Isolation and characterization of a cDNA clone for the complete protein coding region of the δ subunit of the mouse acetylcholine receptor

(bacteriophage λgt10/sequence homology profiles/BC3H-1 cells)

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ABSTRACT   A mouse cDNA clone has been isolated that contains the complete coding region of a protein highly homologous to the δ subunit of the Torpedo acetylcholine receptor (AcChoR). The cDNA library was constructed in the vector λgt10 from membrane-associated poly(A)⁺ RNA from BC3H-1 mouse cells. Surprisingly, the δ clone was selected by hybridization with cDNA encoding the γ subunit of the Torpedo AcChoR. The nucleotide sequence of the mouse cDNA clone contains an open reading frame of 520 amino acids. This amino acid sequence exhibits 59% and 50% sequence homology to the Torpedo AcChoR δ and γ subunits, respectively. However, the mouse nucleotide sequence has several stretches of high homology with the Torpedo γ subunit cDNA, but not with δ. The mouse protein has the same general structural features as do the Torpedo subunits. It is encoded by a 3.3-kilobase mRNA. There is probably only one, but at most two, chromosomal genes coding for this or closely related sequences.

The nicotinic acetylcholine receptor (AcChoR) is a postsynaptic integral membrane protein complex composed of four subunits, denoted α, β, γ, and δ, with gel electrophoretic molecular weights ranging from 40,000 to 65,000. The receptor is very abundant in the electric organ of the ray Torpedo and it has been extensively characterized at the biochemical, functional, and sequence levels (1). The amino acid sequences of the four Torpedo subunits have been determined from nucleotide sequences of full-length cDNA clones (2–7).

Nicotinic AcChoRs occur in lower overall abundances per unit mass of tissue at the neuromuscular junctions of vertebrate striated muscle and in various muscle-like cell systems in culture. Vertebrate systems are of greater interest than Torpedo for most electrophysiological studies and for cell biological studies of assembly (see ref. 8). The subunits of the vertebrate receptor are similar in general properties to those of Torpedo, but they are clearly somewhat divergent at the amino acid sequence level (9).

Full-length cDNA clones for the α subunit of a bovine receptor and chromosomal genes for the α subunit of humans and chickens have been characterized (10, 11), as has a partial cDNA clone for the α subunit of the mouse AcChoR (12). As a further step toward the complete characterization of the vertebrate genes, we report here the isolation and characterization of a cDNA clone containing the complete protein-encoding region of the δ subunit of the mouse AcChoR.

MATERIALS AND METHODS

Preparation of Membrane-Associated Polysomal Poly(A)⁺ RNA. BC3H-1 mouse cells were grown and induced to undergo differentiation on reaching confluency so as to express AcChoR as described (13). A membrane pellet enriched in membrane-bound polyribosomes was prepared by a modification of the method of Merlie et al. (14). Our procedure differed in some details, including omission of the nuclear separation step and use of emetine·HCl in all isolation buffers to prevent ribosome run-off. The final membrane pellet was dissolved in 6 M guanidine·HCl/20 mM sodium acetate, pH 5/1 mM dithiothreitol (15). RNA was purified by four cycles of precipitation with ethanol (0.55 vol) and redissolution in the same buffer. Poly(A)⁺ RNA was isolated by two cycles of oligo(dT)-cellulose chromatography.

cDNA Cloning in λgt10. A cDNA library was prepared in the bacteriophage vector λgt10 by a method devised by Charles Rice of this institution (personal communication), based in part on the procedure of Okayama and Berg (16). First strand synthesis was carried out with 10 μg of the membrane-bound poly(A)⁺ RNA essentially as described (16) except that dT₍₁₂₋₁₈₎ was used for priming. Four micrograms of actinomycin D and 40 units of human placental ribonuclease inhibitor were included in the 100-μl reaction volume. Second strand synthesis by replacement of RNA from 4 μg of the RNA·DNA hybrid was carried out without addition of primer by adding Escherichia coli DNA ligase and polymerase I, ribonuclease H, dNTPs, and other components as described (16). Internal EcoRI sites in the resulting duplex cDNA were then protected by methylation with EcoRI methylase. The duplex cDNA was blunt-ended with T4 DNA polymerase and all four dNTPs. Two micrograms of phosphorylated EcoRI linkers (12-mers) was added with T4 DNA ligase. After digestion with EcoRI, free linkers and their fragments were removed by four cycles of precipitation from 2 M ammonium acetate with 0.6 vol of isopropyl alcohol at 25°C. Approximately 2.4 μg of the final cDNA preparation was then ligated to 4.6 μg of EcoRI-digested λgt10 DNA in 25 μl, using T4 DNA ligase. The reaction mixture was incubated at 15°C for 15 hr. Recombinant λgt10 DNA was packaged in vitro (ref. 17, pp. 264–268). Packaged phage were plated on E. coli strains C600 (ref. 17, p. 504) to assess the proportion of recombinants in the library or on C600 ΔHfl (provided by Carol Nottenburg of the University of California, San Francisco) for screening purposes. An unamplified library of approximately 1 × 10⁶ recombinants was obtained.

Library Screening. We screened 1 × 10⁵ recombinant plaques in duplicate by plaque filter hybridization (ref. 17, pp. 324–328) at a density of 2 × 10⁴ recombinant phage per 150-mm (diameter) plate. Full length Torpedo cDNA clones, kindly provided by D. Noonan (α subunit), T. Claudio (β and δ), and S. Heinemann (γ; see ref. 5), were nick-translated to specific activities of approximately 1.0 × 10⁸ cpm/μg. The hybridization solution contained 50% (vol/vol) formamide,

Abbreviations: AcChoR, acetylcholine receptor; kb, kilobase(s); bp, base pair(s).

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0.9 M NaCl/50 mM sodium phosphate, pH 7.4/5 mM EDTA (5× SSPE) (ref. 17, p. 447), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% sarcosine, denatured salmon sperm DNA at 100 μg/ml, poly(rA), poly(rC), and poly(rG) at 2 μg/ml each, and 1 μg of labeled plasmid probe. Hybridization was carried out at 42°C for 48 hr. Filters were washed in 30 mM NaCl/3 mM sodium citrate/0.05% sarcosine at 50°C. Positive plaques were purified and rescreened at least three times.

RESULTS AND DISCUSSION

Source of mRNA. Differentiation of the myogenic nonfusing mouse cell line BC3H-1 results in the biosynthesis of relatively large amounts of AcChoR (13). An additional enrichment of AcChoR mRNA by a factor of about 5 is achieved by isolation of membrane-bound polysomes (14).

Screening the cDNA Library. The cDNA library was screened at moderate stringency with hybridization probes prepared from full-length cDNA clones for each of the four subunits of the *Torpedo* AcChoR. The α, β, and γ probes each identified between 10 and 25 apparent positive plaques. No positives were obtained with the *Torpedo* δ probe, even when screened under less stringent conditions [33% (vol/vol) formamide/5× SSPE, 42°C]. We chose to characterize first the mouse cDNA clones selected by using the *Torpedo* γ probe.

Restriction Enzyme Analysis of the Mouse cDNA Clones. Five clones that gave strong signals with the *Torpedo* γ probe in gel blots and contained inserts of length greater than 1.0 kilobase (kb) were analyzed in detail. Their restriction endonuclease maps are shown in Fig. 1. Indicated on the figure for reference purposes are the sites of the initiator methionine and the terminator codon as determined by nucleotide sequence analysis (see Fig. 3). Several features bear noting. First, all five clones possess identical restriction maps in their coding regions, indicating they were derived from the same or closely related messages. Second, the inserts have widely different 3' ends. Nucleotide sequence determination revealed A-rich regions in the coding strand of the λ58 insert corresponding to the 3' ends of λ46 and λ53. Oligo(dT) priming from A-rich regions in the mRNA could be responsible for this phenomenon. Third, the 5' ends of all five inserts are within 33 nucleotides of the initiator ATG codon (determined by sequence analysis; data not shown). λ60 extends the furthest upstream from the initiator ATG (33 nucleotides). The sequence of λ58, the clone subjected to detailed sequence analysis, extends five nucleotides upstream of the ATG. λ53 begins at position 7 on Fig. 3 and λ61 begins at position 14 on Fig. 3. (None of these values includes the *Eco*RI linker sequence.) Fourth, the inserts from λ58 and λ60 have different restriction maps near their 3' ends. RNA gel blot experiments suggest that this difference is due to a cloning artifact in the 3' untranslated region of λ58 (see Fig. 6 below).
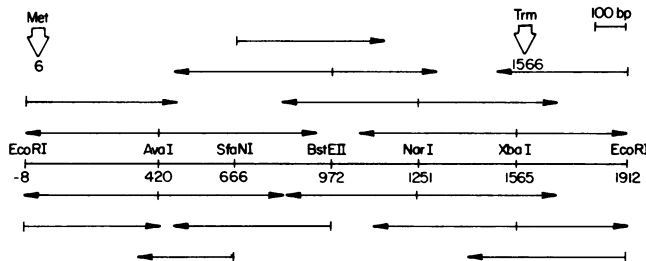

FIG. 1. Restriction maps of inserts from recombinant λ cDNA clones. Positions of the initiator methionine (Met) and the termination codon (Trm) were determined from nucleotide sequence analysis (see Fig. 3). bp, Base pairs.
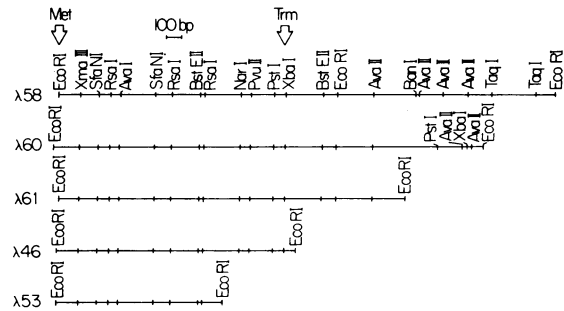

FIG. 2. Sequencing strategy for the protein-encoding region *Eco*RI fragment from λ58 subcloned in pUC9. *E. coli* strain TB1 (provided by T. O. Baldwin, Texas A & M University) was used for transformation. Plasmid DNA was isolated by the alkaline lysis/CsCl gradient method (ref. 17, pp. 90–91). The small closed arrows show the direction of sequencing. Protruding 5' ends were labeled by using T4 DNA kinase and [γ-^{32}P]dATP (>7000 Ci/mmol, ICN; 1 Ci = 37 GBq). Recessed 3' ends were labeled by using *E. coli* DNA polymerase I Klenow fragment (Bethesda Research Laboratories) and the appropriate α-^{32}P-labeled dNTPs (≈3000 Ci/mmol, Amersham). The vertical lines on the ends of the arrows indicate the position of the radioactive label. The nucleotide sequence was determined by the method of Maxam and Gilbert as modified by Smith and Calvo (19). The numbers indicate the position in nucleotides (from Fig. 3) of the restriction sites. The large open arrows show the locations of the initiator methionine (Met) and the termination (Trm) codons.

Nucleotide Sequence Determination. The *Eco*RI fragments in the five cDNA clones shown in Fig. 1 were subcloned separately in the plasmid vector pUC9 (18). Fig. 2 shows the strategy used to determine the nucleotide sequence of the *Eco*RI fragment from λ58 that had been characterized as hybridizing to the coding region of the *Torpedo* AcChoR γ subunit cDNA. Both strands of this fragment were completely sequenced.

Fig. 3 gives the nucleotide sequence of the coding (mRNA sense) strand of this fragment and the deduced amino acid sequence of the complete polypeptide chain. The first base of mouse cDNA from λ58 is at position 1 of the nucleotide sequence in Fig. 3. The initiator ATG codon occurs at position 6. A signal peptide of 24 amino acid residues is encoded by nucleotides 6–77. The first amino acid of the mature protein begins at position 78 of the nucleotide sequence, as judged by comparison with the protein sequences of AcChoR subunits from fetal calf (9). There is an open reading frame of 1560 nucleotides (beginning at position 6), coding for a polypeptide chain of 520 amino acids and terminated by a TAG codon at position 1566. There are two additional termination codons in phase with the reading frame at positions 1581 and 1605. There is no sequence corresponding to the *Eco*RI linker at the 3' end of this fragment, indicating that this *Eco*RI site is a natural internal *Eco*RI site in the mouse sequence. The calculated molecular weights of the mature polypeptide chain and its precursor are 57,104 and 59,393, respectively.

Sequence Comparisons. In Fig. 4 we have aligned the deduced amino acid sequence of the cloned mouse polypeptide chain with the amino acid sequences of the *Torpedo* AcChoR γ and δ subunits. The similarity of the mouse amino acid sequence to the sequences of both of the *Torpedo* subunits is striking. The surprising result is that the mouse amino acid sequence is actually more similar overall to the *Torpedo* AcChoR δ subunit (59% homology) than it is to the *Torpedo* γ subunit (50% homology). (In these calculations, gaps inserted into the sequences for alignment purposes are ignored by the computer.) When the individual amino acids were replaced by their functional groups (nonpolar, polar, acidic, and basic) and the same comparison made, the mouse poly-
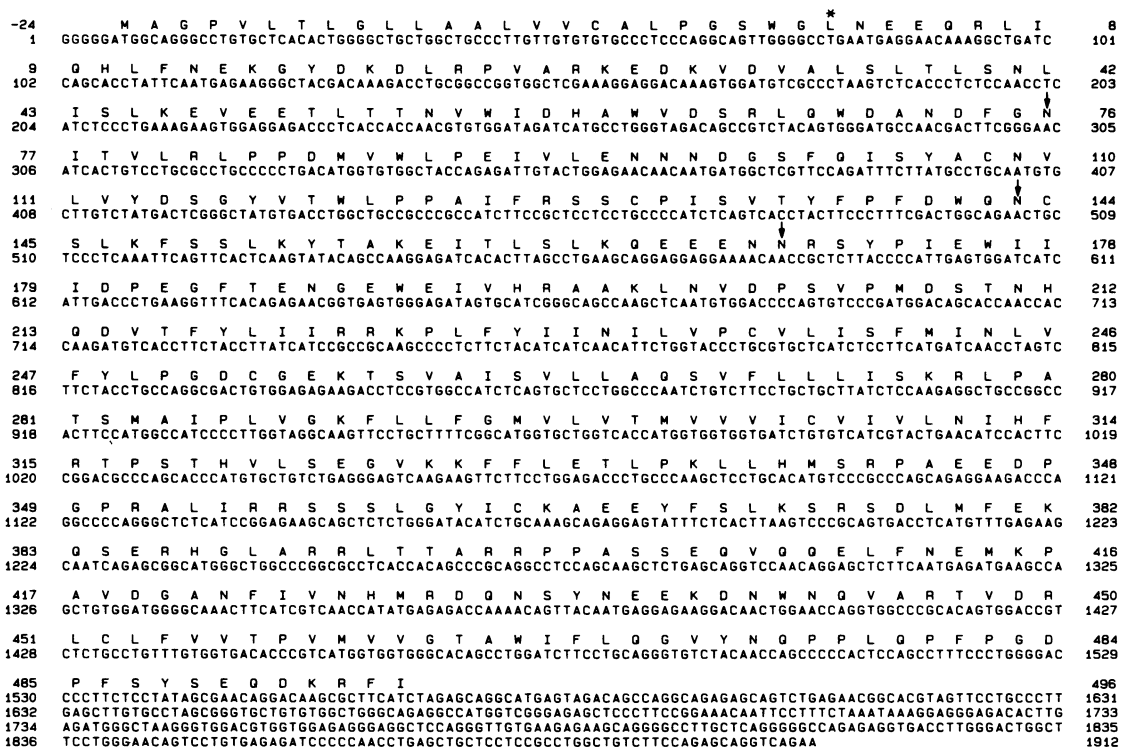
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 -24         M  A  G  P  V  L  T  L  G  L  L  A  A  L  V  V  C  A  L  P  G  S  W  G  L  N  E  E  Q  R  L  I         8
   1  GGGGGATGGCAGGGCCTGTGCTCACACTGGGGCTGCTGGCTGCCCTTGTTGTGTGTGCCCTCCCAGGCAGTTGGGGCCTGAATGAGGAACAAAGGCTGATC    101

   9   Q  H  L  F  N  E  K  G  Y  D  K  D  L  R  P  V  A  R  K  E  D  K  V  D  V  A  L  S  L  T  L  S  N  L        42
 102  CAGCACCTATTCAATGAGAAGGGCTACGACAAAGACCTGCGGCCGGTGGCTCGAAAGGAGGACAAAGTGGATGTCGCCCTAAGTCTCACCCTCTCCAACCTC    203
                                                                                                        ↑
  43   I  S  L  K  E  V  E  E  T  L  T  T  N  V  W  I  D  H  A  W  V  D  S  R  L  Q  W  D  A  N  D  F  G  N        76
 204  ATCTCCCTGAAAGAAGTGGAGGAGACCCTCACCACCAACGTGTGGATAGATCATGCCTGGGTAGACAGCCGTCTACAGTGGGATGCCAACGACTTCGGGAAC    305

  77   I  T  V  L  R  L  P  P  D  M  V  W  L  P  E  I  V  L  E  N  N  N  D  G  S  F  Q  I  S  Y  A  C  N  V       110
 306  ATCACTGTCCTGCGCCTGCCCCCTGACATGGTGTGGCTACCAGAGATTGTACTGGAGAACAACAATGATGGCTCGTTCCAGATTTCTTATGCCTGCAATGTG    407

 111   L  V  Y  D  S  G  Y  V  T  W  L  P  P  A  I  F  R  S  S  C  P  I  S  V  T  Y  F  P  F  D  W  Q  N  C       144
 408  CTTGTCTATGACTCGGGCTATGTGACCTGGCTGCCGCCCGCCATCTTCCGCTCCTCCTGCCCCATCTCAGTCACCTACTTCCCTTTCGACTGGCAGAACTGC    509
                                                                                            ↑
 145   S  L  K  F  S  S  L  K  Y  T  A  K  E  I  T  L  S  L  K  Q  E  E  E  N  N  R  S  Y  P  I  E  W  I  I       178
 510  TCCCTCAAATTCAGTTCACTCAAGTATACAGCCAAGGAGATCACACTTAGCCTGAAGCAGGAGGAGGAAAACAACCGCTCTTACCCCATTGAGTGGATCATC    611

 179   I  D  P  E  G  F  T  E  N  G  E  W  E  I  V  H  R  A  A  K  L  N  V  D  P  S  V  P  M  D  S  T  N  H       212
 612  ATTGACCCTGAAGGTTTCACAGAGAACGGTGAGTGGGAGATAGTGCATCGGGCAGCCAAGCTCAATGTGGACCCCAGTGTCCCGATGGACAGCACCAACCAC    713

 213   Q  D  V  T  F  Y  L  I  I  R  R  K  P  L  F  Y  I  I  N  I  L  V  P  C  V  L  I  S  F  M  I  N  L  V       246
 714  CAAGATGTCACCTTCTACCTTATCATCCGCCGCAAGCCCCTCTTCTACATCATCAACATTCTGGTACCCTGCGTGCTCATCTCCTTCATGATCAACCTAGTC    815

 247   F  Y  L  P  G  D  C  G  E  K  T  S  V  A  I  S  V  L  L  A  Q  S  V  F  L  L  L  I  S  K  R  L  P  A       280
 816  TTCTACCTGCCAGGCGACTGTGGAGAGAAGACCTCCGTGGCCATCTCAGTGCTCCTGGCCCAATCTGTCTTCCTGCTGCTTATCTCCAAGAGGCTGCCGGCC    917

 281   T  S  M  A  I  P  L  V  G  K  F  L  L  F  G  M  V  L  V  T  M  V  V  V  I  C  V  I  V  L  N  I  H  F       314
 918  ACTTCCATGGCCATCCCCTTGGTAGGCAAGTTCCTGCTTTTCGGCATGGTGCTGGTCACCATGGTGGTGGTGATCTGTGTCATCGTACTGAACATCCACTTC   1019

 315   R  T  P  S  T  H  V  L  S  E  G  V  K  K  F  F  L  E  T  L  P  K  L  L  H  M  S  R  P  A  E  E  D  P       348
1020  CGGACGCCCAGCACCCATGTGCTGTCTGAGGGAGTCAAGAAGTTCTTCCTGGAGACCCTGCCCAAGCTCCTGCACATGTCCCGCCCAGCAGAGGAAGACCCA   1121

 349   G  P  R  A  L  I  R  R  S  S  S  L  G  Y  I  C  K  A  E  E  Y  F  S  L  K  S  R  S  D  L  M  F  E  K       382
1122  GGCCCCAGGGCTCTCATCCGGAGAAGCAGCTCTCTGGGATACATCTGCAAAGCAGAGGAGTATTTCTCACTTAAGTCCCGCAGTGACCTCATGTTTGAGAAG   1223

 383   Q  S  E  R  H  G  L  A  R  R  L  T  T  A  R  R  P  P  A  S  S  E  Q  V  Q  Q  E  L  F  N  E  M  K  P       416
1224  CAATCAGAGCGGCATGGGCTGGCCCGGCGCCTCACCACAGCCCGCAGGCCTCCAGCAAGCTCTGAGCAGGTCCAACAGGAGCTCTTCAATGAGATGAAGCCA   1325

 417   A  V  D  G  A  N  F  I  V  N  H  M  R  D  Q  N  S  Y  N  E  E  K  D  N  W  N  Q  V  A  R  T  V  D  R       450
1326  GCTGTGGATGGGGCAAACTTCATCGTCAACCATATGAGAGACCAAAACAGTTACAATGAGGAGAAGGACAACTGGAACCAGGTGGCCCGCACAGTGGACCGT   1427

 451   L  C  L  F  V  V  T  P  V  M  V  V  G  T  A  W  I  F  L  Q  G  V  Y  N  Q  P  P  L  Q  P  F  P  G  D       484
1428  CTCTGCCTGTTTGTGGTGACACCCGTCATGGTGGTGGGCACAGCCTGGATCTTCCTGCAGGGTGTCTACAACCAGCCCCCACTCCAGCCTTTCCCTGGGGAC   1529

 485   P  F  S  Y  S  E  Q  D  K  R  F  I                                                                        496
1530  CCCTTCTCCTATAGCGAACAGGACAAGCGCTTCATCTAGAGCAGGCATGAGTAGACAGCCAGGCAGAGAGCAGTCTGAGAACGGCACGTAGTTCCTGCCCTT   1631
1632  GAGCTTGTGCCTAGCGGGTGCTGTGTGGCTGGGCAGAGGCCATGGTCGGGAGAGCTCCCTTCCGGAAACAATTCCTTTCTAAATAAAGGAGGGAGCACTTG   1733
1734  AGATGGGCTAAGGGTGGACGTGGTGGAGAGGGAGGCTCCAGGGTTGTGAAGAGAAGCAGGGGCCTTGCTCAGGGGGCCAGAGAGGTGACCTTGGGACTGGCT   1835
1836  TCCTGGGAACAGTCCTGTGAGAGATCCCCCAACCTGAGCTGCTCCTCCGCCTGGCTGTCTTCCAGAGCAGGTCAGAA                             1912
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FIG. 3. Nucleotide sequence of the *Eco*RI fragment from mouse cDNA clone λ58 containing the protein coding region. The deduced amino acid sequence (standard one-letter code) of the open reading frame is given above the nucleotide sequence. Nucleotide no. 1 is the first nucleotide of the cDNA. Eight nucleotides d(A-A-T-T-C-G-G-G) present at the 5' end of the sequence were derived from the *Eco*RI linker used in the cloning procedure and have been deleted from the figure. Amino acid no. 1 (*) is that assigned as the first amino acid of the mature protein. The arrows indicate potential sites of asparagine N-glycosylation.

peptide chain was again more homologous overall to the *Torpedo* AcChoR δ subunit (77%) than it was to the *Torpedo* γ subunit (72%). When the nucleic acid sequences were aligned as in Fig. 4, the mouse sequence was also more similar overall to the *Torpedo* δ subunit sequence (60%) than it was to the *Torpedo* γ subunit sequence (55%) (data not shown). It is important to reiterate at this point that the *Torpedo* AcChoR γ subunit cDNA was used to isolate this putative mouse AcChoR cDNA clone and that no positives were obtained when the *Torpedo* δ subunit cDNA was used as hybridization probe.

A clue to the cause for this paradox is provided by the percent homology profiles for both the nucleotide and amino acid sequences (Fig. 5). This analysis displays areas of high and low homology between two sequences as peaks and troughs, respectively. From the nucleic acid sequence comparisons it is apparent that the *Torpedo* γ subunit cDNA sequence contains longer regions of high homology with the mouse cDNA sequence (Fig. 5A) than does the *Torpedo* δ subunit cDNA (Fig. 5C). We estimate that only sequences in the peaks of length *ca.* 75 nucleotides and homology greater than 65–70% in Fig. 5A for the *Torpedo* γ cDNA probe

would hybridize with the mouse sequence under the conditions used (50% formamide/5× SSPE, 42°C), whereas no such regions occur in the *Torpedo* δ cDNA.

In contrast to the nucleic acid homology profiles just described, the amino acid homology profiles show longer stretches of high homology for mouse vs. *Torpedo* δ than for mouse vs. *Torpedo* γ (Fig. 5 D and B), in agreement with the overall percent homology calculations. These data indicate that the subunit of the mouse AcChoR that we have cloned is generally more similar to the *Torpedo* AcChoR δ subunit than to the γ subunit. The nucleic acid sequence homology profiles explain the technical result of selecting a mouse δ cDNA clone with a *Torpedo* γ probe; however, the biological and evolutionary significance of these homology relationships is unclear.

Conti-Tronconi *et al.* (9) have reported NH₂-terminal amino acid sequence data for all four subunits of the fetal calf AcChoR. The data clearly showed sequence homology among the four subunits of the fetal calf AcChoR, similar to that observed in *Torpedo* and *Electrophorus*, and they established the subunit stoichiometry in fetal calf to be 2:1:1:1. However, the data did not permit subunit molecular weights

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                  *
M   -24  MAGPVLTLGLLAALVVCALPGSWGLNEEQRLIQHLFNEKGYDKDL-RPVARKEDKVDVALSLTLSNLISLKE-VEETLTTNVWIDHAWVDS   65
T γ -17  .VL---..L.IIC.-ALEVR---SE...G...EK.L-GD-...RII-.AKTLDHII..T.K...T.....N.K-..A.......EIQ.N.Y   63
T δ -21  .GNIHFVYL.ISC.YYSGCS---.V....E...ND.LIVNK.N.HV-...KHNNEV.NI.............-TD....S...M....Y.H    65

M    66  RLQWDANDFGNITVLRLPPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTWLPPAIFRSSCPISVTYFPFDWQNCSLKFSSLKYTAK   156
T γ  64  ..S.NTSEYEG.DLV.I.SELL...DV.....V..Q.EVA.YA.....ND.SMY.......Y..T...A...........V.R.QT.N.H    154
T δ  66  ..T.N.SEYSD.SI.....EL..I.D...Q....QYHVA.F.....RPN.................N.L............TA.N.D.N    156

M   157  EITLSLKQEEENNRSYPIEWIIIDPEGFTENGEWEIVHRAAKLNV-DPSVPMDSTNHQDVTFYLIIRRKPLFYIINILVPCVLISFMINLV   246
T γ 155  .VN.Q.--SA.EGE--AV...H....D.........T.R..P..K.YNWQLTK-.D.DF.EII.F....Q..........IA......SLVV.   240
T δ 157  ...MD.MTDTIDGKD...........A.........I.KP..K.I-Y.DKFPNG..Y................V..FIT........LAS.A   246

M   247  FYLPGDCG-EKTSVAISVLLAQSVFLLLISKRLPATSMAIPLVGKFLLFGMVLVTMVVV-ICVIVLNIHFRTPSTHVLSEGVKKFFLETLP   335
T γ 241  YF..AQA.GQ.CTLS........TI..F..AQKV.E..LNVS.I..Y.I.V.-F.S.LI.MN......VSL...N..S...KI.HL..GF..   330
T δ 247  ....AES.-..M.T.......A....T.Q...E.AL.V..I.Y.M.I.S...G.I.-N.G....F..........TR..QI...K..      246

M   336  KLLHMSRPAEEDPGPRA---L-IRRSSSLGYICKAEEYFSLKSRSDLMFEKQSERHGLAR-RL---TTARRPPASSEQVQQE--LFN----   412
T γ 331  .Y.G.Q-LEPSEET.E---KPQP..R..F.IMI......ILK.P..E.....E.KD.....-K.VNKM.SDIDIG------TTVDLYKDLANF   409
T δ 336  RI.....AD.SEQ-.DWQND.KL......V...S..Q...NI.....E...........VP-.V----.PRIGFGNNN.NIAASDQ.HD----   417

M   413  --EMKPAVDGANFIVNHMRDQNSYNEEKDNWNQVARTVDRLCLFVVTPVM-VVGTAWIFLQGVYNQPPLQPFPGDPFSYSEQDKRFI       496
T γ 410  AP.I.SC.EAC...AKSTKE..DSGS.NE..VLIGKVI.K-AC.WIALLLFSI..LA...T.HF..V.EF......RK.VP------      489
T δ 418  --.I.SGI.ST.Y..KQIKEK.A.D..VG....L.GQ.I...SM.II....-.L..IF..VM.NF.H..AK..E....D..SDHP.CA      501
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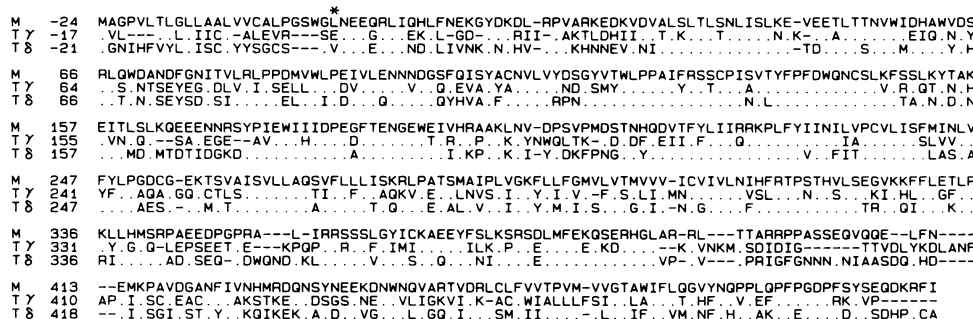
FIG. 4. Alignment of the amino acid sequences of the mouse (M) polypeptide and the *Torpedo* (T) AcChoR γ and δ subunits. The alignment of the latter is from Noda *et al.* (4). Dots in the *Torpedo* sequences indicate identity with the mouse sequence. Dashes indicate positions at which there are no homologous amino acids. In all three sequences, amino acid no. 1 (*) is the first amino acid of the mature protein.

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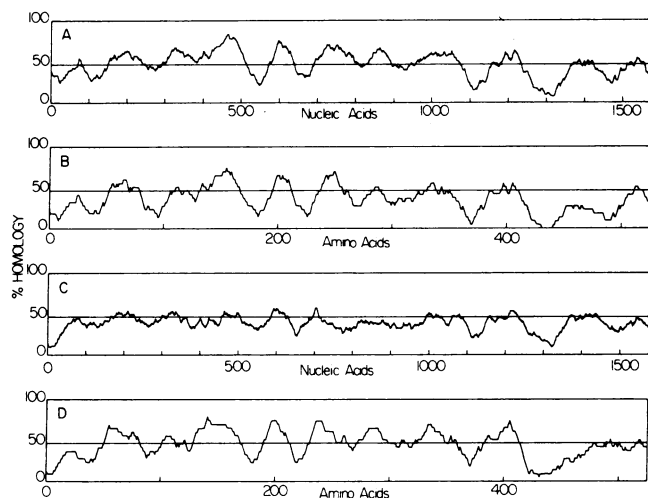
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FIG. 5. Percent homology profiles for the mouse and *Torpedo* AcChoR subunits. In this analysis, the computer determines the percent homology between any two sequences (nucleic acid or amino acid) over a relatively short length of sequence (the search string). The computer begins the analysis at the 5' or NH$_2$ termini of the sequences to be compared and repeats the analysis over the entire length. A percent homology value is calculated over the search string for every residue in the sequences being compared. The nucleic acid sequence search string was 50; the amino acid sequence search string was 17. Sequences were aligned as in Fig. 4. Only the protein-encoding regions of the nucleic acid sequences were compared. In this analysis, gaps inserted in the sequences for alignment purposes were scored as regions of 0% homology. (*A*) Mouse vs. *Torpedo* γ cDNA; (*B*) mouse vs. *Torpedo* γ amino acids; (*C*) mouse vs. *Torpedo* δ cDNA; (*D*) mouse vs. *Torpedo* δ amino acids.

to be associated with two of the four sequences. The amino acid sequence deduced from the mouse cDNA clone described here shows a high degree of similarity to the NH$_2$-terminal amino acid sequence of the fourth subunit of the fetal calf AcChoR (labeled "γ" in ref. 9). Over the first 24 amino acids of the mature proteins, the sequences are identical at at least 12 residues. Many of the changes represent conservative substitutions.

As indicated by arrows in Fig. 3, there are three possible sites for N-glycosylation, at asparagine residues 76, 143, and 169 of the mouse polypeptide. The site at position 143 is precisely conserved among mouse and all four *Torpedo* subunits. In both *Torpedo* γ and δ, there is a conserved site six amino acids toward the amino terminus relative to the mouse site at residue 76.

The mouse polypeptide contains two cysteine residues at positions 130 and 144 which are also precisely conserved in all four subunits of the *Torpedo* receptor (2–5) and in the α subunits of the human and bovine receptors (10). These residues may be involved in disulfide bridge formation (2).

The calculated hydrophobicity profile (20) of the mouse subunit is very similar to the profiles calculated for the several *Torpedo* AcChoR subunits (4, 5). In particular, the lengths and positions of the several proposed hydrophobic membrane-spanning segments are highly conserved. For the mouse δ subunit the residue positions of these segments are M1, 225-251; M2, 257-275; M3, 291-312; and M4, 451-469 (data not shown).

Finer-Moore and Stroud (21) have proposed recently that all four subunits of the *Torpedo* AcChoR contain an amphipathic α-helical membrane-spanning segment that constitutes part of the ion channel. The salient feature of this domain is that one side of the proposed α-helix is composed predominantly of hydrophobic residues that interact with the other hydrophobic domains of the subunit polypeptides, whereas another side is composed of hydrophilic residues,

which constitute part of the wall of the ion channel (21). To examine the possibility that such a domain may exist in this subunit of the mouse AcChoR, an α-helical wheel was constructed beginning with residue 407 (Fig. 3) and ending with residue 428. The overall average hydrophobicity (20) of this region was calculated to be −0.34, a weakly hydrophilic value. The α-helical wheel revealed a strongly hydrophilic surface on the putative α-helix composed of residues 408, 412, 415, 419, 422, and 426 (Fig. 3), whose average hydrophobicity was −3.57. The average hydrophobicity of all the other residues in this region was 0.88. This preliminary analysis suggests that an amphipathic domain may exist in this subunit of the mouse AcChoR as well.

RNA Blot Analysis. Fig. 6A shows an RNA gel blot with membrane-associated poly(A)$^+$ RNA from BC3H-1 cells probed by hybridization with subcloned *Eco*RI fragments from three of the λ recombinants (see Fig. 1). When the coding region subclone 6H was used to probe RNA from differentiated BC3H-1 cells, a predominant band at 3.3 kb was observed. Weaker bands at 4.6 and 2.0 kb were also detected. The major RNA band at 3.3 kb probably represents the mRNA species encoding this subunit of the mouse AcChoR.

The nature of the bands at 4.6 and 2.0 kb is not known. The band at 4.6 kb could represent a partially spliced precursor of the 3.3-kb species. (Nuclei were not removed from the cell homogenate prior to preparation of the membrane pellet.) The band at 2.0 kb could be a messenger related by sequence to the coding region of the mouse AcChoR δ subunit.
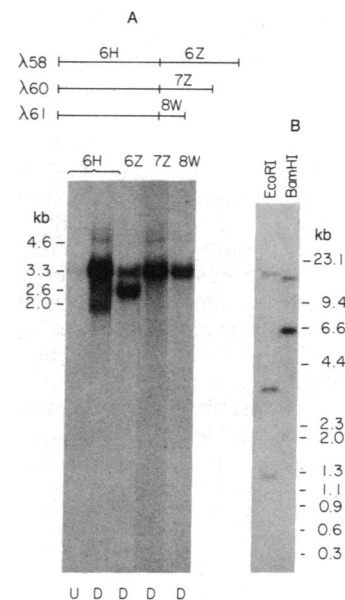

FIG. 6. (*A*) RNA blot analysis of mouse membrane poly(A)$^+$ RNA. RNA was prepared from differentiated BC3H-1 cells (D). Undifferentiated RNA (U) was prepared from BC3H-1 cells harvested after 3–4 days of growth in 20% fetal calf serum. Poly(A)$^+$ RNA was electrophoresed through a 1.5% agarose gel containing 2.2 M formaldehyde and then transferred to nitrocellulose paper (see ref. 22). Nick-translated *Eco*RI fragments 6H, 6Z, 7Z, and 8W (each subcloned in pUC9) were used as hybridization probes. (Subclone 6H contains the protein-encoding region.) Hybridization was carried out as described (22), except that dextran sulfate was omitted. Length standards were denatured *Hin*dIII-digested λ DNA and *Hae* III-digested φX174 DNA. (*B*) Gel blot analysis of mouse genomic DNA probed by hybridization with the *Eco*RI fragment from λ58 containing the protein-encoding region. BALB/c mouse genomic DNA (12 μg) was digested exhaustively with *Eco*RI or *Bam*HI and run on a 0.7% agarose gel. The DNA was transferred to nitrocellulose and hybridized to nick-translated mouse cDNA (see ref. 17, pp. 383–389). Length standards were *Hin*dIII-digested λ DNA and *Hae* III-digested φX174 DNA.

It is tempting to speculate that the 2.0-kb RNA could be the message for the γ subunit of the mouse AcChoR, although there is no evidence at this time to support this conjecture.

In gel blot analysis of membrane-bound poly(A)$^+$ RNA from undifferentiated BC3H-1 cells the intensity of hybridization to the 3.3-kb RNA is about 1/50 that for differentiated cells, confirming that expression of this subunit is induced by differentiation.

All three 3' untranslated region subclones (6Z, 7Z, 8W) also hybridized to the 3.3-kb RNA band. However, subclone 6Z also hybridized intensely to a band at 2.6 kb not detected with any of the other probes. This result suggests that a cloning artifact has occurred in the 3' EcoRI fragment of the λ58 insert. The fact that the restriction maps of the λ58 and λ60 inserts diverge after the first (5') Ava II site suggests that, somehow in the course of cloning, an unrelated piece of cDNA became attached to the 3' end of the λ58 insert at a point between this Ava II site and the Ban I site (see Fig. 1).

Genome Blot Analysis. To obtain some insight into the number of genes coding for this subunit of the mouse AcChoR, the genome blot shown in Fig. 6B was prepared. Multiple genes for one or more subunits of the mammalian AcChoR may explain the observed kinetic and drug sensitivity differences among AcChoRs at the neuromuscular junction and those in other parts of the nervous system. The simple pattern of hybridization obtained (three bands in the EcoRI lane and two in the BamHI lane) is consistent with probably only one and at most two genes for this subunit of the mouse nicotinic AcChoR.

Further Discussion. We have isolated and sequenced a cDNA clone coding for a subunit of the mouse AcChoR. Comparison of this sequence with the sequences for Torpedo and other species confirms the general high degree of amino acid sequence conservation among the several AcChoR subunits in evolution. Although the mouse clone was selected by hybridization to a Torpedo γ subunit cDNA probe, amino acid sequence comparison indicates a slightly higher degree of homology with the Torpedo δ subunit than with γ. On this basis, we tentatively identify our clone as that of the mouse δ subunit. We note that at present the only distinguishing criterion between Torpedo γ and δ subunits is the small difference in gel electrophoretic mobility. A decisive functional or structural criterion for the role of each subunit in the assembled AcChoR awaits discovery.

Just when this manuscript was completed, we learned of the work of Nef *et al.* (23) with full sequence information on a genomic clone coding for the chicken AcChoR γ and δ subunits. Our mouse amino acid sequence is more similar to that assigned by these authors to chicken δ than to that for chicken γ.

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