridization signals for each of the probes. In view of the above-discussed reports on the effect of acetylcholine analogs and CHE inhibitors in the induction of megakaryocytopoiesis and the production of platelets in the mouse, we further searched for such phenomena in nonleukemic patients with platelet-production disorders. Amplifications of both ACHE and BCHE genes have been found in three of the five patients so far examined. Pronounced co-amplification of these two related but distinct genes in correlation with pathological production of blood cells, therefore, suggested a functional role for members of this gene family in megakaryocytopoiesis, and raised the question of whether the co-amplification of these genes could be casually involved in the etiology of hemocytopoietic disorders.

7.3 CHE genes co-amplify with oncogenes in ovarian carcinomas

Several mechanisms have been proposed to account for gene amplification in mammalian genomes. One of these is the insertion and subsequent amplification of a retroviral-DNA-containing host sequence (291), perhaps in the form of a processed cDNA. However, the amplified CHE genes in ovarian tumors appear to contain introns and are not processed. A second mechanism which has been proposed is the "incidental" amplification of a gene residing close to an amplifiable protooncogene. The core amplification unit, including the N-MYC protooncogene, is, for example, 3000 kb in size (292), and the HSTF1-transforming factor which maps to chromosome band 11q13 co-amplifies with the INT2 gene in multiple cancerous cells (293). A third possibility is that our amplified sequences contain internal origins of replication, enabling their independent amplification under certain circumstances; this is similar to the mechanism proposed for the developmentally regulated amplification of the chorion gene clusters in *Drosophila* (294). It is unclear which of the two latter mechanisms, if any, might be applicable to the amplification of the BCHE and ACHE genes in blood cell disorders and ovarian carcinomas. Furthermore, it is unclear whether these genes are subject to linked but independent amplification events, or whether they co-amplify as a single unit. The observation that restriction fragments of similar lengths from both genes hybridize with both of the non-cross-hybridizing cDNA probes could reflect shared genomic sequences and argues for co-amplification. Nonetheless, and unlike the HSTF1/INT2 example, there does not appear to be a direct 1:1 correlation between the number of copies of the two genes in various tumors. Molecular cloning and further chromosomal mapping of the amplified ACHEDNA and BCHEDNA in ovarian tumors and their flanking sequences will be required to clarify this issue.

All of the tumors which displayed extensive amplification in the C-RAF1, V-SIS, and C-FES oncogenes also had high levels of BCHE/ACHE genes and high ratios between copy numbers of BCHE:ACHE sequences. Other tumor samples had high extents of BCHE/ACHE genes but low levels of oncogenes, perhaps indicating that the CHE genes are amplified earlier in tumorigenesis than the oncogenes and that BCHE gene amplification precedes that of ACHE. Interestingly, the BCHE gene is expressed earlier than ACHE also in embryogenesis (60,61). In addition, future measurements of ACHE gene amplification will have to be carried out with regional probes, including the attenuator domain (111), to avoid underestimations of the amplification extent.

RNA blot hybridization, immunochemical and cytochemical staining, and *Xenopus* oocyte microinjection with tumor RNA all demonstrated that the amplified CHE genes

are expressed within tumor tissues to yield their catalytically active enzyme products. Regarding the amplified ACHE genes, we could show transcription products, but remained uncertain as to whether these were translationally active. It is not yet known which promoter directs the expression of the ACHE and BCHE genes in these tumors or why they are expressed only in a minor fraction of the cells. A large body of information suggests that ACHE and/or BCHE activities are transiently expressed during cell growth and/or proliferation in multiple systems (290). If, indeed, this reflects a growth-related role for these enzymes, amplified and expressed ACHE and BCHE genes could confer a selective advantage in localized areas of rapidly growing cells within the tumor tissue. This notion is supported by our finding that the ACHE and BCHE genes are co-amplified in leukemias (145). Furthermore, enhanced and altered modes of expression of CHEs were biochemically demonstrated in various brain tumors (40,41) as well as in the serum of patients suffering from different types of carcinomas (141). These could potentially reflect similar co-amplification events, suggesting that the ACHE and BCHE genes amplification might be correlated with the etiology of the disease, in analogy with the neu oncogene amplification, which is related with relapse and survival in breast cancer (295).

7.4 Ecological implications

Variable modes of expression and gene amplification were observed for CHEs in multiple types of developing and tumor human tissues. Table XI presents examples for such alterations. Altogether, these studies indicate an involvement for this family of proteins in cell division and/or growth mechanisms. In view of parallel studies on the amplification of genes producing target proteins to cytotoxic inhibitors, this raises the question of whether OP poisons could induce selection pressure for CHE gene amplifications.

A considerable body of information, accumulated over the years, demonstrates cytotoxic and mutagenic effects for OP poisons. Pesticide-induced DNA damage and repair processes were shown by several research groups in cultured human cells (296, 297, 298). Sister chromatid exchange and cell-cycle delay were further demonstrated in cultured mammalian cells treated with eight different OP/insecticides (299), including the common agricultural insecticide malathion (300). Animal studies have, in parallel, shown similar effects *in vivo*, in the mouse (301), guinea pig (302), and rat (303). Damaging effects of OP poisons were also found in humans: workers producing OP insecticides displayed transient chromosome aberrations (304), which were also found in patients under acute organic phosphate insecticide intoxication (305) and in lymphocytes from agricultural workers during extensive occupational exposure to pesticides (306). However, the damage may not be limited to these high-risk groups of humans, since pesticide residues were found in basic food products (307), which implies continuous subacute exposure to the entire human population.

Chromosome breakage occurring in germline cells may induce hereditary changes in the affected genes. OP insecticides have been shown to inhibit testicular DNA synthesis (308) and to induce sperm abnormalities in mice (309), findings which may explain the hereditary CHE gene amplification in the OP-exposed H family (146). Animal studies have demonstrated that methyl parathion administration suppressed growth and induced ossification in both mice and rats and high mortality and cleft palate in the mouse (reviewed by Soreq and Zakut, 290). In humans, malformations of the extremities and fetal death were correlated with exposure to methyl parathion in 18 cases (310). In addition, a neonatal lethal syndrome of multiple malformations was reported in women exposed to unspecified insecticides during early pregnancy (311). All of these findings most probably reflect the developmental function(s) of CHEs being impaired by these poisons, and may be related with BCHE and ACHE gene amplifications and chromosome breakage in these individuals.

The molecular mechanism leading to CHE gene amplifications is not yet clear. It is possible that the BCHE and AChE genes contain internal origins of replication, making them appropriate subjects for gene amplification, and that this explains the overexpression of these genes also in brain tumors (41) and the modified CHE properties that we previously found in carcinomas (141). Further studies should be performed to examine whether such alterations are correlated with the occurrence of the "silent" or the "atypical" BCHE phenotypes and with the appearance of BCHE/AChE gene co-amplification, to find out the scope and abundance of this phenomenon in various tumors and to examine whether the exposure to additional ecological poisons induces similar amplifications in other human genes.

Assuming a relationship between the exposure to OP insecticides and CHE gene amplifications implies a fundamental importance for cholinergic responses in cell division and growth, as well as in reproduction and embryogenesis. This assumption is strongly supported by recent findings demonstrating the stimulation of DNA synthesis in brain-derived cells exposed to acetylcholine analogs (147). In this system, the response occurred in fetal and neonate astrocytes and depended on the expression of specific muscarinic receptor subtypes and the hydrolysis of phosphatidyl inositol. Similar induction of DNA synthesis may occur in other cell types under exposure to OP insecticides. These block acetylcholine hydrolysis by covalently interacting with CHEs and thus induce increased concentrations of the G-protein-linked neurotransmitter. It has previously been shown that acetylcholine analogs induce phosphatidyl inositol hydrolysis in *Xenopus* oocytes (reviewed in refs. 223 and 312) and promegakaryocytopoiesis in mouse bone marrow cultures (reviewed in ref. 36). The findings presented in this report suggest that cholinergic intercellular communication, in addition to its important function in communicating between cholinergic neurons and in neuromuscular junctions, may play pivotal role(s) in developing cells.

7.5 Are the mutability and amplification of CHE genes related?

Having been led from abnormal CHE gene expression to CHE gene amplification and finally to a previously undemonstrated role for BCHE in normal cellular processes, we may now suggest a correlation between these phenomena and the long-noted allelic polymorphism of the BCHE gene in man. Ten different point mutations have been discovered in the major coding exon of the BCHE gene within the past 2 years (16,17,155,167,290). These imply amino acid changes at various positions within the catalytic subunit, at least eight of which confer measurable biochemical alterations in the mature BCHE protein. As many as six of these mutations occurred in G residues, which constitute only 15% of the nucleotide composition in this gene. Figure 37 summarizes the currently available data on BCHE gene polymorphism. Several of these mutations appear to be genetically linked to each other (155,167), and have been shown to confer resistance to various CHE inhibitors (268,269). However, at least one of these mutants appears to give rise to an unstable mRNA, resulting in deficient production of active enzyme (155). If indeed CHEs are metabolically or developmentally essential, the combination of variant BCHE genotypes and sustained exposure to CHE inhibitors could create conditions favoring CHE gene amplification. Considering however, factors such as the developmental regulation of these genes, the abundance of allelic polymorphs, haploid vs. diploid genomes, and the infinite possibilities for exposure to CHE inhibitors, dissecting a singular pathway leading to CHE gene amplification is complex indeed. In fact, there are likely multiple independent or interrelated factors capable of generating a stable amplification event, resulting in beneficial or deleterious effects, depending on the circumstances.

Table XI. Expression of altered CHEs in various human tissues

Source	Alteration in CHE Protein	Detected by	Ref. No.
1. Glioblastoma primary tumors	Overexpression of CHE tetramers	Sucrose-gradient centrifugation & biochemical measurements	41
2. Meningioma primary tumors	Overexpression of CHE monomers	Sucrose gradient centrifugation & biochemical measurements	41
3. Ovarian carcinomas	Overexpression of CHE; focal intense-labeling sites	Cytochemical staining;	37
		immunocyto-chemical staining	39
		cytochemical labelling	39
		<u>In situ</u> hybridization	
4. Serum from patients with various carcinomas	Enzyme dimers sensitive to both BW284C51 & iso-OMPA, selective inhibitors of ACHE and BCHE, respectively	Sucrose gradient centrifugation & biochemical measurements	141
5. Individuals with postanesthetic apnea	Low catalytic activity; insensitivity to dibucaine; CHE gene amplification	Biochemical measurements in serum; DNA blot hybridization	146
6. cDNA libraries from glioblastoma & neuroblastoma	Asp70 -Gly Ser425 - Pro	cDNA sequencing	155
7. Leukemias and platelet disorders	Potential over-production from amplified genes	DNA blot hybridization	145

Available examples of gene amplification events in mammals are generally classified into those which are induced under exposure to toxic compounds (313, 314, 315) and those providing ample gene products during cellular differentiation stages, when such products improve cell growth or division rate (294,316). In contrast with findings on insects and amphibia, there are no examples in mammals for the third type of gene amplification, which is developmentally induced during normal embryogenesis or germ cell formation. This could be due to technical difficulties, however, and the possibility that this amplification occurs in mammals should, therefore, not be excluded. Interestingly, CHEs could fit any one of these classifications because of their being targets to toxic inhibitors and since they are transiently expressed during embryogenesis and germ cell development (290). The already available examples of CHE gene amplifications in normal and tumor cells support both notions.

There is no oncogene in the vicinity of the CHE gene (290), suggesting that it amplified on its own merit. Also, both the ACHE and the BCHE genes appear to include the inverted repeats and hairpin structures assumed to be prerequisite to the initiation of gene amplification (317). In addition, their expression in embryogenesis is concomitant with, or immediately follows, phases of DNA synthesis (38,60,61), a period when over-replication of DNA has been noted (318). This is particularly intriguing in view of the drastically different base composition in these two genes, which probably reflects a major evolutionary distance between them, and predicts for the ACHE and the BCHE genes distinct localization on different chromosomal bands and various replication and transcription times during the cell cycle (172). This could, in essence, suggest that either one of these genes, or the two of them together, could be prone to amplify in different cells and cell-cycle phases.

7.6. Current prospects for the production of recombinant human acetylcholinesterase

Human AChE and BCHE are both rather big proteins, 583- and 574-amino-acids-long, respectively. Furthermore, several posttranslational modifications become apparent from the protein sequences. These include S-S bond formation, N-linked glycosylations, and subunit assembly, any or all of which may be prerequisite to the acquisition of catalytic activities and/or to the maintenance of stability in cholinesterases.

Microinjected oocytes of the frog Xenopus laevis constitute the first heterologous expression system in which recombinant cholinesterases could be produced. Studies of total mRNA microinjections were followed by use of the oocytes for production of recombinant human BCHE and ACHE. When synthesized in oocytes, cloned human CHEs display correct biochemical properties regarding substrate specificity and interaction with selective inhibitors. In contrast, both the assembly pattern of molecular forms and their mode of association with subcellular fractions are different from the in vivo patterns, and may be altered by co-injection of tissue mRNAs. This implies that faithful production of ACHE in large quantities will have to be performed in carefully selected cell types.

Production of human BCHE in bacteria (in collaboration with M. Gorecki, Ness Ziona) has so far resulted in totally inactive protein, in agreement with findings of others. This may imply that some or all of the posttranslational processing events are indeed essential to produce active CHEs, so that vertebrate cell systems will be more valuable for future large-scale production of these enzymes.

A most promising system for these purposes is the human embryonic kidney 293 cell line, in which large quantities of human ACHE may be produced following transfection with the above-described cloned human ACHEcDNA (in collaboration with A. Shafferman, Ness-Zion).

7.7 Future directions for human cholinesterase research

Isolated cDNA probes provide the prerequisite tools with which to return to the yet-unanswered biological questions related to the role and regulation of CHEs in humans. It is conceivable that in the near future, mapping by in situ hybridization will reveal the chromosomal localization of the yet-unmapped ACHE gene and the cell types in which and developmental states at which the CHE genes are transcriptionally active. Search for yet-uncovered variations in the human genes encoding for ACHE and BCHE will be complemented by expression studies, in which the biochemical significance of each mutation will be revealed. Structure-function relationship studies using in vivo mutability as well as site-directed mutagenesis

will map the major domains involved in the various aspects of CHE activities, and X-ray crystallography will delineate the three-dimensional structure of the active enzymes. The detailed molecular mechanism leading to the polymorphism of CHEs in various tissues will be searched for in different expression systems. The origin and mechanisms responsible for the surprising phenomenon of CHE gene amplifications in germ and tumor tissues will be pursued. Last, but not least, the combination of multiple scientific approaches and model systems will, hopefully, reveal the biological function(s) of CHEs in specific cell types and at specific developmental stages.

V. Notification of Patent Applications

This is to notify that the Hebrew University of Jerusalem wishes to retain title on our molecularly cloned human acetylcholinesterase cDNA and its putative uses. The rights to the above invention were assigned to Yissum R & D Company, Ltd., at the Hebrew University, an organization which has as its primary function the management of inventions conceived by the Hebrew University's employees, and the filing of patent applications in respect thereto. Yissum R & D Company, Ltd., has filed the following patent application in respect to the above invention.

In addition, Yissum has reached an understanding with Yeda, the parallel company affiliated with the Weizmann Institute of Science, to cooperate with respect to related inventions regarding which patent applications were filed by Yeda (Letter and list enclosed).

<u>Country</u>	<u>Patent Appl. No.</u>	<u>Date filed</u>
Israel	089703	03.21.89
Canada	-	03.21.90
USA	071476,554	03.20.90
EPO	90105274	03.20.90.

The Patent Application includes, as requested, a statement regarding Government Support awarded by the U.S. Army Medical Research and Development Command.

Appendix A

Patent status-

GENETICALLY ENGINEERED PROTEINS HAVING HUMAN CHOLINESTERASE ACTIVITY

<u>Country</u>	<u>Filing date</u>	<u>Application No.</u>	<u>Remarks</u>
Israel	18.06.85	75553	
Canada	16.06.86	511,663	
Denmark	18.06.86	2859/86	
*E.P.O.	16.06.86	86 108189.1	Pub. No. 0206200 30.12.86
Japan	18.06.86	142454/86	Pub. No. 111683/87 22.05.87
U.S.A.	18.06.86	875,737	Abandoned
U.S.A. CIP	21.08.87	087,724	

* Designating: AT,BE,FR,DE,IT,CH,LI,LU,NL,UK,SE.

LETTER OF UNDERSTANDING

This Letter of Understanding is between Yeda Research and Development Co. Ltd. at the Weizmann Institute of Science ("Yeda") and Yissum Research Development Company of The Hebrew University of Jerusalem ("Yissum"), regarding cooperation with respect of inventions by Professor H. Soreq, as described below.

Professor Soreq, while employed by the Weizmann Institute of Science, performed research in the area of genetically engineered proteins having human cholinesterase activity. The title, rights and interest in the results of this research are vested in Yeda. Yeda has pending patent applications with regard to this subject, as more specifically detailed in Appendix A of this Letter and which is an integral part of this Letter. (Hereinafter: Yeda's Patent).

Professor Soreq is presently employed by the Hebrew University of Jerusalem. She is carrying on research in an area related to that described above, and presently is performing research on genetically engineered human cholinesterases. Yissum is pursuing a patent/s application/s with regard to this subject. (Hereinafter: Yissum's Patent/s).

In that both Yeda and Yissum are interested in the promotion of the commercial exploitation of these inventions, and in that the parties agree that Yissum promote the commercial exploitation of these inventions, including signing of Licence Agreements with third parties and taking all needed actions on behalf of Yeda, and itself, the parties agree as follows:

- 1) Each party will continue to maintain patents/patent applications for the inventions as described above.
- 2) In that the above inventions are related, Yisum will seek to commercialize both of the inventions described above, including Yeda's Patent and Yisum's Patent/s.
- 3) In the event that Yisum will commercialize Yeda's patent, Yeda agrees to grant to Yisum a worldwide exclusive license to its patents/patent applications, in exchange for the reimbursement of all expenses, past and future, incurred in pursuit of Yeda's patents/patent applications in this regard and a percent to be agreed between the parties of all income derived from the commercialization of Yeda's Patent and/or Yisum's Patent/s, with the exception of research funds provided to Professor Soreq, to which she will be entitled in full. Said exclusive license will include the license to develop and manufacture, sales, marketing and the right to sub-license.
- 4) In the event that either party's patent is deemed to infringe upon the patent of the other party, the parties agree to make a good faith attempt to resolve this conflict in order to remove such infringement.
- 5) Yeda will provide Yisum with any needed document and/or information in its possession relating to Yeda's patent, to enable Yisum to take appropriate commercialization action.

Y E D A

By

Joel Kish

Title

Deputy General Manager

Date

13.12.85

Y I S U M

By

YISUM
RESEARCH DEVELOPMENT COMPANY
OF THE
HEBREW UNIVERSITY OF JERUSALEM

Title

Managing Director

Date

10 Dec 1985

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- 312 Soreq, H.; Malinger, G.; Zakut, H. Expression of cholinesterase genes in human oocytes revealed by in-situ hybridization. *Human Reproduction* 2: 689-693 (1987).

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VII. List of Personnel Receiving Pay

Beeri, R. (1989-1990)	Loewenstein, Y. (1990)
Ben Aziz, R., M.Sc. (1989-1990)	Neville, L. Ph.D. (1989-1990)
Ehrlich, G. (1989-1990)	Prody, C. Ph.D. (1987-1988)
Gnatt, A. M.Sc. (1987-1990)	Seidman, S., M.Sc. (1987-1990)
Lev-Lehman, E. (1989-1990)	Soreq, H., Ph.D. (1987-1990)
Lieman-Hurwitz, J. Ph.D. (1987-1988)	Zamir, R., M.Sc. (1987-1988)

VIII. Graduate Degrees Resulting from Contract Support

1. Ari Ayalon, M.D., 1988
Basic Sciences Thesis on: Expression of human cholinesterase genes in fetal and neoplastic brain tissue as detected by in situ hybridization.
2. Patrick Dreyfus, M.D., 1986-1989
Ph.D. Thesis on: Multileveled regulation of the human cholinesterase genes and their protein products.
INSERM exchange visitor.
3. Averell Gnatt, 1985-1990
Ph.D. Thesis on: Structure-function relationships in the human cholinesterase genes and their protein products. (1990) Landau Award, 1990.
4. Gustavo Malinger, M.D., 1986
Basic Sciences Thesis on: The Expression of Human Cholinesterase Genes in Normal and Malignant ovary.
Israel Fertility Association Award, 1986.
5. Eduardo Schejter, M.D., 1987
Basic Sciences Thesis on: The expression of human cholinesterase genes in muscle.
6. Shlomo Seidman, 1987-1990
M.Sc. Thesis on: Expression and tissue-specific processing of cloned human butyrylcholinesterase in mRNA injected Xenopus laevis oocytes (1989). Currently proceeds for Ph.D.
7. Ronit Zamir, 1986-1988
M.Sc. Thesis on: Chromosomal mapping of human cholinesterase genes.

IX. LIST of PUBLICATIONS

Supported by Contract DAMD 17-87-C-7169

1. Soreq, H., Zamir, R., Zevin-Sonkin, D. and Zakut, H. (1987)
Human cholinesterase genes localized by hybridization to chromosomes 3 and 6.
Human Genetics, 77, 325-328.
2. Soreq, H., Malinger, G. and Zakut, H. (1987)
Expression of cholinesterase genes in developing human oocytes revealed by in situ hybridization.
Human Reproduction 2, 689-693.
3. Zakut, H., Even, L., Birkenfeld, S., Malinger, G., Zisling, R. and Soreq, H. (1988)
Modified properties of serum cholinesterases in primary carcinomas.
Cancer, 61, 727-737.
4. Mollgard, K., Dziejielewska, K. M., Saunders, N. R., Zakut, H. and Soreq, H. (1988)
Synthesis and localization of plasma proteins in the developing human brain: Integrity of the fetal blood-brain barrier to endogenous proteins of hepatic origin.
Dev. Biol., 128, 207-221.
5. Livneh, A., Sarova, I., Michaeli, D., Pras, M., Wagner, K., Zakut, H. and Soreq, H. (1988)
Antibodies against acetylcholinesterase and low levels of cholinesterases in a patient with an atypical neuromuscular disorder.
Clinical Immunology and Immunopathology, 48, 119-131.
6. Dreyfus, P., Zevin-Sonkin, D., Seidman, S., Prody, C., Zisling, R., Zakut, H. and Soreq, H. (1988)
Cross homologies and structural differences between human cholinesterases revealed by antibodies against cDNA-produced human butyrylcholinesterase peptides.
J. Neurochem. 51, 1858-1867.
7. Malinger, G., Zakut, H. and Soreq, H. (1989)
Cholinoceptive properties of human primordial, pre-antral and antral oocytes: In situ hybridization and biochemical evidence for expression of cholinesterase genes.
J. Molec. Neuroscience, 1, 77-84.
8. Prody, A. C., Dreyfus, P., Zamir, R., Zakut, H. and Soreq, H. (1989)
De novo amplification within a "silent" human cholinesterase gene in a family subjected to prolonged exposure to organophosphorous insecticides.
Proc. Natl. Acad. Sci. USA. 86, 690-694.
9. Soreq, H., Seidman, S., Dreyfus, P. A., Zevin-Sonkin, D. and Zakut, H. (1989)
Expression and tissue-specific assembly of human butyrylcholinesterase in microinjected Xenopus oocytes. *J. Biol. Chem.*, 264, 10608-10613.

10. Lifson-Lapidot, Y., Prody, C. A., Ginzberg, D., Meytes, D., Zakut, H. and Soreq, H. (1989)
Co-amplification of human acetylcholinesterase and butyrylcholinesterase genes in blood cells: Correlation with various leukemias and abnormal megakaryocytopoiesis. *Proc. Natl. Acad. Sci. USA*, 86, 4715-4719.
11. Dreyfus, P. A., Seidman, S., Pincon-Raymond, M. Murawsky, M., Rieger, F., Schejter, E., Zakut, H. and Soreq, H. (1988)
Tissue-specific processing and polarized compartmentalization of nascent cholinesterase in microinjected Xenopus oocytes.
Molec. Cell. Neurobiol. 9, 323-341.
12. Ludgate, M., Dong, P., Dreyfus, P. A., Zakut, H., Taylor, P., Vassart, G. and Soreq, H. (1989)
Definition, at the molecular level, of a thyroglobulin-acetylcholinesterase shared epitope: Study of its pathophysiological significance in patients with Grave's ophthalmopathy.
Autoimmunity, 3, 167-176.
13. Zakut, H., Zamir, R., Sindel, L. and Soreq, H. (1989)
Gene mapping on chorionic villi chromosomes by hybridization in situ: refinement of cholinesterase genes localization to chromosomes 3q21, 3q26-ter and 16q-21.
Human Reproduction, 4, 941-946.
14. Gnatt, A., Prody, C.A., Zamir, R., Lieman-Hurwitz, J., Zakut, H. and Soreq, H. (1990)
Expression of alternatively terminated unusual cDNA transcripts mapping to chromosome 3q26-ter in nervous system tumors.
Cancer Research, 50, 1983-1987.
15. Ben Aziz, R. and Soreq, H. (1990) Improving poor in vitro transcription from G,C-rich genes.
Nucleic Acids Research. 18, 3418.
16. Gnatt, A., Ginzberg, D., Lieman-Hurwitz, J., Zamir, R., Zakut, H. and Soreq, H. (1991)
Human acetylcholinesterase and butyrylcholinesterase are encoded by two distinct genes.
Cell. Molec. Neurobiol. 11, 91-104
17. Zakut, H., Ehrlich, G., Ayalon, A., Prody, C. A., Malinger, G., Seidman, S., Kehlenbach, R., Ginzberg, D. and Soreq, H. (1990)
Acetylcholinesterase and butyrylcholinesterase genes co-amplify in primary ovarian carcinomas.
J. Clin. Invest., 86, 900-908
18. Patinkin, D., Seidman, S., Eckstein, F., Benseler, F., Zakut, H. and Soreq, H. (1990)
Manipulations of cholinesterase gene expression modulate Murine Megakaryocytopoiesis in vitro, *Molec. Cell. Biol.*, 10, 6046-6050.

19. Velan, B., Konnan, C., Grosfeld, H., Leitner, M., Gozes, Y., Flashner, Y., Sery, T., Cohen, S., Ben-Aziz, T., Seidman, S., Shafferman, A. and Soreq, H. (1991).
Recombinant human acetylcholinesterase is secreted from transiently transfected 293 cells as a soluble globular enzyme.
Cell. Molec. Neurobiol., 11, 143-156.
20. Neville, L.F., Ghatt, A., Padan, R., Seidman, S. and Soreq, H. (1990)
Anionic site interactions in human butyrylcholinesterase disrupted by two adjacent single point mutations.
J. Biol. Chem., 265, 20735-20739.
21. Neville, L.F., Ghatt, A., Lowenstein, Y. and Soreq, H. (1990) Aspartate 70 to glycine substitution confers resistance to naturally occurring and synthetic anionic site ligands on in ovo produced human butyrylcholinesterase. J. Neurosci. Res., 27, 461-471.

Monographs

H. Soreq and H. Zakut (1990)
Cholinesterase genes: Multileveled regulation
Monographs in Human Genetics, Vol. 13
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REVIEWS AND BOOK CHAPTERS

1. Soreq, H. and Prody, C. (1989)
Sequence similarities between human acetylcholinesterase and related proteins: Putative implications for therapy of anticholinesterase intoxication.
In: Computer-assisted modeling of receptor-ligand interactions, theoretical aspects and applications to drug design. (Golombek, A. and Rein, R., eds.), Alan R. Liss, New York, pp. 347-359.
2. Soreq, H. and Zakut, H. (1990)
Amplification of butyrylcholinesterase and acetylcholinesterase genes in normal and tumor tissues: Putative relationship to organophosphorous poisoning.
Pharmaceutical Research, 7, 1-7.
3. Seidman, S. and Soreq, H. (1990)
Co-injection of Xenopus oocytes with cDNA-produced and native mRNAs: a Molecular Biological approach to the tissue specific processing of human cholinesterases. Int. Rev. Neurobiol. 32, 107-139.
4. Ayalon, A., Zakut, H., Prody, C.A. and Soreq, H. (1990) Preferential transcription of acetylcholinesterase over butyrylcholinesterase mRNAs in fetal human cholinergic neurons. In: Gene Expression in the nervous system. (A.M. Giuffrida-Stella, Ed.) Alan R. Liss, New York.
5. Soreq, H. and Zakut, H. (1990)
Expression and in vivo amplification of the human cholinesterase genes. Progress in Brain Research. Vol. 84. S.-M. Aquilonius and P.-G. Gillberg, Eds. Elsevier Science Publishers B.V. (Amsterdam) pp. 51-61.

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Modified ligand-binding properties of butyrylcholinesterase muteins produced in microinjected Xenopus oocytes.
In press in: Proceedings of the Third International Meeting on Cholinesterases. ACS books, Washington, D.C., F. Bacu et al., eds.
7. Ben Aziz, R., Gnatt, A., Prody, C.A., Lev-Lehman, E., Neville, L., Seidman, S., Ginzberg, D., Soreq, H., Lapidot-Lifson, Y. and Zakut, H. (1990)
Differential codon usage and distinct surface probabilities in human acetylcholinesterase and butyrylcholinesterase.
In press in: Proceedings of the Third International Meeting on Cholinesterases. ACS books, Washington, D.C., F. Bacu et al., eds.
8. Dreyfus, P.A., Pincon-Raymond, M., Zakut, H., Seidman, S. and Soreq, H. (1990)
Search for the molecular origins of butyrylcholinesterase polymorphism by cDNA screening, deletion mutagenesis and Xenopus oocytes co-injections.
In press in: Proceedings of the Third International Meeting on Cholinesterases. ACS books, Washington, D.C., F. Bacu et al., eds.
9. Soreq, H., Neville, L., Gnatt, A., Ben-Aziz, R., Lapidot-Lifson, Y., Ehrlich, G., Seidman, S., Lev-Lehman, E., Beerli, R., Ginzberg, D. and Zakut, H. (1990)
Structure-function relationships, in vivo mutagenesis and gene amplification in human cholinesterases, targets for organophosphorous poisons.
In press in: Advances in Applied Technology. Kluwer Academic Publishers, Dordrecht. Bar-Tov et al., Eds.
10. Soreq, H., Ehrlich, G., Lapidot-Lifson, Y., Gnatt, A., Neville, L., Ben-Aziz, R., Seidman, S., Ginzberg, D. and Zakut, H. (1990) Amplification and mutagenesis of the acetylcholinesterase and butyrylcholinesterase genes in primary human tumors. In press in: Gene Amplification in Mammalian Cells. R.E. Kellems, ed. Marcel Dekker, Inc., N.Y.