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MOLECULAR CLONING OF
HUMAN GENE(S) DIRECTING
THE SYNTHESIS OF NERVOUS SYSTEM CHOLINESTERASES

Annual/Final Report

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Hermona Soreq, Ph.D.

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19. ABSTRACT
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Several genes encode for human cholinesterase. In situ hybridization of [^{35} S]-labeled ChEcDNA to spread human chromosomes revealed sites for ChE genes on two different chromosomes, No. 3 and 16. Screening of several genomic DNA libraries, using [^{32} P]-ChEcDNA as a probe, enabled the isolation of genomic DNA fragments derived from at least two different genes. When combined with published genetic linkage evidence, these findings imply that at least two genes may direct the synthesis of active ChE in humans.

mRNA transcripts for human cholinesterases were found in mRNA preparations from brain, indicating the in situ production of various ChEs in the brain. This was demonstrated by in vitro and in ovo translation of brain mRNA followed by crossed immunoelectrophoretic autoradiography.

Biochemical alterations in serum cholinesterases were observed in carcinoma patients, indicating altered regulation of ChE genes either due to the cancerous state or to the treatment protocols employed.



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SUMMARY

Cholinesterases (ChEs) are highly polymorphic proteins, capable of rapidly hydrolyzing the neurotransmitter acetylcholine and involved in terminating neurotransmission in neuromuscular junctions and cholinergic synapses. In an attempt to delineate the structure and detailed properties of the human protein(s) and the gene(s) coding for the acetylcholine hydrolyzing enzymes, a human cDNA coding for ChE was isolated by the use of oligodeoxynucleotide screening of cDNA libraries. For this purpose, a method for increasing the effectiveness of oligonucleotide screening by introducing deoxyinosine in sites of codon ambiguity and using tetramethyl-ammonium salt washes to remove false positive hybrids was employed. The resulting isolated 2.4 kilobase ChEcDNA sequences encode for the entire mature secretory protein, preceded by an N-terminal signal peptide. The human ChE primary sequence shows almost no homology to other serine hydrolases, with the exception of an hexapeptide at the active site. In contrast, it displays extensive homology with acetylcholinesterase (AChE) from Torpedo californica and Drosophila melanogaster as well as with the c'-terminal part of bovine thyroglobulin. These extensive homologies probably suggest the need of the entire coding sequence for the physiological function(s) fulfilled by the enzyme, and further suggest a common unique and ancient ancestral gene for these cDNAs.

The isolated ChEcDNA was used as a probe to isolate genomic DNA sequences from the 5'-region of the human ChE gene. The genomic DNA fragment encoding part of the 5'-region of ChEcDNA was detected by DNA blot hybridization, enriched 70-fold by gel electrophoresis and electroelution, cloned in lambda phage and isolated. Sequencing of the cloned DNA revealed that it did indeed include part of the 5'-region of ChEcDNA, starting at an adjacent 5' position to the nucleotides coding for the initiator methionine, and ending with the hexanucleotide GAATTC, specifically cleaved by the restriction endonuclease EcoRI and inherent to the ChEcDNA sequence. The isolated fragment of the human cholinesterase gene is currently employed to complete the structural characterization of this and related genes.

In order to map the structural human ChE genes to distinct chromosomal regions, to be defined as the CHE loci, the cloned human cDNA for cholinesterase was used as a probe for in situ hybridization to chromosomes. For this purpose, lymphocyte chromosome spreads were prepared following short-term culture, and hybridized with ChEcDNA fragments labeled with [³⁵S] by different techniques. These included direct labeling of electrophoretically purified cDNA inserts by nick-translation, synthesis of a labeled second DNA strand to this cDNA subcloned into the single-stranded M13 phages, in vitro transcription of such cDNA into labeled RNA and the use of random oligonucleotides as primers for DNA polymerase I synthesis. Following the hybridization procedure, chromosome spreads were exposed under photography emulsion to create silver grains in sites of hybridization and were then R-banded to enable karyotype analysis and to localize specific silver grains on particular chromosome regions. Using this analysis, the recent genetic linkage assignment of the CHE1 locus to the long arm of chromosome 3 was confirmed and further refined to 3q21-q26, in close proximity to the genes coding for transferrin (TF) and transferrin receptor (TRFC). The CHE1 locus localized to a 3q region that is commonly aberrated, and related with abnormal megakaryocyte proliferation, in acute myelodysplastic anomalies. In view of earlier findings that ChE inhibitors induce megakaryocytopoiesis in culture, this localization may indicate that ChEs are involved in regulating the differentiation of megakaryocytes. Cumulative histograms of in situ hybridization of ChEcDNA to chromosome 3

further suggest that there might be two sites carrying ChE sequences on the long arm of this chromosome.

A third site for ChEcDNA hybridization was found on chromosome 16q11-q23, demonstrating that the CHE2 locus of the cholinesterase gene, which directs the production of the common C5 variant of serum ChE, also codes for a structural subunit of the enzyme and is localized on the same chromosome with the haptoglobin (HP) gene, both genes found on the long arm of chromosome 16. Further in situ hybridization experiments using human haptoglobin cDNA revealed that these two genes are at least 1000 kb apart from each other. The finding of various sites for ChEcDNA hybridization suggests that the different loci coding for human ChEs may include non-identical sequences, responsible for the biochemical differences between ChE variants.

The synthesis of various cholinesterases in different fetal human tissues was studied using in vitro and in ovo translation of poly(A) +RNA followed by crossed immunoelectrophoretic autoradiography. When unfractionated poly(A)+mRNA from fetal brain, muscle or liver was translated in vitro, in the reticulocyte lysate cell-free system, polypeptides were synthesized which reacted with antibodies against either "true" acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) or "pseudo," butyrylcholinesterase (BuChE, acylcholine acylhydrolase, EC 3.1.1.8). The two nascent cholinesterases could be separated by crossed immunoelectrophoresis followed by autoradiography, suggesting that acetylcholinesterase and butyrylcholinesterase are produced in all three tissues from nascent polypeptides containing different, but also homologous immunological domains. To examine whether the biosynthesis of cholinesterases includes post-translational processing events, Xenopus oocytes were microinjected with mRNA from these tissues. Immunoelectrophoretic analysis of oocyte intracellular homogenates, extracts of membrane-associated proteins and secreted proteins localized in the incubation medium revealed various precipitation arcs, reflecting the synthesis and post-translational processing of multiple forms of tissue-specific exported and intracellular acetylcholinesterase and butyrylcholinesterase. These findings demonstrate that polymorphic cholinesterases are produced from nascent polypeptide products which undergo further post-translational processing events in a tissue-specific manner before they become mature compartmentalized cholinesterases.

The biochemical properties of serum Cholinesterases were characterized in the serum of 77 patients with primary carcinomas and 21 healthy volunteers who served as controls. In most of the samples, BuChE accounted for almost all ChE activity and was inhibited by the organophosphorous poison tetraisopropyl pyrophosphoramidate (iso-OMPA). In samples from the tumor-bearing patients, ChE degraded 110 ± 10.9 nmole acetylcholine/hr/ μ g protein, lower than the 144 ± 26.2 nmole/hr/ μ g levels measured in controls. Tumor serum ChEs exhibited elevated sensitivity to BW284C51, the selective bisquaternary inhibitor of "true" AChE, with no correlation to age, sex, staging of tumor, presence of metastases or treatment protocol, and with a different distribution pattern from the decrease in ChE-specific activity or the sensitivity to iso-OMPA. In sucrose gradients, ChE sedimented as 12S in controls, whereas in tumor serum samples an additional component of 6-7 S, inhibited by both iso-OMPA and BW284C51 at final concentrations of 1.10^{-5} M, was also detected. These findings suggest that occurrence of primary carcinomas is accompanied by appearance of a soluble ChE form, with properties of both AChE and BuChE, which accumulates in the serum.

Studies at the levels of the genomic DNA, the tissue mRNAs and the catalytically active cholinesterases are currently combined to reveal the

structure, the chromosomal localization and the organization of the human genes coding for cholinesterases. Genomic DNA fragments and cDNA clones with sequence homologies to the above-described ones are being selected by filter hybridization from various libraries and characterized by DNA sequencing. Subcloning into the SP₆ transcription vector, preparation in vitro of synthetic ChEmRNA and oocyte microinjection are used to study post-translational events contributing to the synthesis and polymorphism of cholinesterases, and in situ hybridization to frozen tissue sections and cells in culture is employed to search for cells and tissues rich in ChEmRNA transcripts, with the long-term goal to characterize in detail the family of genes coding for human cholinesterases and their modes of expression and regulation.

FOREWORD

1. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

2. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

3. For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

4. The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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I. INTRODUCTION: HUMAN CHOLINESTERASES AND THE REGULATION OF THEIR EXPRESSION

I.A. Cholinesterase Classes and Their Physiological Significance

Cholinesterases (ChEs) are scarce polymorphic proteins capable of rapidly degrading the neurotransmitter acetylcholine (1). Two major classes of the enzyme exist, that differ in the nature of their preference for substrate: acetylcholine for acetylcholinesterase (AChE, TrueChE, acetylcholine acetylhydrolase, EC 3.1.1.7) and butyryl/propionylcholine for pseudocholinesterase (butyrylcholinesterase, BuChE, pseudoChE, acylcholine acylhydrolase, EC 3.1.1.8). Both are generally distinguished by their susceptibility to inhibitors (2). Acetylcholinesterase is the major class in neuromuscular junctions ranging from fish to human and a key element in certain cholinergic synapses. At the neuromuscular junction and cholinergic synapse, it terminates the electrophysiological response to acetylcholine.

True AChE occurs in multiple molecular forms, exhibiting different sedimentation coefficients on sucrose gradients (3). Further polymorphism can be distinguished according to the interactions of such forms with non-ionic detergents (4). In mammalian nervous tissue there exist forms of AChE that are secreted, cytoplasmic, membrane-associated (3) and bound to the basal lamina (5). Heavier ChE forms have been shown to be associated with a collagen-like tail (6). The hydrophobic properties of some forms might be explained by the finding of hydrophobic domains (possibly a C-terminal peptide) (7) or tight interactions with phospholipid moieties (i.e. phosphatidyl inositol (8,9)). Although such subcellular segregation suggests the existence of different domains in various ChE classes, pharmacological and kinetic evidence show similar catalytic properties (10). BuChE is similarly polymorphic and many BuChE forms can be said to be homologous to the true AChE forms (11).

The existence of a ChE form which differs from AChE was shown by Alles and Hawes (12), who found that human serum and red blood cell enzymes are qualitatively different. Mendel and Rudney (13) have shown that the serum ChE (later designated as BuChE), hydrolyzes butyrylcholine and propionylcholine faster than the cell bound AChE. This class is the principal one found in the serum and until recently, no known biological role has been found for it. However, it has recently been shown that the sole ChE in the Torpedo marmorata heart muscle is BuChE (14), although in higher vertebrates both classes exist in the heart. Humans having non-functional BuChE in the serum show no known symptoms of illness (15) and exhibit normal muscular and neuronal activity. Altogether this may indicate that in ancestral species each enzyme had specific localized roles, and that with the evolution of the two enzyme classes, the acetylcholine binding site in serum BuChE became less physiologically important.

I.B. Tissue and Cell Type Specificity in the Expression of Cholinesterases

Although ChEs are abundant in nerve and muscle, they may be found in other tissue and cell types, including the erythrocyte (1), adrenal medulla, ovarian follicles (16) and megakaryocytes (17). ChEs have been reported in a number of embryonic tissues (18). In addition, considerable levels of ChEs were detected in various neoplastic tissues such as ovarian carcinomas (18) and brain tumors of glial and mesenchymal origin (19,20). The various ChEs localized in different tissues and cell types vary in their sedimentation properties, hydrophobicity and glycosylation patterns (3,20-22). The physiological role of the enzymes in tissues other than brain or muscle remains unknown.

I.C. Regulation of Expression of Cholinesterase Genes

To comprehend the regulation of biosynthesis of ChEs fully, it is important to understand the genetic makeup of the ChE genes. In the nematode earthworm *Caenorhabditis elegans*, two forms of ChEs were shown to exhibit different kinetic properties, though both enzyme classes (A and B) appear to be AChEs (23). Two distinct genes, *ace-1* and *ace-2* (probably structural genes), were shown to be responsible for the expression of these classes (24,25). Mutations occurring in either the A class alone or the B class alone do not change the phenotype. If, however, mutations occur in both genes, a new uncoordinated phenotype is observed (25). The double mutations do not have a lethal effect, as shown for mutations in the *Drosophila* *ace* locus (the only AChE locus in *Drosophila*: for a review see 26). This observation led to the discovery of a third class of AChE, the C class, whose kinetic properties differ greatly from those of A and B, suggesting the existence of a different active site (24,23,27,28).

In humans, two codominant alleles at a single locus, which has not been genetically mapped, are responsible for phenotypic variants of erythrocyte AChE (29). In contrast, two genetically independent loci, namely, CHE1 and CHE2, have been genetically linked to alterations in serum BuChE (1,30). Genetic linkage studies suggest that the generally expressed CHE1 locus is situated on the long arm of chromosome No. 3 (31), in linkage with the transferrin (TF) gene (32), mapped at 3q31-q26.1 (33), the caeruloplasmin gene and the transferrin receptor gene (TFRC) (reviewed by Kidd and Gusella, 34). Mutations in this gene result in the appearance of the "atypic" and "silent" forms of serum BuChE. The CHE2 gene is expressed in 8% of the Caucasian population and is responsible for the production of the common C5 variant of serum BuChE. This form has been suggested to be the result of the CHE2 gene protein binding to the CHE1 ChE protein, which causes a change in the mobility of BuChE. It also increases the activity of BuChE by up to 48% (35). Genetic linkage studies suggested a possible linkage between the CHE2 gene and the alpha-haptoglobin gene (36), which maps in a region on chromosome No. 16, distal to the fragile site 16q22 (37).

Recent *in situ* hybridization to human chromosomes, using cDNA probes encoding human ChE, revealed that two structural ChE genes exist in the areas where the CHE1 and CHE2 genes have been shown to reside (38). This suggests that the CHE2 gene might by itself encode for a catalytic subunit of ChE, which causes increased activity either due to its own enzymatic properties or by affecting the E1 subunit conformation, creating an increase in the activity of the composite enzyme molecule.

Oocyte microinjection experiments (39) and *in vitro* translation of ChEmRNAs (40,41) suggest that the polymorphism of the ChE proteins extends to the level of mRNA. Crossed immunoelectrophoretic analysis of ChEmRNA

products indicates that various ChEmRNA species encode the biosynthesis of electrophoretically distinct ChE polypeptides in a tissue-specific manner (42,43). However, it is not clear yet what are the differences are between the various species of ChEmRNAs in general, and particularly in humans. Furthermore, it is impossible yet to link particular ChE forms and specific ChEmRNA species to defined ChE genes included in the human genetic repertoire.

II. OBJECTIVE: WHY SEARCH FOR HUMAN CHOLINESTERASE GENES?

II.A. Cholinesterase as a Neurobiological Model for Termination of Neurotransmission

The presence of an acetylcholine-hydrolyzing protein seems to be an essential requirement in all cholinergic synapses, as is evident from the genetic experiments described above as well as from the lethal effects of cholinesterase inhibitors (see following sections for details). In addition, the structure of ChE molecular forms is related to the nature of the synapse. For example, neuromuscular junctions are rich in collagen-tailed asymmetric AChE, whereas muscarinic brain synapses contain mainly slightly hydrophobic AChE tetramers (3,22). This divergent distribution of ChEs may be relevant to the physiological properties of particular synapses.

Recently accumulated evidence reveals that the primary structures of several major proteins play important roles in regulating the pace and mode of function of particular types of synapses. For example, molecular cloning of the nicotinic (44-46) and the muscarinic (47) cholinergic receptors has shown that these two proteins, both of which bind acetylcholine, have completely different primary sequences. Production and mutagenesis of the synthetic nicotinic acetylcholine receptor from cloned DNA in heterologous expression systems such as microinjected *Xenopus* oocytes (48) have been performed by genetic engineering techniques. These studies linked many of the electrophysiological properties characteristic of the nicotinic synapse to primary sequence epitopes in the various subunits of the nicotinic acetylcholine receptor molecule. In contrast with this advanced stage of study of receptor proteins, little has been done to investigate the precise involvement of ChE's functioning as the turning-off signal in regulating cholinergic transmission. The extensive similarities between polymorphic cholinesterases suggests considerable homologies also at the level of nucleotides. However, the various ChEmRNAs coding for the different ChEs present in fast and slow-twitch muscles (49) may carry form-specific differences contributing to the electrophysiological properties of such synapses. This suggestion and parallel questions may today be approached by gene transfection and site-directed mutagenesis studies to determine the significance of the structure of the ChE protein in termination of neurotransmission.

II.B. Cholinesterases as a Model System for Regulation of Protein Polymorphism

As mentioned previously, ChEs are extremely polymorphic proteins. They exist as monomers, dimers and tetramers. Some forms are globular and others, which are asymmetric, are associated with a collagen-like tail [for a review of the forms, see (3)]. In an attempt to understand the underlying causes of such polymorphism of proteins, high-level production of the various ChE forms in cell culture would be of use. It seems that the information localizing the asymmetric AChE within the quail muscle membrane is acquired in the Golgi apparatus (50), although the mechanism of this polymorphic determination is unknown. The polymorphism of ChEs could in principle be a result of different genes encoding various primary structures. However, this seems less likely than other possibilities because *in situ* chromosomal mapping (38) and genomic DNA blot analysis using isolated human ChEcDNA (51,52) suggest that the structural cholinesterase genes do not exist in as many copies as the number of existing molecular forms. Other possibilities include post-transcriptional (see 53) and/or post-translational processing events, such as glycosylation, formation of intramolecular and inter-subunit S-S bonds,

distinct assembly patterns and binding of the collagen-like tail. Since all of these processes do take place during biosynthesis, the ChEs are appropriate to be used as a model system to study the regulation of protein polymorphism.

II.C. Clinical Potential of the Study of Cholinesterase Genes

1. Prolonged Apnea Following Succinylcholine Administration

Succinylcholine, which acts as a competitive analog of acetylcholine, is often used in surgery as a short-term muscle relaxant. Since the drug is hydrolyzed by BuChE, its administration into individuals carrying genetically abnormal BuChE causes prolonged apnea (54). The most common variant with this problem is the atypical variant CHE^{al} , for which 3-6% of the of the Caucasian population is heterozygous and about 0.05% is homozygous (55). This enzyme hydrolyzes acetylcholine but not succinylcholine (30). Another variant, CHE^{s} , which causes the complete absence of catalytically active serum BuChE in homozygotes, is also associated with this clinical problem (15). This type of "silent" enzyme cannot hydrolyze any ChE substrate, nor can it bind organophosphorous compounds (56). High frequency of atypical and silent BuChE genes was reported among Iraqi and Iranian Jews (11.3% for heterozygotes and 0.08% for homozygotes, respectively) (57). This could explain the high frequency of reports of prolonged apnea following surgery in Israel. It is likely that BuChE could be used intravenously to rid the body of the succinylcholine in cases of prolonged apnea. For such use large amounts of the purified functional human ChE would be necessary. Since human ChEs cannot be purified in sufficient quantities, a cloned product would be necessary for such a purpose.

2. Poisoning by Organophosphorus Compounds

Complete inhibition of ChEs, for example, by the administration of organophosphorus (OP) poisons, is lethal (58). This inhibition is achieved by formation of a stable stoichiometric (1:1) covalent conjugate with the active site serine (59), followed by a sequential reaction, termed "aging," which renders the inhibited ChE refractory to regeneration by the commonly used reactivators (59), such as active-site directed nucleophiles (e.g., quaternary oximes) which detach the phosphoryl moiety from the hydroxyl group of the active site serine (60). The aging process is believed to involve dealkylation of the covalently bound OP group (59), and renders therapy of intoxication by certain organophosphorus compounds such as sarin, diisopropyl-fluorophosphate (DFP), and soman, exceedingly difficult (61). The cloned BuChE protein could potentially be produced in large quantities and used prophylactically (e.g., in people who handle agricultural organophosphorus insecticides) to prevent intoxication. This prospect is strengthened by the recent observation that injection of highly purified fetal bovine serum AChE into mice protects them against organophosphorus poisoning (56) and by the positive effects of injected purified serum cholinesterase on patients suffering from alkyl phosphate poisoning (62).

3. Open Neural Tube in Human Embryos

Neural tube defects in human embryos are biochemically characterized by secretion of a 10S tetrameric form of AChE into the amniotic fluid (63). The detection of alterations in the level and in the isoform composition of AChE is currently carried out by sucrose gradient sedimentation of amniotic fluid, followed by enzymatic assays of ChE activity (64). In an alternative, less quantitative method, amniotic fluid samples are separated by gel electrophoresis and AChE activity is detected by specific staining (65). It would be particularly desirable to have a simple specific

procedure to determine the level of specific ChE forms in the amniotic fluid. This could be made possible by amino acid sequence determination, molecular cloning and the expression of the peptide regions specific to this cloned protein in sufficient quantities to allow production of type-specific antibodies. Such antibodies could possibly be used in a radioimmunoassay to detect the existence of the enzyme isoform and thereby the open neural tube itself.

4. Detection of Cholinesterases in Other Disorders

Modifications of human brain AChE have been reported in several neurological or genetic disorders, such as Alzheimer's disease (66) and Down's syndrome (67). In the brains of patients with presenile dementia of the Alzheimer type (SDAT; about 5% of the population above 65), the levels of AChE in cholinergic brain areas drop by about 50% (64). This particularly refers to soluble AChE tetramers. However, it is not clear at what step in the pathway of gene expression this decrease is controlled. Probes for ChE genes, and good antibodies for the various forms of AChE affected in such disorders, will help to shed more light on this issue.

5. Cholinesterases in Embryonic and Tumor Cells

In addition to their functional sites at neuromuscular junctions and cholinergic synapses, ChEs are noted for appearance at various other loci and also for their unexplained transient expression in proliferating tissues of epithelial and mesenchymal origin (2). Appearance of ChEs early in the development of various embryonic tissues has been correlated with regulation of cell proliferation (17) and with morphogenetic cell migration (18,68), and it occurs also in tissues that do not express synaptic ChEs, such as the chondrogenic core of the chicken limb bud or rat promegakaryocytes (17). Expression of ChEs is an early differentiation marker in the entire brain of chicks (68), of the macaque monkey (69) and of man (22). High levels of ChEs have also been detected in various types of primary tumors, including ovarian carcinomas (18), glioblastomas (19,20) and meningiomas (20).

In the human blood, soluble tetra isopropylpyrophosphoramidate (iso-OMPA)-sensitive tetramers of BuChE reside in the serum (56,70-72), whereas hydrophobic, 1,5-bis[4-(allyldimethylammonium)phenyl]pentan-3-one dibromide (BW)-sensitive dimers of AChE (73) are covalently attached to the membrane of erythrocytes through phospholipid moieties (74). In malignant and embryonic tissues, both BW-sensitive and iso-OMPA-sensitive ChEs were detected, with independent patterns of expression. The term "embryonic cholinesterase" has been proposed for these activities by Drews (18) and later supported by Layer (68). However, it is not known yet whether the heterogeneity of ChEs in general stems from different structural genes or from post-transcriptional and post-translational processing (for a recent review see (75).) Thus it has also remained unclear whether the term "embryonic ChE" refers to a biochemically distinct entity and what are its characteristic properties. In previous studies, we found indications for the expression of a ChE form which is sensitive to both iso-OMPA and BW in primary brain tumors and in fetal human brain (20,22). To further examine this possibility, we set out to characterize the ChEs which accumulate in the serum of patients suffering from carcinomas of different tissue origins or with various rates of cell proliferation and differentiation levels. Our findings indicate that the occurrence of primary carcinomas is accompanied by a modified route of expression for ChE genes, resulting in the accumulation in the serum of an embryonic type of ChE, susceptible to both BW and iso-OMPA inhibition.

III. TECHNICAL APPROACHES AND METHODOLOGY

III.A. The Use of Synthetic Oligodeoxynucleotides as Probes and/or Primers for DNA Sequencing

Peptide sequencing information is often used to devise synthetic oligodeoxynucleotide probes, which may in turn be employed in various ways for hybridization with particular cDNA sequences (76). Throughout the studies described in this report, oligodeoxynucleotides were prepared by phosphoramidite chemistry, first based on published peptide sequencing information (71) and then based on DNA sequences derived in our laboratory for ChEcDNA (51,52). The sequences and origin of part of these probes are detailed below, in Table 1. For example, the three probe mixtures designated OPSYN include all of the possibilities which could encode for the active site ChE hexapeptide (71). It was initially decided to limit the active site probe length to this hexapeptide, since this is a consensus sequence, identical in human BuChE and in Torpedo AChE (71,77). Further publications revealed that the limitation of the length of these probes was crucial to the success of the screening project, since the human BuChE active site sequence that was published in 1986 (56) differed from that published in 1984 (71) by 4 out of 29 amino acids.

TABLE 1

Oligonucleotides used as probes and/or primers for sequencing. Part of the synthetic oligodeoxynucleotides employed throughout this work as probes and/or primers are presented. (+): the mRNA (coding) strand; (-): the cDNA (non-coding) strand. Underlined nucleotides represent mistakes due to primary sequencing only from one strand, which were found after extensive sequencing. The polypeptides encoded by these oligodeoxynucleotides are numbered according to the nucleic acid residue numbers in the full-length ChEcDNA. Origin refers to the source of information by which these sequences were determined. Probes labelled 1 were synthesized according to nucleotide sequences found in the isolated ChEcDNA. Those labelled 2 were deduced from peptide sequencing data published by Lockridge (71). Those labelled 3 were determined from genomic nucleotide sequences, included in phages hybridizing with ChEcDNA probes, and those labelled 4 follow the sequence of Haas and Rosenberry (73) for the N-terminal peptide of human erythrocyte AChE.

TABLE 1
Oligodeoxynucleotides

Name	DNA sequences 5'—3'	Encoded polypeptide	Origin
Pseudo-C-term (-)80	A A C AGCCCNAC CA CT TC G G T	1864-1881	1
Opsyn (-) 151	A A C CT CT G G T A AC IGCIGCICCGC TCICC AA IGI C G IGA T	742-770	1
Opsyn I (-)	A A C G C C A CC GC CT TC CC AA G A T G G T T	742-758	2
Opsyn II(-)	A A C G C C A CC GC GA TC CC AA G C T G G T T	None	2
Opsyn III(-)	A A C A C C A CC GC GA TC CC AA G T T G G T T	None	2
N-term (-) 200	TGTTGCAATTATGATGTCATCTTC	160-183	1
SP (+) 212	GGATTCTTAGCTTTGCC	613-629	1
TH2 (+) 214	GGATCAGAGATGTGGAA	391-407	1
SP (+) 216	TTGGAGAAAGTGCAGGA	743-759	1

(continued on next page)

Table 1. Oligonucleotides Used as Probes and/or Primers for Sequencing
(continued)

TABLE 1 (continued)

Oligodeoxynucleotides

Name	DNA sequence, 5'—>3'	Encoded polypeptide	Origin
N-term (+) 217	GATGACATCATAATTGC	163-179	1
SP (-) 220	AGCCCIACICAICTTTC	1864-1880	1
SP (+) 226	GAIAGITGIGTIGGGCT	1864-1880	1
SP (+) 232	AAAGATGAAGGGACAGC	1126-1142	1
SP (+) 233	AATTATCAGTGCTCTGC	2187-2203	1
SP1424 (-) 250	AAAGGCATTATTTCCCC	1424-1440	1
SP1175 (-) 253	AGGAGCACCATAGACTA	1175-1191	1
SP1715 (-) 254	GCACGTAGTTTCGTCAT	1715-1732	1
SP2012 (-) 252	TCCTTCTGGCATTGTG	2012-2028	1
308IV (-)	ACTTAACCAAGGCTGAA	Genomic	3
309IV (+)	TTCAGCCTTGGTTAAGT	Genomic	3
Nt-true 121	A A A C C C C GG TC TC GG CC TC G T G G T T T	None	4

Because of codon ambiguity, a total of 384 possibilities existed for the composition of the particular 17-long active site oligonucleotide. Therefore, it was essential to divide the OPSYN mixture of probes into three groups, to ensure that the specific activity of each individual sequence would be high enough to be detected in an autoradiogram. The ChEcDNA-derived oligonucleotide probes prepared later had the advantage of representing a single sequence each, which allowed a much higher specific activity in their use for hybridization experiments and eliminated problems of false-positive hybrids.

To be used as probes in hybridization reactions, the synthetic oligodeoxynucleotides were end-labelled at their 5'-ends with [^{32} P] using the enzyme polynucleotide kinase (51). Hybridization and washing of hybrids using such probes is carried out with special caution because of the short nucleotide chains (76); this also increases the probability of false-positive hybrids. To minimize such errors, 3 M tetramethylammonium chloride ((CH₃)₄NCl) was employed to discriminate against short GC-rich hybrids in a base composition-independent manner (78).

III.B. Isolation of Complementary DNA Clones Coding for Human Cholinesterase

Poly(A)+RNA was extracted from fetal human brain and liver (18 weeks' gestation) and was tested for the presence of cholinesterase mRNA by oocyte microinjection followed by cholinesterase bioassay (39,79). Complementary DNA libraries (constructed by Dr. Axel Ullrich of Genentech, Inc.) were prepared from these RNA preparations and were inserted into the EcoRI site of lambda gt10, using a polylinker containing restriction sites for EcoRI, XhoI, Sal I and Sst I. The fetal brain lambda gt10 library (1.6 x 10⁶ plaque-forming units) was plated out, and nitrocellulose filter copies were prepared and screened with two overlapping oligodeoxynucleotide probes (51,52). Each probe was designed to complement the predicted mRNA sequence as follows: Probe OPSYN, [3'-AA(A,G)CCNCT(C,T){[TC(A,G)], [AG(G,C)]}CGNC where N equals A,C,G,T, a 17-mer with a 25-fold degeneracy which represents the consensus peptide sequence, Phe-Gly-Glu-Ser-Ala-Gly, present in human serum BuChE (71) and in "true" AChE from Torpedo electric organ (41); Probe OPSYN0, [3'-AA(A,G)CCICT(C,T){[TC(A,G)], [AGI]}CGICICGICGI{[TC(A,G)]}, [A I]}C a 29-mer with a 3-fold degeneracy which codes for the peptide Phe-Gly-Glu-Ser-Ala-Gly-Ala-Ala-Ser-Val found in human serum BuChE (71) and which differs from the parallel peptide of Torpedo AChE by one amino acid (No. 7 in this peptide, Gly in Torpedo) (77). The limitation of codon degeneracy in probe OPSYN0 was made possible by insertion of deoxyinosine in positions where codon ambiguity permits all four nucleotides (80). Both probes were manually synthesized using phosphoramidite chemistry. Oligodeoxynucleotides were 5'-end-labelled with [^{32} P]ATP (5,000 Ci/mole, Amersham) using polynucleotide kinase (New England Nuclear). Unreacted [^{32} P]ATP was removed using DEAE52₆cellulose columns (Whatman), resulting in specific activities of > 1x10⁶ dpm/pmole. Hybridization was performed in 6xSSC (1xSSC=0.015M sodium citrate and 0.15 M NaCl), 5xDenhardt (1xDenhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.05 mg/ml denatured herring sperm DNA and 3x10⁶ dpm/ml of [^{32} P]-labelled oligonucleotide probe, for 1 hr at 40°C. Washing at 40°C in 3xSSC was followed in the third screen by 3M tetramethylammonium (TMA) chloride washes at 53°C. Under these conditions, only a single clone in the fetal brain library gave a significant hybridization signal with both probes. DNA was prepared from this clone (51) and digested with XhoI (Boehringer) to separate the 765-nucleotides long-cDNA insert, designated FBChE12, which was purified by preparative gel electrophoresis and

electrolution. The FBChE12 DNA was then [^{32}P]-labelled by nick-translation and employed as a probe to screen the fetal brain and liver libraries. Hybridization and washing were performed as previously described (81). Rescreening the fetal brain library resulted in finding three more positive inserts, none of which was longer than FBChE12. Of 1.4×10^6 fetal liver phages, 23 gave positive hybridization signals with [^{32}P]-labelled FBChE12. Of these, 4 phages contained 2.4 kb-long inserts with identical restriction maps. These hybridized with a C-terminal probe, [3'-CT(C,T)[[AGN], [TC(G,A)]]AC(G,A)CANCCCGA], a 17-mer with a 96-fold degeneracy which codes for the peptide Glu-Ser-Cys-Val-Gly-Leu found in the C-terminus of human serum BuChE (71). One of these cDNA clones, designated FL39, was used for further characterization and DNA sequencing.

Two cDNA clones were isolated from the fetal brain library by screening with the OPSYN II probe (75). These could represent members of the cholinesterase family or be false-positives. To distinguish between these possibilities and to characterize the oligodeoxy-nucleotide-cDNA hybridization, DNA sequencing was performed by the Sanger dideoxy sequencing technique (82), with the single-stranded vectors M13mp10, M13mp11 and M13mp19 (83). Similar techniques were employed for the characterization of other clones, including those coding for cholinesterase from fetal brain and liver ((51,52); see further sections for details).

III.C. Isolation and Characterization of Genomic DNA Fragments Hybridizing with Cholinesterase cDNA Probes

ChEcDNA was employed as a probe for the screening of genomic DNA libraries, to detect and isolate DNA fragments from the human ChE gene(s). In addition, special attention was paid to the 5'-terminal region of such genes. In genomic DNA blots, EcoRI (Bio Labs) digested DNA from all of the tissue origins checked to date gave seemingly identical patterns when hybridized with ChEcDNA probes (see section IV for details). When this hybridization was carried out using the 5'-terminal fragment of the human brain cDNA coding for ChE, one major band of approximately 4.7 ± 0.5 kb was visible and was therefore presumed to contain DNA fragments complementary to the 5'-terminal domain of the ChE gene. The DNA fragment containing the 5'-terminus is of particular interest to us, since it should lead to the promotor region and possibly other sequences that might shed light on the regulation of ChE expression.

In order to isolate the 5'-terminal domain of the ChE gene, genomic DNA fragments cut with EcoRI were separated electrophoretically and the electrophoresed DNA fragments in the size range of 3.7-4.7 kilobase were electroeluted and concentrated by chromatography on a DEAE cellulose minicolumn. The enriched DNA was ligated with dephosphorylated EcoRI-digested lambda gt10 DNA and packaged using the Gigapack Plus packaging kit (Stratagen). Phage colonies containing DNA sequences which hybridized with [^{32}P]-labelled ChEcDNA were isolated and further characterized (73). Genomic double-stranded DNA fragments packaged in the lambda gt10 phages were then subjected to direct sequencing by the dideoxy Sanger technique using the enzyme reverse transcriptase and synthetic oligodeoxynucleotide primers. Sequencing was carried out essentially as described by Zagursky et al. (84), with minor modifications, or by use of the Klenow fragment of DNA polymerase. This was done using the M13 sequencing kit (Amersham), with minor adjustments in the reaction conditions.

III.D. Mapping of Human Cholinesterase Genes by In Situ Hybridization with Lymphocyte Chromosomes

Chromosome spreads from peripheral blood lymphocytes treated with 5-bromodeoxyuracil (85) were pre-incubated in 2xSSC for 30 min at 37°C. RNA was hydrolyzed by 60 min incubation at 37°C in 0.1 mg/ml of pancreatic ribonuclease (Sigma), followed by successive washes of 5 min in 2xSSC and 70, 80 and 100% ethanol. DNA was denatured by 4 min incubation at 70°C in 70% formamide, 2xSSC and 10 mM potassium phosphate buffer at a final pH of 7.0. The chromosome spreads were immediately transferred to frozen 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 µl drop of hybridization solution, containing 50% formamide, 10% dextran sulfate, 1 x Denhardt's solution, and 8 ng of the preboiled DNA probe, labelled by nick-translation with [³⁵S]adenosine and [³⁵S]-cytosine to a specific activity of 1x10⁸ cpm/µg and purified by three successive precipitations in ethanol, in the presence of 10 W:W of salmon sperm DNA as a carrier. Hybridization was for 18 hr at 37°C, in a humid chamber under cover slides. The chromosomes were then washed with 50% formamide and 2xSSC (1 hr, 37°C), 2xSSC (15 min, 37°C), 2xSSC and 20 mM β-mercaptoethanol (15 min, 37°C), 2xSSC (15 min, 50°C) and 0.15xSSC (15 min, 50°C), dehydrated by successive 5 min incubations in 70, 80 and 100% ethanol at room temperature and 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 D-19 Kodak developer.

Slides were then stained for 15 min in 150 µg/ml Hoechst 33258 (Aldrich), rinsed in distilled water and dried. To create the R-bands (85), stained slides were mounted in 2xSSC under coverslips and were illuminated for 30 min by a mercury vapour lamp at a 56 cm distance maintaining a temperature of 47°C-50°C, rinsed in distilled water and restained in 4% buffered Giemsa (Gurr-R-66) at pH 6.8. Results of experiments carried out with several ChEcDNA probes were essentially similar. High concentrations of grains were located on the chromosome regions 3q21-q26 and 16q11-16q23, while labelling on all other chromosomes was insignificant.

III.E. Translation In Vitro and In Ovo of the mRNA Species Coding for Human Cholinesterases

Fetal human tissues (16-22 weeks' gestation) were obtained within 2 hr of death, having been stored at 4°C. Samples were taken from abortion material with no apparent genetic or pathological defects, and the size and weight of the fetuses corresponded to gestational age in weeks. Brain, muscle and liver were carefully dissected on ice, immediately frozen in liquid nitrogen and stored at -70°C until used. In several studies, we confirmed that the yield and the translational efficiency of mRNA extracted from such tissues were similar to those obtained using parallel rodent tissues, dissected immediately after the animals were killed.

Polyadenylated RNA was phenol-extracted and purified by oligo(dT)-cellulose chromatography as described elsewhere (39,79). The purity and integrity of mRNA preparations were tested by agarose gel electrophoresis, followed by staining with ethidium bromide (86). General translational activity was examined in vitro, in the reticulocyte lysate cell-free system (87). The presence of intact ChEmRNAs was verified by the oocyte microinjection bioassay (39). Only mRNA preparations which appeared to contain intact poly(A)+RNA and to be translationally active in both translation systems were employed.

Translation in vitro was carried out in the nuclease-treated reticulocyte lysate cell-free system (87) and as previously described (88). mRNA-directed incorporation of [³⁵S]-methionine (730 Ci/mole, Radiochemical Center, Amersham) into polypeptides was determined by trichloroacetic acid precipitation. Reaction mixtures of 25.0 µl contained about 0.4 µg of mRNA and were incubated for 1 hr at 30°C. Phenylmethylsulfonylfluoride was then added to a final concentration of 1 mM, and samples were stored at -70°C until used.

Oocytes of adult Xenopus laevis females were injected as described previously (39,79), with 50 ng of mRNA together with 25 uCi/oocyte of lyophilized [³⁵S]-methionine. Incubation was in the presence of protease inhibitors, at 21°C for 24 hr (39). Incubation medium was then separated and oocytes homogenized in 100 µl/10 oocytes of a buffer containing 10 mM Tris-HCl pH 7.0, 1.0 M NaCl, 1% Triton X-100 and protease inhibitor mixture. Oocyte homogenates were centrifuged for 2 min in a microcentrifuge and the transparent interphase was collected. Oocyte fractions were stored at -70°C until used.

III.F. Crossed Immuno-electrophoretic Autoradiography of Nascent ChE

Nascent polypeptides were separated from in vitro translation mixtures and from homogenates and incubation medium of microinjected oocytes by crossed immuno-electrophoresis (89) as described previously (43,90). The mRNA translation products were applied to the wells together with human fetal plasma (2.5 µl, 30 weeks' gestation), which served as unlabelled carrier protein; we have recently found that human fetal plasma contains both AChE and BuChE activities. Intermediate gels contained 2.5 µl/cm² of rabbit anti-human erythrocyte membrane antibody (Dakopatts, Denmark) and top gels contained 2.5 µl/cm² of rabbit anti-human pseudocholinesterase antibody (Dakopatts, Denmark). Electrophoresis was in 1%, w/v agarose (Litex) in Tris-barbitone buffer at pH 8.6 on Plastic Gelbond (Marine Colloids), or glass 10 x 10 cm plates. First-dimension electrophoresis was carried out at 10 v/cm for 60 min. First dimension gel strips were then placed against intermediate and top gels and a voltage of 2 v/cm was applied overnight at room temperature (second dimension) in a direction at

right angles to that of the first dimension. Pressing and washing were according to Axelson (91), after which plates were air-dried and stained for cholinesterase (16). Autoradiograms were prepared by exposing dried plates to Agfa Curix RP film for 1-3 weeks. Plates from each experiment were all exposed together, so that the immunoprecipitated ChE curves within each set of autoradiograms reflect the relative amount of [^{35} S]-methionine incorporation into ChE immunoreactive polypeptides.

IV. CURRENT STATE OF EXPERIMENTAL OBSERVATIONS

IV.A. cDNA Screening and Sequence Analysis of Positively Hybridizing Phages

In an attempt to isolate ChEcDNA clones, four screens were performed on a fetal brain cDNA library using the OPSYN probes (51). The findings obtained in one of these screens are described below as an example for the procedures involved. The screen using the oligonucleotide probe mixture designated OPSYN II yielded two positively hybridizing phages, neither of which formed stable hybrids with the longer OPSYN0 probe [(51); see Table 1 for the detailed sequences included in each of these probes.] In order to determine whether these cDNAs contained the nucleotide sequence coding for the active site hexapeptide and to examine the hybridization properties of the isolated cDNAs, OPSYN II-hybridizing fragments derived from phages isolated from the OPSYN II screen were inserted into M13 single-stranded phages and their nucleotide sequences determined. Both sequences proved to contain open reading frames. The sequence of the OPSYN-II hybridizing region of clone 7b and its translation in an open reading frame were as follows:

```

0                                     51
AGG TGC GCA GGC CGA TTC TCC AAA GAA CTT GCA TTT AAG CTA AGG TGC GCA
Arg Cys Ala Gly Arg Phe Ser Lys Glu Lys Ala Phe Lys Leu Arg Cys Ala

52                                     84
GGC CGA TTC TCC AAA GAA CTT GCA TTT AAG CTT
Gly Arg Phe Ser Lys Glu Leu Ala Phe Lys Leu

```

Thus the mRNA from which this clone was reverse-transcribed codes for a protein which does not contain the ChE active site hexapeptide. Comparison of the nucleotide sequence in this clone with that of the OPSYN II probe revealed a base-pairing with a single mismatch, as shown in Table 2.

TABLE 2

Proposed base pairing probe-DNA, clone 7bxOPSYN II. See text for details.

TABLE 2

Proposed Base Pairing Probe-DNA, Clone 7bxOPSYN II

5'→3' PROBE	C	C	G	G	C	C	G	A	T	T	C	T	C	C	A	A	A
3'→5' DNA	G	T	C	C	G	G	C	T	A	A	G	A	G	G	T	T	T

The sequence of the OPSYN II - hybridizing region of clone 24 and its translation in an open reading frame were as follows:

```

0                                     51
AAG CAT AGC CAT CAG TTA GAA GTT TTT TAT TTT TGG GGA TGT CGG CAG GAG
Lys His Ser His Gln Leu Glu Val Phe Tyr Phe Trp Gly Cys Arg Gln Glu

52                                     96
GAA TTT CCT TTA AAG GAG CAT ATA TAT ACG TCA GGA TTT GTC TTA
Glu Phe Pro Leu Lys Glu His Ile Tyr Thr Ser Gly Phe Val Leu

```

In this case as well, the ChE active site hexapeptide is not included in the protein encoded by the positively hybridizing cDNA, and the proposed base-pairing of the hybrids contained a single unpaired base (Table 3).

TABLE 3

Proposed base pairing probe-DNA, clone 24xOPSYN II. See text for details.

TABLE 3

Proposed Base Pairing Probe-DNA, Clone 24xOPSYN II

```

5'→3' PROBE C C T G C C G A C T C C C C A A A
3'→5' DNA   G G A C G G C T G A G G G G T T T
                    T
                    *

```

IV.B. Cholinesterase mRNAs Revealed by RNA Blot Hybridization and *Xenopus* Oocyte Microinjection

To study the in vivo biosynthesis of the protein encoded by these clones, the cDNA inserts were [^{32}P]-labelled and hybridized with human RNA and DNA. In low-stringency RNA blots loaded with 10 μg of poly(A)+RNA/lane, [^{32}P]-labelled FBChE12 interacted with a single 2.5 kb band of RNA, of a similar size as the FL39 clone. This mRNA was present in fetal brain and liver, but not in the cholinesterase-deficient human epidermoid (HEp) carcinoma, which does not express any type of cholinesterase activity (39). The exposure time needed to visualize the hybridization was relatively long (10 days), suggesting that cholinesterase mRNA exists at rather low concentrations even in positive tissue sources such as fetal brain and liver.

The levels of the mRNAs coding for particular types of cholinesterase in fetal brain and liver were analyzed in parallel by mRNA microinjection into *Xenopus* oocytes, where AChEmRNA and BuChEmRNA are translated to yield their catalytically active enzyme products (39). Considerable production of iso-OMPA-insensitive AChE was observed in oocytes injected with either fetal brain or liver RNA but not with HEpRNA. In contrast, only liver mRNA was capable of producing significant levels of BW284C51-insensitive BuChE (51). Thus the pattern revealed in the RNA blot hybridization is compatible with the levels of fetal AChEmRNA, or with both species of cholinesterase mRNA together, but not with BuChEmRNA alone.

IV.C. Comparative Analysis of Cholinesterase cDNAs of Various Genetic Origins

1. Primary Structure of Human Cholinesterase cDNA

In addition to the false-positive hybrids detailed above, the initial screening procedure described under Technical Approaches and Methodology also resulted in the isolation of a single true-positive, in the form of a fetal brain cDNA clone, 765 nucleotides in length, designated FBChE12. This cDNA clone hybridized with both OPSYN and OPSYNO probes (51). The nucleotide sequence of FBChE12 that is complementary to probes OPSYN and OPSYNO corresponded exactly to the peptide sequence used to design these oligodeoxynucleotide probes (Fig. 1, amino acid residues encoded by nucleotides 742-759 and 742-771, respectively). FBChE12 was then used as a probe to screen the fetal brain and liver cDNA libraries. Four clones of 2.4 kb in length were isolated from the fetal liver library and one of these, designated FL39, was further characterized in comparison with FBChE12. It was found that both clones contained an identical sequence of 693 nucleotides, with the 5'-end of the FL39 insert starting at nucleotide No. 73 of FBChE12 (Fig. 1), suggesting that both cDNAs were derived from similar mRNA transcripts (52). When the amino acids predicted from the FBChE12 and the FL39 sequence are aligned with the available peptide sequence of human BuChE (72), the entire coding region for the mature enzyme is defined, starting at residue 1 (nucleotide 150), which corresponds to the N-terminal peptide, and ending at residue 574 (nucleotide 1881), which is the last amino acid residue in the C-terminal tryptic peptide of BuChE as determined from amino acid sequencing (56). This sequence also includes the active site tryptic peptide of human ChE, which contains a serine residue that can be labelled by DFP (71) (Fig. 1, enclosed by a hexagon). The polypeptide inferred by the FBChE12 and the FL39 sequences is identical to the BuChE polypeptide. In contrast, the amino acid composition of the FL39-coded protein clearly differed from the parallel composition derived for erythrocyte AChE (92). In addition, the N-

terminus of the cholinesterase encoded by FBChE12 and FL39 differs from the peptide reported for erythrocyte AChE (73). Altogether, this proves that both FBChE12 and FL39 code for BuChE. It should be noted that the amino acid sequence of nervous system ChEs has not been approached as yet, because of difficulties in purifying sufficient quantities of the active proteins. Thus there is no indication at present regarding correlations between the isolated ChEcDNA and nervous system ChEs in humans.

FIGURE 1 - Primary structure of the fetal human cholinesterase encoded by FBChE12 and FL39.

The 2.4 kilobase composite nucleotide sequence of clones FBChE12 and FL39 (52) was translated into its encoded amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, and the predicted amino acids are shown below the corresponding nucleotide sequence. Boxed sequences indicate three amino acid sequences that were found to match peptides present in human serum BuChE as shown by peptide sequencing (71). These are the N-terminal peptide (nucleotides 160-228), the active-site peptide [nucleotides 730-765, with a hexagon enclosure indicating the active-site Ser residue No. 198, where the count of amino acid residues begins at the N-terminal Glu residue, nucleotide no. 160-166 in Fig. 1, according to the peptide sequencing results of the mature serum protein (71)] and the C-terminal peptide (nucleotides 1864-1881). The amino acid sequence of the active-site peptide served as a basis for designing the oligodeoxynucleotide probes with which these cDNA clones were selected (Table 1). Also boxed are a presumptive ribosome binding site (nucleotides 30-36) and a signal peptide (nucleotides 88-150). Three polar amino acid residues appear at both ends of the signal peptide (93), and seven potential sites for N-linked glycosylation (starting at nucleotides 208, 475, 880, 925, 1180, 1600 and 1615), predicted by the sequence Asn-X-Thr/Ser, where X represents any amino acid except proline (94), are boxed. His 77 and Asp 129, which are the best candidates to be involved in the active site by comparison with other serine esterases (95), are enclosed by hexagons. The FL39 sequence also includes a long 3'-untranslated region, ending with a polyadenylation site and a poly(A)-tail. The cysteine residues in the coding sequence are circled.

Figure 1. Primary structure of the fetal human cholinesterase encoded by FBChE12 and FL39.

```

ATT TCC CCG AAC TAT TAC ATG ATT TTC ACT CCT TGC AAA GTT TGC CAT CTT TGT TGC AGA
30
GAA TCG GAA ATC AAT ATG CAT AGC AAA GTC ACA ATC ATA TGC ATC AGA TTT CTC TTT TGG
90
Phe Leu Leu Leu Cys Met Met His Ser Lys Val Thr Ile Ile Cys Ile Arg Phe Leu Phe Trp
120
TTT CTT TTG CTC TGC ATG CTT ATT GGG AAA TCA CAT ACT GAA GAT GAC ATC ATA ATT GCA
150
Phe Leu Leu Leu Cys Met Leu Ile Gly Lys Ser His Thr Glu Asp Asp Ile Ile Ile Ala
180
ACA AAG AAT GGA AAA GTC AGA GGG ATG AAC TTG ACA GTT TTT GGT GGC ACG GTA ACA
210
Thr Lys Asn Gly Lys Val Arg Gly Met Asn Leu Thr Val Phe Gly Gly Thr Val Thr Ala
240
TTT CTT GGA ATT CCC TAT GCA CAG CCA CCT CTT GGT AGA CTT CGA TTC AAA AAG CCA
270
Phe Leu Gly Ile Pro Tyr Ala CAG Pro Pro Leu Gly Arg Leu Arg Phe Lys Lys Pro Gln
300
TCT CTG ACC AAG TGG TCT GAT ATT TGG AAT GCC ACA AAA TAT GCA AAT TCT TGC TGT
330
Ser Leu Thr Lys Trp Ser Asp Ile Trp Asn Ala Thr Lys Tyr Ala Asn Ser Cys Cys Gln
360
AAC ATA GAT CAA AGT TTT CCA GGC TTC CAT GGA TCA GAG ATG TGG AAC CCA AAC ACT
390
Asn Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn Pro Asn Thr Asp
420
CTC AGT GAA GAC TGT TTA TAT CTA AAT GTA TGG ATT CCA GCA CCT AAA CCA AAA AAT GCC
450
Leu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp Ile Pro Ala Pro Lys Pro Lys Asn Ala
480
ACT GTA TTG ATA TGG ATT TAT GGT GGT GGT TTT CAA ACT GGA ACA TCA TCT TTA CAT
510
Thr Val Leu Ile Trp Ile Tyr Gly Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val
540
TAT GAT GGC AAG TTT CTG GCT CGG GTT GAA AGA GTT ATT GTA GTG TCA ATG AAC TAT
570
Tyr Asp Gly Lys Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg
600
GTG GGT GCC CTA GGA TTC TTA GCT TTG GGA AAT CCT GAG GCT CCA GGG AAC ATG
630
Val Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro Gly Asn Met Gly
660
TTA TTT GAT CAA CAG TTG GCT CTT CAG TGG GTT CAA AAA AAT ATA GCA GCC TTT GGT
690
Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln Lys Asn Ile Ala Ala Phe Gly Gly
720
AAT CCT AAA AGT GTA ACT CTC TTT GGA GAA AGT GCA GGA GCA GCT TCA GTT AGC CTG
750
Asn Pro Lys Ser Val Thr Leu Phe Gly Glu Ser Ala Gly Ala Ala Ser Val Ser Leu His
780
TTG CTT TCT CCT GGA AGC CAT TCA TTG TTC ACC AGA GCC ATT CTG CAA AGT GGA TCC
810
Leu Leu Ser Pro Gly Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Phe
840
AAT GCT CCT TGG GCG GTA ACA TCT CTT TAT GAA GCT AGG AAC AGA ACG TTG AAC TTA
870
Asn Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr Leu Asn Leu Ala
900
AAA TTG ACT GGT TGC TCT AGA GAG AAT GAG ACT GAA ATA ATC AAG TGT CTT AGA AAT
930
Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu Ile Ile Lys Cys Leu Arg Asn Lys
960
GAT CCC CAA GAA ATT CTT CTG AAT GAA GCA TTT GTT GTC CCC TAT GGG ACT CCT TTG
990
Asp Pro Gln Glu Ile Leu Leu Asn Glu Ala Phe Val Val Pro Tyr Gly Thr Pro Leu Ser
1020
GTA AAC TTT GGT CCG ACC GTG GAT GGT GAT TTT CTC ACT GAC ATG CCA GAC ATA TTA
1050
Val Asn Phe Gly Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu
1080
GAA CTT GGA CAA TTT AAA AAA ACC CAG ATT TTG GTG GGT GTT AAT AAA GAT GAA GGG
1110
Glu Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys Asp Glu Gly Thr
1140

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Figure 1. Primary structure of the fetal human cholinesterase encoded by FBChE12 and FL39 (continued).

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1170
TTT TTA GTC TAT GGT GCT CCT GGC TTC AGC AAA GAT AAC AAT AGT ATC ATA ACT AGA
Phe Leu Val Tyr Gly Ala Pro Gly Phe Ser Lys Asp Asn Asn Ser Ile Ile Thr Arg
1200

1230
GAA TTT CAG GAA GGT TTA AAA ATA TTT TTT CCA GGA GTG AGT GAG TTT GGA AAG GAA
Glu Phe Gln Glu Gly Leu Lys Ile Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu
1260

1290
ATC CTT TTT CAT TAC ACA GAC TGG GTA GAT GAT CAG AGA CCT GAA AAC TAC CGT GAG
Ile Leu Phe His Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu
1320

1350
TTG GGT GAT GTT GTT GGG GAT TAT AAT TTC ATA TGC CCT GCC TTG GAG TTC ACC AAG
Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu Glu Phe Thr Lys
1380

1410
TTC TCA GAA TGG GGA AAT AAT GCC TTT TTC TAC TAT TTT GAA CAC CGA TCC TCC AAA
Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr Tyr Phe Glu His Arg Ser Ser Lys
1440

1470
CCG TGG CCA GAA TGG ATG GGA GTG ATG CAT GGC TAT GAA ATT GAA TTT GTC TTT GGT
Pro Trp Pro Glu Trp Met Gly Val Met His Gly Tyr Glu Ile Glu Phe Val Phe Gly
1500

1530
CCT CTG GAA AGA AGA GAT AAT TAC ACA AAA GCC GAG GAA ATT TTG AGT AGA TCC ATA
Pro Leu Glu Arg Arg Asp Asn Tyr Thr Lys Ala Glu Glu Ile Leu Ser Arg Ser Ile
1560

1590
AAA CGG TGG GCA AAT TTT GCA AAA TAT GGG AAT CCA AAT GAG ACT CAG AAC AAT AGC
Lys Arg Trp Ala Asn Phe Ala Lys Tyr Gly Asn Pro Asn Glu Thr Gln Asn Asn Ser
1620

1650
AGC TGG CCT GTC TTC AAA AGC ACT GAA CAA AAA TAT CTA ACC TTG AAT ACA GAG TCA
Ser Trp Pro Val Phe Lys Ser Thr Glu Gln Lys Tyr Leu Thr Leu Asn Thr Glu Ser
1680

1710
AGA ATA ATG ACG AAA CTA CGT GCT CAA CAA TGT CGA TTC TGG ACA TCA TTT TTT CCA
Arg Ile Met Thr Lys Leu Arg Ala Gln Gln Cys Arg Phe Trp Thr Ser Phe Phe Pro
1740

1770
GTC TTG GAA ATG ACA GGA AAT ATT GAT GAA GCA GAA TGG GAG TGG AAA GCA GGA TTC
Val Leu Glu Met Thr Gly Asn Ile Asp Glu Ala Glu Trp Glu Trp Lys Ala Gly Phe
1800

1830
CGC TGG AAC AAT TAC ATG ATG GAC TGG AAA AAT CAA TTT AAC GAT TAC ACT AGC AAG
Arg Trp Asn Asn Tyr Met Met Asp Trp Lys Asn Gln Phe Asn Asp Tyr Thr Ser Lys
1860

1890
GAA AGT TGT GTG GGT CTC TAA TTA ATA GAT TTA CCC TTT ATA GAA CAT ATT TTC CTT
Glu Ser Cys Val Gly Leu
1920

1950
ATC AAG GCA AAA ATA TCA GGA GCT TTT TTA CAC ACC TAC TAA AAA AGT TAT TAT GTA
1980

2010
GAA ACA AAA ATG CCA GAA GGA TAA TAT TGA TTC CTC ACA TCT TTA ACT TAG TAT TTT
2040

2070
TAG CAT TTC AAA ACC CAA ATG GCT AGA ACA TGT TTA ATT AAA TTT CAC AAT ATA AAG
2100

2130
TAC AGT TAA TTA TGT GCA TAT TAA AAC AAT GGC CTG GTT CAA TTT CTT TCT TTC CTT
2160

2190
AAA TTT AAG TTT TTT CCC CCC AAA ATT ATC AGT GCT CTG CTT TTA GTC ACG TGT ATT
2220

2250
ATT ACC ACT CGT AAA AAG GTA TCT TTT TTA AAT GAA GTT AAA TAT TGA AAC ACT GTA
2280

2310
CAT AGT TTA CAA TAA TTA GTG TTT CCT AAG TTA AAA TAA GAA TTG AAT GTC AAT AAT
2340

2370
AAT AAT TAA AAT AAG CAC AGA AAA TCA CAA AAA AAA ACA AAA AAA AAA AAA AAA
2400

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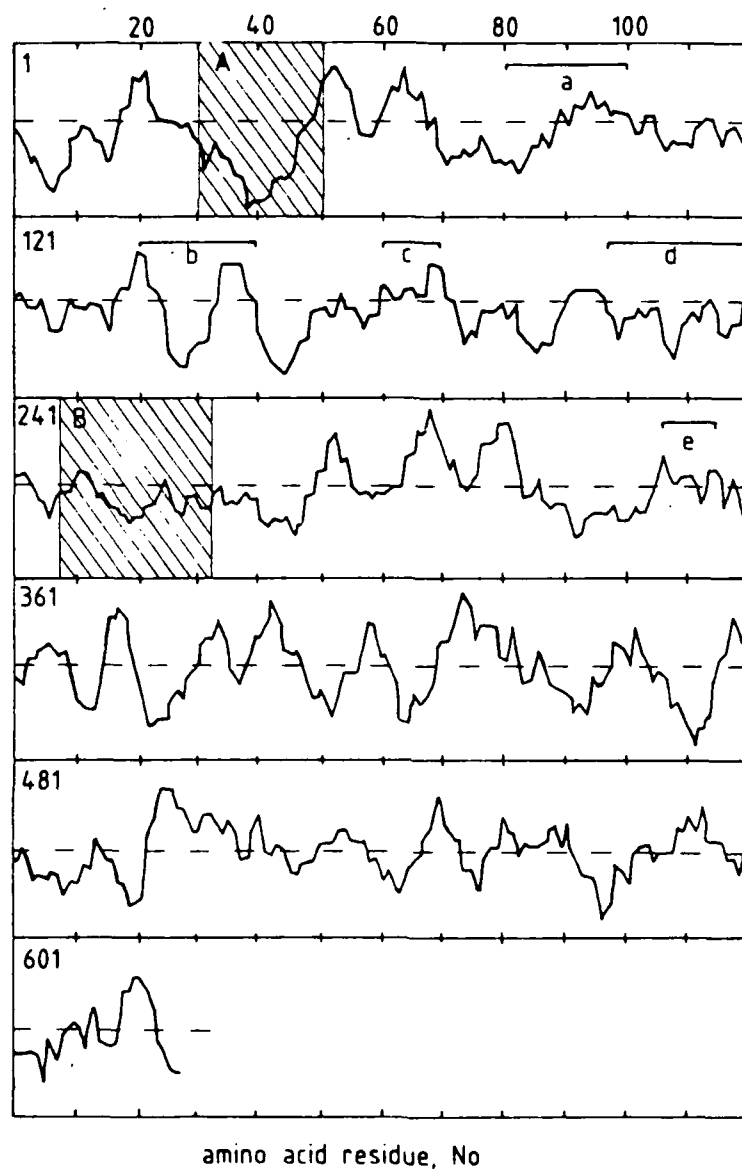
The region upstream of the BuChE amino-terminal residue (nucleotides 88-150) in FBChE12 codes for 20 amino acids characteristic of leader peptides of membrane-associated and exported protein precursors (96). The hydrophobic sequence in this region is rich in large nonpolar amino acids. It is preceded by the tripeptide His Ser Lys, and terminates with Lys-Ser-His, both composed of polar amino acids. Further upstream, the cDNA sequence consists of a fully open reading frame without stop codons, and includes a presumptive ribosome binding site and an additional ATG triplet (Fig. 1), perhaps indicating that this mRNA is subject to translational control at the level of initiation.

To examine further the molecular properties of the protein encoded by the human cholinesterase cDNA, we subjected it to hydrophobicity analysis according to Hopp and Woods (97). The results of this analysis are presented in Fig. 2. The hydrophobicity pattern is consistent with a globular protein, in which a clearly hydrophobic region, that of the signal peptide, can be observed (Fig. 2).

FIGURE 2 - Hydrophobicity pattern of the complete cholinesterase protein.

Represented is the prediction of the hydrophobic and hydrophilic regions of human ChE protein, using the algorithm of Hopp and Woods (97). The baseline represents a hydrophilicity value of 0, increasing hydrophilicity is in the upward direction and increased hydrophobicity is in the downward direction. "A" represents the putative signal peptide, "B" the active site region; and a,b,c,d and e represent regions that show high homology to the amino acid sequence of bovine thyroglobulin (see text for details). These are possible epitopes for autoimmune antibodies (see Discussion).

Figure 2. Hydrophobicity pattern of the complete cholinesterase protein.



2. Comparison of Human cholinesterase cDNA to cDNAs of Other Proteins

The coding region in the cDNA and inferred amino acid sequence of the FL39 clone were compared to the parallel sequences recently published for cDNA clones coding for AChE from Torpedo californica electric organ (41) and from Drosophila melanogaster (98). This analysis revealed considerable homologies between the corresponding parts of the cholinesterases from Torpedo californica, Drosophila melanogaster and human, strongly suggesting that they have a common ancestral origin. A higher level of conservation was consistently found at the amino acid level than at the DNA level. Significant homology was also observed with the DNA and the amino acid sequence of bovine thyroglobulin (99). These homologies are presented in Fig. 3a-d as computer-derived matrices. The intrinsic differences between the cDNAs encoding these proteins have also been approached by analysing their nucleotide composition and distribution of codons and dinucleotides, as shown in Table 4. In contrast with the pronounced species-specific differences in codon usage and nucleotide frequencies, the amino acid composition of the various cholinesterases remained surprisingly conserved, as displayed in Table 5.

FIGURE 3 - Nucleotide and amino acid matrix homologies between the coding regions of cDNAs for cholinesterases from human, Torpedo, Drosophila, and part of bovine thyroglobulin.

Nucleotide (a) and amino acid sequence (b) data for FL39 (52) were compared with the parallel sequences published for a cDNA clone coding for AChE from Torpedo electric organ (4). Regions of homologies were searched for by the dot matrix approach (100) as modified by Unger and Sussman (personal communication). Match values that yielded clear homology regions and minimal background noise are presented (12 out of 15 matches for nucleotide sequence and 4 out of 5 matches for amino acid residues). Nucleotides are numbered in the 5' to 3' directions and amino acids in the N' to C' directions for the cDNAs in a, b, c and d. The homologies start from around nucleotide 110 in the FBChE12 region that matches the beginning of the Torpedo cDNA clone. Note the presence of regions where both the nucleotide sequence and the primary structure of amino acids are homologous (see, for example, nucleotides 1450-1500 in the human cholinesterase cDNA), as compared with regions with amino acid similarities but no nucleotide match (such as nucleotides 1000-1050 in the human cDNA) and with the short domains where the similarities in both nucleotide and amino acid sequences were lower than the match frequency of choice (for example, nucleotides 310-340 in the human cDNA). Parallel analyses are presented for cDNAs coding for Drosophila AChE (98) and for bovine thyroglobulin (99). Arrow denotes an insert in the Drosophila AChE protein that is missing from the human ChE protein.

Figure 3. Nucleotide and amino acid matrix homologies between the coding regions of cDNAs for cholinesterases from human, Torpedo, Drosophila and part of bovine thyroglobulin.

Fig. 3a.

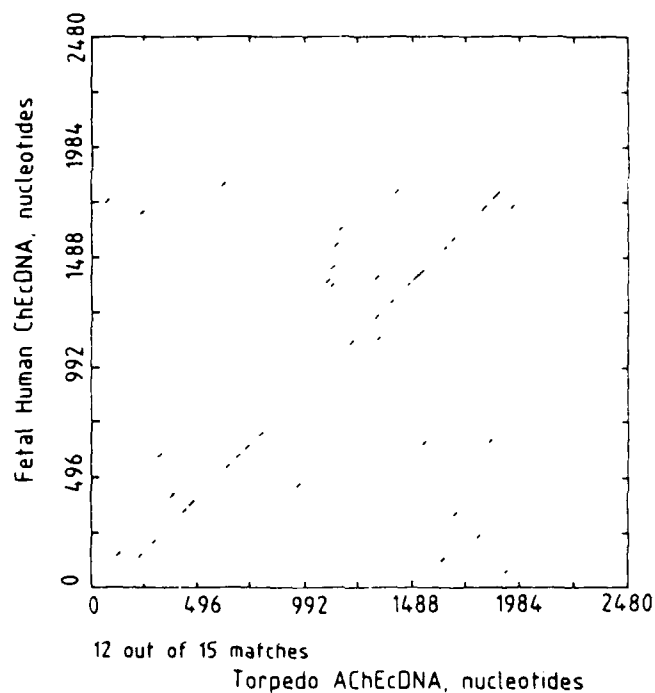


Fig. 3b.

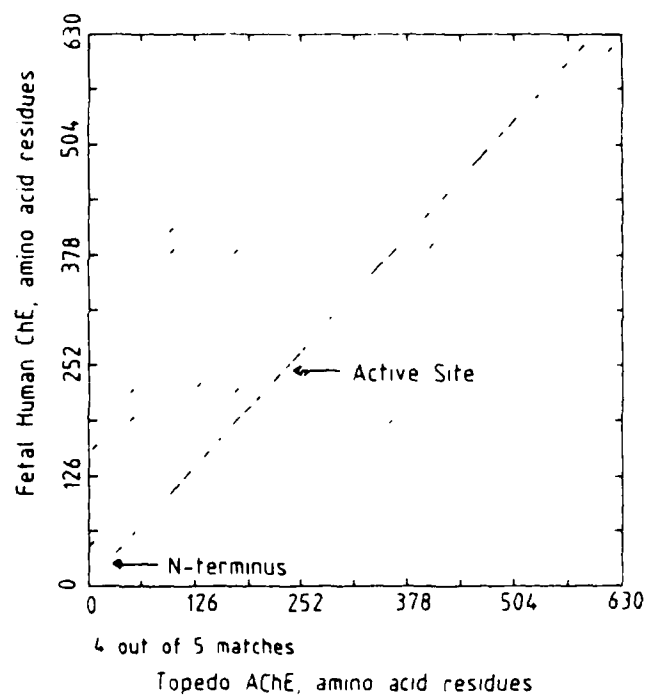


Figure 3. Nucleotide and amino acid matrix homologies between the coding regions of cDNAs for cholinesterases from human, Torpedo, Drosophila and part of bovine thyroglobulin (continued).

Fig. 3c

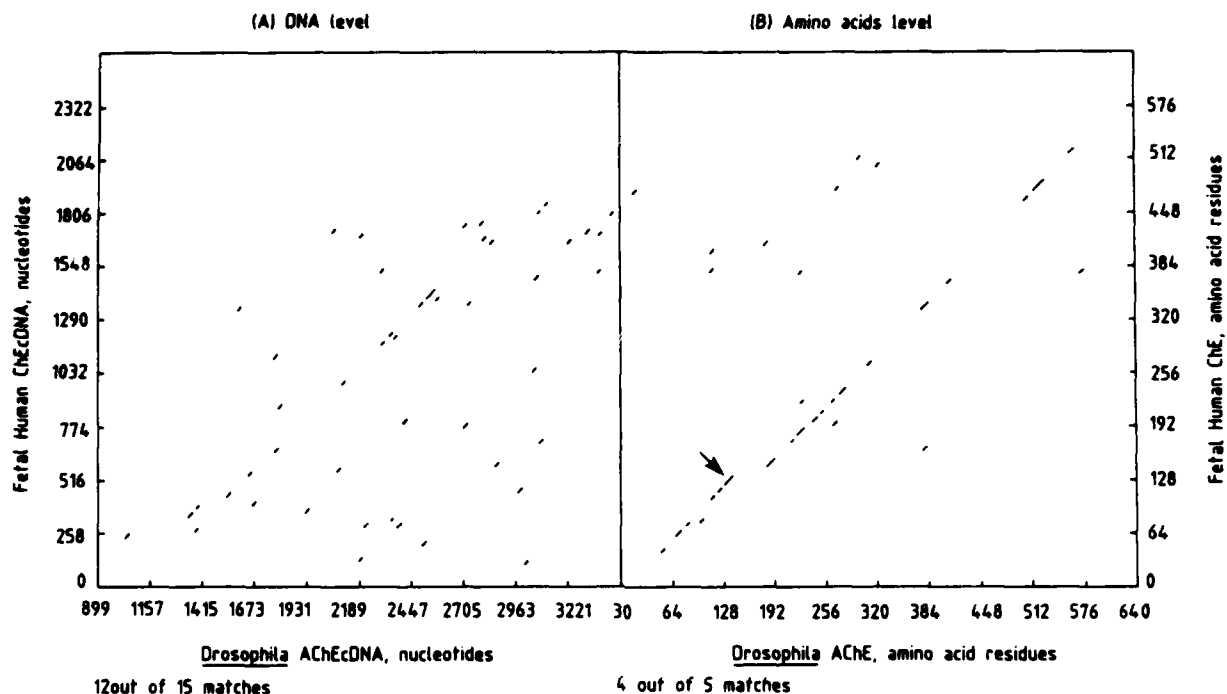


Fig. 3d

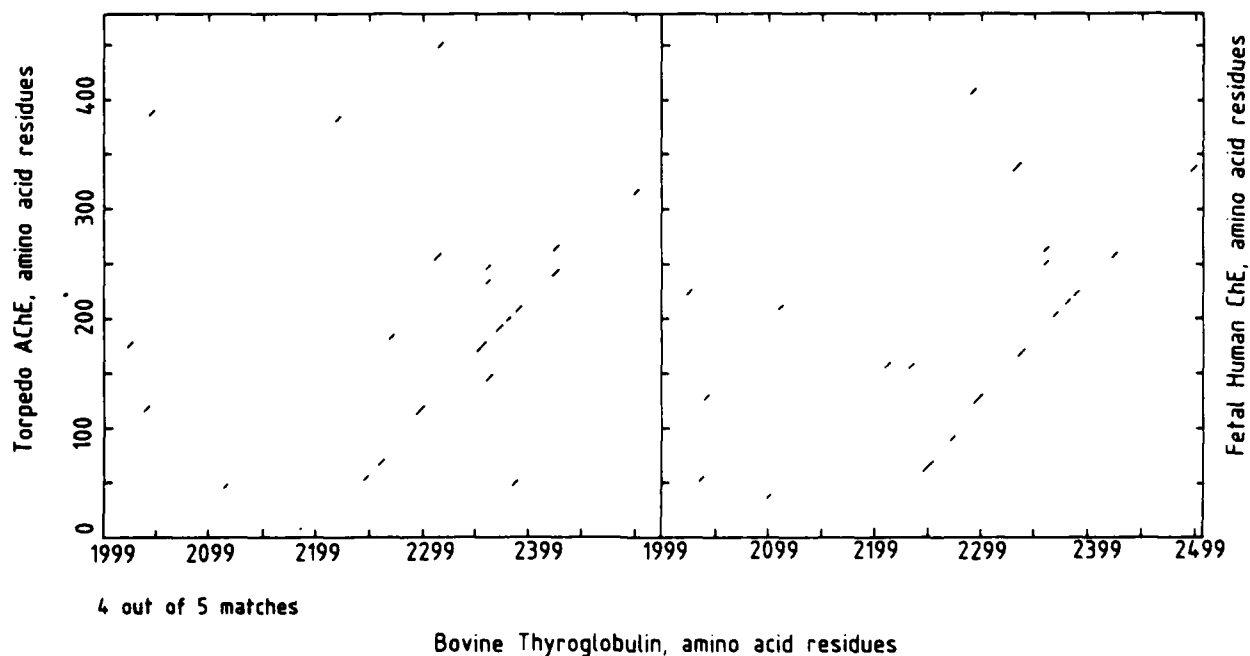


TABLE 4

Nucleotide compositions and frequencies in various cholinesterase cDNAs. Data were derived by computerized analysis of the specific cDNA sequences coding for human, Torpedo, and Drosophila ChEs. See text for details regarding the sources of the sequences.

TABLE 4
Nucleotide Compositions and Frequencies in Various Cholinesterase cDNAs

I. Human ChEcDNA mature protein encoding sequence

The sequence contains 1722 nucleotides		Nucleotide No.	%
		A	535 (31.1)
		C	308 (17.9)
		G	388 (22.5)
		T	491 (28.5)
		A + T	1026 (59.6)
		C + G	696 (40.4)

The dinucleotide frequency is

AA	195	(11.3)	CA	118	(6.9)	GA	139	(8.1)	TA	83	(4.8)
AC	84	(4.9)	CC	68	(4.0)	GC	60	(3.5)	TC	96	(5.6)
AG	117	(6.8)	CG	17	(1.0)	GG	105	(6.1)	TG	148	(8.6)
AT	139	(8.1)	CT	104	(6.0)	GT	84	(4.9)	TT	89	(9.5)

I_b Distribution of codons in the open reading frame

TTT	Phe	27(4.7)	TCT	Ser	7(1.2)	TAT	Tyr	14(2.4)	TGT	Cys	5(0.9)
TTC	Phe	12(2.1)	TCC	Ser	5(0.9)	TAC	Tyr	6(1.0)	TGC	Cys	3(0.5)
TTA	Leu	9(1.6)	TCA	Ser	9(1.6)	TAA	End	0(0.0)	TGA	End	0(0.0)
TTG	Leu	15(2.6)	TCG	Ser	0(0.0)	TAG	End	0(0.0)	TGG	Trp	18(3.1)
CTT	Leu	12(2.1)	CCT	Pro	12(2.1)	CAT	His	8(1.4)	CGT	Arg	2(0.3)
CTC	Leu	4(0.7)	CCC	Pro	3(0.5)	CAC	His	1(0.2)	CGC	Arg	1(0.2)
CTA	Leu	4(0.7)	CCA	Pro	13(2.3)	CAA	Gln	10(1.7)	CGA	Arg	3(0.5)
CTG	Leu	6(1.0)	CCG	Pro	2(0.3)	CAG	Gln	9(1.6)	CGG	Arg	2(0.3)
ATT	Ile	12(2.1)	ACT	Thr	13(2.3)	AAT	Asn	25(4.4)	AGT	Ser	9(1.6)
ATC	Ile	5(0.9)	ACC	Thr	6(1.0)	AAC	Asn	13(2.3)	AGC	Ser	7(1.2)
TAT	Ile	11(1.9)	ACA	Thr	15(2.6)	AAA	Lys	25(4.4)	AGA	Arg	13(2.3)
ATG	Met	11(1.9)	ACG	Thr	3(0.5)	AAG	Lys	8(1.4)	AGG	Arg	3(0.5)
GTT	Val	10(1.7)	GCT	Ala	11(2.3)	GAT	Asp	17(3.0)	GGT	Gly	17(3.0)
GTC	Val	6(1.0)	GCC	Ala	10(1.7)	GAC	Asp	7(1.2)	GGC	Gly	5(0.9)
GTA	Val	8(1.4)	GCA	Ala	12(2.1)	GAA	Glu	26(4.5)	GGA	Gly	18(3.1)
GTG	Val	8(1.4)	GCG	Ala	1(0.2)	GAG	Glu	11(1.9)	GGG	Gly	6(1.0)

(Table continued next page)

TABLE 4
Nucleotide compositions and frequencies in various cholinesterase cDNAs (continued).

TABLE 4 (continued)

II_a *Torpedo californica* ChEcDNA mature protein-encoding sequence

The sequence contains 1725 nucleotides:			Nucleotide	No.	%
			A	417	(24.2)
			C	460	(26.7)
			G	481	(27.9)
			T	367	(21.3)
			A + T	784	(45.4)
			C + G	941	(54.6)

The dinucleotide frequency is

AA	101	(5.9)	CA	133	(7.7)	GA	148	(8.6)	TA	35	(2.0)
AC	121	(7.0)	CC	120	(7.0)	GC	96	(5.6)	TC	123	(7.1)
AG	130	(7.5)	CG	84	(4.9)	GG	146	(8.5)	TG	120	(7.0)
AT	65	(3.8)	CT	123	(7.1)	GT	90	(5.2)	TT	89	(5.2)

II_b Distribution of codons in the open reading frame

TTT	Phe	11(1.9)	TCT	Ser	10 (1.7)	TAT	Tyr	2 (0.3)	TGT	Cys	4 (0.7)
TTC	Phe	25(4.3)	TCC	Ser	7 (1.2)	TAC	Tyr	17 (3.0)	TGC	Cys	4 (0.7)
TTA	Leu	2(0.3)	TCA	Ser	5 (0.9)	TAA	End	0 (0.0)	TGA	End	0 (0.0)
TTG	Leu	8(1.4)	TCG	Ser	5 (0.9)	TAG	End	0 (0.0)	TGG	Trp	17 (3.0)
CTT	Leu	4(0.7)	CCT	Pro	7 (1.2)	CAT	His	2 (0.3)	CGT	Arg	0 (0.0)
CTC	Leu	18(3.1)	CCC	Pro	11 (1.9)	CAC	His	16 (2.8)	CGC	Arg	3 (0.5)
CTA	Leu	2(0.3)	CCA	Pro	5 (0.9)	CAA	Gln	1 (0.2)	CGA	Arg	4 (0.7)
CTG	Leu	17(3.0)	CCG	Pro	8 (1.4)	GAG	Gln	17 (3.0)	CGG	Arg	5 (0.9)
ATT	Ile	6(1.0)	ACT	Thr	3 (0.5)	AAT	Asn	8 (1.4)	AGT	Ser	5 (0.9)
ATC	Ile	11(1.9)	ACC	Thr	9 (1.6)	AAC	Asn	30 (5.2)	AGC	Ser	15 (2.6)
ATA	Ile	3(0.5)	ACA	Thr	7 (1.2)	AAA	Lys	7 (1.2)	AGA	Arg	6 (1.0)
ATG	Met	18(3.1)	ACG	Thr	4 (0.7)	AAG	Lys	19 (3.3)	AGG	Arg	9 (1.6)
GTT	Val	7(1.2)	GCT	Ala	6 (1.0)	GAT	Asp	5 (0.9)	GGT	Gly	5 (0.9)
GTC	Val	20(3.5)	GCC	Ala	9 (1.6)	GAC	Asp	23 (4.0)	GGC	Gly	16 (2.8)
GTA	Val	1(0.2)	GCA	Ala	4 (0.7)	GAA	Glu	11 (1.9)	GGA	Gly	12 (2.1)
GTG	Val	12(2.1)	GCG	Ala	6 (1.0)	GAG	Glu	29 (5.0)	GGG	Gly	12 (2.1)

(Table continued next page)

TABLE 4
Nucleotide compositions and frequencies in various cholinesterase cDNAs (continued).

TABLE 4 (continued)

III. *Drosophila melanogaster* ChEcDNA protein encoding sequence

The sequence contains 1945 nucleotides:			Nucleotide	No.	%
			A	406	(20.9)
			C	576	(29.6)
			G	567	(29.2)
			T	396	(20.4)
			A + T	802	(41.2)
			C + G	1143	(58.8)

The dinucleotide frequency is

AA	89	(4.6)	CA	133	(6.8)	GA	136	(7.0)	TA	48	(2.5)
AC	109	(5.6)	CC	160	(8.2)	GC	176	(9.1)	TC	131	(6.7)
AG	94	(4.8)	CG	153	(7.9)	GG	174	(9.0)	TG	145	(7.5)
AT	114	(5.9)	CT	130	(6.7)	GT	81	(4.2)	TT	71	(3.7)

II. Distribution of codons in the open reading frame

TTT	Phe	6	(0.9)	TCT	Ser	1	(0.2)	TAT	Tyr	7	(1.1)	TGT	Cys	1	(0.2)
TTC	Phe	21	(3.2)	TCC	Ser	19	(2.9)	TAC	Tyr	19	(2.9)	TGC	Cys	10	(1.5)
TTA	Leu	1	(0.2)	TCA	Ser	2	(0.3)	TAA	End	0	(0.0)	TGA	End	0	(0.0)
TTG	Leu	8	(1.2)	TCG	Ser	11	(1.7)	TAG	End	0	(0.0)	TGG	Trp	15	(2.3)
CTT	Leu	2	(0.3)	CCT	Pro	5	(0.8)	CAT	His	4	(0.6)	CGT	Arg	4	(0.6)
CTC	Leu	9	(1.4)	CCC	Pro	19	(2.9)	CAC	His	11	(1.7)	CGC	Arg	11	(1.7)
CTA	Leu	2	(0.3)	CCA	Pro	3	(0.5)	CAA	Gln	3	(0.5)	CGA	Arg	5	(0.8)
CTG	Leu	32	(4.9)	CCG	Pro	13	(2.0)	CAG	Gln	20	(3.1)	CGG	Arg	4	(0.6)
ATT	Ile	7	(1.1)	ACT	Thr	4	(0.6)	AAT	Asn	15	(2.3)	AGT	Ser	5	(0.8)
ATC	Ile	23	(3.5)	ACC	Thr	17	(2.6)	AAC	Asn	18	(2.8)	AGC	Ser	6	(0.9)
ATA	Ile	4	(0.6)	ACA	Thr	4	(0.6)	AAA	Lys	5	(0.8)	AGA	Arg	1	(0.2)
ATG	Met	19	(2.9)	ACG	Thr	9	(1.4)	AAG	Lys	17	(2.6)	AGG	Arg	7	(1.1)
GTT	Val	2	(0.3)	GCT	Ala	11	(1.7)	GAT	Asp	19	(2.9)	GGT	Gly	8	(1.2)
GTC	Val	14	(2.2)	GCC	Ala	24	(3.7)	GAC	Asp	14	(2.2)	GGC	Gly	30	(4.6)
GTA	Val	2	(0.3)	GCA	Ala	6	(0.9)	GAA	Glu	7	(1.1)	GGA	Gly	14	(2.2)
GTG	Val	21	(3.2)	GCG	Ala	16	(2.5)	GAG	Glu	27	(4.2)	GGG	Gly	4	(0.6)

*Data were derived by computerized analysis of the specific cDNA sequences coding for human, *Torpedo*, and *Drosophila* ChEs (see text for details regarding the sources of these sequences).

TABLE 5

Amino acid composition and primary protein properties in various cholinesterases. The amino acid sequences of each of the ChEs detailed under this table were deduced from the published cDNA data and compared by computerized analysis. Note the high similarities in general amino acid composition as compared with distinct differences in nucleotide composition and dinucleotide frequencies and codon usage (Table 4).

TABLE 5

Amino Acid Composition and Primary Protein Properties in Various Cholinesterases^a

I Amino acid composition of the mature human ChE

Amino acids	No. of residues	%	Amino acids	No. of residues	%
Ala	34	5.9	Leu	51	8.7
Arg	24	4.2	Lys	26	5.7
Asn	38	6.6	Met	18	1.9
Asp	24	4.2	Phe	36	6.8
Cys	8	1.4	Pro	31	5.2
Gln	19	3.3	Ser	23	6.4
Glu	37	6.4	Thr	17	6.4
Gly	46	8.0	Trp	19	3.1
His	9	1.6	Tyr	17	3.5
Ile	28	4.9	Val	27	5.6
Acidic (Asp + Glu)				68	10.6
Basic (Arg + Lys)				53	9.9
Aromatic (Phe + Trp + Tyr)				72	13.4
Hydrophobic (Aromatic + Ile + Leu + Met + Val)				201	34.5

Mol wt = 65,087. Total amino acids = 574

II Amino acid composition of the mature T. californica AChE

Amino acids	No. of residues	%	Amino acids	No. of residues	%
Ala	25	4.3	Leu	54	8.9
Arg	27	4.7	Lys	22	4.5
Asn	38	6.6	Met	19	3.1
Asp	28	4.9	Phe	27	6.3
Cys	8	1.4	Pro	40	5.4
Gln	18	3.1	Ser	44	8.2
Glu	40	7.0	Thr	34	4.0
Gly	45	7.8	Trp	15	3.0
His	18	3.1	Tyr	26	3.3
Ile	20	3.5	Val	39	7.0
Acidic (Asp + Glu)				67	11.8
Basic (Arg + Lys)				54	9.2
Aromatic (Phe + Trp + Tyr)				68	12.5
Hydrophobic (Aromatic + Ile + Leu + Met + Val)				214	35.0

Mol wt = 65,596. Total amino acids = 575

(Table continued next page)

TABLE 5
Amino acid composition and primary protein properties in various cholinesterases (continued).

TABLE 5 (continued)

III Amino acid composition of the <i>D. melanogaster</i> AChE					
Amino acids	No. of residue	%	Amino acids	No. of residues	%
Ala	57	8.8	Leu	54	8.3
Arg	32	4.9	Lys	22	3.4
Asn	33	5.1	Met	19	2.9
Asp	33	5.1	Phe	27	4.2
Cys	11	1.7	Pro	40	6.2
Gln	23	3.5	Ser	44	6.8
Glu	34	5.2	Thr	34	5.2
Gly	56	8.6	Trp	15	2.3
His	15	2.3	Tyr	26	4.0
Ile	34	5.2	Val	39	6.0
				67	10.3
Acidic (Asp + Glu)				54	8.3
Basic (Arg + Lys)				68	10.5
Aromatic (Phe + Trp + Tyr)				214	33.0
Hydrophobic (Aromatic + Ile + Leu + Met + Val)					
Mol wt = 71,662. Total amino acids = 648					

IV.D. Preliminary Characterization of the Structural Human Cholinesterase Genes

To identify the genomic DNA fragments encoding various regions of the ChE protein, segments of the molecularly cloned full-length cholinesterase cDNA cut by the enzyme EcoRI were employed as probes for DNA blot hybridization of genomic DNA from postnatal and fetal brain as well as from primary meningioma tumors. This analysis revealed a 10 kb DNA band that hybridized with the 3'-end of the cDNA, a 2.3 kb DNA fragment that appears to carry most of its central part and a 4.3 kb fragment that codes for at least part of the 5'-terminal region of this cDNA, as shown in Fig. 4.

FIGURE 4 - DNA blot hybridization using cholinesterase cDNA probes

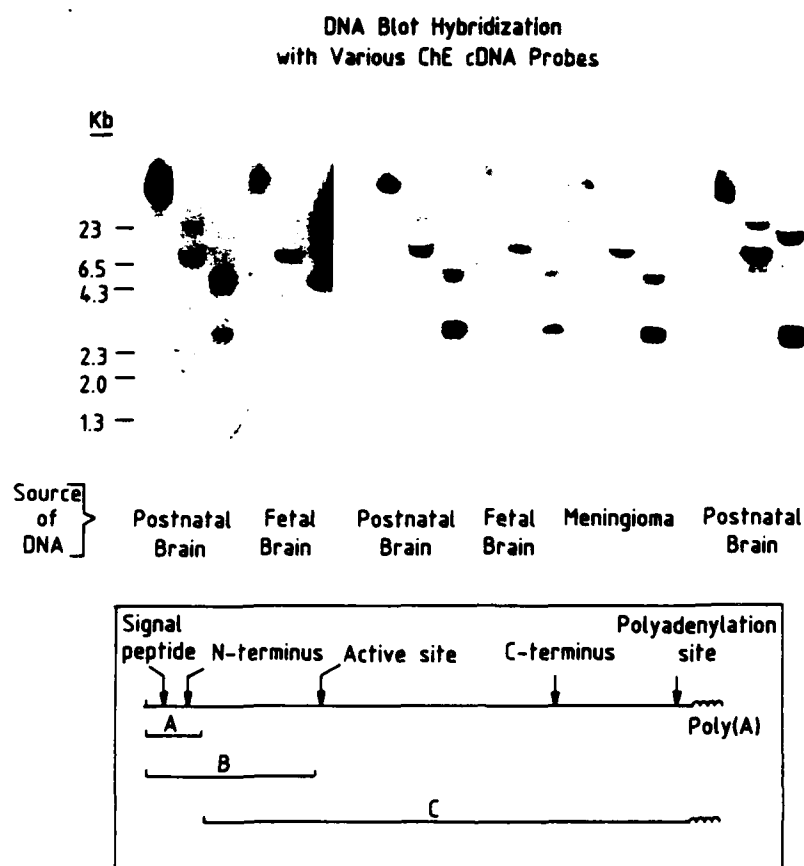
A. Schematic drawing of the DNA probes employed. The full-length human ChEmRNA (upper line) contains 2500 nucleotides, is expressed in both fetal brain and liver (51,52) and includes sequences coding for a signal peptide and the N-terminal, active site, and C-terminal peptides found in human serum BuChE (71). Probe A represents a 250 nucleotides long EcoRI fragment of a cDNA insert isolated from a lambda gt10 library of fetal brain origin and spanning from the 5'-end region of the ChEcDNA through the N-terminal peptide that appears in the BuChE encoding sequences. Probe B represents

the original FBChE12 cDNA clone isolated by use of oligodeoxynucleotides (51). This clone contains a 765 nucleotides long insert from which probe A was derived, beginning at the same point as probe A, but reaching the active site region of the human serum BuChE sequence. Probe C represents a 2230 long cDNA fragment isolated from a lambda gt10 library of fetal liver origin. It contains a stretch of 590 nucleotides overlapping with probe B and it spans from an EcoRI restriction site within probe B through the active site and C-terminal regions of BuChE as well as the 3'-untranslated region and polyadenylation site of ChEcDNA.

B. ChEcDNA blot hybridization reveals various fragments of genomic DNA.

Twenty µg of genomic DNA from human fetal brain, postnatal brain or a primary meningioma tumor as noted below the figure was restricted with the following enzymes: EcoRI (E), MspI (M), and HpaII (H) (Bio Labs), separated by agarose gel electrophoresis, and hybridized with a human ChEcDNA probe (B). The autoradiogram was washed in denaturing solution to "peel off" the probe and was then divided into two parts, which were then hybridized with probes A and C. Note the enhanced appearance of a strong 4.3 kb EcoRI-cut band with probe A but not C, while a 2.3 K_v EcoRI-cut band is visible only with probes B and C. A large (10 kb) EcoRI-cut band can be detected only with probe C.

Figure 4. DNA blot hybridization using cholinesterase cDNA probes.



To obtain the 5'-region of the ChE gene with its flanking region, DNA blot hybridization was performed with enzymatically restricted human genomic DNA and a [^{32} P]-labelled fragment derived from the 5'-terminus of the cloned ChEcDNA. A 4.7 ± 0.5 -kilobase-long cDNA fragment was detected and enriched 70-fold by preparative restriction with EcoRI (Bio Labs), gel electrophoresis and electroelution. The enriched DNA fraction was ligated with lambda gt10 DNA and packaged in the lambda coat. The resultant genomic library was screened using ChEcDNA probes and positive phages containing genomic ChEDNA fragments were isolated. DNA-sequencing analysis of one of these phages revealed that the 4.2 kilobase DNA fragment inserted into the lambda gt10 DNA included in its 3'-terminus a stretch of 180 nucleotides similar to the sequence that has previously been found in the 5'-terminal part of ChEcDNA.

Our findings at present indicate the existence of at least one intervening sequence in the 5'-region of the isolated human choline-terase gene, included in a 4.2 kb DNA fragment that ends with an EcoRI site. A large portion of the coding sequence then appears to be included in a 2.5 kb fragment. This fragment contains an exon at least 585 nucleotides in length, which starts with an EcoRI site and ends with a BamHI site, both of which are inherent to the ChEcDNA sequence (Nucleotides No. 248-253 and 833-839, respectively, Fig. 1). Finally, the C-terminal part of the cDNA seems to hybridize with another (10 kb) fragment of DNA. It should be stated that molecular cloning and detailed sequence analysis of these DNA fragments will be required to establish whether they are indeed parts of the actively expressed BuChE gene or whether they are derived from genes coding for other cholinesterases or related proteins or from transcriptionally inactive and processed BuChE pseudogenes.

IV.E. Chromosomal Mapping of Cholinesterase Genes by In Situ Hybridization

Two loci related to inherited alterations in serum ChE (1,30) were genetically linked with the transferrin (31,32) and haptoglobin (36) genes on chromosomes 3 and 16, respectively, but it remained unclear whether they represent structural ChE genes. During the period covered by this report, we demonstrated by in situ chromosomal hybridization that both chromosome 3 and 16 carry sequences hybridizing with ChEcDNA. One of these ChE genes localizes to a 3q site that is commonly aberrated, and related to abnormal megakaryocyte proliferation, in acute myelodysplastic anomalies (10).

In situ hybridization experiments were performed using Q-banded and R-banded (85) chromosome preparations from peripheral blood lymphocytes and either a 760 nucleotides-long [^{35} S] cDNA probe coding for about half of the catalytic subunit of cholinesterase isolated from fetal human brain (51) or a 2230 nucleotides-long EcoRI fragment derived from a full-length cholinesterase cDNA from fetal liver origin (Fig. 1). Hybridization was carried out according to Rabin et al. (102) with some modifications (see Technical Approaches and Methodology for details).

Of a total of 52 cells from 8 unrelated volunteers having normal karyotypes which were scored, 53 copies of chromosome No. 3 in 43 cells and 37 copies of chromosome No. 16 in 30 cells gave positive hybridization signals. These carried 98 and 77 grains on chromosomes 3 and 16, respectively, altogether 175 grains out of a total of 646 which were associated with chromosomes, with 45 (87%) cells being positive for either one or both chromosomes (38).

The cumulative distribution of autoradiographic silver grains observed

over photographed chromosome spreads 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 μ m). This analysis revealed that 63 (64%) of the grains on chromosome 3 were concentrated within the region 3q21-->q26, with two clear peaks around 3q21 and 3q26. On the shorter chromosome 16, radioactivity concentrated around the 16q12 band, with 49 (64%) of the grains within the region 16q11-->q23. Statistical evaluation of the number of silver grains per unit chromosome length, assuming a Poisson distribution, indicated that the localization on chromosomes 3 and 16 was significant in both cases ($p \leq 0.025$ and $p \leq 0.01$, respectively). The labelling over all other chromosomes was not significant. Several fragments from the ChEcDNA probes gave essentially similar results, further confirming the significance of the above-mentioned hybridization experiments.

IV.F. Polymorphism of Nascent Cholinesterases Demonstrated by mRNA Translation Studies

Electrophoretic separation and immunoprecipitation of human AChE and BuChE isoforms was obtained by crossed immunoelectrophoresis. Rabbit antibodies against human erythrocyte membrane proteins, which specifically interact with "true" AChE (103), were included in the intermediate gel, while the top gel contained anti-human BuChE antibodies. Plates were stained for ChE activities by the acetylthiocholine protocol (16), in the presence or absence of selective ChE inhibitors (2). Fetal plasma "true" AChE, which was resistant to iso-OMPA but susceptible to BW284C51, created an immunoprecipitable peak of activity in the intermediate gel (42,43). Anti-rat brain AChE antibodies, received from J. Massoulie, reacted similarly with human true AChE when included in the intermediate gel. In contrast, BuChE continued to migrate through the intermediate gel and into the top gel, where it formed a prominent precipitation arc, mostly resistant to BW284C51 and quantitatively inhibited by iso-OMPA. Both ChE forms were negatively charged under these electrophoresis conditions and, in the first dimension gel, moved towards the anode at similar rates, suggesting that their amino acid composition and structure involve similar charges. Coomassie Brilliant Blue staining failed to show the ChE immunoprecipitation arcs, stressing the high sensitivity of the acetylthiocholine staining and the low concentration of ChE proteins.

The synthesis of nascent ChE polypeptides from mRNA was followed by resolving [³⁵S]-methionine-labelled translation products by immunoelectrophoresis, as described above. Fetal human plasma was used as a carrier source for active ChEs, and processed plates were stained for ChE activities and then autoradiographed. Polypeptides immuno-reactive with either of the specific anti-ChE antibodies employed created labelled precipitation arcs in corresponding gels.

Using this method, brain, muscle and liver mRNA preparations induced in vitro, in the reticulocyte lysate cell-free system, the formation of visible immunoprecipitable ChE polypeptides with different intensities, reflecting the capacity of these fetal tissues for the synthesis of ChEs (42). In contrast, there was very faint labelling in the control plates, loaded with reticulocyte lysate devoid of mRNA, showing that the radioactive immunoprecipitation arcs represented translation products specific for the fetal tissue mRNAs. mRNA from all three tissues produced separable immunoreactive similarly charged AChE and BuChE polypeptides, which precipitated either as sharp bands or as diffuse spots in the corresponding gels. Fetal brain mRNA was most potent in producing immunoprecipitable AChE, with lower amounts of BuChE. This is in agreement

with our earlier biochemical results (22). In contrast, the amount of newly synthesized BuChE was higher in fetal muscle. It is interesting to note that fetal liver mRNA clearly produced both BuChE and "true" AChE, implying that the nascent AChE and BuChE polypeptides probably contain domains which differ in their amino acid sequence, since the post-translational processing which takes place in vitro is very limited.

To examine whether post-translational processing is involved in the biosynthesis of ChEs, and whether brain, muscle and liver mRNAs differ in their capacity to induce intracellular or exported ChEs, we injected Xenopus oocytes with such mRNAs. ChE-immunoreactive polypeptides in oocyte homogenates and incubation media were then monitored by crossed immunoelectrophoresis. The in ovo mRNA-directed ChE polypeptides appeared to be polymorphic at both the first and second dimensions of the crossed immunoelectrophoresis gels, with more distinct precipitation arcs in oocyte extracts and diffuse curves in the incubation medium (42). The difference between these patterns and the ones obtained in vitro suggests that post-translational processing events modify the properties of newly synthesized ChEs. Glycosylation is probably one of these post-translational processing events, since we found that immunoprecipitable oocyte-produced ChEs bind to Sepharose-bound Lens Culinaris lectins similarly to chick muscle ChEs (50). ChEs produced in oocytes injected with mRNAs from the various fetal tissues exhibited different electrophoretic migration coordinates, further suggesting that these post-translational processing events are tissue-specific. Faint labelling could also be detected in homogenates of oocytes that were injected with [³⁵S]-methionine and buffer only, and where no mammalian mRNA was present. This demonstrates that Xenopus ChEs share some immunological domains with human ChEs.

IV.G. Modified Properties of Cholinesterases in the Serum of Carcinoma Patients Suggest that Anti-tumor Therapy Alters the Expression of the Cholinesterase Gene(s)

Several earlier reports have noted changes in the expression of cholinesterases in cases of carcinoma tumors (18,30). To find out whether carcinomas are accompanied by changes in the biochemical properties of serum ChE, we set out to determine inhibition curves for the ChEs in 77 serum samples drawn from patients suffering from carcinomas of various tissue origins and in 21 serum samples from healthy volunteers, using selective inhibitors specific for particular types of ChEs. Three compounds were used: iso-OMPA, an organophosphorous poison with high specificity towards serum BuChE (2), BW284C51, a bisquaternary reversible inhibitor of "true" AChE, and succinylcholine, a substrate analog of acetylcholine which is rapidly hydrolyzed by normal BuChE but cannot be degraded by the "atypic" or "silent" types of serum BuChE (30). Each inhibitor was added at six different dilutions, covering a wide range of concentrations.

The enzyme in all of the serum samples was clearly sensitive to iso-OMPA, as expected from human BuChE, with 1×10^{-6} up to 1×10^{-4} M of the inhibitor sufficient for quantitative block of acetylcholine hydrolysis. The inhibition curves of the tumor serum samples could be divided into three major groups:

- 1) Curves which are indistinguishable from controls, with high sensitivity towards iso-OMPA and considerably lower sensitivity to BW
- 2) Curves displaying high sensitivity to BW and normal inhibition by iso-OMPA
- 3) Curves with the same level of sensitivity towards both inhibitors (102).

In some of the samples examined, low concentrations of particular

inhibitors caused an increase in the rate of acetylcholine hydrolysis. This may be due to the blocking action of these inhibitors on the competitive or inhibitory activity of other serum proteins.

About 75% of the samples examined were drawn post-surgery from patients under irradiation therapy, chemotherapy or hormone treatment. Metastases were diagnosed in a few patients only, and the age group examined ranged between 18 and 90 years. Specific values from the above-described inhibition curves (the total ChE specific activity and the inhibitions caused by 50 ng/ml succinylcholine, 1×10^{-5} M BW and 1×10^{-4} M iso-OMPA) were selected as representative data. In most of these measurements, the total ChE activity was lower and the sensitivity to BW higher in tumor samples as compared with controls. In contrast, there were no differences in the total protein concentration or in the sensitivity to iso-OMPA or succinylcholine. The enhanced sensitivity to BW was not significantly correlated to age, sex, ethnic origin or mode of treatment, and did not differ between patients suffering from distinct types of carcinomas. Because of insufficient histological information, it remained unclear whether the modified ChE properties are related to the state of differentiation of particular carcinomas.

Summary of the biochemical observations revealed that the average specific activity of ChE was significantly lower by about 25% ($P \leq 0.001$) in the serum of tumor patients, in agreement with previous findings of others (reviewed in ref. 30). This phenomenon is generally attributed to decreased functioning of the liver in patients under anti-tumor therapy. The sensitivity to iso-OMPA was generally similar in tumor serum samples to that measured in control samples. In contrast, 1×10^{-5} M BW284C51 was sufficient to cause a $29 \pm 6\%$ decrease in ChE activity in the serum samples from patients but only $6 \pm 16\%$ in controls ($P \leq 0.0005$). These included two samples in which BW inhibition was exceptionally high (103). Succinylcholine, in turn, showed relatively low variability in the extent of inhibition in tumor serum samples. However, there was no significant difference between the inhibition observed in diseased and healthy sera, and 50 ng/ml of this analog was essentially sufficient to cause substantial block in all serum samples.

Analysis of the distribution of the modified ChE properties in various tumor samples indicated that the enhanced sensitivity to BW was not related to the decrease in total ChE activity or to the stable susceptibility to iso-OMPA. This implied that the increased sensitivity to BW could reflect the appearance of another form of ChE in the serum samples from cancer patients. To further test this possibility, we subjected several serum samples to sucrose gradient centrifugation and determined the sedimentation profile of ChE forms in these samples in the absence and presence of BW and iso-OMPA. The major ChE form in all of these gradients displayed a sedimentation value of ca. 12S, as expected from soluble BuChE tetramers and in agreement with previous investigators (20). The 12S form was the principal one in control serum samples. In contrast, we detected an additional minor but reproducible peak of activity sedimenting as ca. 6-7 S in gradient profiles of serum samples from patients suffering from various tumor types. This tumor-characteristic ChE form was quantitatively blocked by both BW and iso-OMPA, which implied that it was neither AChE nor BuChE, but another form of ChE with combined properties of both. This form of ChE could be responsible for the considerable enhancement in sensitivity to BW in serum ChEs which also displayed normal sensitivity to iso-OMPA.

V. DISCUSSION

V.A. Oligonucleotide Stability in Screening of Libraries -- non-perfect hybrids: stability in the presence of tetramethylammonium salts

Pools of oligodeoxynucleotides ranging in length from 14-20 bases are commonly used to screen libraries of cloned cDNA to isolate desired DNA sequences (76). The oligonucleotide mixtures contain probes of equal size that represent all of the DNA possibilities encoding for a specific polypeptide. Because of the ambiguity of the codon, there might be many such possibilities for a given peptide, which requires the preparation of probe mixtures of high complexity. Such high complexity probes often contain sequences with varying G-C content, creating a problem in determining the stringency of hybridization and wash, since the stability of G-C pairs is higher than that of A-T pairs (78). To overcome this difficulty, a selective method has been developed in which TMA salts are employed to allow the stringency of hybridization to be controlled as a sole function of probe length (78). The effect of TMA was suggested to cancel the stabilizing effect of G-C pairs on the dissociation temperature (t_d) of DNA hybrids. Thus the length factor of the probe should remain as a single determining element (104). Since all of the unique probes in the pool are of identical size, the t_d of true positives should be identical under TMA washes and higher than the t_d of all the non-exact matches, regardless of their G-C content. This should exclude non-exact false-positives, which remain stable under standard washing conditions.

Our findings indicate that the use of TMA is efficacious but not perfect, since some of the mismatched hybrids remain stable even following TMA washes (51,75). If it is simply assumed that the TMA washes cancel the G-C effect, both of the OPSYN II-positive clones described under Current State of Experimental Observations should not have been stable under the TMA washes used in the third screen. However, clone 7b was stable and included a single CxT mismatch, whereas clone 24 represents a perfect homology with an additional unpaired thymidine base. Furthermore, an additional OPSYN I positive clone proved to be stable to TMA washes, although it included three successive mismatches (C. Prody, unpublished data).

Studies of others confirm the ability of oligodeoxynucleotides to form a double helix with an additional unpaired adenine base, stacked into the duplex (105), or an additional thymidine base forming a single base loop (106). Mismatched base pairs in oligodeoxynucleotide duplexes have also been shown to exist and might play a role in mutagenesis (105,107-109). However, in all of the reported cases of mismatched or additional unmatched base pairs in duplexes, the stability of hybrids was found to be lower than in perfectly matched duplexes. In contrast, clones 7b and 24 displayed high stability hybridizations with the mismatched oligodeoxynucleotide probes. In light of the present studies, we would like to suggest that sequence-specific elements also contribute to the stability of probe-DNA hybrids in the presence of TMA. These could be structural effects related to interactions of the probe-DNA hybrids with the TMA itself. The stabilizing factor contributed by the specific sequences would thus be apparent in the presence of TMA, with each TMA-DNA complex having unique physico-chemical properties. It must be noted that 78.4% of the positively hybridizing mismatched clones behaved as expected and were not TMA-stable (51). The TMA treatment therefore appears to be an effective method for screening with large pools of oligonucleotide probes, but it does not exclude all of the mismatched oligonucleotides.

V.B. Use of Base Substitution in Designing Oligonucleotide Probes

In place of mixtures of oligonucleotides that cover all of the alternatives allowed by codon ambiguity, it is possible to use nucleic acid analogs when polypeptide sequences are used to design probes for screening cDNA libraries. In this approach, the nucleotide analogs may substitute for more than one of the Watson-Crick bases, thus allowing the limitation of probe complexity in highly ambiguous mixtures. Deoxyinosine (dI) is the preferred analog for such substitutions and was successfully used in places of codon ambiguity to isolate a human cholecystokinin gene (80) and many others. The thermal stability of oligodeoxyribonucleotide duplexes containing dI was analyzed (110). The results suggest that dI is useful at A/C, G/T, three- and four-fold ambiguities. It seems that deoxyguanosine (dG) might be preferable in cases of T/C ambiguities, since dI is less stabilizing on the average. We can conclude from our results using dI in the OPSYN0 probe (51) that it very specifically labelled the human ChEcDNA and was useful in verification of the "true positive." Since dI might not contribute to the stability of the hybrids, it would be advantageous to use dI-containing probes longer than the 17-mers, such as those employed in the high complexity OPSYN probes.

V.C. Codon Usage Specificity as a Tool for Designing Probes

An alternative approach in the use of oligonucleotide probes is to exploit the fact that different taxa have been found to display different codon preferences (111). Instead of synthesizing a pool of probes, one can synthesize a single or a few probes whose codons are taxa-specific (112). This method has been used successfully in the search for several cDNAs. However, it involves a "statistical risk." Even if, statistically, a specific codon were in general preferable, it might occur that an unpredicted codon is used in the particular site for which the probe is synthesized. This would cause the synthesis of an incorrect probe. Therefore, it was decided in our case to use inosine probes and oligodeoxynucleotide pools; in fact, the codon usage of the particular OPSYN0 sequence that served for the preparation of our oligodeoxynucleotide probes appears to be nonfavorable according to the general codon usage tables for human genes (111). Thus in this specific case the statistical risk was also a practical one and the choice of probe mixtures and deoxyinosine was therefore justified.

V.D. Human Cholinesterase Homology to Other Proteins

The primary sequence of human ChE as encoded by the isolated cDNA can clearly be distinguished from those of other serine hydrolases (95), although they share common amino acids in the immediate vicinity of the organophosphorous-binding serine (51). Thus the genes coding for cholinesterases most probably have arisen from a unique gene. As shown above, ChEs from various species such as Torpedo californica, Drosophila melanogaster, and human, share extensive sequence homologies throughout the polypeptide sequence of the enzyme proteins. This conservation suggests that most of the regions in the ChEs are necessary for biological functions and that the ancestral gene for ChE has developed very early in evolution with essentially the same properties as those observed in most species today. One may postulate several important domains within the ChE protein. These include one for binding of the collagen-like "tail," another responsible for membrane binding, and clearly the active site and the anionic site domains (3). In addition, sites of contact between subunits and S-S bonds may be postulated. Since all of these properties should be displayed by ChEs in all species, it seems logical to expect to find such high homology between ChEs of genetically remote species. The exceptional, additional amino acids found in the Drosophila ChE might hence be necessary for the Drosophila enzyme alone, but probably do not create any steric hindrance of activity.

To our surprise, homology searches with both human ChE and the Torpedo californica protein sequences determined from their nucleic acid cDNA sequences show extensive homology with bovine thyroglobulin, as already noted by Schumacher et al. (41). This suggests that these two proteins contain in part a shared common ancestral origin. The accepted role of thyroglobulin is that of a carrier protein (99); however, this homology suggests that there may exist another as yet undetermined function in common for both proteins. The divergence of the ancestral gene is intriguing, considering the different functions of these proteins. Furthermore, the conservation of the primary structure in the thyroglobulin protein may actually become a "physiological nuisance" in cases of hyperthyroidism (such as Graves' ophthalmopathy). In this disorder, there exists an overproduction of the hormone thyroxin. It was suggested (113) that antibodies raised against thyroglobulin, the thyroxin precursor, could cross-react with ChEs due to the homology in their primary sequence. These new antibodies, recognizing epitopes shared by both proteins, would then cause an autoimmune effect, responsible for at least some of the symptoms observed in hyperthyroidism. The location of primary sequences of human ChE showing high homology to bovine thyroglobulin (presented in Fig. 3d) is close to the area of the active site of the human ChE. This implies that anti-thyroglobulin antibodies cross-reacting with the ChE protein would be likely to inhibit its enzymatic activity. It remains to be shown, though, that the thyroglobulin reaches the vascular system in this disorder, and whether such autoimmune antibodies exist (114).

V.E. Preliminary Findings on the Structure of the Human ChE Gene

1. Composition and Structure of the Cholinesterase cDNA

The polypeptide inferred by the nucleotide sequences of FBChE12 and FL39 shares common amino acid sequences with human BuChE (71), which is present in the fetal human brain (22) and liver (1). In addition, the FBChE12 and FL39 fragments appear to be derived from a relatively small region in the human genome. Under low stringency conditions, they hybridized with equal efficiency with mRNA from preparations rich either in fetal AChEmRNA or in BuChEmRNA. The most straightforward explanation for these findings is that these clones were reverse-transcribed from BuChEmRNA. An alternative explanation, implied from the DNA and RNA blot hybridizations, was that FBChE12 and FL39 were derived from a cholinesterase mRNA that is fetus-specific (52). This putative fetal cholinesterase may share amino acid sequences with both AChE and BuChE, while the DNA sequence directing its synthesis may be sufficiently different from those coding for other cholinesterases to prevent cross-hybridization under the high-stringency conditions employed. An example for such a situation is observed in the homologies between FL39 and the Torpedo AChE cDNA clone (41) (Fig. 1).

According to the findings presented in this report, the cholinesterase(s) encoded by the FBChE12 and the FL39 clones may be produced from several genes but not from a multigene family. Differential splicing or alternative transcription of a single gene, or independent expression of a few related genes, can direct the synthesis of several highly homologous cholinesterase mRNAs, differing in limited domains (e.g., N-terminus (73)). This is compatible with the finding of intervening sequences within this human gene.

Microinjection experiments have suggested that several broad peaks of size-fractionated fetal brain mRNA can induce production of catalytically active cholinesterases in Xenopus oocytes (39). The heavy size class of these mRNAs was similar in size to mRNA from fetal brain, which hybridized with a DNA fragment from the vicinity of the ace locus in Drosophila (81). However, FBChE12 and FL39 hybridize with mRNA of the size of the second broad peak of cholinesterase-inducing mRNAs, and do not hybridize with either the Drosophila ace DNA fragment or its counterpart human DNA fragment. It is possible, therefore, that both the heavy size class of cholinesterase-inducing mRNA and the ace-homologous fragment could be involved in the biosynthesis of a brain-enriched protein controlling the production of active cholinesterases from their nascent polypeptide precursors via post-translational processing. An example is the production in oocytes of EGF from its 28 heavy mRNA by a salivary-gland-specific protease encoded by a small mRNA (86).

The full-length cholinesterase cDNA fragment contains 2400 nucleotides with a high (63.3%) content of pyrimidines and a very low content (0.9%) of C-G pairs, a dinucleotide frequency characteristic of the human genome (112). Its nucleotide sequence displays high levels of homology to the cDNA clones coding for Torpedo AChE (41) and for Drosophila AChE (98). Furthermore, it resembles bovine thyroglobulin cDNA sequence (99), with a similarly open reading frame preceding the signal peptide. The fractional codon utilization of this cDNA is compatible with other human protein-coding sequences (112), and differs considerably from that found in the Drosophila and the Torpedo AChE cDNA clones, yet its inferred protein displays 53% and 35% matches with the parallel protein sequences of Torpedo and Drosophila AChE, respectively. In the few domains where the amino acid

sequence was not conserved (for example, the region encoded by nucleotides 310-340 in FBChE12), there are also no similarities in the nature of the corresponding residues, as classified by Doolittle (115). This confirms our previous assumption (52) that most of the sequence inferred by these cDNA clones is required as such for maintaining the yet undefined biochemical properties characteristic of BuChE.

2. Genomic DNA Fragments Hybridizing with Cholinesterase cDNA Probes

Human DNA restricted with the EcoRI enzyme includes fragments of 4.2, 2.3 and 10 kb in length, hybridizing with the 5'-terminal, active site region and 3'-terminal parts of the cDNA encoding the cholinesterase protein. The 2.3 kilobase fragment is not homologous to the 5' region of the gene, since it does not hybridize with a 5'-terminal probe. When human DNA is restricted with EcoRI and BamHI together, this 2.3 kb band does not appear, and instead a fragment of 560 bases can be seen. The cDNA restriction pattern reveals a similar size fragment using both restriction enzymes. Therefore, it seems likely that this 2.3 kb fragment contains a 560-nucleotide-long fragment that appears in exactly the same form in the cDNA. The 10 kb fragment contains sequences homologous to the 3'-terminal area. Finally, The 4.2 kb fragment was enriched from genomic DNA and cloned into lambda gt10. Its sequence analysis revealed that it indeed contains a portion of the 5' region of the cDNA, in addition to at least one intervening sequence, separating the 5'-regulatory region of the ChEcDNA from the nucleotides coding for the initiator methionine (116). The existence of an intervening sequence at this important region of the ChEcDNA sequence may reflect an alternative splicing phenomenon, which could regulate the production of various ChEmRNAs from a single gene (53). Further characterization of genomic and cDNA sequences encoding for human ChEs would be required to examine these possibilities.

3. DNA Methylation in the Cholinesterase Genes

A ChEcDNA-positive EcoRI fragment of 4.3 kb has been detected in DNA from various human tissue sources (75). The cloned phage, though it originally contained a 4.3 kb fragment, appeared to include an additional EcoRI restriction site that gives rise to two fragments when the phage-amplified DNA was examined. This site, found in the intron, near the initiator methionine, seems to be protected against enzymatic restriction in DNA from all of the tissue sources examined to date (75). The inevitable conclusion is that the novel EcoRI site appeared in the cloned DNA because of a structural change in the DNA sequence, for example, lack of methylation in the phage cloned insert as opposed to high methylation level in brain DNA.

Accumulating evidence suggests that DNA modification may play an important part in gene regulation (117). Experiments using methylation-sensitive and zeta-insensitive restriction enzymes suggest a correlation between DNA undermethylation and expression, such as exists with the β -globin gene (118). Expression is generally observed in undermethylated DNAs whereas in DNA that is heavily methylated, little or no expression exists (118,119,120). Furthermore, it was shown that demethylation of DNA accompanies the switching-on of immunoglobulin heavy chain class genes (121). The importance of the methylation site in the human ChE gene might be implied by its proximity to the splice junction. It is possible that methylation in this site is a structural requirement for processes involved in the "splice mechanism," such as lowering the melting temperature in the splice junction. In this case, methylation in this site could be important for regulating the expression of the human ChE gene in vivo and in

transfected cells, where large quantities of the authentic human enzyme can be produced for neurotoxicological studies.

4. Two Chromosomal Sites Carry Cholinesterase Genes

Three phenotypic variants for erythrocyte AChE were reported to reflect two codominant alleles at a single locus (29) which has never been genetically mapped. In contrast, serum BuChE displays multiple variants which have been genetically linked to two independent loci, CHE1 and CHE2. The E1 locus has recently been assigned to a chromosome 3q region (31), in linkage with the TF gene, mapped at 3q26-q31, the TFRC and the ceruloplasmin gene, with no further order of these four loci (reviewed in 34). The CHE2 locus, directing the production of the common C5 variant of serum BuChE (1,30,35), has been genetically linked to the alpha-haptoglobin gene (36). Recent *in situ* hybridization experiments localized the haptoglobin-coding sequences to a region distal to the fragile site at 16q22. Our present findings thus localize structural cholinesterase-coding sequences to two regions in the CHE1 locus at 3q21-q26, central to the transferrin gene on the long arm of chromosome 3, and to the CHE2 locus at a 16q11-q23 position, central to that of the haptoglobin gene on chromosome 16.

Accumulated genetic evidence suggests that the ChE gene in the CHE1 locus is generally expressed, with non-frequent CHE1 variants directing the production of defective ("silent" or "atypic") enzyme (1,30,34). In contrast, the ChE gene in the CHE2 locus leads to the expression of an active serum ChE in ca. 8% of the Caucasian population only (35,36). Thus the E2 gene might be a transcriptionally inactive pseudogene in the majority of individuals. Alternatively, mutations in this gene could produce catalytically active forms of the serum ChE protein. Further experiments will be needed to distinguish between these possibilities and determine whether any of these genes also codes, perhaps by alternate promoters or differential splicing, for other ChEs, such as the membrane-associated AChE in neuromuscular junctions (3) or soluble brain AChE tetramers (20,22).

Apart from acetylcholine hydrolysis, the roles of ChEs in general and of serum BuChE in particular are still unknown. Recently, several clinical reports have linked cytogenetic anomalies in the 3q21; 3q26 region in acute nonlymphocytic leukemia (122,123) with thrombocytopenia (124) (reviewed in ref. 100). In view of the physiological effect of carbamylcholine in inducing megakaryocytopoiesis in culture (17,125), as combined with our present localization of the CHE1 ChE gene to this particular chromosomal region, it would be intriguing to propose that humoral ChE may take part in directing progenitor cells of the hemopoietic family to become committed promegakaryocytes.

V.F. Evidence for Post-translational Processing of Nascent Cholinesterases

The findings presented in this report demonstrate that various post-translational alterations of nascent ChE peptides contribute to the extensive polymorphism of cholinesterases in fetal human tissues. This has been shown by detecting newly synthesized labelled ChEs, translated from ChEmRNAs, in the sensitive two-dimensional separation system of crossed-immunoelectrophoresis followed by autoradiography.

Three levels of ChE heterogeneity were found. First, ChEmRNAs from various fetal tissues were demonstrated to be translated *in vitro*, in the reticulocyte lysate cell-free system, into distinguishable AChE and BuChE

polypeptides interacting with anti-ChE antibodies. This is in agreement with our recent findings of different mRNAs inducing the synthesis of fetal brain AChE and BuChE (39). Recently, Sikorav et al. (40) and Schumacher et al. (41) found that ChEmRNAs from Torpedo marmorata and Torpedo californica, respectively, produce in the reticulocyte lysate system two distinct immunoprecipitable ChE polypeptides. Our present observations therefore imply that this heterogeneity of ChEmRNAs was retained with evolution. The separation between the in vitro translated nascent ChEs suggests that AChE and BuChE are produced from distinct peptides with at least partially distinguishable immunological domains. Both nascent ChE peptides appeared to create rather diffuse precipitation patterns under conditions in which mature ChEs form clear, sharp arcs. This probably implies that post-translational processing, which does not take place in vitro, also contributes to the immunological properties of mature ChEs, against which the antibodies were raised.

A second origin for ChE polymorphism was observed when fetal ChEmRNAs were translated in microinjected Xenopus oocytes, where post-translational processing events and correct subcellular distribution take place (79,86). Extracellular and intracellular forms of newly synthesized ChEs differed in their migration in both dimensions of the electrophoretic separation. This suggests that in addition to differences inherent to the nascent ChE polypeptides, which could be observed in vitro, post-translational modifications alter the charge of the newly made ChEs. It has previously been shown that mild papain digestion induces charge-shifts in purified asymmetric AChE from both Torpedo marmorata and human erythrocytes (102). Thus new forms of active AChE which migrate faster in crossed immunoelectrophoresis are created by removal of short hydrophobic peptides. Following such papain digestion, amphipathic erythrocyte AChE becomes soluble and hydrophilic, and ceases to form micelles with non-ionic detergents (7,102). It is therefore probable that a similar process occurs naturally in microinjected oocytes. Oocytes were incubated and homogenates processed in the presence of potent protease inhibitors (39), indicating that such mild proteolysis should have taken place prior to secretion, probably in the rough endoplasmic reticulum or the Golgi apparatus, where maturation of ChE has been reported to take place (50). This is also supported by the observation that the faster migrating forms were found both within the oocytes and in the incubation medium, whereas the slower migrating, hydrophobic forms were not secreted (42). Together with previously reported information (7), this experiment suggests that proteolytic removal of terminal hydrophobic peptides contributes to the formation of at least part of the globular, secretory ChE forms from nascent, more hydrophobic peptides.

The third and most extensive level of polymorphism appears to be tissue-specific. The migration patterns of ChEs induced in oocytes by unfractionated mRNAs from various tissues were not similar. However, human cholinesterase cDNA (51) appears to hybridize with mRNA of the same size in brain, muscle and liver. Furthermore, similar ChEcDNA clones were isolated from libraries from all these tissue sources (unpublished observations). This indicates that post-translational events are probably necessary to create the tissue-specific patterns of migration of the ChE peptides and that additional, tissue or species-specific mRNAs, missing in the oocytes, may be necessary for these yet undefined processes. An example for this is the incomplete processing of preproEGF peptides in oocytes microinjected with size-fractionated salivary gland mRNA (86). Moreover, the protein(s) performing these post-translational event(s) could be the natural rate-limiting step in the production of ChEs from microinjected mRNA. This is also indicated from the seasonal changes in endogenous ChE

levels and in the efficiency of ChEmRNA translation in oocytes (79). Our findings therefore imply that human ChEs are produced from nascent peptide precursors by post-translational processing events which may contribute to the regulation of ChE expression in a tissue-specific manner.

Both the amino acid composition and the length of the protein subunit of the mature human serum BuChE (56) differ from that of erythrocyte AChE (7). Furthermore, genetic evidence suggested that human BuChE is encoded by two genes with multiple different alleles (31,32,36,126), independent multiallele regulation pattern was also suggested for erythrocyte AChE (1,29). This implies that BuChE and AChE in humans are encoded by at least two distinct DNA sequences. In nematodes, the expression of cholinesterase genes (24) has recently been shown to be regulated in a tissue-specific manner (127). It is important to note that tissue-specific expression of distinct DNA sequences can also operate via fine regulation of transcription within a single gene (see, for example, 128). Thus, at this stage, our experiments cannot indicate whether the nascent ChE peptides are produced from fetal and tissue-specific genes or, via alternative transcription and/or differential splicing, from a limited number of human ChE genes which are expressed in all tissues and all developmental stages. Further study will require simultaneous characterization of our recently isolated ChE cDNA clones (51) and genomic DNA sequences, of their descendent ChEmRNA species and of the ChE isoforms themselves.

V.G. Altered Expression of Cholinesterase Genes in Carcinoma Patients under Anti-tumor Therapy

Biochemical characterization of ChE properties in the serum of 77 patients suffering from primary carcinomas of different tissue origins, as compared with control serum of 21 healthy volunteers, revealed the appearance of a new type of ChE. The study was based on the analysis of a single serum sample from each patient, and in most cases serum was drawn after surgery from patients under surveillance and various treatment protocols according to the tissue origin of the tumor. The wide range of tumor types and the wide ranges in the ages of these patients further complicate the interpretation of our findings. However, the reproducibility of inhibition patterns and gradient profiles, supported by the statistical analysis of a rather large group of samples, indicates that the modified properties of ChEs in the serum of these patients reflects a true in vivo phenomenon.

The new soluble serum ChE was found to be susceptible to inhibition by both BW and iso-OMPA and exhibited a sedimentation coefficient of 6-7 S in sucrose gradients. Thus its properties differ both from those of the well-characterized soluble serum BuChE, which is insensitive to BW and sediments as 12S tetramers (71), and from the properties of the dimeric erythrocyte AChE, which under normal conditions is not released to the serum in a soluble form and is not sensitive to iso-OMPA inhibition (73). To the best of our knowledge, partial proteolysis of various ChE forms does not alter their sensitivity to selective inhibitors (129,2,103,19,51). It is therefore unlikely that this tumor-characteristic type of serum ChE results from a disease-related release of AChE from the erythrocyte membranes or from breakdown of BuChE tetramers into dimers. Furthermore, the appearance of this new ChE was not related to the average decrease in total serum ChE activity or to its general level of sensitivity to iso-OMPA and succinylcholine. This may imply that these two serum ChE activities originate from different pools of nascent polypeptides. There is a single report in which the sensitivity of serum ChE to succinylcholine increased in a case of carcinoma (30). However, in view of our present analysis, this seems to be an exception.

BW-sensitive ChE has previously been detected by histochemical techniques in tissue sections derived from various types of primary carcinomas (18). In tissue homogenates from primary glioblastomas and meningiomas, we have found light forms of ChE which were sensitive to inhibition by both BW and iso-OMPA (20). Microinjection of mRNA from glioblastomas, meningiomas and fetal brain into Xenopus oocytes induced the production of ChE activities which could be blocked by both inhibitors (39). Finally, we have recently isolated ChEcDNA clones from fetal brain origin (52) which code for a protein that interacts with anti-AChE antibodies and shares common sequences with human BuChE. Altogether, this evidence raises the possibility that the novel serum ChE fraction which we describe is similar to the less-characterized enzyme for which the term "embryonic ChE" has been proposed (18). However, it cannot be concluded at present whether this putative embryonic ChE is synthesized within and transported from the tumor tissue into the serum, or produced in another tissue and released as a response to the malignant state or to the treatment employed. To find out whether the production of embryonic ChE takes place within the tumor cells and is reduced after surgery, as is the case with many other tumor markers, longitudinal studies should be performed following the course of the disease in a carefully selected group of patients, all suffering from the same type of tumor, and treated and followed by the same protocol.

The primary structure of the soluble embryonic ChE and its relationship to other human ChEs, such as neuromuscular AChE or serum BuChE, are of particular interest. Understanding the regulation of this ChE may reveal the molecular control mechanisms leading to the tissue and cell type specificity of ChE polymorphism and shed light on the unknown physiological function of these serine hydrolases in proliferating and differentiating cells. In pheochromocytoma cells, AChE biosynthesis is induced by nerve growth factor and in glioblastomas we found that enhanced levels of AChE (20) accompany the increase in epidermal growth factor receptor protein (130) and the amplification of the Erb-2 oncogene (131). It would be interesting to examine whether the expression of ChE genes is coregulated with oncogenes and, if so, in what way. To approach this issue, ChEcDNA clones of primary tumor origin are currently being isolated. The nucleotide sequence of such tumor-originated ChEcDNA clones will then be compared to the sequence of human cholinesterase cDNA from non-malignant tissues (75). These clones will subsequently be used as labelled probes to examine whether new types of ChEmRNA exist in carcinoma tissues.

VI. NOTIFICATION OF PATENT APPLICATION

I would like to take this opportunity to notify that the Weizmann Institute wishes to retain title on our molecularly cloned cholinesterase cDNAs and their putative uses. The rights to the above invention were assigned to Yeda R&D Company, Ltd., at the Weizmann Institute of Science, an organization which has as its primary function the management of inventions conceived by Weizmann Institute Scientists, and the filing of patent applications in respect thereto. Yeda R&D Company, Ltd., has filed the following patent applications in respect to the above invention.

<u>Country</u>	<u>Patent Appl. No.</u>	<u>Date Filed</u>
Israel	75553	18.6.1985
	(Title in Israel - A AChE-Like Peptide)	
U.S.A.	875,737	18.6.1986
European Patent Off.	86108189.1	16.6.1986
	(Designating all 11 countries)	
Canada	no number as yet	18.6.1986
Denmark	2859/86	18.6.1986
Japan	no number as yet	18.6.1986

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

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VIII. LIST OF PERSONNEL RECEIVING CONTRACT SUPPORT

1. Soreq, Hermona, Ph.D.
2. Zevin-Sonkin, Dina, Ph.D.
3. Prody, Catherine, Ph.D.
4. Zisling, Rivka, M.Sc.
5. Koch, Regina, M.Sc.
6. Matzkel, Avi, M.D.

Research Students

7. Blatt, Ilan, M.D. Thesis, 1984
8. Avni, Adi, M.Sc. Thesis, 1985
9. Even, Lea, M.D. Thesis, 1986
10. Gnatt, Averell, M.Sc. Thesis, 1986
11. Malinger, Gustavo, Basic Research Thesis, 1987

Theses

7. Developmental variations in the levels of cholinesterases in discrete regions of the human fetal brain.
8. Isolation and partial characterization of a human acetylcholinesterase gene identified by homology to the Drosophila gene.
9. Cholinesterases in the serum of carcinoma patients: Putative marker for tumor occurrence.
10. The isolation and characterization of human cholinesterase cDNA and genomic sequences.
11. Expression of human cholinesterase genes in normal and malignant ovarian tissues.

IX. CHRONOLOGICAL BIBLIOGRAPHY OF ALL PUBLICATIONS SUPPORTED BY THE CONTRACT

1. Soreq, H., Zevin-Sonkin, D., Avni, A., Hall, L. and Spierer, P. (1985) A human acetylcholinesterase gene identified by homology to the Drosophila gene. Proc. Natl. Acad. Sci. USA 82, 1827-1831.
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14. Soreq, H. (1985) Biosynthesis of biologically active proteins in mRNA-microinjected Xenopus oocytes. Critical Reviews in Biochemistry, 18, 199-238.
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