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Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ; primary structure of the precursor of the glycolipid-anchored dimeric form

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In this paper, we show the existence of alternative splicing in the 3′ region of the coding sequence of *Torpedo* acetylcholinesterase (AChE). We describe two cDNA structures which both diverge from the previously described coding sequence of the catalytic subunit of asymmetric (A) forms (Schumacher *et al.*, 1986; Sikorav *et al.*, 1987). They both contain a coding sequence followed by a non-coding sequence and a poly(A) stretch. Both of these structures were shown to exist in poly(A)$^+$ RNAs, by S1 mapping experiments. The divergent region encoded by the first sequence corresponds to the precursor of the globular dimeric form (G_2^a), since it contains the expected C-terminal amino acids, Ala-Cys. These amino acids are followed by a 29 amino acid extension which contains a hydrophobic segment and must be replaced by a glycolipid in the mature protein. Analyses of intact G_2^a AChE showed that the common domain of the protein contains intersubunit disulphide bonds. The divergent region of the second type of cDNA consists of an adjacent genomic sequence, which is removed as an intron in A and G^a mRNAs, but may encode a distinct, less abundant catalytic subunit. The structures of the cDNA clones indicate that they are derived from minor mRNAs, shorter than the three major transcripts which have been described previously (14.5, 10.5 and 5.5 kb). Oligonucleotide probes specific for the asymmetric and globular terminal regions hybridize with the three major transcripts, indicating that their size is determined by 3′-untranslated regions which are not related to the differential splicing leading to A and G^a forms.

Key words: cDNA sequence/differential splicing/disulphide bonds/glycolipid anchor/*Torpedo marmorata* acetylcholinesterase

Introduction

Acetylcholinesterase (AChE, EC 3117) and butyrylcholinesterase (BuChE, EC 3118) exist in a number of molecular forms, classified as asymmetric (A) and globular (G), depending on the presence or absence of a collagen-like tail associated with the catalytic subunits (cf. reviews by Massoulié and Bon, 1982; Massoulié and Toutant, 1988). The A forms may be inserted in extracellular structures (basal lamina) by ionic interactions of their tail with polyanions. The G forms are either amphiphilic (G^a) or non amphiphilic (G^{na}), as indicated by their interaction with non-denaturing detergent micelles (Bon *et al.*, 1988).

Two types of attachment of amphiphilic forms to plasma membranes have been demonstrated, depending on the nature of their hydrophobic domain. Like a number of membrane-bound proteins, such as Thy-1 (Low and Kincade, 1985), the decay acceleration factor (DAF) (Davitz *et al.*, 1986; Medof *et al.*, 1986) and the variant surface glycoproteins (VSG) from *Trypanosoma* (Ferguson *et al.*, 1985), the dimeric forms of AChE from *Torpedo* electric organs (Futerman *et al.*, 1983, 1985), mammalian erythrocytes (Haas *et al.*, 1986; Roberts and Rosenberry, 1986; Roberts *et al.*, 1986) and *Drosophila* brain (Gnagey *et al.*, 1987; Fournier *et al.*, 1988) possess a glycophosphatidylinositol (GPI) anchor. This anchor consists of a phosphatidylinositol linked through a carbohydrate chain and an ethanolamine, to their C-terminal amino acid (for reviews, see Cross, 1987; Silman and Futerman, 1987; Ferguson and Williams, 1988). In the membrane-bound tetrameric enzyme from bovine brain, two of the catalytic subunits are disulphide-linked with a distinct hydrophobic subunit (Inestrosa *et al.*, 1987). The existence of these different types of molecules raises important questions regarding the structure of the catalytic subunits and the mechanism of their biosynthesis.

Torpedo electric organs contain asymmetric forms together with an amphiphilic dimeric form (G_2^a) and the corresponding catalytic subunits are clearly different in their mass, respectively 72 kd and 69 kd. In addition, they contain a third type of catalytic subunit of 76 kd (Witzemann and Boustead, 1983). This subunit seems to correspond to monomers which are inactive but may be labelled by DFP (Stieger *et al.*, 1987).

Multiple mRNAs, derived from a single gene, code for these catalytic subunits in *Torpedo marmorata* (Sikorav *et al.*, 1985, 1987) and *T.californica* (Schumacher *et al.*, 1986). This suggests that these various subunits are generated by alternative splicing.

The primary structure of one type of AChE subunit, deduced from cDNA sequences for *T.californica* (Schumacher *et al.*, 1986) and *T.marmorata* (Sikorav *et al.*, 1987) has been shown to correspond to that determined by direct amino acid sequencing of the A subunit (MacPhee-Quigley *et al.*, 1986). Studies of the genomic structure (Y.Maulet and

P.Taylor, personal communication) have shown that the coding sequence is contained in three exons. A large exon starts shortly after the initiation codon and terminates at position 1511, a second small exon extends from 1512 to 1678, and the C-terminal 40 amino acids are encoded by a third exon. It is noteworthy that introns also exist at precisely corresponding positions in the genes of *Drosophila* AChE (D.Fournier, P.Spierer and J.Bergé, in preparation) and also in the C-terminal domain of mammalian thyroglobulin (Malthiéry and Lissitzky, 1987), which presents a structural and sequence homology with cholinesterases (Schumacher *et al.*, 1986; Swillens *et al.*, 1986).

Although they differ in their molecular weight, the catalytic subunits of the A and amphiphilic G_2^a forms show few differences in their primary structures (Doctor *et al.*, 1983). Since these two subunits have the same N-terminal sequence (MacPhee-Quigley *et al.*, 1985; Bon *et al.*, 1986), their molecular weight difference probably resides in the C-terminal region of the protein, which is linked to a GPI in the amphiphilic G_2^a form. Gibney *et al.* (1988) have recently characterized the C-terminal peptide of the amphiphilic G^a subunit: Leu-Leu-Asn-Ala-Thr-Ala-Cys-ethanolamine-glycophospholipid. This sequence coincides in its five first amino acids with residues 531–535 of the A subunit (numbering the amino acids from the N terminus of the mature enzyme), but the last residues (Ala-Cys) are different.

Both types of subunits form disulphide-linked dimers, and it is interesting to ask whether the disulphide bonds are located in homologous positions in both, especially since MacPhee-Quigley *et al.* (1986) identified interchain bonds at the C-terminal extremity of the A subunit.

Here, we report the isolation of two types of cDNA clones which diverge from the described A sequence. One of them encodes the precursor of the G_2^a form. The other one contains an adjacent genomic sequence, which may function as a third coding sequence. S1 mapping and Northern blot analyses have been carried out to examine the significance of these structures and their correlation with the multiple AChE transcripts. In addition, we have examined inter-subunit disulphide bonds in intact amphiphilic dimers and in their proteolysed non-amphiphilic derivatives, in order to obtain some insight on a possible influence of the different C-terminal regions on the conformation of the common domain of the A and G^a subunits.

Results

Throughout this paper we number nucleotides from the first in-frame initiation codon (Figure 3 in Sikorav *et al.*, 1987); the amino acids are numbered from the N terminus of the mature subunits. We will refer to the distinct C-terminal regions of the A and G^a subunits as A and G^a coding sequences, as opposed to the common coding sequence which constitutes the core of the protein. We also propose to specify the amino acid encoded by the divergent sequences with the corresponding letters (e.g. cysteine G^a-538, cysteine A-572).

S1 mapping indicates the existence of divergent sequences in the coding region of AChE mRNAs, and in the 3′-untranslated region of the mRNA of the A subunit

We investigated the existence of multiple mRNA structures in the 3′ region of the AChE coding sequence, by S1 mapping, using a fragment of the cDNA sequence described previously (Sikorav *et al.*, 1987), which corresponds to the catalytic subunit of asymmetric AChE (Figure 1A and B). The existence of three protected fragments indicates the existence of two divergence sites. One divergence site is located within the coding region, and the other one in the 3′-untranslated region.

We defined the position of the first divergence more precisely by using a shorter, end-labelled probe, as shown in Figure 1C and D. The size of the small fragment identified the first divergence at position 1678–1679. This corresponds exactly to the observed difference between the A and G^a subunits, i.e. just after Cys(G^a-538) (Gibney *et al.*, 1988).

In both experiments, the relative abundance of the protected fragments suggested that the two alternative coding sequences were represented in similar proportions, but the low abundance of the longer fragment indicated that pAChE2 corresponds to a minor type of mRNA. The divergence in the 3′-untranslated region of the A mRNA is located at nucleotide 1830.

Screening for alternative cDNA sequences

We screened several cDNA libraries for AChE clones which would differ from the A sequence downstream from the identified coding divergence position. We used two probes derived from the known cDNA sequences and straddling the divergence. The first probe, a 1208 bp *Pst*I–*Pst*I fragment (nucleotide 606–1813) was expected to detect A clones as well as divergent structures, while the second one, a 22mer oligonucleotide complementary to nt 1668–1689, was designed to hybridize exclusively with the A coding sequence. The screening of 7.5×10^5 cDNA clones allowed the isolation of 35 clones which hybridized with the 1208 bp probe, four of which were negative with the straddling 22mer. Restriction mapping and sequence analysis showed that these clones were of two distinct types.

We sequenced two independent clones, λAchE8 and λAChE9, by the dideoxynucleotide method of Sanger, using a series of complementary oligonucleotides (20mers) in addition to the universal M13 primer.

Structure of clones λAChE8 and λAChE9; predicted C-terminal divergent regions

Upstream of the divergence, both clones contain a fragment of the AChE coding region of the A subunit (Sikorav *et al.*, 1987). Clone λAChE8 contains a 600-bp long *Eco*RI fragment upstream of the *Eco*RI site at nt 976 and clone λAChE9 starts at nucleotide 1574. As shown in Figure 2A, both clones diverge from the sequence of pAChE2 at precisely the expected position, i.e. after 1678. They both contain a poly(A) extension. Although we do not find the canonical AATAAA polyadenylation signal, λAChE8 presents a sequence (ATTAAAATAAT), which consists of two overlapping hexamers considered as potential polyadenylation signals, ATTAAA and AATAAT, starting 17 nt upstream of the poly(A), and λAChE9 contains a hexanucleotide (AATTAT) which resembles a polyadenylation signal, starting 44 nt upstream of the poly(A) (for a review, see Birnstiel *et al.*, 1985). As shown in Figure 2C the 3′-untranslated region of λAChE9 contains two repeated sequences of ~150 nucleotides (88.7% match, according to Kanehisha, 1984).

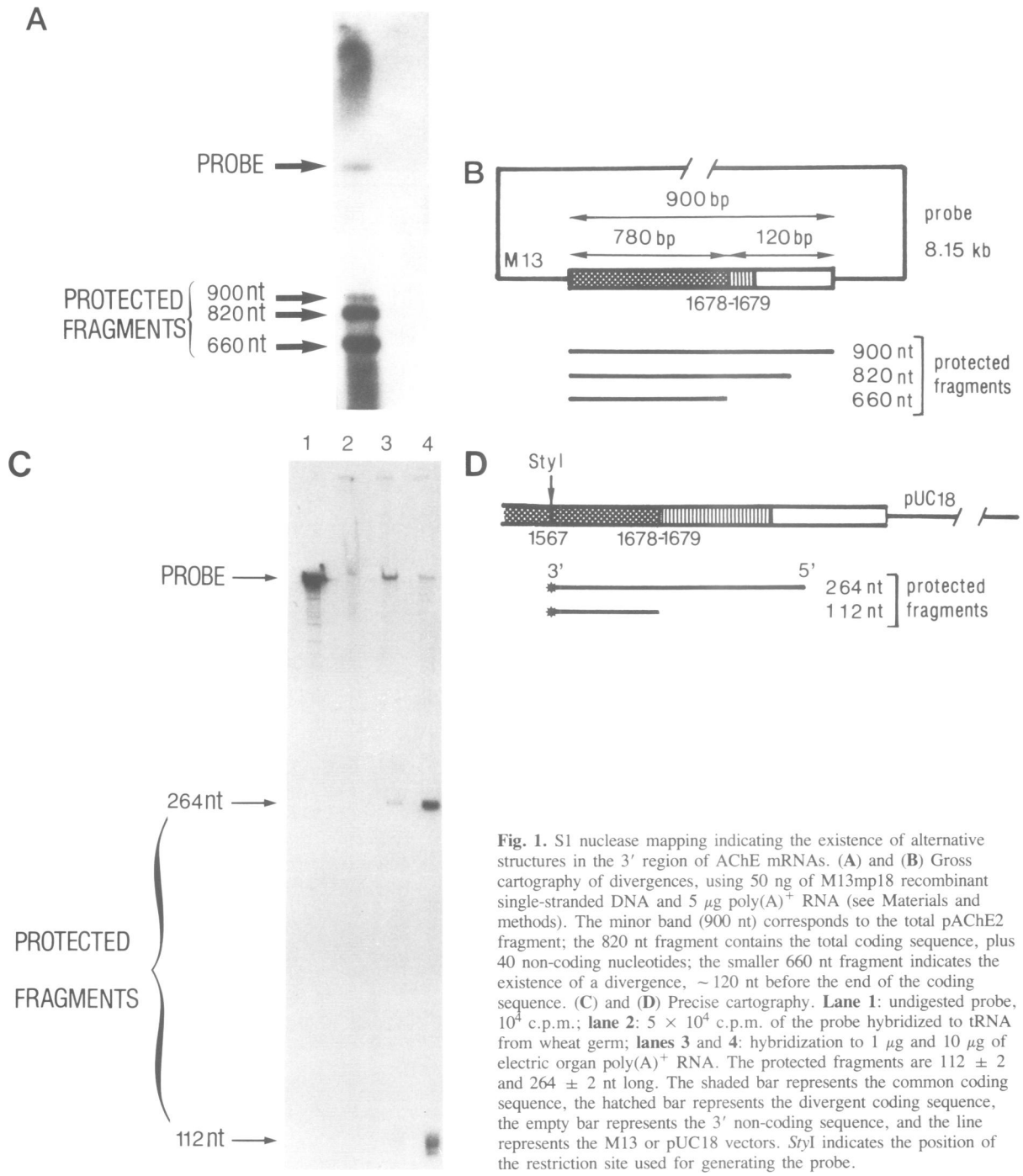


Fig. 1. S1 nuclease mapping indicating the existence of alternative structures in the 3′ region of AChE mRNAs. (**A**) and (**B**) Gross cartography of divergences, using 50 ng of M13mp18 recombinant single-stranded DNA and 5 μg poly(A)$^+$ RNA (see Materials and methods). The minor band (900 nt) corresponds to the total pAChE2 fragment; the 820 nt fragment contains the total coding sequence, plus 40 non-coding nucleotides; the smaller 660 nt fragment indicates the existence of a divergence, ~120 nt before the end of the coding sequence. (**C**) and (**D**) Precise cartography. **Lane 1**: undigested probe, 10^4 c.p.m.; **lane 2**: 5×10^4 c.p.m. of the probe hybridized to tRNA from wheat germ; **lanes 3** and **4**: hybridization to 1 μg and 10 μg of electric organ poly(A)$^+$ RNA. The protected fragments are 112 ± 2 and 264 ± 2 nt long. The shaded bar represents the common coding sequence, the hatched bar represents the divergent coding sequence, the empty bar represents the 3′ non-coding sequence, and the line represents the M13 or pUC18 vectors. *Sty*I indicates the position of the restriction site used for generating the probe.

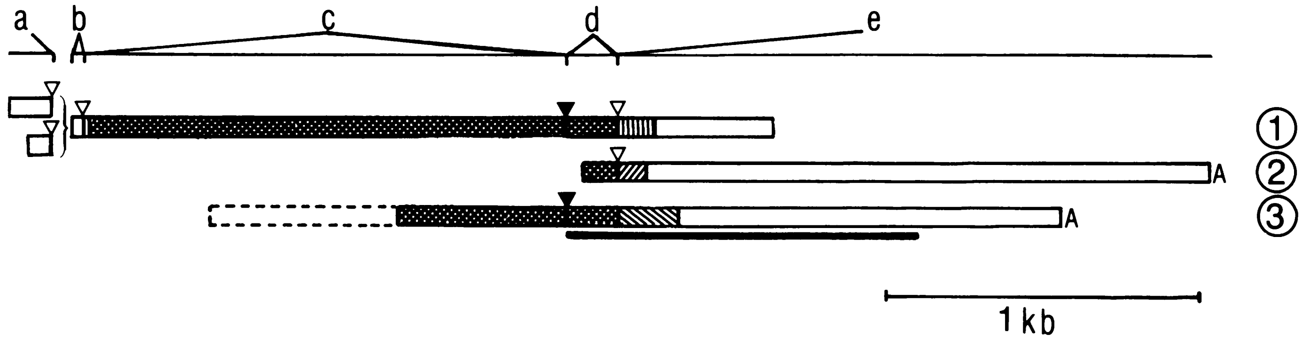
λAChE8 and λAChE9 would encode divergent peptides of respectively 66 and 31 amino acids. The C-terminal peptide encoded by λAChE9 starts with Ala-Cys, and shows a significant homology with the corresponding sequence deduced from the cDNA of *Drosophila* AChE, which also consists of GPI-anchored dimers (Gnagey *et al.*, 1987; Fournier *et al.*, 1988). λAChE9 therefore codes for the precursor of the amphiphilic G^a subunit.

Figure 3 shows the hydropathy profiles of the three divergent C-terminal regions of *Torpedo* AChE, of the homologous region of *Drosophila* AChE, according to Manavalan and Ponnuswamy (1978). The G^a subunits of *Torpedo* and *Drosophila* terminate with a succession of hydrophilic and hydrophobic stretches. The putative sequence deduced from λAChE8 also contains a hydrophobic segment.

Existence of the divergent sequences in mRNAs

S1 nuclease mapping of poly(A)$^+$ RNA from electric organ was performed with uniformly labelled probes derived from clones λAChE8 and λAChE9, containing both common and divergent regions (Figure 4A and B). With the probe derived from λAChE8, we obtained a major protected fragment, corresponding to the common coding region, upstream from the divergence, and a much weaker band, which contained also the divergent region. The divergent sequences corresponding to the A and G^a subunits were much more abundant, and in similar proportions, as indicated

A



B

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                                                                                          E  T  I  D  E
                                                                                          AGACCATTGACGAG    1
F  I  D  L  N  T  E  P  I  K  V  H  Q  R  L  R  V  Q  M  C  V  F  W  N  Q  F  L  P  K  L  L  N  A  T   A  C  D  G  E
TTTATTGACCTCAACACAGAGCCCATAAAAGTCCACCAGCGACTCCGAGTTCAGATGTGCGTATTCTGGAACCAGTTCCTCCCCAAGCTCCTCAACGCCACAG  CTTGTGATGGAGAA    2
                                                                                          G  N  V  F  A
                                                                                          GTAATGTGTTTGCT    3
1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680      1690

A  E  R  Q  W  K  T  E  F  H  R  W  S  S  Y  M  M  H  W  K  N  Q  F  D  Q  Y  S  R  H  E  N  C  A  E  L  *
GCAGAACGCCAGTGGAAGACGGAGTTTCATCGGTGGAGTTCCTACATGATGCACTGGAAGAACCAATTTGACCAGTACAGCAGACACGAGAACTGTGCTGAGCTGTGAGCTCTGCACTGC    1
L  S  S  S  G  T  S  S  S  K  G  I  I  F  Y  V  L  F  S  I  L  Y  L  I  F  Y  *
CTGTCTAGCTCCGGGACATCTAGTTCTAAGGGGATCATTTTCTATGTTTTATTTTCTATTCTGTACTTGATTTTTTATTAAGCAAACGTAGTTCCCAGGGTGTGTGGCATTCTGTGTGAA    2
F  H  M  Q  K  V  R  T  P  A  K  T  Y  H  F  G  V  I  V  A  H  L  L  L  S  R  P  T  A  S  D  V  P  R  L  A  S  S  K
TTCCACATGCAGAAAGTTCGAACCCCCGCCAAAACGTATCACTTTGGTGTAATCGTTGCCCATCTCCTTCTCCTTTCTCTTCCCACGGCATCGGACGTTCCTCGCCTGGCCTCGTCCAAA    3
   1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800      1810

AGGTCGCCTGGTGAGGCAGAGAGCAGAGTCCGATATTGACCAGACACCCAGTCTAGTTCCTGGAGACCCTGCCTGGCCCTCTGAGCCACCCCGCCAAACCTCGCCTTCGAGACTGCCTCC    1
AGGCCATGCAAGAGGAGAAAAGGAGGGCTGTTTGTCAAGAGTTCACATATGATCCAGGACCAGTCAAATTTGGCGAGTGGATTAGGGGGACGTTGGATTGGAGTTATTCAGATGTAGGCC    2
W  W  A  H  S  D  P  L  C  S  R  R  C  W  E  S  W  G  R  I  L  *
TGGTGGGCCCACTCCGACCCCCTGTGCTCTCGCCGGTGTTGGGAGAGCTGGGGTCGGATTCTGTAGCTCTCCTGGCGCGGGGGAAGGGGGATCGAGTATGGCCGTTCCTCTCCCAACCAT    3
   1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920      1930

CTCCCATCCCTCCGCTAACCCCAGGCTGCCGCACCTTGTTCTCTCGCATCATCACTCGTGAATCTGCGGCCACCATTCTTTCGGCCACCCACTCTCGCATCAGCCCAATTCTCTCCTTCC    1
CAAGCTCTCTCCCAACGTCCAGTCCTGAAAAAGTACCCCCGCACATAAGCAAGCCCCCGAAAGCCCCTCGTCACCTGCCAAACGCTGAGCGCTTGTCCCCTTCAAGCCCCCCCATTCCC    2
GGCGAGGACCACACTGCGCAGGCTGGGATCAAGGGCGTGTAGTAGCGTTAGTGCCTCACGGTTCCAGTGACCCAGGGTCATTCCTGACCTCCGTGCTGGCTGTGTGAGTCTCTGTGACTG    3
   1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040      2050

TCTCCCCCCCTATTGCCCACTCTCCACCTGCCGCACTCTCTCTCCCAATACCCGTCAGCAACCATTCCGGTCCCCTTGATCTCTCTCCTGTCACCTATTGACGTTCTACCCAACCCCCC    1
AACGCCTCCCGACCCCTGAACTCCTCCAAAGCCCTCTATCCAACCCCTCCTCAATGCCCGTCACCAAATCCTGAAACATTCCCACCCCACCCCCTCCGACAAAGTTCCAAAATTCAGATT    2
CCACAAATGTTCCGTCTTCCCCCCATTTCCCAAAGAAAGGCGGGTTGGTAGACCAATTGGCCAGTGCAGATTGTCCAGGTGGGTAATATGAACTGTAAGGAATATGGGGAGAACAGATGA    3
   2060      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160      2170

CTACTCGG                                                                                                                    1
TATTGTCAGAGTTCATACATGTATCACATACAACCCCCGAGATTCTTTTTCCCGCGGGCAAAGCAGAATTACCACTTACTGGTGGTGCCAAAAAACTGTGCTCAAGAAGATGCATGTAAA    2
CAGAGAAAATTTGCCTGGGGGAAACGGATAACTTCATGAACTAGCATAGACAGTGGGCTGAATGGCCTCCTACGTTTGTTAAGGCTGGATGGAAAATGTCCAAAACAGGGAACATTTCC    3
   2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280      2290

TAAATGAGGGCGGCATGGTTGGCACGTGGCGGTTAGCGCAACCCTGTTACAGCGCCACAGTCCGGGGTTCGAATCCGGCGCTGTCTGTAAGGGGTTTGTACGTTCTCCCCGTGTCTGTGT    2
GTCTGATTCAGTCTCGACAATGGAGGTCGTCCTCCATCTTCTTCAGGGGGTACCTAATCTCATCTGAGCTTCCGCATCAGTCACAGAGAGCCAGAGCCCATCTGAGGCTTGGCAGAGTTC    3
   2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400      2410

GGGTTTTCCCCGGGGGCTCCGGTTTCCTCCCACCGTTCAAAACGTACCAGGGGTTCTAGGTTATATTTGGGCGACACGGGCTCATGGGCCAGAAGGGCTGTAACCATGCTGTATGTCTAA    2
ACGTTTTACCAATAGTTTCCTTGCGTTTGCCTGTTTACGCAGATAAATATGAATATTAAATTGTTCACCTCCCACCGTGCAATCATATGATGTTCGCAAACGAACACCCAAGAGTTTCCT    3
   2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520      2530

ATTTAAATTTAAACATTACAGCACAGTAAAGGCCCTTCGGCCACGATGTTGTGCCGACCCATATATACCTAAGCAAAAACTAAACCCTCCCTACCTCATAATCCTCTGTACGTTCTCCCC    2
CGATCTGTCAACGCTGCCTTGTCCAACAGTGCTGAAGGAAAATTATAAAGAAAGGCCACGAGAAAGTGAGACATTTGTTAACCGCGTGACAGTTACGGGCAACCGGGATGCGGCCCCCTT    3
   2540      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640      2650

GTGTCTGCGTGGGTTTCCTCCGGGCGCTCCGGTTTCCTCCCACCGTTCAAAATATATACCAGGGGTTGTAGGTTATATTTGGGCGACACGGGCTCATGGGCCGGAAGGTGTTACCGTGCT    2
TAAGGGTCTGTCAATGGCTCAGTTGCCGTGGAAACGGAGTCGTTTCTGGGCTCGAGGTTCCCCAGCCTCCCTTTCTGCCTCGATTTCCCTGCCAGACGAGTCGTGCCTCGTTACCCTCGG    3
   2660      2670      2680      2690      2700      2710      2720      2730      2740      2750      2760      2770

GTATGTCTAAATTTTTTTTTTTAAATGTAAACAACCTGACTGTGCAATACAGAGAAGAAAACAAAATCAATTAAGAGCAAAAGTCTTTACCATATAACCTATATAACATATTACAGCACAG    2
CTTAATACAGAGGAAACGTAGCGATTAGATGCGAGTTGGGTACACGGGTGAGGTCAAGATTGAGTTCAGGATGAGTGGCCCCATGTCCAAACGCCCTGTTAGTATCCCTTGCAAAAATGA    3
   2780      2790      2800      2810      2820      2830      2840      2850      2860      2870      2880      2890

AAACAGGCCAGTTCGGCCTTCTAGTCCATGCCGAACACCTTCTCCCACCTAGTCCCACTGACCGCACCCGGCCCATAACCCTCCGCACCTCTCTCGTCCATATACCTATCCAACTTTTCC    2
GGAAGGGGGAAAGATGAATTATCATCAGACCATGAGATATTGGAGCAGAAATAGGCCATTCAGCCCATCGAGTCTGCCCCGCCATTCAATCATGAGCTGGTCCATTTCCCCACTCAGCCC    3
   2900      2910      2920      2930      2940      2950      2960      2970      2980      2990      3000      3010

TTAAACATTAAAATTGAACCTGCATCTACCACTTCGGCCGGAAGCTCGTTCCACATTCCCACCACCCTCTGAGTGAAGAAATTCCCCTCATGTTTCTCTAAACTTTTCCCCCTTCAATCT    2
CACTGCCCGGCCTTCTCCCCATAACCTTTGATGCTCTGGAGAAAGGGTTCAAATATTAAGTCAGGGAATTATGCATTAAAATAATTTTTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA    3
   3020      3030      3040      3050      3060      3070      3080      3090      3100      3110      3120      3130

CAATCCATGCCCTCTTGTTTGAATCTTCCCCACTCTCAATGGAAAAAGCCTGTCCACATTGACTCTATCTTTCCCCTTCATAATTTTAAATACCTCTATCAAATCACCCCTCAACCTTCT    2
   3140      3150      3160      3170      3180      3190      3200      3210      3220      3130      3240      3250
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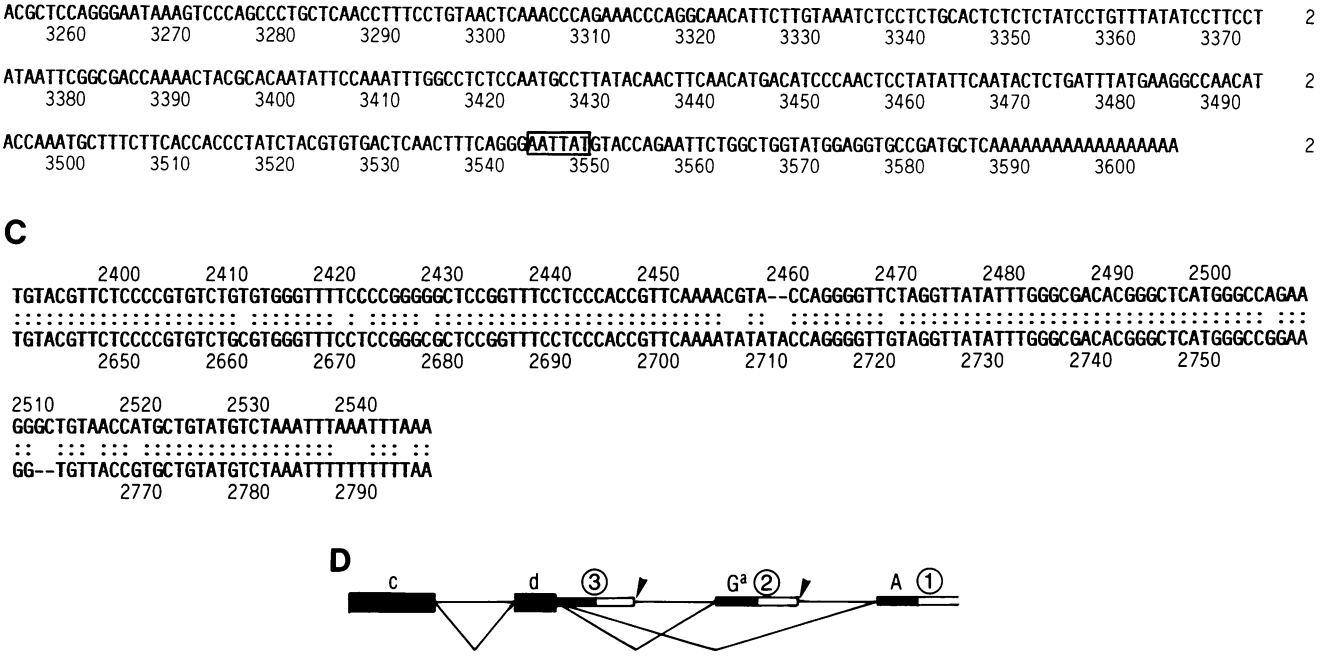

Fig. 2. Structure of the cDNA clones. (1) cDNA structure encoding the A subunit, from Sikorav *et al.* (1987); (2) λAChE9; (3) λAChE8. (**A**) Schematic diagram of the different cDNA structures; empty boxes: non-coding regions; shaded boxes: common coding region; hatched boxes: divergent 3′ coding regions. The box enclosed in a dashed line represents a part of the λAChE8 insert which has not been sequenced. The black triangles indicate sites of splicing, the open triangles, sites of alternative splicing; the letter 'A' represents poly(A) tails. (a) Two possible 5′-untranslated exons, probably corresponding to different transcription origins; (b) alternatively spliced 5′ untranslated exon; (c) first common coding exon; (d) second common coding exon; (e) alternative 3′ regions. The underlined region of λAChE8 has been shown to correspond to a continuous genomic sequence of *T.californica* (Y.Maulet and P.Taylor, personal communication). (**B**) 3′ regions of cDNA clones pAChE2 (1), λAChE8 (3) and λAChE9 (2). The figure shows only a small part of the 5′ regions of the inserts, which correspond exactly to the sequence of clone pAChE2 (Figure 3, Sikorav *et al.*, 1987), from position 976 in the case of λAChE8 and from position 1574 in the case of λAChE9 (numbered from the initiation site); upstream of the *Eco*RI site (976), λAChE8 contains a 600 bp fragment which has not been sequenced. The common sequence, as well as the sequence of λAChE9 and their amino acid translations are indicated in bold type; the divergent parts of pAChE2 and λAChE8 are indicated in normal type. Putative polyadenylation signals are boxed. (**C**) Alignment between two repeated sequences of λAChE9, according to Kanehisha (1984). (**D**) Interpretation of the splicing pattern producing different C-terminal regions. Common exons are represented by wide boxes, alternative exons by narrow boxes and introns by lines; coding regions are black and non-coding regions empty; the arrows indicate polyadenylation sites. This arrangement is very similar to that which produces secreted and membrane-bound forms of immunoglobulin heavy chains, e.g. IgM (Early *et al.*, 1980) and IgG (Rogers *et al.*, 1981), corresponding respectively to (3) and (1) or (2). The existence of additional non-coding 3′ exons might explain the heterogeneity and large size of the mRNAs. Note that this diagram is not drawn to scale.

by S1 analyses using the A sequence (Figure 1A and C), as well as a probe derived from λAChE9 (Figure 4C and D). These experiments establish that both λAChE8 and λAChE9 sequences exist, in different proportions, in the mRNA population. We found no indication of heterogeneity in the mRNAs at the exon−exon boundary which exists between nucleotides 1490 and 1491 (Y.Maulet and P.Taylor, personal communication), as shown in Figure 1, and in experiments with a uniformly labelled probe (data not shown).

Northern blot analyses with A and G^a specific probes

Figure 5 shows Northern blots of electric organ poly(A)$^+$ RNA. As previously reported (Sikorav *et al.*, 1985), a 1208 bp probe corresponding to the common coding sequence hybridized with three bands, at 5.5 kb, 10.5 kb and 14.5 kb. The same bands were also observed with the straddling 22mer oligonucleotide (see above), and with oligonucleotides specific for the A (1689−1714 from pAChE2) and G (1703−1725 from λAChE9) divergent sequences. The existence of multiple minor, smaller mRNAs, from 2−5 kb, can also be detected on overexposed Northern blots (data not shown).

Interchain disulphide bonds

We examined whether the C-terminal cysteine of the G^a subunit, in addition to carrying the GPI anchor, was also responsible for the interchain disulphide linkage of G^a subunits: we compared intact amphiphilic $G_2{}^a$ AChE and its non-amphiphilic derivative ($G_2{}^{na}$) from which a C-terminal peptide had been removed by pronase. To make sure that cleavage was complete, the two forms were isolated by sedimentation, the former sedimenting at 6S in the presence of Triton X-100, and the latter at 7S, with or without detergent. Denaturing polyacrylamide gel electrophoresis of non-reduced and partially reduced enzyme showed that both intact and lytic forms were disulphide-linked (Figure 6). The lytic dimers and monomers show a small but significant decrease in their mass, compared to the intact enzyme.

Discussion

Evidence for complex alternative splicing, generating AChE subunits with distinct C-terminal regions

We have described two distinct cDNA clones, isolated from a *T.marmorata* electric organ library, which contain a common AChE coding sequence, and both diverge from the

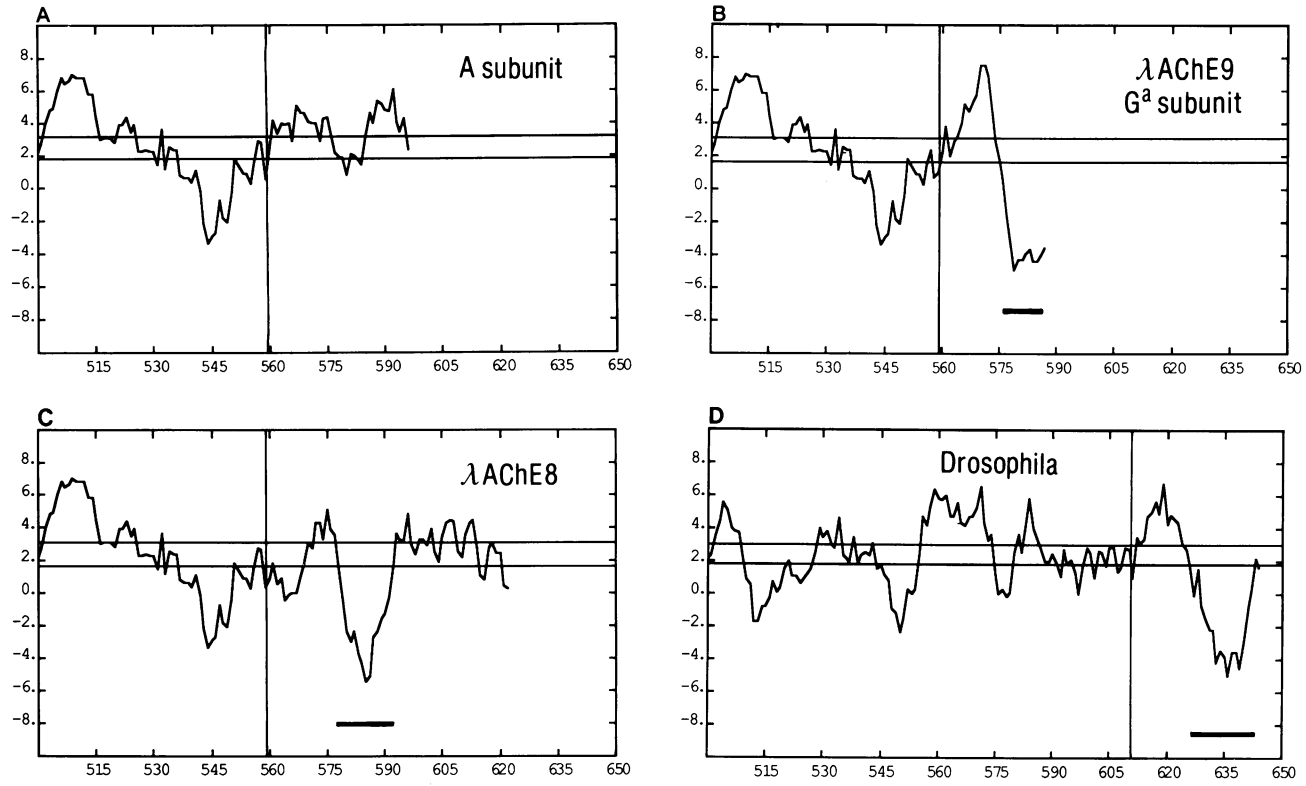

Fig. 3. Hydropathy profiles of AChE, with a comparison of the different possible C-terminal sequences, starting at position 500. The lower line indicates the mean hydrophobicity and the upper line indicates the limit of a significant hydrophilic character, as computed according to the method of Manavalan and Ponnuswamy (1978), using spans of seven amino acids. (**A**) A subunit; (**B**) λAChE9; (**C**) λAChE8; (**D**) *Drosophila* AChE. The vertical line indicates the position of divergence between the three *Torpedo* cDNA sequences, and the corresponding position in the *Drosophila* sequence. Solid bars underline the hydrophobic regions in (B), (C) and (D). The amino acids are numbered from the first methionine, including the signal peptide.

previously established coding sequence of the A subunit after nucleotide 1678 (numbered from the first initiation codon, as in Figure 3 of Sikorav *et al.*, 1987).

The existence of a divergence at this position was predicted and confirmed by S1 mapping experiments (Figures 1 and 4), and is consistent with the polypeptide structure of the A and G^a subunits (MacPhee-Quigley *et al.*, 1986; Gibney *et al.*, 1988). This position coincides with an exon−exon boundary in the AChE genes of *T.californica* (Y.Maulet and P.Taylor, personal communication) and *Drosophila* (D.Fournier, P.Spierer and J.Bergé, in preparation), as well as in the C-terminal domain of thyroglobulin (Malthiéry and Lissitzky, 1987). Because a single gene appears to code for the various AChE catalytic subunits (Sikorav *et al.*, 1987), multiple mRNAs must be generated by alternative splicing.

One of the divergent cDNA clones codes for the glycolipid-anchored G^a subunit

The amphiphilic *Torpedo* G_2^a dimers possess a glycophosphatidylinositol (GPI) anchor: their hydrophobic domain consists of a phosphatidylinositol, linked through a carbohydrate and an ethanolamine to the C-terminal amino acid, cysteine G^a-538. After the divergence position, λAChE9 encodes a 31 amino acid peptide which starts with Ala-Cys, in agreement with the peptidic sequence determined by Gibney *et al.* (1988). In addition, the C-terminal peptide bears a clear homology to that of the putative precursor of *Drosophila* AChE: in this case, the second amino acid encoded by the C-terminal exon is also a cysteine and it is followed by 34 residues, also starting with Asp-Gly (Hall and Spierer, 1986). The structure of clone λAChE9 therefore possesses all the features expected for an mRNA encoding the precursor of the G^a subunit.

The cDNA sequence of clone λAChE9 predicts that the G^a subunits are first synthesized with a 29 amino acid extension, which is probably exchanged very rapidly for the glycolipid, as shown in the case of DAF (Medof *et al.*, 1986) and of the *Trypanosoma* VSG proteins (Ferguson *et al.*, 1986). The molecular weight computed for the non-glycosylated precursor (i.e. including the signal peptide) is of 66 kd, roughly 2 kd lighter than the precursor of the A subunit (Sikorav *et al.*, 1987), in agreement with the values obtained for the products of *in vitro* synthesis directed by *Torpedo* electric organ mRNA (Sikorav *et al.*, 1984).

The only common feature of the C-terminal extensions of GPI-anchored proteins seems to be the presence of a hydrophobic sequence (Ferguson and Williams, 1988) and the structure of the predicted peptide agrees with that rule. It is remarkable that the extensions predicted for *Torpedo* and *Drosophila* G^a subunits terminate with a similar succession of hydrophilic and hydrophobic sequences; the GPI anchor would however be more distant from this motif in *Drosophila*, if it is grafted to the homologous cysteine. This observation raises the question of the protein features which determine this type of processing and define the position of cleavage.

Nuclease S1 protected fragments containing only the common sequence appear approximately equivalent in

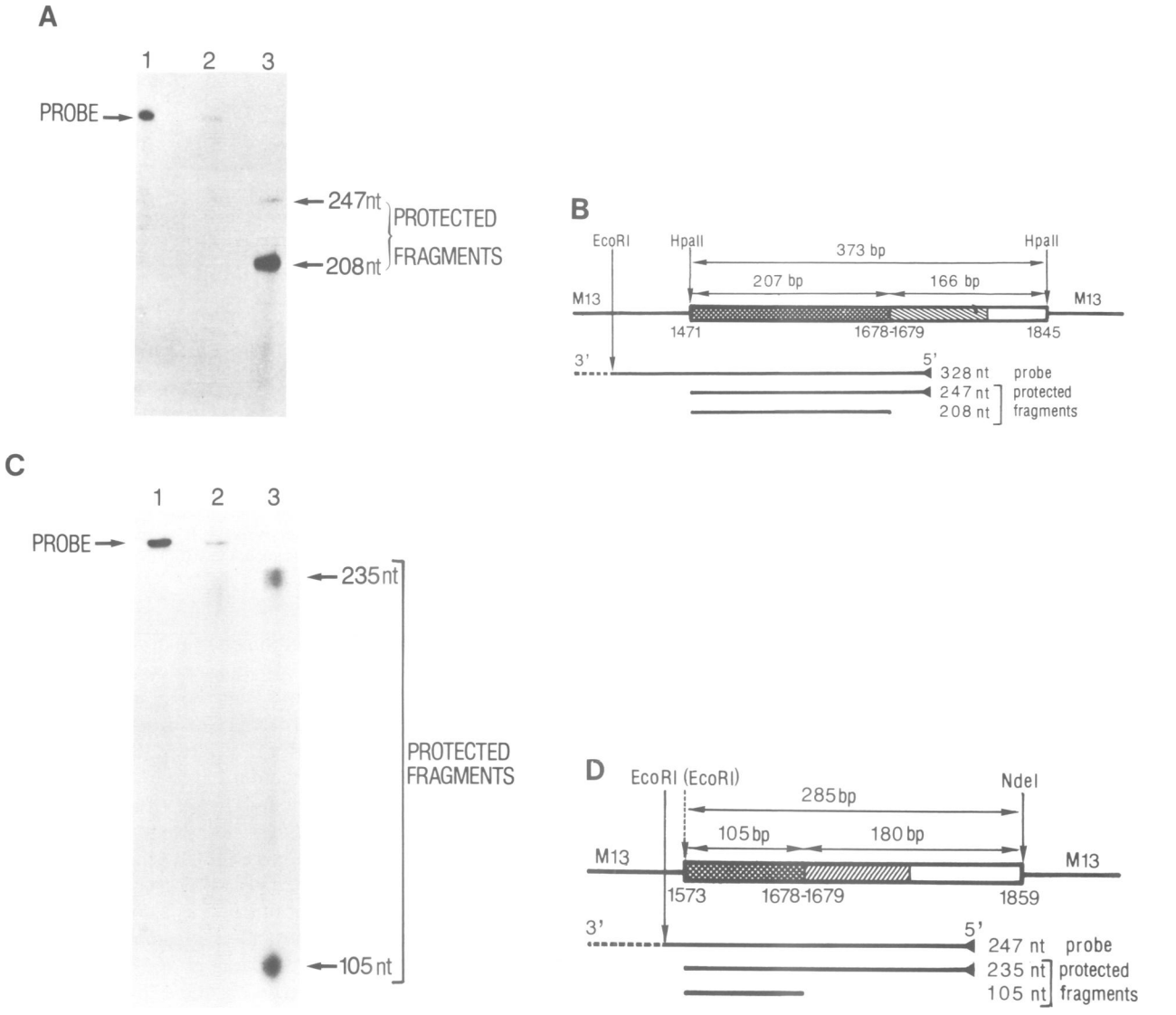

Fig. 4. S1 nuclease mapping with probes derived from λAChE8 and λAChE9. (**A**) and (**B**) Structure of the uniformly labelled probe, derived from an *Hpa*II−*Hpa*II fragment of λAChE8. The phage λAChE8 was digested with *Hpa*II, filled-in and the desired restriction fragment was subcloned in the relevant direction at the filled-in *Bam*HI site of M13mp10. A probe was obtained using an oligonucleotide complementary to nucleotides 1701−1719 (5′-GGGGTTCGAACTTTCTGC-3′), and *Eco*RI was used to cleave the extended product. The shaded bar represents the common coding region, the hatched bar represents the divergent region, the empty bar represents the 3′ non-coding region, and the line represents the M13 vector; the thin vertical lines indicate the restriction sites used to construct the probe; the dashed line corresponds to a filled-in *Eco*RI site; for details, see Materials and methods. **Lane 1**: undigested probe, 10^4 c.p.m.; hybridization of 2×10^5 c.p.m. of the probe to 5 μg of wheat germ tRNA (**lane 2**), and to 5 μg of electric organ poly(A)$^+$ RNA (**lane 3**). (**C**) and (**D**) For λAChE9, an *Eco*RI−*Nde*I fragment was used in the same manner, i.e. filled-in and subcloned at the filled-in *Bam*HI site of M13mp10. The probe was obtained by priming with another oligonucleotide, complementary to 1791−1809. The mol. wts were estimated from a sequencing ladder (not shown).

abundance to fragments which also contain specific A or G^a sequences (Figures 1A,C and 4C). This suggests that the levels of both types of mRNAs are similar. It is likely that the mRNAs are translated with the same efficiency, since their 5′-untranslated regions and most of the coding sequences are identical, and therefore that A and G^a subunits are synthesized at comparable rates in electric organs. This is consistent with *in vitro* translation of *Torpedo* mRNAs, which produces two major precursors of AChE catalytic subunits (Sikorav *et al.*, 1984). A direct demonstration of the synthesis of both asymmetric and $G_2{}^a$ forms has been obtained by heavy-isotope labelling in cultures of embryonic *Torpedo* electric organ (S.Bon, unpublished experiments). It is therefore clear that the $G_2{}^a$ form is not only imported from the motoneurons of the electric lobes by axonal flow to presynaptic membranes (Morel and Dreyfus, 1982; Li and Bon, 1983; Futerman *et al.*, 1985), but is also produced locally in the electric organ.

What is the significance of the second divergent cDNA clone?

S1 mapping analysis rules out the possibility of an artefactual construction of λAChE8. Moreover, the genomic sequence of *T.californica* which follows the second coding exon has been partially determined over 1 kb, and shows at least 90% identity with that of λAChE8, bridging the junction between the common and divergent regions. There is no overlap with the A or G^a cDNA clones in this genomic region. The

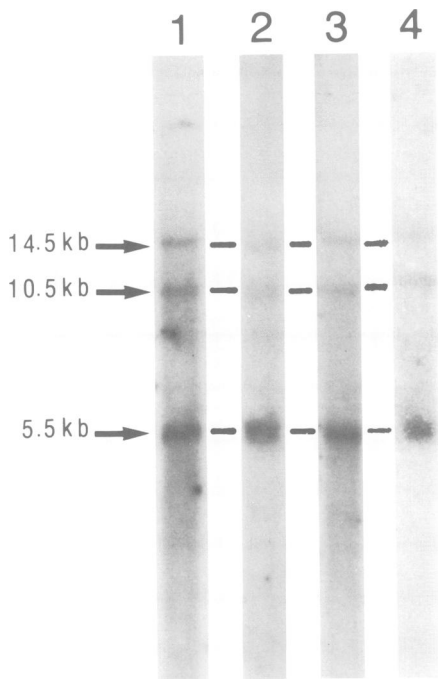

Fig. 5. Northern blots of electric organ poly(A)$^+$ RNA. 5 μg poly(A)$^+$ RNA were deposited in each lane and four different probes were used for hybridization. **Lane 1**: hybridization with the 1208 bp fragment of pAChE2 (nucleotides 606–1813); **lane 2**: hybridization with the 22mer oligonucleotide (1668–1689) specific for the junction of the common exon to exon A; **lane 3**: hybridization with a G^a-specific oligonucleotide (1703–1725 from λAChE9); **lane 4**: hybridization with an A-specific oligonucleotide (1689–1714). The cDNA probe was labelled by random primed synthesis of a complementary strand of the pAChE2 fragment using [α-^{32}P]dCTP (Feinberg and Vogelstein, 1984), and oligonucleotides were labelled by T4 polynucleotide kinase, with [γ-^{32}P]ATP (Richardson, 1965). The specific activity of the probes was $\sim 10^9$ c.p.m./μg.

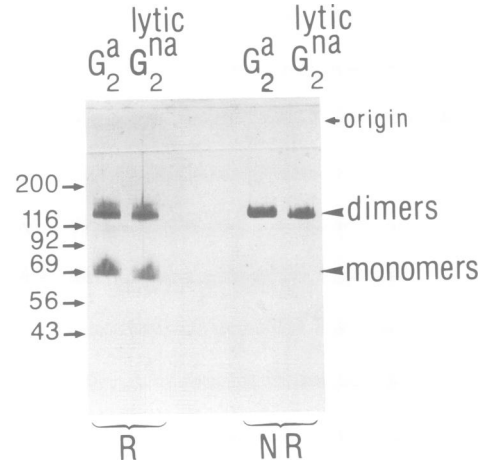

Fig. 6. Existence of disulphide bonds in amphiphilic G_2^a AChE and its proteolytic non amphiphilic derivative. G^a: intact enzyme; G^{na}: non-amphiphilic lytic enzyme. NR: non-reduced; R: partially reduced. Note that the apparent mass of the lytic monomer (67 kd) is smaller than that of the intact subunit (69 kd).

divergent sequence of λAChE8 must therefore be spliced to produce the A and G^a forms. Indeed, this sequence presents some of the characteristics of an intron, because it begins with a canonical intron donor motif (AG/GTAA) (Shapiro and Senapathy, 1987).

In support of the possible intronic nature of the λAChE8 divergent region, it is interesting to note that incomplete splicing has been observed at the same position in BuChE cDNA clones obtained from rabbit liver (A.Chatonnet, personal communication) and human brain (McTiernan *et al.*, 1987), containing the junction of an intronic sequence with the upstream exon and with the A exon, respectively. However, no polymorphism of the BuChE catalytic subunit has been documented so far; in particular, GPI-anchored BuChE forms have not been described.

On the other hand, λAChE8 shows an adequate polyadenylation signal and terminates with a poly(A) tail. We cannot exclude the possibility of an additional splicing, occurring between the 5′ donor site and a putative 3′ acceptor site, which can be found upstream of the poly(A). Our experimental evidence rather supports the hypothesis that the structure of AChE8 exists as such in the mRNA population: nuclease S1 experiments show that it corresponds to a significant proportion of AChE mRNA, whereas the presence of an unspliced intron between the first and second coding exons (nt 1490–1491) was not detectable in similar conditions (data not shown). Thus the simplest interpretation of our results is that 1678–1679 splice site is cryptic in the processing of this mRNA, as illustrated in Figure 2D. An essentially identical situation exists in the case of the secreted and membrane-bound heavy chain variants of immunoglobulins (Early *et al.*, 1980; Rogers *et al.*, 1981), except that we have three possible C-terminal sequences. Interestingly, the DAF protein also presents an alternatively spliced sequence which can function as a coding sequence, and only one of the two structures presumably leads to a glycolipid-anchored protein (Caras *et al.*, 1987).

The proposed mRNA would encode a divergent peptide of 66 amino acids. In *T.californica*, the sequence corresponding to the first 30 codons have been determined with certainty (Y.Maulet and P.Taylor, private communication): it corresponds to the same amino acids, except at four positions. In fact, the predicted C-terminal peptides have the same length and are extremely similar: if we allow for sequence uncertainties which introduce frame shifts, the partially determined divergent peptide contains at least 57 identical residues. Of course, the question of the possible coding function of the divergent region of AChE8 will be finally answered only by the demonstration of the existence of the protein produced by this minor mRNA.

This putative subunit would consist of 600 amino acids (after cleavage of the signal peptide). Its mass would thus be 2.4 kd higher than that of the A subunit, by comparison of the C-terminal domains. In spite of the partially hydrophobic character of the putative C-terminal region, this might correspond to the soluble inactive monomeric 76 kd subunit, which has been reported to exist in electric organ (on the order of 5%), in addition to the 72 kd (A) and 69 kd (G^a) AChE subunits (Witzemann and Boustead, 1983; Stieger *et al.*, 1987). Another possible candidate could be the amphiphilic forms of type II, which are readily soluble without detergent, do not aggregate in the absence of detergent, and do not seem to possess a GPI anchor (Bon *et al.*, 1988).

Are the common domains of the A and G^a subunits identical in conformation? Are the interchain disulphide bonds located in the common or divergent domains?

The primary structure of the A and G^a AChE subunits suggests that the protein is organized in two distinct domains, a large common catalytic domain and a small C-terminal A or G^a domain. The common domain, which contains the catalytic machinery, is probably folded in the same way in all types of subunits. Both types of subunits form disulphide-linked dimers, and MacPhee-Quigley *et al.* (1986) showed that A subunits are associated by cysteine A-572, close to their C-terminal extremity. They also propose that the tail is linked by cysteine 231, in the common domain. If the relationship between associated AChE subunits is equivalent in A and G^a dimers, the G^a subunits should be linked through their C-terminal cysteine (Cys G^a-538).

In agreement with previous observations made on proteinase K-solubilized AChE by Stieger and Brodbeck (1985), we find that the subunits are still disulphide-linked after proteolytic cleavage of a C-terminal peptide, which removes the GPI anchor. It is unlikely that the amide bond which links the GPI ethanolamine to the C-terminal amino acid is sensitive to proteases, as indicated by analyses of proteolytic fragments of other GPI-anchored proteins. Cleavage of Thy-1 by V8 protease, trypsin and pepsin produced glycopeptides containing a variable number of amino acids, including the C-terminal cysteine (which forms an intra-chain disulphide bond) (Campbell *et al.*, 1981; Tse *et al.*, 1985), and digestion of *Trypanosoma* VSG by pronase released the glycolipid anchor attached to the C-terminal amino acid (Asp or Ser) (Ferguson *et al.*, 1985). In the case of human erythrocyte AChE, papain releases a glycopeptide containing the last two amino acids, His-Gly (Haas *et al.*, 1986). In this enzyme, dimers are linked by one inter-subunit disulphide bond, which subsists after cleavage of the anchor by papain (Kim and Rosenberry, 1985), and each subunit binds one detergent micelle (Rosenberry and Scoggin, 1984), suggesting that the two anchors are not in close proximity. In agreement with these findings, our observation that G^a dimers retain their disulphide link after pronase digestion thus implies that it resides in the common domain of the protein. Thus, the nature of the disulphide bonds which link the A subunits as dimers and associate them with the tail raises the question of the identity of quaternary interactions in A and G^a AChE dimers.

Is there a correlation between the size heterogeneity of AChE mRNAs, and the coding of A and G^a subunits?

Northern blots show that A and G^a specific oligonucleotides hybridized with the three major bands (5.5, 10.5 and 14.5 kb) which we previously identified with a common probe (Sikorav *et al.*, 1985). Although we observed a diversity of 5′-untranslated regions, the longest did not exceed 250 bp, as indicated by primer extension (Sikorav *et al.*, 1987). The large size of the three mRNAs may be explained in two ways: either they correspond to incompletely processed transcripts, or they contain very long 3′-untranslated regions. The first explanation cannot be ruled out entirely, but appears unlikely because no pre-messengers could be detected in Northern blots of AChR subunits (Giraudat *et al.*, 1982; Hershey *et al.*, 1983), because S1 nuclease analyses did not show the presence of any unspliced intron between the first and second coding exons, and because the straddling 22mer oligonucleotide, which is specific for the junction between the second coding exon and the A exon, hybridizes with all three bands (Figure 5, lane 2).

The three major bands therefore probably correspond to mature mRNAs, and contain both A and G^a messages (Figure 5). It is likely that the overall length of their 3′-untranslated regions depends either on a choice of exons, or on the use of polyadenylation sites, which are not related to the alternative splicing of the C-terminal coding exons. We have indeed obtained evidence for an alternative sequence in the 3′-untranslated region of the A message (Figure 1).

In addition to the major large mRNA species, smaller mRNAs have been observed in the case of *T.californica* (Schumacher *et al.*, 1986). λAChE8 and λAChE9 might be derived from such minor transcripts, if we assume that their poly(A) stretches represent *bona fide* poly(A) tails, even though we only find approximate polyadenylation signals (Birnstiel *et al.*, 1985): including a 150 bp poly(A) tail, λAChE8 and λAChE9 should correspond to mature transcripts of ~3.5 kb and 4 kb. Experiments designed to identify mRNAs corresponding to λAChE8 are in progress.

Why were cDNA clones for the G^a subunit more difficult to find than for the A subunit?

Several hypotheses could *a priori* be considered to explain a counterselection of AChE G^a clones in cDNA libraries constructed from *Torpedo* electric organs, in our laboratory as well as in P.Taylor's laboratory (personal communication). Firstly, one could suppose that G^a cDNA clones were indeed obtained, but were not recognized as such. They could for example differ only by a short nucleotide sequence from the A clones, so that their restriction fragments would not be readily distinguished. This is the reason why we screened our libraries with the straddling oligonucleotide, which was designed to hybridize only with mRNAs coding for the A subunit, according to a strategy used for cloning DAF (Caras *et al.*, 1987). The structure of clones λAChE8 and λAChE9 however shows that this was not the case.

Another possible explanation could be that the presynaptic component of the G_2^a form is predominant in the electric organ, so that the corresponding mRNAs would be much less abundant than those encoding the A subunits. Here again, our results do not support this hypothesis.

Finally, the existence of long, multiple mRNAs suggested that the G^a subunits would be encoded by the longer mRNAs (10.5 kb and/or 14.5 kb) while the A subunits would be encoded by the relatively shorter 5.5 transcripts. In that case, it would naturally be more difficult to obtain cDNAs containing the G coding sequence, especially in oligo(dT)-primed libraries. Our Northern blots however demonstrate that there is no such bias in the size of the major A and G^a mRNAs. The most likely explanation is that we mostly cloned short, minor mRNAs and that the G^a message is under-represented in this population. Figure 1 indeed shows that pAChE2 does not correspond to an abundant mRNA, in its 3′-untranslated region, and the structure of clone λAChE9 also suggests that it derives from a short mRNA which is not readily detected in Northern blots.

Acetylcholinesterase presents an extremely interesting complexity, from both genetic and biochemical points of view. The AChE gene presents at least two origins of transcription which combine with alternative splicing to produce four possible 5′-untranslated regions (Sikorav *et al.*, 1987). Alternative splicing also operates in the coding sequences, generating two (and possibly three) distinct catalytic subunits. Furthermore, the mRNAs possess three types of very long 3′-untranslated regions, which may be generated by alternative splicing and/or distinct polyadenylation signals. Although the significance of alternative A and G^a coding sequences is clear, the meaning of the diversity in both 5′- and 3′-untranslated regions and the large size of the latter remain puzzling. It is now possible to investigate the relationships between the C-terminal A and G^a domains of AChE, the catalytic activity of the enzyme, and its various modes of attachment; in particular it will be possible to determine which features of the G^a peptide directs the processing which grafts the GPI-anchor, and to search for yet other types of mature AChE subunits.

Materials and methods

The methods used for the preparation of poly(A)$^+$ RNA, the construction and screening of cDNA libraries, sequencing, S_1 nuclease mapping and Northern blots have generally been described previously (Sikorav *et al.*, 1985, 1987). The cDNA libraries were screened with a 1208 bp *PstI*–*PstI* fragment of pAChE2 corresponding to nt 606–1813, and with a 22mer complementary oligonucleotide straddling the alternative exon–exon boundary (1678–1679) (5′-TCAATGGTCTCTGTGGCGTT 3′). Sequencing of the inserts of clones λAChE8 and λAChE9 was achieved by the dideoxynucleotide biosynthetic method (Sanger *et al.*, 1977), using successive oligonucleotides as primers in addition to the M13 universal primer.

Electric organ RNA was isolated according to the method of Cathala *et al.* (1983). The tissue was homogenized in 5 M guanidium monothiocyanate and RNA precipitated by 4 M LiCl. Poly(A)$^+$ RNA was purified on an oligo(dT) column. For Northern blots, electrophoresis of poly(A)$^+$ RNA was performed in 0.6% agarose gels, in a formaldehyde buffer. The mRNAs were transferred to Hybond-C-extra membranes (Amersham) and pre-hybridized and hybridized as previously described (Sikorav *et al.*, 1987).

Mapping

S1 mapping was performed with unlabelled (Figure 1A and B), end-labelled (Figure 1C and D), and uniformly labelled (Figure 5) DNA fragments: *Mapping with an unlabelled DNA fragment.* A pAChE2 fragment of 900 nt containing the 780 last nucleotides of the coding sequence followed by 120 3′-untranslated nucleotides was subcloned in M13mp18 in the relevant direction. The single-stranded infectious form of the phage DNA was purified, and hybridized to poly(A)$^+$ RNA. The products obtained after S1 digestion were fractionated in a 1% agarose gel, transferred to a Nylon membrane, and hybridized to a 1208 bp probe.

Mapping with an end-labelled DNA fragment. pAChE2 DNA was cleaved by *StyI* (at position 1563), labelled at the 3′ end by filling-in with the Klenow fragment of *E.coli* polymerase I, hybridized to poly(A)$^+$ RNA. The fragments obtained after S1 digestion were analyzed in a 6% polyacrylamide sequencing gel.

Mapping with uniformly labelled probes. S1 mapping with uniformly labelled probes was carried out as described previously (Sikorav *et al.*, 1987).

Analysis of disulphite bonds. G_2^a AChE was purified from *T.marmorata* electric organs as described previously (Bon *et al.*, 1986). An aliquot of the pure enzyme was digested with 0.1 mg/ml pronase for 1 h at 20°C; the non-digested amphiphilicdimer and its non-amphiphilic derivative were isolated by sedimentation as described (Bon *et al.*, 1986). Electrophoresis in SDS–polyacrylamide gels, with or without reduction, was performed as described previously (Bon *et al.*, 1986) or with an LKB Phast system and the proteins were visualized with the silver staining method.

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