STRUCTURAL AND FUNCTIONAL STUDIES ON THE SUBUNITS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

Thesis by

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Who wills,

Can.

Who tries,

Does.

Who loves,

Lives.

Anne McCaffrey

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ABSTRACT

An introduction to the work in the study of the nicotinic acetylcholine receptor is presented. The author reviews the field to place the work of the present volume in its proper context. The major developments in studying the protein biochemistry of the receptor are reviewed, including the subunit makeup, ligand binding, and protein sequences. These studies led to the cloning and sequencing of many of the subunits as cDNA or genomic DNA constructions. This wealth of sequence information has allowed the formulation of detailed models of receptor structure. Current work centers on testing various aspects of these models and expanding the scope of the field into different species and tissues that utilize this receptor.

Partial cDNA clones specific for the β and δ subunits of the acetylcholine receptor of *Torpedo californica* were isolated by the following method. A cDNA library was constructed from electric organ poly(A)$^+$ RNA and enriched by screening for clones more abundantly represented in electric organ than in brain or liver mRNA preparations. These clones were tested by hybridization selection of clone specific mRNA which was then translated *in vitro* . Protein products were immunoprecipitated and analyzed by gel electrophoresis. The isolated clones were used to screen a library of *Torpedo* genomic DNA which resulted in the isolation of the gene for the *Torpedo* δ subunit . The δ gene was found to be single copy in *Torpedo* , and it contains at least four introns.

A cDNA library was constructed in λgt10 from membrane bound poly(A)$^+$ RNA from mouse BC3H-1 cells. This library was screened with cDNA encoding the complete protein region of the *Torpedo* γ and δ subunits. Positively hybridizing clones isolated with the *Torpedo* γ subunit were sequenced and compared with published data. The deduced amino acid sequence was more highly homologous to the *Torpedo* δ than to the *Torpedo* γ

and on this basis the mouse clone was tentatively identified as a δ subunit of the acetylcholine receptor. The mouse nucleotide sequence has several stretches of strong homology with the *Torpedo* γ subunit cDNA, but no such homology with the *Torpedo* δ subunit. A genomic blotting experiment indicated that there is probably one, but at most two chromosomal genes encoding this or closely related sequences.

In order to test the assignment of the mouse δ cDNA by a more functional criterion than simple amino acid homology, the following experiment was done. The phage SP6 transcription system was used to transcribe mRNA from the four individual *Torpedo* subunits and from the mouse δ. When the four *Torpedo* subunit specific mRNAs were injected into *Xenopus* oocytes, functional receptors appeared in the oocyte membrane. If the β or γ subunit RNA was omitted, no response to acetylcholine was detected, while a small response was detected if the δ subunit RNA was omitted. When mouse δ specific RNA was injected in place of the *Torpedo* δ, a 3-4 fold larger response was measured in response to acetylcholine under voltage clamp conditions. The replacement of *Torpedo* γ RNA with mouse δ RNA gave no detectable response. Surface binding of α–bungarotoxin was not significantly altered by exchanging the δ subunits, which indicates that the difference is intrinsic to the channel rather than a matter of stability or synthesis rates. Examination of the amino acid sequences of the two δ subunits and the *Torpedo* γ did not identify an obvious region of subunit specific homology. The amino acid features necessary to determine a specific subunit are not obvious from simple homology comparisons.

We have constructed a series of chimeric subunits to try to localize subunit determining regions of the acetylcholine receptor polypeptides. Each chimera was tested in the oocyte system by replacing its RNA for each of the parent RNAs in turn. None of the chimeras we have constructed retained enough of either parental subunit

characteristics to function fully in place of that parent subunit to form an acetylcholine receptor that is responsive to acetylcholine. We conclude that a minimum of two subunit-specific regions are widely dispersed over the subunit length. These data are also consistent with the conclusion that there are no discrete regions that determine subunit identity, but instead that this information is rather evenly distributed along subunit length. In some combinations, the chimeras were incorporated into surface AchRs, although these complexes were only weakly responsive to Ach. We further conclude that there are regions needed for efficient function of these subunits that are not necessary for the formation of surface complexes. We have demonstrated that the α subunits of both mouse and chick form functional receptors in the *Xenopus* oocyte system in combination with the β and γ subunits from *Torpedo* and a δ from either *Torpedo* or mouse. The responses of these hybrid AchRs are smaller than the response from the *Torpedo* AchR. In contrast, the mouse γ subunit did not form functional AchRs in any combination of the subunits mentioned above.

The present author spent the early part of her career studying the molecular biology of the actin genes of *Drosophila melanogaster*. Portions of each of the six actin genes were sequenced. These sequences revealed that the amino acid sequence of actin is highly conserved but that the positions of introns in these genes are strikingly nonconserved. Further, each of the *Drosophila* actins resembles the cytoplasmic isoforms from vertebrates, while none resemble the muscle isoforms.

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CHAPTER 1

The biochemistry of the nicotinic acetylcholine receptor

The nicotinic acetylcholine receptor (AchR) is the best studied of all the neurotransmitter-activated channels. This protein complex has led the way for studies in this field for two reasons. The rich sources of AchR found in the electric organs of the electric fish, *Torpedo* and *Electrophorous*, have made biochemical isolation and study much easier than for most other receptors of this type. The second reason studies of this channel have progressed so far is the existence of potent α-neurotoxins from the venom of Elapid snakes (*Bungarus multicinctus* and several *Naja* species). These toxins, as well as a variety of cholinergic ligands have been invaluable in the study and isolation of these receptors.

The role of the AchR in the neuromuscular junction is to transduce a chemical signal from the nerve into muscle depolarization. When acetylcholine (Ach) is released by the nerve terminal, it diffuses across the synaptic cleft and binds to AchRs on the muscle cell surface. When two Achs bind to each receptor, a conformational change occurs to transiently open a pore in the receptor complex, which is sufficiently large to allow small cations to flow down their electrochemical gradients into the muscle cell. This temporary depolarization of the endplate membrane leads, through several steps, to the contraction of the muscle or to an electrical potential buildup in the asymmetric stack of electrocytes in the electric organ.

The AchR has been studied in a variety of species at the protein biochemistry level. The pioneering work utilized the specialized electric organs of electric fish *Torpedo* (a marine elasmobranch) and *Electrophorous* (a freshwater teleost). Extensive studies were also carried out in mammalian systems, notably rat muscle and rodent cell lines expressing AchR such as BC3H-1. Some recent reviews on the structure and function of the AchR include McCarthy *et al.* (1986), Stroud and Finer-Moore (1985), and Conti-Tronconi and Raftery (1982).

Reynolds and Karlin (1978) used sedimentation analysis to estimate a mass of 250,000d for the AchR of *Torpedo californica.* These authors also proposed that the receptor was composed of four distinct polypeptides in the stoichiometry $\alpha_2\beta\gamma\delta$ based on observations of denaturing gels in previous studies (Karlin *et al.*, 1975) combined with their estimate of the overall complex size. This stoichiometry was first proven unequivocably by the work of Raftery *et al.* (1980). These workers used the quantitation gleaned from N-terminal amino acid sequencing. The sequence data further showed that each of the purified subunits was a homologous but distinct polypeptide. The subunit molecular weights from *Torpedo* are 40,000d (α), 50,000d (β), 60,000d (γ), and 65,000d (δ) for a calculated molecular weight 255,000d for the complex. This stoichiometry is consistent for both *Electrophorous* and calf (Conti-Tronconi *et al.*, 1982a, b).

Each subunit of the *Torpedo* AchR is synthesized as an individual polypeptide as shown by labeling each with the formylated initiator (^{35}S) Met-tRNA (Anderson and Blobel, 1981). These authors have further shown that each of the four subunits is an integral membrane protein by showing that they are incorporated into dog pancreas microsomes as glycosylated and trypsin resistant forms (1981). Froehner (1981) also demonstrated that the subunits each transverse the membrane since they have immunological determinants on both sides of the membrane. The δ subunit requires the signal recognition protein for integration into the endoplasmic reticulum and integration is accompanied by cleavage of an N-terminal signal sequence (Anderson *et al.*, 1982).

Monomers of the complete receptor pentamer $\alpha_2\beta\gamma\delta$ are naturally found crosslinked to form dimers in *Torpedo* and *Narcine* species of electric ray. The crosslink is a disulfide bridge between the δ subunits (Chang and Bock, 1977; Hamilton *et al.*,

1977). Other phyla do not exhibit dimers and their functional role is unclear (reviewed by McCarthy *et al.*, 1986).

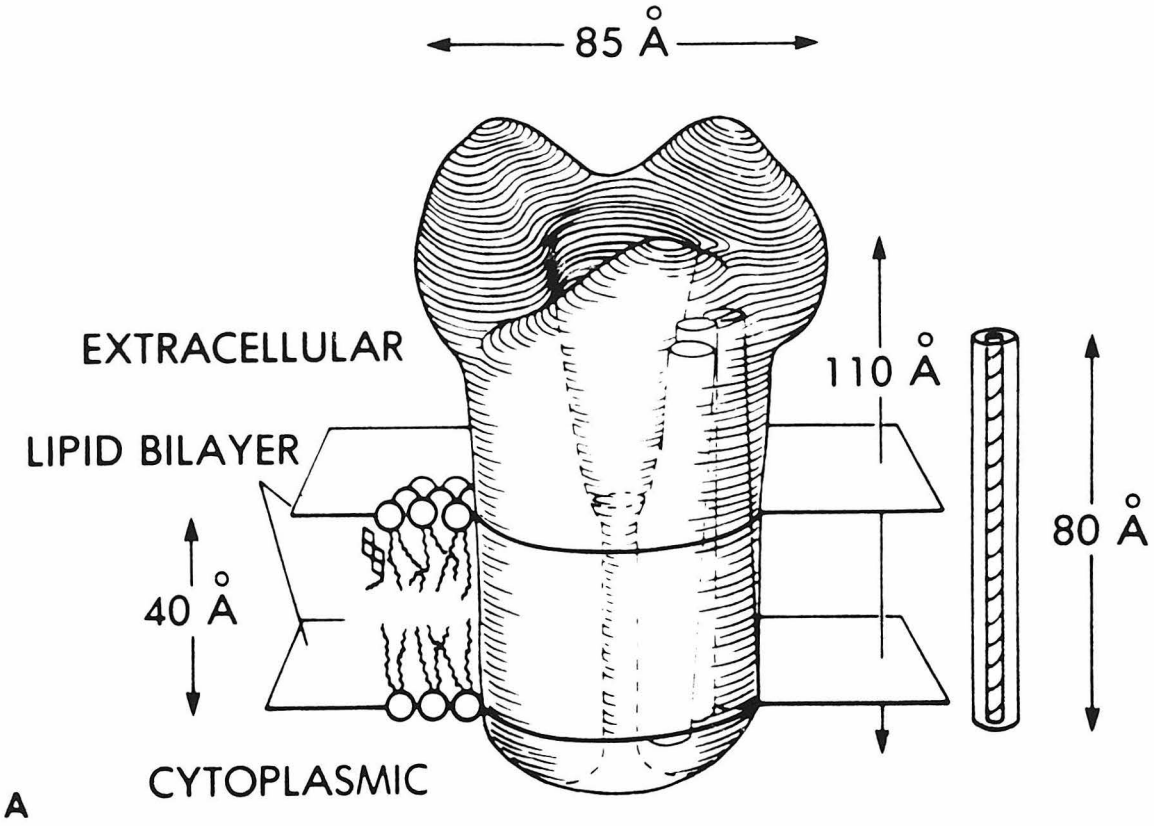
Figure 1 shows a proposed three dimensional model of the AchR, viewed from the side in A and from the synaptic side looking down on the membrane surface in B. A variety of groups (Kistler *et al.*, 1982; Karlin *et al.*, 1983; Brisson and Unwin, 1984) have extensively mapped the surface of the AchR with enhanced electron micrographs. The image generated by all these studies is that of a funnel shaped complex with the larger end on the synaptic side and the ion channel in the middle. The subunits appear as rods parallel to each other around the central well which is presumably the ion channel. This arrangement has been compared with five staves of a barrel.

Brisson and Unwin (1984, 1985) have refined their three-dimensional electron image analyses on tubular crystals of AchR from *Torpedo* . The overall shape is a regular pentamer, but the height varies around the top face. These data indicate that the subunits are symmetrically placed rods with the same cross-sectional area but different lengths. The very high degree of symmetry determined at each level through the bilayer reinforces the conclusions (based on sequence homology) that the subunits have the same tertiary structure except where their lengths differ.

The assignments of the subunits in Figure 1B are tentative since the order of the subunits has not been conclusively shown. Holtzman *et al.* (1982) studied the arrangement of the two α subunits with respect to one another and with respect to the δ-δ crosslink in dimers from *Torpedo californica.* Toxin linked to biotin marked the α subunits followed by avidin binding to visualize the α's in the electron micrographs. The δ-δ crosslink localized the δ's to the line between centers of the two monomers in association as a dimer. Their results show that the two α subunits are separated by one other subunit (also Fairclough *et al.*, 1983) and that this subunit is not δ. β-β dimers were

Figure 1. A three-dimensional model for the funnel shaped AchR complex in the lipid bilayer (from Kistler *et al.*, 1982). (A) AchR as viewed from the side, slightly above the plane of the membrane (B) AchR as viewed from the synaptic side looking down on the AchR surface. The assignment of subunit types is tentative. An $\alpha\gamma\alpha\beta\delta$ arrangement has also been proposed (Karlin *et al.*, 1983).

Figure 1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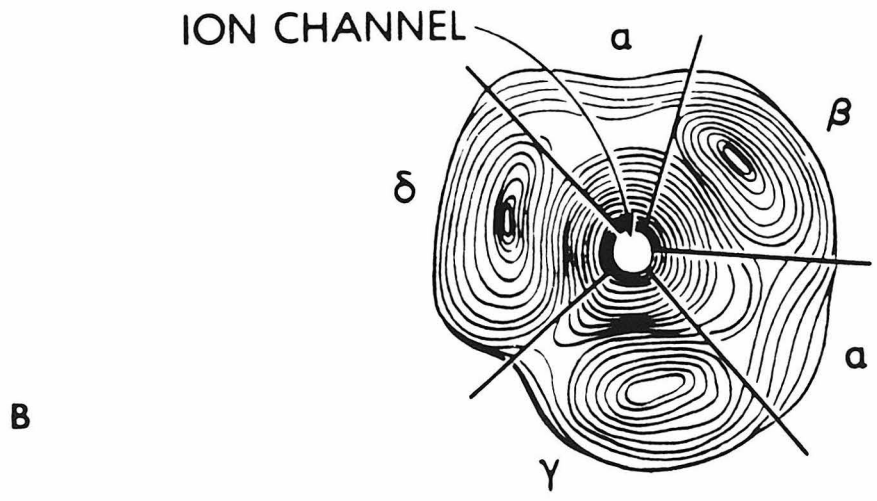
40 Å
CYTOPLASMIC
110 Å
80 Å
A


ION CHANNEL
α
β
δ
α
γ
B

constructed and analyzed similarly. These experiments gave very similar results, implying that the β does not lie between the two α subunits either (Karlin *et al.*, 1983). The order αγαβδ is most consistent with these data, but an αβαγδ arrangement has also been proposed (Kistler *et al.*, 1982; Zingsheim *et al.*, 1982).

There is a great deal of data, sometimes conflicting, on the nature of ligand binding to the AchR. It is clear that there are two high affinity Ach binding sites on each receptor complex and that these reside on the α subunits (reviewed by Conti-Tronconi and Raftery, 1982; Popot and Changeux, 1984). Ligand binding is not cooperative, but at least two Ach molecules must bind per receptor complex to open the channel. Neubig and Cohen (1980) determined a Hill coefficient of 1.97 ± 0.06 for channel opening. The common interpretation is that these two high affinity sites on the α subunits are involved in channel opening. Workers have tried to identify the region of the α subunit that binds ligand in many ways. Reduced AchR preparations are affinity labeled by 4-(N-maleimido)-benzyltri(^{3}H)methylammonium iodide (^{3}H-MBTA, demonstrated by Karlin and Cowburn, 1973). ^{3}H-MBTA specifically alkylates a sulfhydryl group very close to the ligand binding site. This affinity reagent covalently binds only one site per dithiothreitol-reduced 250,000d receptor monomer from *Torpedo californica* (Karlin *et al.*, 1975). In contrast, α-bungarotoxin (α-BTX, isolated from snake venom) binds very tightly to two sites per 250,000d monomer, and blocks labeling by ^{3}H-MBTA. Kinetic experiments (Damle and Karlin, 1978) show that the two toxin binding sites are not identical in that only one of them is available for labeling by ^{3}H-MBTA. The nonequivalence of the two binding sites has been confirmed by many other authors in a variety of assays. Since the two α's are imbeded in a pentameric complex their surrounding subunits must be different. Some workers feel that there are differences in the α's post-translational modifications as well (see below).

The two α subunits in the mature receptor complex of *Torpedo californica* are glycosylated to a different extent at asp-141 (Conti-Tronconi *et al.*, 1984). (All numbering of amino acids in this volume follows that of Stroud and Finer-Moore, 1985.) This may affect ligand binding since cys-142 is one of the two cysteines thought by some workers to form a disulfide bridge close to the high affinity binding site (Noda *et al.*, 1982; Mishina *et al.*, 1985). Thus, a larger carbohydrate on only one of the α subunits of a receptor complex may make these sites non-equivalent (Conti-Tronconi *et al.*, 1984). From the work discussed above it is not clear whether the α with the larger carbohydrate is at a specific location in the complex, or whether (as seems less likely) one of the two α's is chosen at random. Other workers have found no evidence for glycosylation of either of these two peptides since they do not bind to ^{125}I-concanavalin A (see below and Wilson *et al.*, 1985.)

The high affinity Ach binding sites are competitively and equivalently blocked by α-BTX in *Torpedo* receptors (Ellena and McNamee, 1980). Recent work has localized the binding site for α-BTX to a region on the α subunit beyond the glycosylation site at asp-141 (Neumann *et al.*, 1985). Previous authors suggested that the Ach binding site was in the segment of amino acids between the first two cysteine residues, 128 and 142 (Noda *et al.*, 1982), or in the first 20 amino acids (Smythies, 1980). A series of peptide blotting studies have demonstrated that these two regions do not contain detectable ^{125}I-labeled-α-BTX binding sites by this method. Instead, an 18,000d proteolytic fragment retains ^{125}I-α-BTX binding (Neumann *et al.*, 1985). This 18,000d fragment's binding is blocked by prior alkylation with ^{3}H-MBTA (Wilson *et al.*, 1984), which demonstrates that this binding is close to the physiologically relevant site. The α-BTX binding site was localized still further by the demonstration that a 32-amino acid synthetic peptide corresponding to residues 173-204 of the α subunit binds specifically to ^{125}I-α-BTX.

Competition studies with unlabeled toxin and *d*-tubocurarine show this binding to be specific (Wilson *et al.*, 1985).

The affinity label studies discussed above have focused the attention of those looking for ligand binding sites on regions near the four external cysteines at residues 130, 140, 192, and 193 (Noda *et al.*, 1982). The importance of these four was emphasized by the results of Mishina *et al.* (1985), who showed that changing any of these four cysteines to serine resulted in loss of sensitivity to Ach. Cys-130 and -140 are retained in all the subunits sequenced thus far (Stroud and Finer-Moore, 1985) and elimination of either cys-130 or -140 in the α subunit resulted in much lower stability of all the subunits. It seems reasonable to conclude, therefore, that these two form a disulfide bridge of major importance to the three-dimensional structure of the α subunit, through it may be important in assembly rather than Ach binding.

The proposal that adjacent cysteines may form a disulfide bridge is not impossible (Mitra and Chandrasekaran, 1984), but this structure has never been seen in a protein. It might be plausible to suggest that cys-192 and -193 are not bridged at all and that the reduction required for ^{3}H-MBTA binding opens a cys-130/140 disulfide. The resulting change in conformation may expose cys-192 and/or -193 to alkylation by ^{3}H-MBTA. Specific binding of ^{3}H-MBTA must be retained since it is competed by α-BTX (Karlin *et al.*, 1975).

When peptides from previously ^{3}H-MBTA alkylated α subunits were sequenced, contradictory results were reported. Kao *et al.* (1984) found that cys-192 and perhaps cys-193 are labeled by ^{3}H-MBTA while the cysteines at 130 and 140 were unlabeled. In contrast, Cahill and Schmidt (1984) found labeling only at cys-142. These data, plus the finding that removal of any one of these cysteines eliminates sensitivity to Ach (Mishina *et al.*, 1985), may imply that all four are closely juxtaposed to the Ach binding site.

Mutation of cys-192 or -193 to serine does not totally abolish (39-28% remains) α-BTX binding, a surprising result since the α-BTX binding peptide above includes these residues. This may imply that the adjacent cysteine is close enough to function in α-BTX binding or that the structure of serine is close enough to cysteine for that purpose.

Other sites for agonist have been described on the AchR (Dunn and Raftery, 1982). By using a fluorescent probe bound to the receptor, changes in conformation can be observed. A change in conformation occurs with the correct time scale for channel opening, even when the receptor's high affinity sites are blocked by bromo-Ach (Dunn *et al.*, 1983). These authors favor a model in which the low affinity sites are responsible for channel activation, while the high affinity sites are involved in channel desensitization (Dunn *et al.*, 1983).

Sequences and Clones

A wealth of data has recently flooded the AchR field. Complete sequences are now available for nineteen (by this author's count) different subunits from a variety of species, and at least three other partial sequences have been published. This author will not attempt to catalogue them all, since they have been recently reviewed (Stroud and Finer-Moore, 1985). Instead, what follows is an overview of how the clones were isolated, with emphasis on the clones that were used in the work detailed in the rest of this volume.

The first sequence data on any of the AchR subunits that was published was from the α subunit of *Torpedo marmorata* (Devillers-Thiery, 1979). These workers determined 20 amino acids from the N-terminal (amino-terminal) portion of α-subunit. Raftery *et al.* (1980) determined 56 amino acids from the N-terminus of each of the four subunits in *Torpedo californica* electric organ. As discussed above, this work confirmed

the stoichiometry of the subunits as $\alpha_2\beta\gamma\delta$. Conti-Tronconi and coworkers emphasized the conservation of AchR structure by sequence analysis of the amino termini of the *Electrophorous* (1982b) and calf (1982a) subunits. These data showed that the stoichiometry remained $\alpha_2\beta\gamma\delta$ across all three of these species. Further, a unifying picture began to develop; all three species have four subunits which, allowing for protease problems, run approximately at the same positions on denaturing polyacrylamide gels. Moreover, each of the sequences (a total of 12) exhibit marked homology with the strongest homology between examples of analogous subunits (*e.g.*, the three lightest subunits are all more closely related to each other than to any of the others). This homology led the authors to propose that all four subunits were derived by duplications of a single primordial gene very early in animal evolution (Conti-Tronconi *et al.*, 1982a).

A series of reports by Noda *et al.* (1982, 1983a, b) describes the cloning of cDNAs for each of the four polypeptides from *Torpedo californica*, as well as analysis of their sequences and derived amino acid sequences. These clones were isolated by hybridization of mixed sets of oligonucleotides representing all possible coding sequences for short segments of the amino acid sequences determined by Raftery *et al.* (1980). The newly available sequences of all four subunits and the extensive homology between them allowed Noda *et al.* (1983) to assert that the four polypeptides all probably assumed the same or "pseudosymmetric" transmembrane topology (see discussion below).

Ballivet *et al.* (1982) describe the cloning, by another method, of the γ subunit of *Torpedo californica*. These workers used differential hybridization to choose cDNA clones representing messenger RNAs that are more abundant in electric organ RNA than in brain. They further screened the abundantly expressed clones by hybridization-selection of homologous RNAs from electric organ poly $(A)^+$ RNA. This mRNA was then eluted, translated *in vitro*, and the products immunoprecipitated with rabbit antisera

to SDS-denatured AchR. The resulting polypeptides were analyzed by gel electrophoresis and compared to *Torpedo* subunits produced in parallel from poly $(A)^+$ mRNA. Clones isolated in this manner were sequenced (Claudio *et al.*, 1983) and shown to contain the complete polypeptide coding region for the γ subunit. The present author and others used this method to isolate partial cDNA clones for the β and δ subunits from *Torpedo californica* (Hershey *et al.*, 1983, and this volume).

Several labs used the methods detailed above to obtain "first generation" clones from the most abundant source of mRNA: the *Torpedo* electric organ. These cDNAs were then used as hybridization probes to identify "second generation clones" in cDNA libraries made from less abundant sources such as calf, mouse and chicken. An example of this methodology is described in Nef *et al.*, (1984). These workers used a *Torpedo g* subunit cDNA clone as a hybridization probe on a chicken genomic library in bacteriophage λ. Sequence analysis demonstrated that the isolated phage contained the gene for the γ subunit of chick AchR. Nef *et al.* (1984). showed that the same phage isolated with the γ probes also contained the gene for the chick δ subunit. The two protein coding regions are transcribed in the same direction and are separated by only 740 base pairs. Furthermore, the *Torpedo* γ probe hybridizes faintly to the chicken δ gene. The γ and δ subunits are more closely related to each other than to either the α or β subunits (Noda *et al.*, 1983b).

Several clones of other subunits have been isolated by hybridization from one species to another. Some of the more interesting examples were the unexpected cloning of the δ subunits of both mouse and calf using the γ subunits from *Torpedo* and calf respectively. LaPolla *et al.* (1984, and this volume) did not isolate any clones by hybridization with a *Torpedo* δ cDNA probe. Clones isolated with a *Torpedo* γ probe exhibited higher sequence homology with the *Torpedo* δ at the amino acid level and were

therefore tentatively assigned as the mouse δ subunit. This conclusion was confirmed by the subsequent experiments of White *et al.* (1985, and this volume). Clones isolated from the same cDNA library with lighter hybridization signals to the *Torpedo* γ do encode the mouse γ subunit (Yu *et al.*, 1986).

Both the calf δ subunit and a calf ε subunit, which had not been described previously, were isolated using a cDNA clone encoding the calf γ subunit (Takai *et al.*, 1984). The calf δ subunit, like the mouse δ discussed above, was not detected by hybridization with *Torpedo* δ çDNA. Kubo *et al.* (1985) reported that their efforts to clone the calf δ using *Torpedo* δ as a probe were unsuccessful. However, when they hybridized a large number of calf muscle cDNA clones with the calf γ, some of the clones isolated proved to contain the δ subunit dDNA. Sequence analysis showed that local homology at the nucleic acid level allowed detectable hybridization between calf δ and calf γ but not with the *Torpedo* δ. Overall nucleic acid sequence homology in the protein coding regions is the same (60%) for either pair. Takai *et al.* (1985) have isolated a calf cDNA clone (named ε) with their previously described calf γ (Takai *et al.*, 1984) that is similar to all the γ sequences previously described. As this clone can substitute for the *Torpedo* γ in an expression system (described more fully below), it is reasonable to deduce that the ε subunit has a similar function to that of the γ. Northern blot data indicate that the ε subunit is much less prevalent than the γ and is present at an earlier time in fetal development.

One of the many goals of studying electric organ and muscle AchRs is to establish whether the identical subunits are used in these locations as well as in other parts of the nervous system. High affinity α-BTX binding sites in neural tissue do not always correspond to postsynaptic locations in the central nervous system where synaptic AchRs would be expected. (See, for example and review, Jacob and Berg, 1983). In addition,

some AchRs from vertebrate brain have distinct pharmacology from those studied at the neuromuscular junction. Subunits from chicken brain and optic lobe have identical N-terminal sequences that are highly homologous to, but distinct from, the sequence of the chick muscle α subunit (Conti-Tronconi *et al.*, 1985). This work was the first demonstration that subunits with distinct primary sequences are used in different tissues of one species.

Boulter *et al.* (1986) have also reported studies on a neuronally derived α subunit. An α subunit cDNA clone from mouse muscle (Boulter *et al.*, 1985) was used as a low stringency hybridization probe against a cDNA library from a rat chromaffin cell derived cell line. The clone isolated demonstrates strong sequence homology to the mouse muscle α sequence in some regions and provocative differences in other regions. On the basis of the strongly homologous regions and, in particular, the conservation of the four cysteines thought to be important in Ach binding, this protein has been called the rat neuronal α subunit. This mRNA is not expressed in muscle, but instead in regions of the rat brain that are known to contain neuronal nicotinic AchRs that do not bind α-BTX. Genomic blotting data indicates that there are several sequences related to this neuronal subunit in the mouse genome.

Models

Three groups proposed, nearly simultaneously, very similar models for the arrangement of an AchR subunit in the postsynaptic membrane (Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983; Noda *et al.*, 1983). These models are based on amino acid sequences derived from cDNA sequences of *Torpedo* subunits. Each group finds four hydrophobic stretches flanked by charged amino acids which are good candidates for membrane spanning regions. Thus, the structure proposed is a large extracellular region

comprising roughly the N-terminal half of the protein followed by three hydrophobic membrane spanning α-helices (termed M1, M2, M3) in quick succession. The next region ranges from 110 to 143 amino acids in length and is placed on the cytoplasmic side. A final hydrophobic membrane spanning region (M4) follows with a tail of varying length (11 - 27 amino acids) on the extracellular side. Difficulties with this model include the placement of both the C-terminus and the N-terminus of a protein on the same side of the membrane as well as the identification of the parts of the subunits that should contribute to the ion channel. Devillers-Thiery *et al.* (1983) and Noda *et al.* (1983) each suggest that the polar residues of some of the segments would align on one side of the α-helix and perhaps form a polar lining for the channel.

These models were altered substantially by two later proposals that there may be an amphipathic helix (MA) in each subunit that would line the pore (Guy, 1984; Finer-Moore and Stroud, 1984). The authors of both papers searched for an amphipathic region by different methods and each found such a region between the third and fourth hydrophobic membrane spanning regions (M3 and M4). Figure 2 presents a drawing of the subunit topology proposed by these authors. This additional helix answers both major reservations about the previous model. The fifth membrane spanning helix places the C-terminus on the cytoplasmic side of the membrane and also supplies a stripe of strongly charged side chains to line the water-filled pore.

Guy (1984) presents a very detailed model placing all five of the above α-helices of each subunit quite precisely in relation to each other. The model he presents places the MA helices from each subunit as a ring around the pore. The other four helices are arranged in two concentric layers, determined by side chain packing calculations that fit the "knobs into holes." Each subunit has the same overall structure in this model, and either of the two subunit orders discussed above (αβαγδ or αγαβδ) works equally well.

Figure 2. A drawing of the membrane topology proposed by Guy (1984) and Finer-Moore and Stroud (1984) for each of the AchR subunits. The curved line represents the polypeptide from N-terminus (N) to C-terminus (C). The five proposed membrane spanning helices are marked M1, M2, M3, and M4 for hydrophobic helices and MA for the amphipathic one,which is also marked with + and- charge symbols.

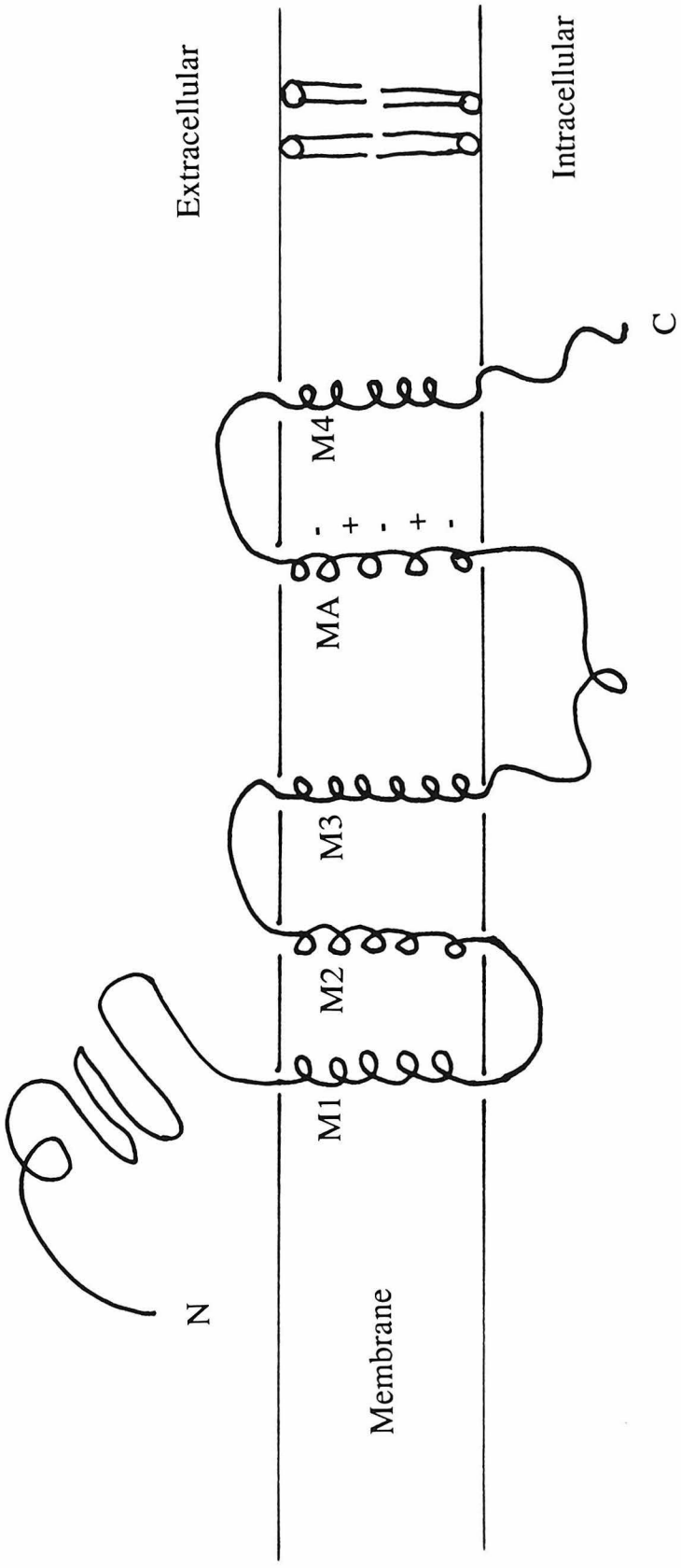


Figure 2

M2 and M3 have amino acid side chains that interact well with proteins and are placed next to each other and against the uncharged side of MA as the second concentric layer. This layer has 10 helices, with two being contributed from each subunit. Portions of the outer surface of the second layer appear to interact directly with the lipid bilayer, while some parts are covered by M4 and M1 which form the third concentric layer. Some compelling features of this model are that the side groups for each helix of each subunit are paired to form salt bridges between charged residues and hydrogen or disulfide bonds where appropriate. Important roles are proposed for the proline kinks that break M1 and for the interesting conservation of cystine residues in some helices. Different open and closed conformations are presented by Guy (1984) that may involve the interchange of disulfide bridges.

Model Testing

Some experiments designed to test the above models have been presented. Lindstrom *et al.* (1984) and Young *et al.* (1985) have both presented immunological evidence that the C-termini of the AchR subunits are on the cytoplasmic side of the membrane. Antibodies that bind the C-terminal peptides are not able to bind to AchR-rich vesicles until the vesicles are permeabilized by detergent. Electron micrographs using colloidal gold-conjugated second antibodies further demonstrate that the determinants are actually intracellular and not merely buried on the extracellular side prior to detergent treatment (Young *et al.*, 1985). These data all support the presence of MA as a membrane spanning region and the general structure shown in Figure 2.

A recent series of experiments has attempted to locate the position of determinants of monoclonal antibody determinants to particular peptides on the AchR polypeptides. These monoclonal antibodies were then characterized for binding to the cytoplasmic or

extracellular sides of AchR membranes containing AchR (Criado *et al.*, 1985; Ratnam *et al.*, 1986a, b). The data presented in these studies cast doubt on the previously described models of Guy (1983) and Stroud and Finer-Moore (1984).

Xenopus oocytes have been used as an expression system for studying the AchR. Initial reports showed that injected mRNA from *Torpedo* electric organ caused faithful surface expression of AchRs (Sumikawa *et al.*, 1981) that were also functional by electrophysiological criteria (Barnard *et al.*, 1982).

Mishina *et al.* (1984) demonstrated that mRNA derived from cloned subunit cDNAs could express AchRs when injected in *Xenopus* oocytes. These workers used COS monkey cells to express mRNA for each subunit cloned into a pKCR derived expression vector. These studies showed that the four *Torpedo* cDNAs contain all the necessary information for AchR assembly. Similar systems have been described by Mishina *et al.* (1985) and White *et al.* (1985, and this volume). Both these groups have adopted the phage SP6 transcription system for *in vitro* transcription of RNA from cloned cDNAs (Melton *et al.*, 1984; Krieg and Melton, 1984).

Two other systems are in use for studying the biosynthesis of receptor subunits. Fujita *et al.* (1986) described the surface expression of the α subunit in yeast cells transformed with an expression plasmid bearing the complete α cDNA. Similarly transformed animal cells are also useful for AchR studies. COS cells, described above as a source for mRNA, make the α subunit protein but do not appear to incorporate the α into the plasma membrane (D. Noonan and S. Taylor, personal communication). These transformation-dependent systems are somewhat more difficult for studying electrophysiological effects because of their small size and because it is difficult to get all four subunits expressed at a high rate in a single population of cells. Studies of receptor

biosynthesis and assembly may well be easier in tissue culture cells than in oocytes (Merlie and Lindstrom, 1983).

The *Xenopus* systems have been quite fruitful in testing various portions of the models described above. Mishina *et al.* (1985) showed that the α and β subunits are crucial for Ach sensitivity. A very low level of response to Ach was demonstrated by oocytes injected with all but either the γ or δ subunit mRNA. These results were extended by White *et al.* (1985), presented in detail in this volume. These workers demonstrated that a mouse AchR subunit that had been isolated by hybridization with the *Torpedo* γ cDNA was, in fact, the mouse δ. RNA transcribed *in vitro* from the mouse δ gave large responses to Ach when used as a replacement for *Torpedo* δ, but not for *Torpedo* γ. This clear functional distinction was not discernible at the level of overall sequence homology nor by the homology profile studies presented in LaPolla *et al.* (1984) and White *et al.* (1985). Thus, predictions of subunit specific regions of each polypeptide may require more careful analysis of the side chain packing structures as discussed by Guy (1984). An attempt to locate periodic homology that would fall along one side of the hydrophobic helices is discussed in White *et al.* (1985). The experiments of White *et al.* (1985) and also Takai *et al.* (1985) demonstrate another rationale for doing expression studies - that of identifying subunits of the AchR that have unclear identities or functional roles. Another subunit for which such studies may prove very informative is the neuronal α subunit from mouse described by Boulter *et al.* (1986).

Mishina *et al.* (1985) have reported an initial series of experiments aimed at locating functional regions of the α subunit by site-directed mutagenesis and small deletions. Each altered α subunit is transcribed by SP6 polymerase and injected into *Xenopus* oocytes with similarly transcribed mRNAs for the other three subunits. Sensitivity to Ach is then measured as a percent of the wild type value under voltage

clamp. Any deletion that removed a major portion of one of the proposed membrane spanning helices resulted in the loss of any sensitivity to Ach. An exception was a large deletion of MA which retained only 3% of the Ach response. This deletion seems to replace the MA amphipathic helix with another helix of somewhat lower but still discernable amphipathy. Deletions in the proposed cytoplasmic region between M3 and M4 have only slight effects on Ach responses. The site-directed mutants generated in the α subunit by Mishina *et al.* (1985) also yielded some very interesting results. Each of the cysteine residues thought to be near the Ach binding site (discussed in more detail above) was changed to a serine. Each one of these four mutations destroyed responsiveness to Ach in the oocyte patch clamp assay. Interestingly, the single glycosylation on the α subunit at Asn-143 is also crucial for the Ach response.

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CHAPTER 2

Structure and expression of genomic clones coding for the δ-subunit of the *Torpedo* acetylcholine receptor

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Structure and Expression of Genomic Clones Coding for the δ-Subunit of the *Torpedo* Acetylcholine Receptor

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Many important problems concerning the structure and the function of nicotinic acetylcholine receptors (AChR) can be advantageously approached by molecular cloning. From the nucleic acid sequences of the cDNA clones, one can conveniently determine the amino acid sequences of the polypeptide subunits of this integral membrane complex. This goal has now been accomplished in several laboratories for the *Torpedo* AChR (Noda et al. 1982, 1983; Sumikawa et al. 1982; Claudio et al. 1983; Devillers-Thiery et al. 1983). Important conclusions about the external, transmembrane, and cytoplasmic domains of each subunit and about their evolutionary relatedness can be deduced from these data.
data.

In addition, one can address the problem of the number of nonallelic genes for each subunit by the study of genomic clones. An important question to be answered is whether any particular subunit is itself a member of a multigene family, with different members being expressed in different muscle, nerve, or other tissues. Furthermore, the determination of the structures of the genomic clones is an essential initial step for the study of the developmental regulation of expression of these genes.

It will be of interest to determine whether the intron-exon structures of the natural genes correlate with domain structures deduced from cDNA sequences. These results will provide information on the evolution of these related genes and on the general question of the relationship of exons to protein domains in evolution.

MATERIALS AND METHODS

Isolation of electric organ RNA (Chirgwin et al. 1979) and its subsequent in vitro translation and immunoprecipitation (Mendez et al. 1980) were performed essentially as described previously.

Cloning of electric organ poly(A) RNA into the *Pst*I site of pBR322 (Efstratiadis and Villa-Komaroff 1979), screening of the resulting cDNA library (Hanahan and Meselson 1980), and hybridization selection analysis (Ricciardi et al. 1979) of electric-organ-specific cDNA clones were performed as described previously.

High-molecular-weight *Torpedo californica* DNA was obtained by grinding of the frozen electric organ, isolation of nuclei, lysis with Sarkosyl and proteinase K, banding in cesium chloride, RNase-A digestion, and deproteinization.

The resulting high-molecular-weight DNA (avg. length >100 kb) was partially digested with restriction endonuclease *Mbo*I. Aliquots from several different degrees of digestion were analyzed by gel electrophoresis, and appropriate samples were mixed to help obtain a representative sample. The pooled fragments were subjected to sucrose gradient velocity sedimentation. Fractions that were approximately 20 kb long were isolated, purified, and used for library construction.

Cohesive ends of bacteriophage λL47 DNA (Loenen and Brammar 1980) were annealed, the DNA was digested with *Bam*HI, and the ends were separated from the stuffer fragment by sucrose gradient velocity sedimentation. Standard methods (Maniatis et al. 1982) were used for ligation of the vector to *Torpedo* DNA. In vitro packaging was carried out as described by Mullins et al. (1981). Typical efficiencies were in the range of 5×10^5 pfu/μg of *Torpedo* DNA. Libraries were screened by standard Benton-Davis screening (Maniatis et al. 1982).

RESULTS

Isolation of cDNA Clones

cDNA libraries were prepared as described from *Torpedo* electric organ poly(A) RNA. The library was screened for clones that reacted strongly with cDNA made from electric organ poly(A) RNA and weakly with cDNA from liver or brain poly(A) RNA. Several hundred clones were isolated and tested by hybrid selection of RNA from the electric organ followed by in vitro translation in a rabbit reticulocyte system. The resulting polypeptides were immunoprecipitated with antisera specific to the SDS-denatured subunits or total receptor (Claudio and Raftery 1977) and analyzed by gel electrophoresis. Clones that code for parts of the β- and δ-receptor subunits were positively identified by this procedure. They are denoted pT50 and pT65, respectively.

By comparison with the DNA sequence determined by Noda et al. (1983) from the full-length cDNA clones, it has been determined that the clone denoted pT65 extends from nucleotide 353 to nucleotide 1715 in the coordinate system given by Noda et al. and thus includes the codons extending from amino acid residue 118 to the carboxyl terminus of the mature protein. Clone pT50 extends from coordinate 130 to about 830

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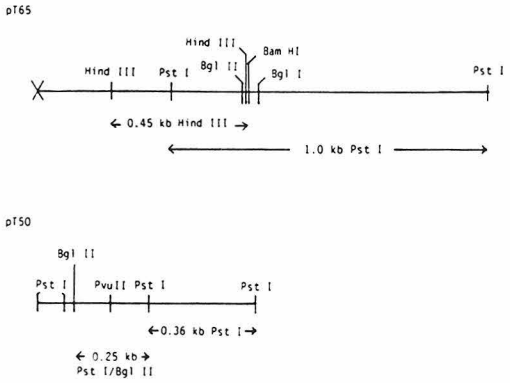

Figure 1. Restriction endonuclease maps of the cDNA clones for the δ(pT65) and the β(pT50) subunits. The *Pst*I sites at the extreme ends of the clones were introduced by the cloning procedure of Efstratiadis and Villa-Komaroff (1979). The site at the left end of the insert in pT65 was not reconstructed during cloning. In both cases, the left-to-right orientation of the clones is 5′ to 3′.

nucleotides in the coordinate system of the β-subunit (Noda et al. 1983), corresponding to codons 44 to 277 of the amino acids in the mature β-subunit. Maps of these clones are shown in Figure 1.

RNA Blots

Standard RNA blots to determine the lengths of the electric organ poly(A) RNAs that are complementary to the β and δ cDNA sequences were carried out. We observe that the β-subunit is represented by a single RNA transcript of about 1600 nucleotides in length (Fig. 2). This length is only slightly greater than that necessary to code for a polypeptide with 460 amino acids (1370 nucleotides of protein-coding sequence).

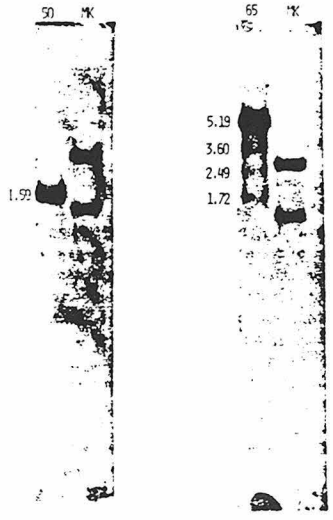

Figure 2. Autoradiographs of RNA gel blots of electric organ poly(A) RNA probed with the pT50 (*left*) and pT65 (*right*) cDNA clones. The markers (MK) are *E. coli* 16S and 23S rRNAs.

In contrast, four transcripts with lengths of 5.2, 3.6, 2.4, and 1.7 kb are observed when the δ cDNA clone is used as a probe (Fig. 2). Similar results are reported by Noda et al. (1983). At the level of sensitivity used, these RNA species are specific to electric organ and are not detected in RNA extracted from brain, liver, or muscle. The relative intensities of the weaker bands vary from preparation to preparation; the 5.2-kb transcript was always predominant, the 1.7-kb transcript was the next in intensity but much weaker, and the two transcripts of intermediate length were fainter still.

Genome Blots

Southern blots of gel-fractionated, restriction-endonuclease-digested *Torpedo* DNA have been probed with β and δ cDNA clones to obtain a preliminary indication of the number of chromosomal genes coding for each subunit and to determine their restriction endonuclease pattern. To avoid problems due to nonspecific hybridization and stickiness associated with the G·C homopolymer sequences at the junctions of the cDNA inserts with vector DNA, the central 450-bp *Hin*dIII fragment of pT65 and the 250-bp *Pst*I-*Bgl*II fragment of pT50 were used as probes. The δ probe hybridizes to only one *Hin*dIII fragment, which has a length of 5.9 kb, and to *Eco*RI fragments with lengths of 4.4 kb and 4.0 kb. The β probe hybridizes to a single 17.7-kb *Hin*dIII fragment, to *Pst*I fragments with lengths of 4.2, 3.1, 1.9, and 1.5 kb, and to *Eco*RI fragments with lengths of 1.7 kb and 3.8 kb (Fig: 3).

These results show that the δ-probe sequence used is present as a single copy in the *Torpedo* genome. The resolution of the 1% agarose gel in the 18-kb region is not high. Therefore, it is probable that the observation that there is only a single band 17.7 kb long for the

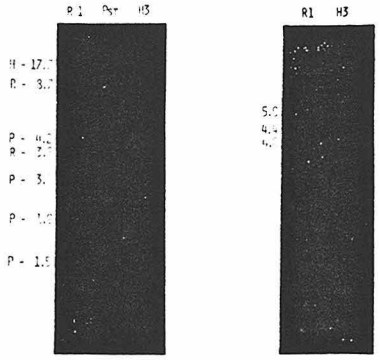

Figure 3. Gel blots of *Torpedo* genomic DNA digested with the indicated enzymes and probed with cDNA clones for the β-subunit (*left*) and the δ-subunit (*right*). These genome blots were carried out with the central 450-bp *Hin*dIII fragment of pT65 and the central 250-bp *Pst*I-*Bal*II fragment of pT50 as probes.

*Hin*dIII digest that hybridizes with the β-subunit probe indicates that this sequence is also only single copy in the genome; but the possibility remains that there are two or three unresolved bands in the genome that correspond to several nonallelic copies of this sequence. We therefore conclude that the δ gene is single copy and that the β gene is certainly not a member of a large multigene family and may be a single-copy gene.

δ Genomic Clones

The *Torpedo* haploid genome comprises 6.5×10^6 kb of DNA (Hinegardner 1976). Thus, for an average insert size of 12 kb as observed for the present λL47 library, 5.5×10^5 phage are equivalent to one haploid genome. A phage library consisting of about 1.0×10^6 plaques was screened by the standard Benton-Davis procedure with a probe prepared by nick translation of the 0.45-kb *Hin*dIII fragment of the δ-subunit cDNA clone. Four clones hybridizing to this fragment have been isolated and characterized.

Restriction maps of these clones were determined by standard methods. These maps (Fig. 4) show that the four clones are overlapping, that they define a total of 20 kb of contiguous genomic *Torpedo* DNA, and that they contain the 4.4-kb and 4.0-kb *Eco*RI fragments and the 5.9-kb *Hin*dIII fragment, which were observed to hybridize to the pT65 probe in the genome blots.

By blot hybridization of the genomic clones with the several *Hin*dIII fragments of pT65, whose orientation was determined by DNA sequence data, the 5′ to 3′ orientation of the gene was determined to be from left to right in Figure 4.

*Eco*RI fragments from the left and right ends of the cloned region were gel-isolated and hybridized to electric organ RNA gel blots. (These fragments are depicted as 14 and 10 in Fig. 4.) Both of these probes hybridized to the same four RNA bands shown in Figure 2, with the relative intensities noted above. The intensity of hybridization by probe 14 was substantially less than that for probe 10, suggesting that the exon-to-intron ratio within this particular segment is low. Thus, all four RNAs contain sequences from the far left and far right of the cloned region.

The map in Figure 4 also shows those regions of the genomic clones that do and do not hybridize with the entire pT65 probe. It should be noted particularly that, reading from left to right, there is a 1.2-kb *Eco*RI fragment at 3–4 kb from the left end, a 1.65-kb *Hin*dIII fragment at 5–7 kb from the left, and a 0.54-kb *Eco*RI-

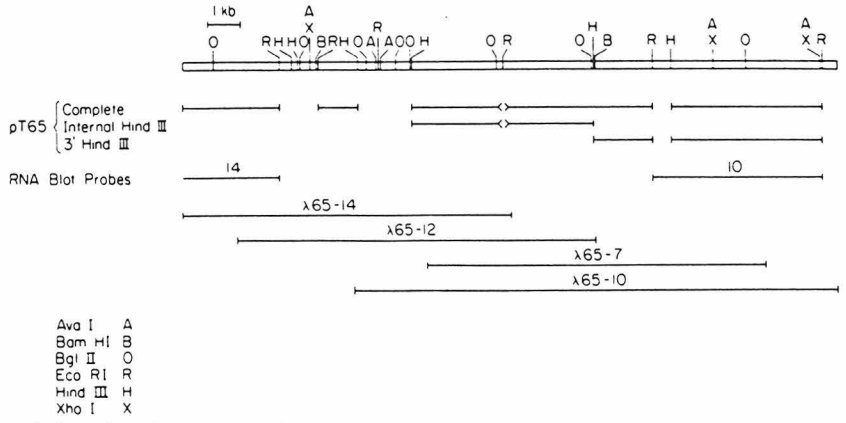

Figure 4. Restriction endonuclease map and summary of blotting experiments with the cloned inserts for the chromosomal gene for the δ subunit.

*Hin*dIII fragment at about 14 kb, all of which do not hybridize with the cDNA probe. Therefore, these fragments must lie entirely within introns. In addition, there is an *Eco*RI site in the genomic clone at 9.6 kb on the map of Figure 4 that is not present in the cDNA clone. Therefore, there must be an additional intron in this region. This observation is confirmed by the fact that the 5.9-kb genomic *Hin*dIII fragment that contains this *Eco*RI site is the only genomic segment that hybridizes with the 0.45-kb cDNA *Hin*dIII segment. Therefore, there must be about 5.45 kb of intron sequences within this genomic fragment. Thus, there are at least four introns within the 20-kb region that has been cloned.

DISCUSSION

In this preliminary report, we describe the isolation of 20 kb of overlapping genomic clones coding for part of the δ-subunit of the AChR. This region probably does not include either the 5′ end or the 3′ end of the complete gene. It contains at least four introns and at most 1 kb of exon sequences. Thus, the ratio of intron-to-exon sequences in this particular example of a structural gene in the *Torpedo* genome is approximately 20:1.

One of the more surprising results is the observation that the most abundant δ RNA transcript has a length of about 5 kb. The intensity of this band on the RNA blots is comparable to that of the 1.7-kb β-band. Since the amounts of β- and of δ-peptide synthesized by in vitro translation of total electric organ RNA in a rabbit reticulocyte system are comparable, it is highly probable that the 5-kb transcript is the mRNA for the δ-subunit in spite of its seemingly excessive length. Whether the fainter, shorter transcripts have biological significance or whether they are the results of degradation at nuclease-sensitive points during extraction remains to be determined.

The preliminary evidence presented here indicates that there is a single copy of the δ-subunit in the *Torpedo* haploid genome. Nicotinic AChRs occur in the electric organ for *Torpedo* and at skeletal muscle synapses and in the CNS for vertebrates in general. Presumably there are nicotinic AChRs in the skeletal muscles and CNS of *Torpedo*. There is evidence for pharmacological differences between such receptors in the chick (Carbonetto et al. 1978). If there is diversity in the δ-subunit of *Torpedo*, there would have to be alternate processing of mRNA transcripts derived from a single gene, as observed for the myosin heavy chain of *Drosophila* (C. Rozek and N. Davidson, pers. comm.). Alternatively, any diversity in receptors may reside in the other subunits. These important questions remain to be studied.

ACKNOWLEDGMENTS

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CHAPTER 3

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

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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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Contributed by Norman Davidson, August 20, 1984

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 *Eco*RI sites in the resulting duplex cDNA were then protected by methylation with *Eco*RI methylase. The duplex cDNA was blunt-ended with T4 DNA polymerase and all four dNTPs. Two micrograms of phosphorylated *Eco*RI linkers (12-mers) was added with T4 DNA ligase. After digestion with *Eco*RI, 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 *Eco*RI-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 Δ*Hfl* (provided by Carol Nottenburg of the University of California, San Francisco) for screening purposes. An unamplified library of approximately 1×10^6 recombinants was obtained.

Library Screening. We screened 1×10^5 recombinant plaques in duplicate by plaque filter hybridization (ref. 17, pp. 324–328) at a density of 2×10^4 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 1.0×10^8 cpm/μg. The hybridization solution contained 50% (vol/vol) formamide,

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

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).

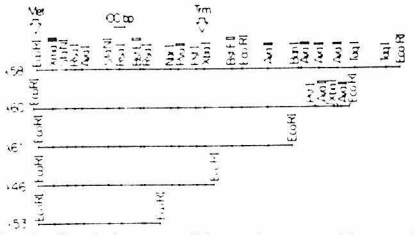

FIG. 1. Restriction maps of inserts from recombinant λ cDNA clones. Positions of the initiator methionine (Met) and the termination codon (Trm) were determined from nucleotide sequence analysis (see Fig. 3). bp, Base pairs.

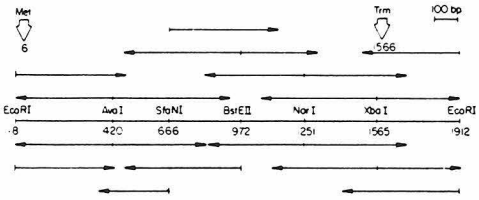

FIG. 2. Sequencing strategy for the protein-encoding region *Eco*RI fragment from λ58 subcloned in pUC9. *E. coli* strain TB1 (provided by T. O. Baldwin, Texas A & M University) was used for transformation. Plasmid DNA was isolated by the alkaline lysis/CsCl gradient method (ref. 17, pp. 90–91). The 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-

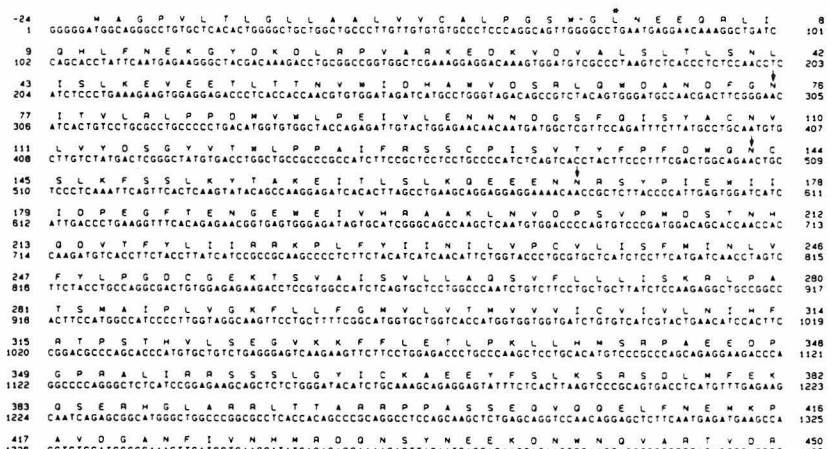

FIG. 3. Nucleotide sequence of the *Eco*RI fragment from mouse cDNA clone λ58 containing the protein coding region. The deduced amino acid sequence (standard one-letter code) of the open reading frame is given above the nucleotide sequence. Nucleotide no. 1 is the first nucleotide of the cDNA. Eight 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. 5*A*) than does the *Torpedo* δ subunit cDNA (Fig. 5*C*). We estimate that only sequences in the peaks of length *ca.* 75 nucleotides and homology greater than 65–70% in Fig. 5*A* 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 NH_2-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

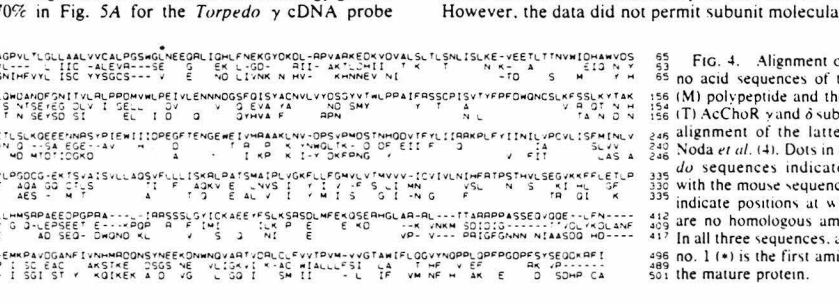

FIG. 4. Alignment of the amino acid sequences of the mouse (M) polypeptide and the *Torpedo* (T) AcChoR γ and δ subunits. The alignment of the latter is from Noda *et al.* (4). Dots in the *Torpedo* sequences indicate identity with the mouse sequence. Dashes indicate positions at which there are no homologous amino acids. In all three sequences, amino acid no. 1 (*) is the first amino acid of the mature protein.

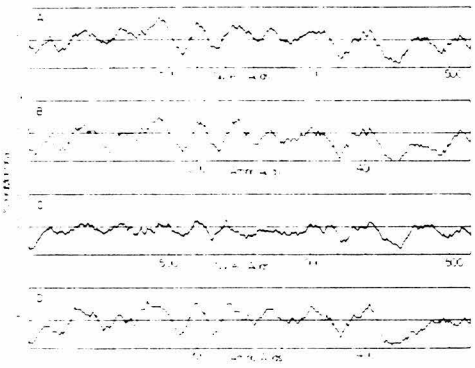

FIG. 5. Percent homology profiles for the mouse and *Torpedo* AcChoR subunits. In this analysis, the computer determines the percent homology between any two sequences (nucleic acid or amino acid) over a relatively short length of sequence (the search string). The computer begins the analysis at the 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. 6*A* 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.

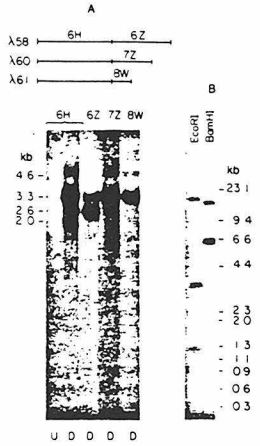

FIG. 6. (*A*) RNA blot analysis of mouse membrane poly(A)$^+$ RNA. RNA was prepared from differentiated BC3H-1 cells (D). Undifferentiated RNA (U) was prepared from BC3H-1 cells harvested after 3–4 days of growth in 20% fetal calf serum. Poly(A)$^+$ RNA was electrophoresed through a 1.5% agarose gel 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′ *Eco*RI 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. 6*B* 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 *Eco*RI lane and two in the *Bam*HI 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.

We are grateful to Dr. Dan Noonan for much help and advice and for providing a *Torpedo* α cDNA clone, to Dr. Toni Claudio for *Torpedo* β and δ clones, and to Dr. Steve Heinemann for the *Torpedo* γ clone (5). Dr. J. P. Merlie provided the BC3H-1 cells. Dr. Barbara Wold provided λgt10 DNA; she and Dr. Charles Rice contributed helpful advice about the cDNA cloning procedure. Tim Hunkapiller provided valuable assistance in the computer analysis of the data. This research has been supported by research grants to N.D. from the National Institutes of Health and the Muscular Dystrophy Association, by a National Institutes of Health Fellowship to R.L.P., and by a National Institutes of Health Predoctoral Traineeship to K.M.M.

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CHAPTER 4

Mouse-*Torpedo* hybrid acetylcholine receptors: Functional homology does not equal sequence homology

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Mouse–*Torpedo* hybrid acetylcholine receptors: Functional homology does not equal sequence homology

(*Xenopus* oocytes/SP6 RNA polymerase/cDNA clones/ion channels/*in ovo* translation)

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Contributed by Norman Davidson, April 5, 1985

ABSTRACT **The nicotinic acetylcholine (AcCho) receptor (AcChoR) is a multisubunit protein complex of stoichiometry $\alpha_2\beta\gamma\delta$. The several subunits show homology with each other within a given species; in addition, homology is found between analogous subunits between species. We have used the phage SP6 RNA polymerase transcription system to produce single-species RNA *in vitro* for various AcChoR subunits from cDNAs. Injection of an equimolar mixture of RNA for the α, β, γ, and δ subunits of *Torpedo californica* AcChoR into *Xenopus* oocytes results in the appearance of functional receptors in the oocyte membrane. No response to AcCho is detected when the β or γ subunit RNA is omitted, and a small response is seen when the δ subunit RNA is omitted. Replacement of *Torpedo* δ subunit RNA by the mouse BC3H-1 cell line AcChoR δ subunit RNA leads to the formation of functional receptors that show a 3–4-fold greater response to AcCho than does the full *Torpedo* complex. No response is seen when the mouse δ RNA replaces *Torpedo* γ RNA. By amino acid homology profile comparisons, the mouse δ subunit appears to be moderately but not highly similar to the *Torpedo* δ subunit; the apparent similarity to the *Torpedo* γ subunit is only slightly less. Therefore, the features of the primary sequence that determine the functional δ character of the mouse polypeptide are not revealed by simple homology comparisons.**

The nicotinic acetylcholine receptor (AcChoR) is involved in vertebrate neuromuscular transmission and the generation of electrical impulses by the electroplax of electric fish. Extensive electrophysiological, biochemical, structural, and molecular biological studies have made it the best-characterized membrane channel from excitable cells (for recent reviews, see refs. 1–5). The receptor complex consists of four subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. The binding of AcCho to each of the two α subunits results in the opening of a transmembrane channel that is permeable to small cations, resulting in the transmission of an impulse at the nerve–muscle synapse or in a high-voltage pulse from electric fish.

The recent cloning of cDNAs for the subunits of the *Torpedo* electric organ AcChoR (6–11) makes it possible to apply the powerful techniques of molecular biology to the study of the structure, evolution, biosynthesis, and mechanisms that underlie the operation of the complex. Our interest lies in the mechanism of ligand activation and ion permeation through the channel. Through the use of site-directed mutagenesis (12), we hope to identify the structural features and, thus, the mechanisms involved in the functioning of the receptor. Since the mutagenesis involves manipulations at the DNA level, a suitable expression system must be developed to study the properties of these "mutant" receptors.

Xenopus oocytes have proved to be an attractive system for the expression of proteins coded for by exogenous nucleic acids. Nuclear injection of DNA (13) or cytoplasmic injection of mRNA (14) results in the biosynthesis of functional products. Barnard and co-workers (15, 16) have shown that when *Torpedo* electric organ mRNA is injected into oocytes, functional *Torpedo* AcChoRs appear in the oocyte membrane. Mishina *et al.* (17) injected mRNA isolated from COS cells transfected with expression vectors containing cDNAs coding for each of the subunits of *Torpedo* AcChoR and obtained functional AcChoRs, an indication that the cDNAs contained all of the *Torpedo*-specific information required for assembly of functional receptors.

In this report, we describe another approach to the expression of *Torpedo* AcChoRs in *Xenopus* oocytes. We utilize the highly efficient phage SP6 RNA polymerase *in vitro* transcription system developed by Melton and colleagues (18, 19). This system allows the synthesis of microgram quantities of pure RNA from cDNA. When used with cDNAs for the individual subunits of the AcChoR, injection of the *in vitro* transcripts into oocytes gives rise to functional *Torpedo* AcChoRs in large quantities that can be studied readily by both biochemical and electrophysical techniques.

While this manuscript was in preparation, Mishina *et al.* (20) described an expression system essentially similar to that described here. They have used this system to study the effects of segment deletion or single amino acid changes introduced into the *Torpedo* AcChoR α subunit on receptor function. For our first study, we have chosen to test the effect of a much larger "mutation" in that we have asked whether a hybrid receptor containing the *Torpedo* α, β, and γ subunits and the mouse δ subunit is functional. This study necessarily required examination of the effects of one-by-one deletion of each individual *Torpedo* subunit on receptor assembly and function.

MATERIALS AND METHODS

Plasmids. Full-length *Torpedo* AcChoR cDNA clones were provided by D. Noonan of Scripps Research Institute (α subunit), T. Claudio of Yale University (β and δ subunits) and S. Heinemann of Salk Institute (γ subunit; ref. 10). The mouse BC3H-1 cell line AcChoR δ subunit cDNA clone was isolated in this laboratory (21). The cDNA inserts were excised from the vector and inserted into plasmid pSP62-PL (provided by D. Melton of Harvard University), which contains the phage SP6 RNA polymerase promoter followed by a polylinker. The resulting plasmids were maintained in *Escherichia coli* strain HB101.

***In Vitro* Transcription.** Plasmids containing the appropriate AcChoR subunit were linearized by digestion with either *Aat* II (*Torpedo* α, γ, and δ subunits), *Xmn* I (*Torpedo* β subunit), or *Sca* I (mouse δ subunit) (New England Biolabs) according to the supplier's recommendations. The digestion mixture

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Abbreviations: AcChoR, nicotinic acetylcholine receptor; AcCho, acetylcholine; GpppG, diguanosine triphosphate.

was extracted with phenol, and the DNA was precipitated with ethanol.

The transcription reaction contained 40 mM Tris (pH 7.5); 10 mM NaCl; 10 mM dithiothreitol; 6 mM $MgCl_2$; 4 mM spermidine; 0.5 mM each of ATP, CTP, and UTP; [α-^{32}P]CTP at 50 μCi/ml (Amersham; 1 Ci = 37 GBq), 0.1 mM GTP; 0.5 mM diguanosine triphosphate (GpppG; Pharmacia P-L Biochemicals), RNasin at 1000 units/ml (Promega Biotec, Madison, WI) SP6 RNA polymerase at 160 units/ml (Promega Biotec); and linearized DNA at 25 μg/ml. Total volume was 100 μl. Transcription was carried out for 70 min at 37°C. The reaction was stopped by the addition of RNase-free DNase (grade DPRF, Worthington) to a concentration of 20 μg/ml, and incubation was for 10 min at 37°C. The reaction mixture was extracted with phenol, unincorporated nucleotides were removed by the spun-column method of Penefsky (22) with 10 mM Na-phosphate (pH 7.0), and the RNA was recovered by precipitation by ethanol. A portion of the reaction product was treated with glyoxal and analyzed by gel electrophoresis (23).

Preparation of Oocytes and RNA Injection. Mature *Xenopus* females were obtained from Nasco (Ft. Atkinson, WI) and anesthetized by immersion in water containing 0.15% tricaine (3-aminobenzoic acid ethyl ester). An incision was made in the abdomen, and a portion of the ovary was removed and placed in ND-96 solution (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes/NaOH, pH 7.6). Follicle cells were removed by incubating the tissue in Ca^{2+}-free OR-2 solution (24) containing collagenase (type IA, Sigma) at 2 mg/ml for 30–45 min at room temperature. The oocytes were transferred to Ringer's solution (116 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/mM $MgCl_2$/5 mM Hepes/NaOH, pH 7.6), and the adhering follicular tissue was removed with forceps. Isolated stage V and VI oocytes were then transferred to ND-96 supplemented with 2.5 mM Na pyruvate.

RNA was dissolved in distilled water at 0.2 mg/ml, and 30–50 nl were injected into the cytoplasm by using a device similar to that described by Contreras *et al.* (25). The oocytes were incubated at room temperature for 48–72 hr in 70% L-15 medium (26) supplemented with 10 μg of penicillin and streptomycin per ml and 0.5 mM theophylline.

Electrophysiology. Individual oocytes were transferred to the recording chamber and studied under voltage-clamp conditions by using a standard two-microelectrode voltage clamp (model 8500, Dagan Instruments). The electrodes were filled with 3 M KCl/100 mM potassium EGTA, pH 7.0, and had resistances of 0.5–3 MΩ. The chamber was continuously perfused with 96 mM NaCl/2 mM KCl/5 mM $MgCl_2$/0.3 mM $CaCl_2$/0.3 μM atropine/5 mM Hepes/NaOH, pH 7.6. Holding potential in all experiments was −60 mV.

RESULTS AND DISCUSSION

Transcription of Subunit-Specific RNAs. *In vitro* transcription using plasmids containing the highly efficient SP6 promoter and SP6 RNA polymerase produces large quantities of single-species RNA. Fig. 1 shows the products of transcription reactions using linearized plasmids containing cDNA of the α, β, γ, and δ subunits of the *Torpedo* AcChoR and the δ subunit of the mouse AcChoR. Each reaction produces a single RNA species that carries the coding sequence for the appropriate subunit. Under the conditions described, 2–5 μg of RNA are produced per μg of DNA, which corresponds to 10–30 transcripts per DNA template.

The 5′ cap structure found in eukaryote mRNAs is an absolute requirement for mRNA stability in the cytoplasm (27). In their initial studies, Green *et al.* (28) capped their *in vitro* SP6 transcripts by subsequent treatment of the RNA

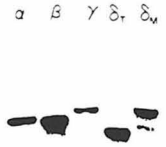

FIG. 1. *In vitro* synthesis of AcChoR subunit RNA by SP6 RNA polymerase. Plasmids containing the α, β, and γ AcChoR subunits of *Torpedo* and the δ AcChoR subunits of *Torpedo* and mouse, designated δ_T and δ_M, respectively, were linearized and transcribed as described. An aliquot from each reaction was treated with glyoxal and electrophoresed through 1% agarose. The transcripts are 2.5(α), 2.3(β), 2.65(γ), 2.1(δ_T), and 2.6(δ_M) kilobases long.

with vaccinia virus guanyltransferase. We obtained capped transcripts by inclusion of the cap analog diguanosine triphosphate (GpppG) in the transcription reaction. Contreras *et al.* (29) discovered that the cap analogs GpppA and GpppG can be incorporated at the 5′ end of RNAs produced *in vitro* with *E. coli* RNA polymerase. The capped transcripts are not rapidly degraded after injection into *Xenopus* oocytes and are efficiently translated. Konarska *et al.* (30) found that SP6 RNA polymerase also can incorporate the cap analog GpppG at the 5′ end of the transcripts. Whereas the cap analogs reduce the efficiency of the *E. coli* polymerase transcription reaction, we found that inclusion of GpppG stimulates transcription by SP6 RNA polymerase by 20–50%. Under the conditions described >95% of the transcripts are capped at the 5′ end (data not shown). We have not found it necessary to prepare transcripts with a 7-methylguanosine cap structure, which is not surprising because *Xenopus* oocytes contain a cytoplasmic methyltransferase (27). In addition, it is not necessary to include a polyadenylate tract at the 3′ end for translation of the RNA in oocytes, in agreement with Krieg and Melton (19).

Expression of *Torpedo* AcChoRs in *Xenopus* Oocytes. When the *Torpedo* subunit-specific RNAs are mixed in equimolar proportions and injected into *Xenopus* oocytes, functional AcChoRs are synthesized and inserted into the oocyte plasma membrane. Fig. 2*A* shows the response of a voltage-clamped oocyte previously injected with *in vitro* synthesized RNA to bath application of various concentrations of AcCho. Atropine (300 nM) was included in the perfusion solution to block the endogenous muscarinic AcCho receptors (31), and the Ca^{2+} concentration was kept low (0.3 mM) to obtain dose–response data without the complications introduced by desensitization (32). When a physiological Ca^{2+} concentration (1.8 mM) is used, the receptors do desensitize (data not shown). Membrane current increases as AcCho activates the receptor; the time course of this increase is limited by perfusion mixing rather than by the molecular events of channel gating. The response is nonlinear; doubling the AcCho concentration from 330 nM to 660 nM results in a 5-fold increase in current. No response was seen in uninjected oocytes. Furthermore, *d*-tubocurarine (5 μM), a competitive inhibitor of the nicotinic receptor, blocked the response (data not shown). Fig. 2*B* shows the results of many measurements of this type plotted on double-logarithmic coordinates. Data from each experiment are normalized for comparison to a response of 100 for 1 μM AcCho. The data are fit well by a relation with a Hill coefficient, n, of 2.0 ± 0.1.

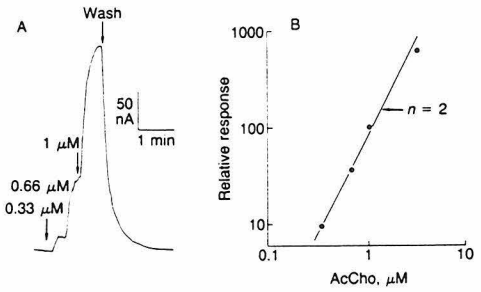

FIG. 2. *In vitro* transcribed RNA directs the synthesis of AcChoRs in *Xenopus* oocytes. (*A*) An oocyte injected with 10 ng of RNA 48 hr previously was voltage-clamped, and membrane current was monitored in response to bath application of 0.33, 0.66, and 1.0 μM AcCho. Note the nonlinear dependence of the response on AcCho concentration. (*B*) Pooled data from measurements on four oocytes. Data from measurements like those in *A* were normalized to a response of 100 for 1 μM AcCho. Each data point represents the mean ± SEM of four to six determinations; the error bars are smaller than the points. The solid curve is drawn according to the relation:

$$\text{response} = 100 \cdot \left(\frac{[\text{AcCho}]}{1\ \mu M}\right)^2.$$

The slight deviation from $n=2.0$ for 3.3 μM AcCho may be due to the effect of the small amount of desensitization seen at this concentration. The same quadratic dependence of response on ligand concentration has been observed both in electrophysiological dose–response curves using nerve-muscle and nerve-electroplaque preparations (33, 34) and in ligand-activated flux measurements on isolated membrane vesicles (35). This basic finding is consistent with the hypothesis that two AcCho molecules must bind to the receptor complex to effect a response.

These data indicate that the *in vitro* transcribed RNAs are capable of directing the synthesis of *Torpedo* AcChoRs in *Xenopus* oocytes. The pharmacological porperties (activated by AcCho, blocked by curare, unaffected by atropine), the desensitization, and the functional stoichiometry ($n=2$) are all hallmarks of normal receptors. In particular, the requirement for two agonist molecules is thought to manifest an interaction between the two liganded α subunits within an individual receptor molecule. Thus biosynthesis of the protein complex in oocytes appears to have all of the features of the *in vivo* process in *Torpedo* electroplax and muscle cells.

Subunit Deletion Experiments. The amino acid sequences of the four *Torpedo* subunits show homology among each other (8). In addition to this intrareceptor homology, each subunit also exhibits homology to the corresponding subunit from other species (21, 36–40). We have taken advantage of the flexibility of our expression system to determine if this sequence homology is also a functional homology. Fig. 3 and Table 1 show the results obtained from oocytes injected with various mixtures of *in vitro* transcribed RNAs. No current is elicited by bath application of 1.5 μM AcCho when either the *Torpedo* β or γ subunit RNA is omitted from the injection mixture. When the *Torpedo* δ subunit RNA is omitted, the elicited current is small (3% of control) but nonetheless present. This result differs somewhat from that of Mishina *et al.* (17), who detected a response in only 3 of 105 oocytes injected with an αβγ mixture prepared by synthesis in COS cells. We ascribe this difference to a higher sensitivity in our measurements, rather than any real difference in expression.

Formation of Hybrid Receptors. LaPolla *et al.* (21) have described the isolation of a cDNA clone for the δ subunit of the mouse BC3H-1 cell line AcChoR. It is probable that this

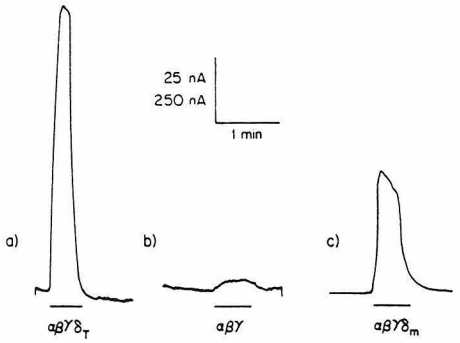

FIG. 3. Responses of oocytes injected with various combinations of AcChoR subunit RNAs. Oocytes were injected with the indicated mixture of AcChoR subunit RNAs, incubated, and voltage-clamped as described. The current elicited by bath application of 1.5 μM AcCho (solid bar below current traces) was determined. The current scale is 25 nA for traces a and b and 250 nA for trace c.

is the cDNA for the gene expressed in the skeletal muscles of the mouse. These authors reported that the amino acid sequence of the mouse δ subunit (δ_M) shows 59% overall amino acid homology to the *Torpedo* δ subunit (δ_T). The clone also shows 50% homology to the *Torpedo* γ subunit. Using a somewhat refined sequence alignment, we find that δ_M shows 59% homology to δ_T and 48% homology to the *Torpedo* γ subunit. We find that when γ RNA is replaced by δ_M RNA, no response is seen (Table 1). On the other hand, when δ_T RNA is replaced by δ_M RNA, the elicited current is 3- to 4-fold greater than the control (all *Torpedo* subunits; Fig. 3 and Table 1). The difference in the response of the hybrid receptor compared to the control is not due to a greater number of receptors on the surface of the oocyte, as we do not detect a significant difference in the number of surface α-bungarotoxin biding sites for the two populations (data not shown). The enhanced response is due to a change in the intrinsic properties of the receptor complex. Possibilities include a 4-fold increase in the single-channel conductance, a 2-fold decrease in the AcCho concentration for half-maximal activation, or an increased channel mean open time.

Whatever similarity exists between δ_T and δ_M that is not present in the *Torpedo* γ subunit is not obvious from comparison of the amino acid sequences. Fig. 4 presents homology profiles for δ_M vs. δ_T, δ_M vs. γ, and δ_T vs. γ subunits. There are no obvious differences between the profiles to suggest a region that defines the mouse subunit as a δ rather than a γ. It is possible that the regions of sequence nonhomology contain structural features that determine the

Table 1. Electrophysiological responses of oocytes after injection of various AcChoR mRNAs

Mixture	Response, nA ± SEM
$\alpha\beta\gamma\delta_T$	235.6 ± 25.5
$\alpha\gamma\delta$	0
$\alpha\beta\gamma$	6.5 ± 1.1
$\alpha\beta\delta_T$	0
$\alpha\beta\gamma\delta_M$	792.0 ± 153.9
$\alpha\beta\delta_T\delta_M$	0

Oocytes were injected with various combinations of AcChoR subunit-specific RNAs as indicated, incubated, and voltage-clamped as described. The current elicited by bath application of 1.5 μM AcCho was determined. Each value represents the mean ± SEM of 10–20 determinations.

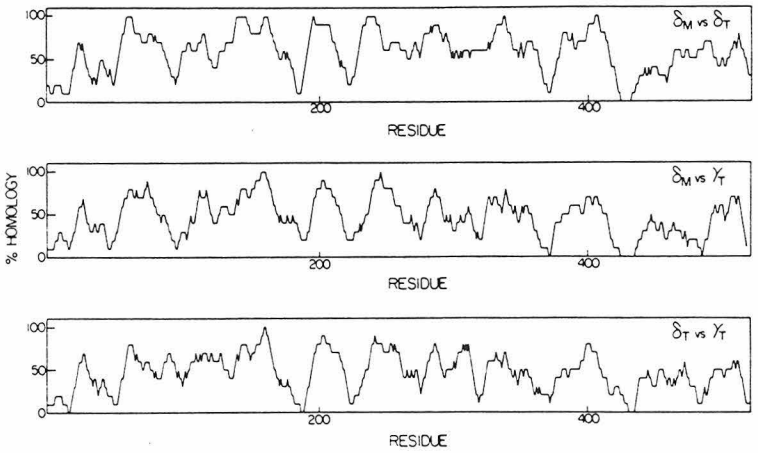

FIG. 4. Homology profile comparisons. Percent homology values for strings of 10 amino acids were calculated by using refinements of the sequence alignments described in ref. 21. In this analysis, the gaps inserted in the sequences for alignment purposes were scored as regions of 0% homology.

"deltaness" of the mouse subunit. Such structural/functional homology despite a lack of sequence homology is by no means uncommon. For example, the structure of the NAD-binding domain of NAD-dependent dehydrogenases is remarkably conserved despite a lack of sequence homology (41). In the absence of high-resolution structural data concerning the AcChoR, this possibility remains a viable hypothesis. However, another possibility does exist. There may be sequences conserved in the two δ subunits but not in the γ subunit that are not detected by the overall sequence analysis shown in Fig. 4. If subunit–subunit interactions are the forces that hold the complex together, then the points of contact could define the identity of each subunit. If one considers interactions between α helices, then these contact points should repeat every 3.6 residues (the periodicity of the helix). This periodicity may easily be missed in the plot shown in Fig. 4. We have examined the sequences of the four putative hydrophobic transmembrane domains of the subunits for such a periodicity. Fig. 5 shows helical net projections of these four domains. Residue positions in which δ_M, δ_T, and γ have identical amino acids are outlined with rectangles, and residue positions in which δ_M and δ_T but not γ have identical amino acids are outlined with ovals. Our hope was to find a stripe of sequence conservation between the two δ subunits that is not in the γ subunit. We have discounted such benign differences as valine to isoleucine, etc., and searched for rather drastic differences. Helix IV appears to be the best candidate for such a difference. A stripe of sequence conservation runs down the length of the helix for the δ subunits. The γ sequence differs in various positions with substitutions of tryptophan for phenylalanine, leucine for proline, and phenylalanine for methionine. These major

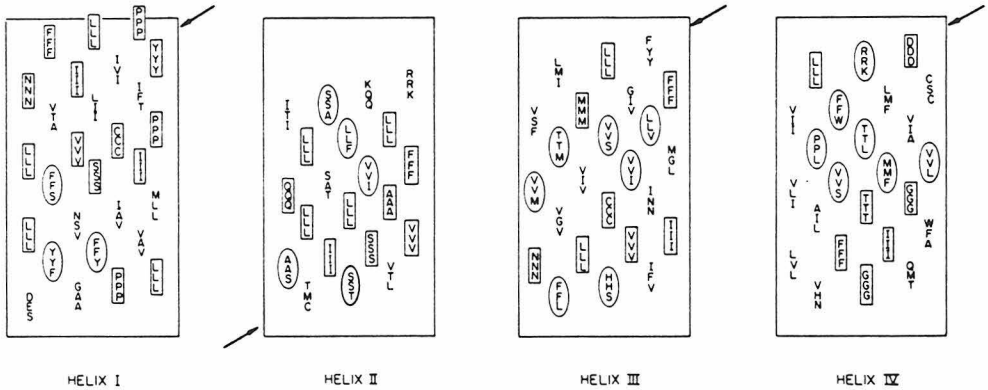

FIG. 5. Helical net representation of the four transmembrane hydrophobic regions. The residues are displayed as triplets in the order (top to bottom) δ_M, δ_T, and γ. Triplets in which all subunits have identical amino acids are outlined with a rectangle; triplets in which the two δ subunits but not the γ subunit have identical amino acids are outlined with an oval. The arrows indicate the amino terminus of each helix. Note the stripe of δ homology in helix IV.

side-chain changes could well prevent the neighbor–neighbor interactions that hold the δ subunit in its normal position.

This study illustrates the principle that the subtle ways in which conservation of function is encoded in the primary sequence of evolutionarily related proteins may not be revealed by a simplistic sequence homology comparison.

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CHAPTER 5

Functional studies on chimeric subunits of the acetylcholine receptor indicate that subunit-specific regions are widely dispersed over the subunit length

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HENRY A. LESTER and NORMAN DAVIDSON

Abstract

Acetylcholine receptor (AchR) subunits have been cloned and sequenced from a variety of species. There is a high level of sequence homology among all the subunits studied. The strongest homology is among analogous subunits between species. Still, analyses of this type have not elucidated a region or regions of subunit-specific homology that could mandate subunit identity. We have constructed a series of chimeric subunits to try to localize subunit determining regions of the AchR polypeptides. Each chimera was tested in the oocyte system by replacing its RNA for each of the parent RNAs in turn. None of the chimeras we have constructed retained enough of either parental subunit characteristics to completely function in place of that parent subunit to form an AchR that is responsive to acetylcholine. We conclude that a minimum of two subunit-specific regions are widely dispersed over the subunit length. These data are also consistent with the conclusion that there are no discrete regions that determine subunit identity, but instead that this information is rather evenly distributed along subunit length. In some combinations, the chimeras were incorporated into surface AchRs, though these complexes were at best weakly responsive to Ach. We conclude that there are regions needed for efficient function of these subunits that are not necessary for the formation of surface complexes. We have also demonstrated that the α subunits of both mouse and chick form functional AchRs in the *Xenopus* oocyte system in combination with the β and γ subunits from *Torpedo* and a δ from either *Torpedo* or mouse. The responses of these hybrid AchRs are smaller than the response from the *Torpedo* AchR. In contrast, the mouse γ subunit did not form functional AchRs in any combination of the subunits mentioned above.

The nicotinic acetylcholine receptor (AchR) is the best studied of all the neurotransmitter-activated channels. The AchR is a protein complex composed of four homologous transmembrane subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. AchRs are very abundant in the electric organs of *Torpedo* where they have been well characterized at the structural, biochemical, and sequence levels (reviewed by Conti-Tronconi and Raftery, 1982; Stroud and Finer-Moore, 1985).

The role of the AchR in the neuromuscular junction is to transduce a chemical signal from the nerve into depolarization of the muscle. When acetylcholine (Ach) is released by the nerve terminal it diffuses across the synaptic cleft and binds to AchRs on the muscle cell surface. When two Achs bind to each receptor, a conformational change occurs to open transiently a large pore in the receptor complex. This pore allows small cations to flow down their electrochemical gradients into the muscle cell. This temporary depolarization of the endplate membrane leads, through several steps, to the contraction of the muscle or to an electrical potential buildup in the asymetric stack of electrocytes in the electric organ.

Considerable sequence homology is demonstrated by the cDNA clones for many AchR subunits isolated recently for *Torpedo* , calf, human, mouse and chick (reviewed by Stroud and Finer-Moore, 1985). These clones can be used to study all aspects of AchR structure, biosynthesis, assembly and mechanisms of function of the channel with the powerful tools of molecular biology. *Xenopus* oocytes are an efficient system for expressing AchRs from *Torpedo* electric organ RNA (Sumikawa *et al.*, 1981; Barnard *et al.*, 1982) and from *in vitro* transcribed RNAs (Mishina *et al.*, 1985; White *et al.*, 1985). Subunit specific RNAs are transcribed *in vitro* by the phage SP6 transcription system. A mixture of these RNAs is injected into the oocyte cytoplasm where it is translated. The polypeptide products are processed and inserted into the oocyte membrane where they can

be assayed electrophysiologically. Voltage clamp studies show that the surface receptors resulting from injection of all four *Torpedo* mRNAs are AchRs by the criteria of Ach responsiveness with a Hill coefficient of 2.0, atropine insensitivity, and *d*-tubocurarine sensitivity (Mishina *et al.*, 1984, 1985; White *et al.*, 1985).

Our earlier studies on the isolation and identification of a δ subunit cDNA clone from mouse (LaPolla *et al.*, 1984; White *et al.*, 1985) encouraged us to investigate the nature of subunit-specific structure in the AchR. We isolated a mouse δ clone (δ_M) by hybridization with a *Torpedo* γ (γ_T) cDNA. On the basis of slightly higher amino acid sequence homology to the *Torpedo* δ (δ_T) than to γ_T we tentatively identified the mouse clone as a δ (LaPolla *et al.*, 1984). This assignment was confirmed by a much more decisive functional assay (White *et al.*, 1985).

Homology profile comparisons did not identify a region of subunit-specific homology that seemed to define "deltaness" of the mouse subunit. We reasoned that the closest contacts between subunits may occur between the membrane spanning α-helices if the models proposed by Guy (1984) and Finer-Moore and Stroud (1985) are correct. Since the amphipathic helices (abbreviated, MA) are regions of relative divergence among the various subunits (Stroud and Finer-Moore, 1985), we focused our attentions on the proposed hydrophobic membrane spanning regions (M1, M2, M3 and M4). A stripe of subunit-specific homology down one side of one or more of these helices might represent the line of contact between subunits. M4 offers the most promising stripe of sequence conservation between δ_M and δ_T that is not present in γ_T. Individual residues in the other three helices show subunit-specific identity, but the apparent subunit-specific residues in M4 are more clustered. When the corresponding residues from the mouse γ (γ_M) (Yu *et al.*, 1986) are included in helical net projections like those used in White *et al.* (1985) the differences between the pair of δ's and the pair of γ's are more pronounced. M4 is the

least conserved of all the hydrophobic α-helices, even when comparing sequences from a specific subunit type (Stroud and Finer-Moore, 1985).

In this field, the term "hybrid" has been used to describe AchRs that are composed of complete subunits from two or more species (White *et al.*, 1985; Sakmann *et al.*, 1985). We have constructed a series of subunits that are the product of fusing an N-terminal portion of one subunit to the C-terminal portion of another subunit. We refer to these constructions as "chimeras" in order to avoid confusion with the interspecies hybrid AchRs defined above.

Chimeric proteins are commonly used in a variety of systems to study functions ascribed to a discrete portion of a protein molecule. Protein sorting sequences have been studied by fusing them at the DNA level to a detectable protein such as β-galactosidase followed by transformation of the fusion gene into bacteria (Emr *et al.*, 1980), yeast (Douglas *et al.*, 1984), or animal cells (Guan and Rose, 1984; van Loon *et al.*, 1986). The DNA binding portion of LexA, an *E. coli* represssor protein, has been fused to the yeast transcriptional activator GAL4 to generate a transcriptional activator that binds at a specific bacterial operator (Brent and Ptashne, 1985). Fusion studies involving homologous proteins include the "exon shuffling" experiments used to determine the function of different domains of mouse histocompatability antigens (Evans *et al.*, 1982; Germain *et al.*, 1985). In these experiments genomic clones were "recombined" in vitro by intronic ligation of exon-containing fragments. The resulting genes were transfected into L cells and the proteins assayed by antibody binding and T-cell restriction. The *trpA* genes of *E. coli* and *S. typhimurium* have been recombined *in vivo* to produce hybrid proteins (Schneider *et al.*, 1981). The hybrid *trpA* genes produce the α polypeptide subunit of the $\alpha_2\beta_2$ tryptophan synthetase complex. These fusion proteins were assayed for catalytic activity to determine the significance of amino acid substitutions between

these highly homologous genes. Catalytic activity was retained in every α hybrid, but many had decreased thermal stability. These data suggest that the differences in the individual subunits coded by *trpA* do not have special roles nor "balance" each other in the active site structure. The loss of one of a "balancing" pair of amino acid differences may, however, explain the loss of stability at elevated temperature.

We have constructed chimeric subunits to try to localize subunit determining regions of the AchR polypeptides. The oocyte expression system is a very convenient one for these experiments, since individual subunit RNAs can be substituted very easily in the injection mixture. We have also studied the formation of hybrid AchRs with several subunit combinations including the chick muscle α subunit (α_C) and the mouse muscle α (α_M) as well as δ_M and γ_M.

Materials and Methods

Plasmids. Full-length *Torpedo* AchR cDNA clones were provided by D. Noonan of Scripps Research Institute (α), T. Claudio of Yale University (β and δ), and S. Heinemann of The Salk Institute (γ). The cDNA containing fragments were inserted into plasmid pSP65 (Melton *et al.*, 1984) or pSP64·f1$^-$ (D. Mead *et al.*,1985). The mouse cell line BC3H-1 δ and γ subunits were isolated in this laboratory (LaPolla *et al.*, 1984; Yu *et al.*, 1986) and were subcloned into pSP64T (Krieg and Melton, 1984) and pSP65 respectively. The chick muscle α cDNA clone (Beeson *et al.*, 1986) was provided by Drs. J. Jackson and E. Barnard of Imperial College and MRC Molecular Neurobiology unit and was subcloned into pSP64 (Melton *et al.*, 1984). The mouse α subunit from BC3H-1 cells was provided by J. P. Merlie of Washington University, St. Louis as a subclone in pGEM1 (Promega Biotec).

Oligonucleotides. Oligonucleotides were gel purified from 20% acrylamide gels with 8M urea and excised following visualization by UV-shadowing. The DNA was eluted by diffusion (Maxam and Gilbert, 1980). Precipitated oligonucleotides were phosphorylated with polynucleotide kinase with ATP for use in ligations or (γ^{32}P) ATP for use as probes.

Gel isolation of DNA fragments (M. Hu, personal communication). All the cDNA fragments and vectors were isolated from gels by the following method. Restriction digested DNA was electrophoresed in 1% Sea Plaque agarose (Marine Colloids) in 80 mM Tris-phosphate, 8mM EDTA, pH8, with recirculation. The gel was briefly stained in 50 ng/ml ethidium bromide. Bands were visualized briefly on a long-wave UV transilluminator and excised in a minimum-sized slice of agarose. The volume of the slice was estimated (*e.g.*, 200 µl) and an equal volume of 100 mM Tris-Cl, 10 mM EDTA, pH8, was added to the slice in a 1.5 ml microfuge tube. The slice was completely melted by incubating the capped tube at 70°C for 5 minutes. An equal volume of room temperature buffered phenol (*e.g.*, 400 µl) was added. The tube was quickly capped, vortexed, and microfuged for 10 minutes at room temperature. The supernate was removed, avoiding the white, agarose-containing interface, and re-extracted twice with phenol-chloroform (1:1) followed by chloroform, and ethanol precipitated twice. DNA isolated in this manner worked very efficiently in all procedures attempted.

Construction of cDNA chimera plasmids. Early ligation reactions without salt were unsuccessful, so several steps were added to allow the oligonucleotides time to hybridize with salt and at higher temperature than the 15°C ligation conditions allow. The phosphorylated oligonucleotides were heated to 50°C in 100 mM NaCl and allowed to cool to 40°C over 1 hour. Other components and DNAs were added to give a final reaction that was 50 mM Tris (pH 7.6) 50 mM NaCl, 10 mM $MgCl_2$, 10 mM

dithiothreitol, 1mM spermidine, 1mM ATP in a total of 20 μl. The mixture was held at 40°C for 5 minutes before chilling to 15°C. 400 units T4 DNA ligase (Boehringer Mannheim) was added and the reaction was incubated at 15°C overnight. Each ligation contained approximately 1 μg of vector DNA, 0.5 - 0.2 μg of each cDNA fragment, and 10 ng of each oligonucleotide, all of which were previously gel isolated individually. The ligations were diluted and an aliquot was used to transform fresh DH1 (δ_M-γ_T 1 and 1A) or frozen HB101 (all other constructions) competent cells. Frozen competent HB101 were obtained from Bethesda Research Labs. Transformation efficiencies were higher when the ligation mixture was diluted, extracted with phenol, followed by chloroform, and passed through a 1 ml spun column of Sephadex G-50 (Maniatis *et al.*, 1982; Penefsky, 1977).

Ampicillin resistant transformants were replica plated and screened with three ^{32}P-labeled probes: each nick-translated cDNA fragment and one of the oligonucleotides (KM1, KM8, KM10, KM16). Hybridizations were performed at 42°C in 50% formamide, 900 mM NaCl, 50 mM sodium phosphate, pH 7.4, and 5 mM EDTA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% sarkosyl (sodium n-lauroylsarcosine), 100 μg/ml sonicated denatured salmon sperm DNA, plus radioactive probe at 10^5 cpm/ml. For KM16 hybridizations the formamide was omitted. The filters were washed at 50°C in 2X SSC, 0.1% sarkosyl followed by 0.2 X SSC, 0.1% sarkosyl (2X SSC is 300 mM NaCl, 30 mM sodium citrate). KM16 hybridized filters were washed in 6X SSC, 0.1% sarkosyl at 42°C. Greater than 90% of all transformants hybridized to all three probes except for the α_T-δ_T ligations, as described in the text. Selected positively hybridizing clones were rescreened by the same procedure and then tested by restriction endonuclease digestion. A few promising clones were grown up and

the sequences confirmed by the method of Maxam and Gilbert (1980). Seven out of twelve clones sequenced matched the desired sequence exactly.

Replica plating (L. Garfinkel, personal communication). Replica filters for colony hybridization were prepared using standard 82 mm diameter nitrocellulose filters (Millipore) and L-broth agar plates supplemented with 25 μg/ml ampicillin. The original transformation plate can be used as the master, or colonies can be transferred to grid patterns on fresh plates. A labeled filter was laid on the surface of the colony-containing plate and keyed with India ink in an 18 gauge needle. After the filter was completely wet, it was removed and laid, colonies up, on Whatman 3 MM paper. This master filter was dried at room temperature for 10 minutes.

A fresh nitrocellulose filter (replica filter) was premoistened by laying it on a sterile L-broth plus ampicillin plate. This filter was then laid on the master filter and covered with a piece of Whatman 3 MM paper. A lucite block was placed on the filter sandwich and pressure applied (~ 100 lbs). The replica filter was keyed with holes to match the master. The two filters were separated and the replica filter was laid on a fresh L-broth plus ampicillin plate and grown overnight at 37°C. The master filter was stored, colonies up, on an L-broth plus ampicillin plate, at 4°C. This filter or the original colony plate could both be used to pick colonies identified by hybridization. Colonies were lysed and the filters were prepared for hybridization by standard methods.

Site-directed mutagenesis. An attempt to correct the frameshift mutation in chimera α_T-δ_T C was made. The internal 800 base pair Pst I fragment from α_T-δ_T C was cloned into the Pst I site of M13 phage mp18. Mutagenesis protocols were as described by Zoller and Smith (1983); however, no specific hybridization or priming by oligonucleotide KM17 was observed, regardless of the orientation of the Pst I fragment. The sequence of KM17 was confirmed by DNA sequencing (Maxam and Gilbert, 1980).

In vitro Transcription. Plasmid DNAs were linearized by restriction endonuclease digestion at sites within the plasmid vectors. The DNA was prepared and transcribed essentially as described in White *et al.* (1985), with the following changes. Diguanosine triphosphate (GpppG; Pharmacia P-L Biochemicals) was found to be very acidic if re-suspended in water and was therefore adjusted to pH 7.5 in 100 mM Tris. Other nucleotide triphosphateswere had been adjusted to pH 7.5 in 70 mM Tris and were diluted from a 10X stock to 0.5 mM (ATP, UTP, CTP), or 0.1mM (GTP). The overall concentration of Tris in the transcription reaction remained 40 mM. The concentration of radioactive tracer was dropped from 50 to 20 μCi/ml, and spermidine was omitted from the reaction. The phenol extraction was eliminated. Reaction volumes can be increased to 300 or 500 μl with commensurate increase in the spun column volume to 3 ml or 5 ml. Phage T7 polymerase transcriptions of α_M were performed under identical buffer conditions.

Preparation of oocytes and RNA injection. Mature *Xenopus* females were obtained from commercial sources and anesthetized by immersion in water containing 0.17% tricaine (3-aminobenzoic acid ethyl ester). An incision was made in the abdomen, and a portion of the ovary was removed and placed in 82.5 mM NaCl, 2.0 mM KC1, 1.0 mM MgCl, 5.0 mM HEPES/NaOH, pH 7.5. Follicle cells were removed by incubating the tissue in this solution containing collagenase (type 1A, Sigma) at 2 mg/ml for 3 hours at room temperature.

RNA was dissolved in distilled water at 1 mg/ml, and 50 nl were injected into the cytoplasm with a microdispenser (Drummond Scientific Co., Boomall, PA) through glass needles having tip diameter of about 20 μm held by a micromanipulator. The oocytes were transferred to ND-96 solution (96 mM NaCl, 2 mM KC1, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/NaOH, pH 7.6) supplemented with 100 units of penicillin and

100 μg of streptomycin per ml, 0.5 mM theophylline, 2.5 mM pyruvic acid, and incubated at room temperature for 48-72 hours. Surface binding of α–bungarotoxin (α-BTX) was measured by incubation of oocytes, the same day as they were tested electrophysiologically, in 5 nM ^{125}I-α-BTX in ND-96 solution with 1 mg per ml bovine serum albumin and 0.1 mg per ml cytochrome C for 1 hour at room temperature.

Electrophysiology. Individual oocytes were transferred to the recording chamber which was connected to six funnels through a rotary stopcock. The oocytes were perfused with 96 mM NaCl, 2 mM KC1, 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.3 μM atropine, 5 mM HEPES/NaOH, pH 7.6, and stimulated with various concentrations of ACh solutions by switching the rotary stopcock. The responses were measured under voltage-clamp conditions by using a standard two-microelectrode voltage clamp (model 8500, Dagan Instruments). The electrodes were filled with 3 M KC1, 100 mM potassium EGTA, 20 mM HEPES/KOH, pH 7.4 and had resistances of 0.5-1 MΩ. Holding potential in all experiments was -60 mV.

Results

Construction of chimeras. An alignment of the amino acid sequences from all AchR subunits used in this study is shown in Figure 1. The alignment and numbering follows that of Stroud and Finer-Moore (1985) with the addition of the α_M, α_C, and γ_M sequences. Chimeric cDNAs were constructed that encode intersubunit fusion proteins as shown schematically in Figure 2. In Figure 2 the membrane spanning helices (M1, M2, M3, MA, and M4) as proposed by Guy (1984) and Finer-Moore and Stroud (1985) are included as landmarks for the locations of the chimeric protein breakpoints. "Breakpoint," as used here, refers to the exact point of transition in the amino acid sequence, without regard to the way the DNA molecules were manipulated to achieve that

Figure 1. Alignment of the amino acid sequences of the AchR subunits used in this work. The alignment scheme used is that of Stroud and Finer-Moore (1985), with the addition of the α_M, α_C, and γ_M subunits. Amino acid number 1 is the first amino acid of the mature protein (determined for *Torpedo* and chick subunits (Raftery *et al.*, 1980; Conti-Tronconi *et al.*, 1985), predicted for mouse). Dots in the lower seven sequences indicate identity with the δ_M sequence. Predicted membrane spanning amphipathic (MA) and hydrophobic (M1, M2, M3, and M4) helicies are indicated.

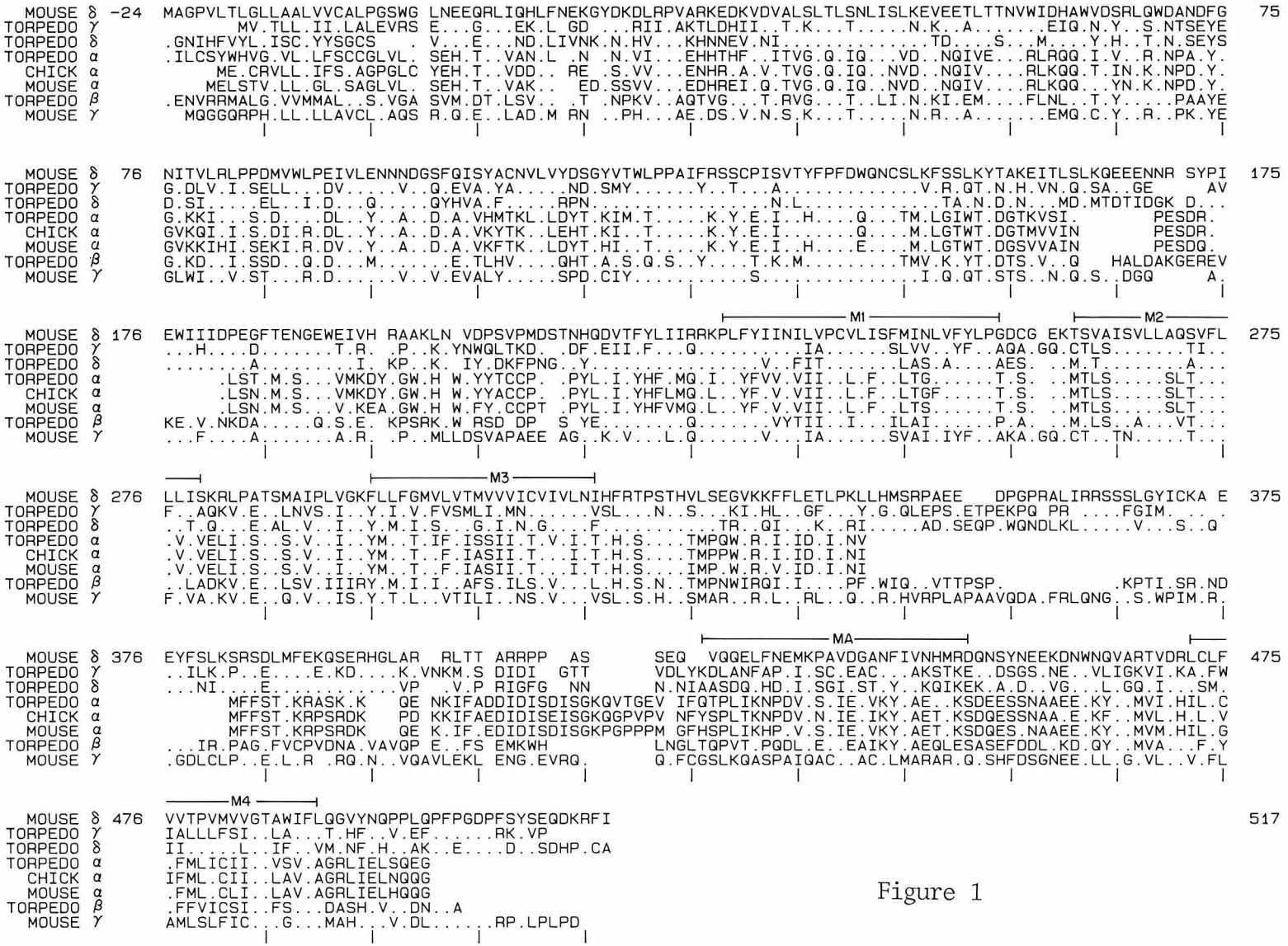
MOUSE δ -24 MAGPVLTLGLLAALVVCALPGSWG LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTLSNLISLKEVEETLTTNVWIDHAWVDSRLQWDANDFG 75
TORPEDO γ MV.TLL.II.LALEVRS E...G...EK.L GD ...RII.AKTLDHII..T.K...T.....N.K..A.....EIQ.N.Y..S.NTSEYE
TORPEDO δ .GNIHFVYL.ISC.YYSGCS . V...E...ND.LIVNK.N.HV...KHNNEV.NI.....TD....S...M....Y.H..T.N.SEYS
TORPEDO α .ILCSYWHVG.VL.LFSCCGLVL. SEH.T..VAN.L .N .N.VI...EHHTHF..ITVG.Q.IQ...VD..NQIVE...RLRQQ.I.V..R.NPA.Y.
CHICK α ME.CRVLL.IFS.AGPGLC YEH.T..VDD.. RE .S.VV...ENHR.A.V.TVG.Q.IQ..NVD..NQIV....RLKQQ.T.IN.K.NPD.Y.
MOUSE α MELSTV.LL.GL.SAGLVL. SEH.T..VAK.. ED.SSVV...EDHREI.Q.TVG.Q.IQ..NVD..NQIV....RLKQQ...YN.K.NPD.Y.
TORPEDO β .ENVRRMALG.VVMMAL..S.VGA SVM.DT.LSV.. .T .NPKV..AQTVG...T.RVG...T..LI.N.KI.EM....FLNL..T.Y.....PAAYE
MOUSE γ MQGGQRPH.LL.LLAVCL.AQS R.Q.E..LAD.M RN ..PH...AE.DS.V.N.S.K...T.....N.R..A.....EMQ.C.Y..R..PK.YE
MOUSE δ 76 NITVLRLPPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTWLPPAIFRSSCPISVTYFPFDWQNCSLKFSSLKYTAKEITLSLKQEEENNR SYPI 175
TORPEDO γ G.DLV.I.SELL...DV.....V..Q.EVA.YA.....ND.SMY.....Y..T...A.....V.R.QT.N.H.VN.Q.SA..GE AV
TORPEDO δ D.SI....EL..I.D...Q.....QYHVA.F.....RPN.....N.L.....TA.N.D.N...MD.MTDTIDGK D...
TORPEDO α G.KKI...S.D....DL..Y..A..D.A.VHMTKL.LDYT.KIM.T.....K.Y.E.I..H....Q...TM.LGIWT.DGTKVSI. PESDR.
CHICK α GVKQI.I.S.DI.R.DL..Y..A..D.A.VKYTK..LEHT.KI..T.....K.Y.E.I.....Q....M.LGTWT.DGTMVVIN PESDR.
MOUSE α GVKKIHI.SEKI.R.DV..Y..A..D.A.VKFTK..LDYT.HI..T.....K.Y.E.I..H....E....M.LGTWT.DGSVVAIN PESDQ.
TORPEDO β G.KD..I.SSD..Q.D...M.....E.TLHV....QHT.A.S.Q.S..Y....T.K.M.....TMV.K.YT.DTS.V..Q HALDAKGEREV
MOUSE γ GLWI..V.ST...R.D.....V..V.EVALY.....SPD.CIY.....S.....I.Q.QT.STS..N.Q.S..DGQ A.
M1
M2
MOUSE δ 176 EWIIIDPEGFTENGEWEIVH RAAKLN VDPSVPMDSTNHQDVTFYLIIRRKPLFYIINILVPCVLISFMINLVFYLPGDCG EKTSVAISVLLAQSVFL 275
TORPEDO γ ...H....D.....T.R. .P..K.YNWQLTKD. .DF.EII.F...Q.....IA.....SLVV..YF..AQA.GQ.CTLS.....TI..
TORPEDO δ .....A.....I. KP..K. IY.DKFPNG..Y.....V..FIT.....LAS.A....AES. ..M.T.....A...
TORPEDO α .LST.M.S...VMKDY.GW.H W.YYTCCP. .PYL.I.YHF.MQ.I..YFVV.VII..L.F..LTG.....T.S. ..MTLS....SLT...
CHICK α .LSN.M.S...VMKDY.GW.H W.YYACCP. .PYL.I.YHFLMQ.L..YF.V.VII..L.F..LTGF.....T.S. ..MTLS....SLT...
MOUSE α .LSN.M.S...V.KEA.GW.H W.FY.CCPT .PYL.I.YHFVMQ.L..YF.V.VII..L.F..LTS.....T.S. ..MTLS....SLT...
TORPEDO β KE.V.NKDA.....Q.S.E. KPSRK.W RSD DP S YE.....Q.....VYTII..I...ILAI.....P.A. ..M.LS..A...VT...
MOUSE γ ...F....A.....A.R. .P..MLLDSVAPAEE AG..K.V...L.Q.....V...IA.....SVAI.IYF..AKA.GQ.CT..TN.....T...
M3
MOUSE δ 276 LLISKRLPATSMAIPLVGKFLLFGMVLVTMVVVICVIVLNIHFRTPSTHVLSEGVKKFFLETLPKLLHMSRPAEE DPGPRALIRRSSSLGYICKA E 375
TORPEDO γ F..AQKV.E..LNVS.I..Y.I.V.FVSMLI.MN.....VSL...N..S...KI.HL..GF...Y.G.QLEPS.ETPEKPQ PR ....FGIM.....
TORPEDO δ ..T.Q...E.AL.V..I..Y.M.I.S...G.I.N.G....F.....TR..QI...K..RI.....AD.SEQP.WQNDLKL.....V...S..Q
TORPEDO α .V.VELI.S..S.V..I..YM..T.IF.ISSII.T.V.I.T.H.S....TMPQW.R.I.ID.I.NV
CHICK α .V.VELI.S..S.V..I..YM..T..F.IASII.T...I.T.H.S....TMPPW.R.I.ID.I.NI
MOUSE α .V.VELI.S..S.V..I..YM..T..F.IASII.T...I.T.H.S....IMP.W.R.V.ID.I.NI
TORPEDO β ..LADKV.E..LSV.IIIRY.M.I.I..AFS.ILS.V...L.H.S.N..TMPNWIRQI.I....PF.WIQ..VTTPSP. .KPTI.SR.ND
MOUSE γ F.VA.KV.E..Q.V..IS.Y.T.L..VTILI..NS.V...VSL.S.H..SMAR..R.L..RL..Q..R.HVRPLAPAAVQDA.FRLQNG..S.WPIM.R.
MA
MOUSE δ 376 EYFSLKSRSDLMFEKQSERHGLAR RLTT ARRPP AS SEQ VQQELFNEMKPAVDGANFIVNHMRDQNSYNEEKDNWNQVARTVDRLCLF 475
TORPEDO γ ..ILK.P..E....E.KD....K.VNKM.S DIDI GTT VDLYKDLANFAP.I.SC.EAC...AKSTKE..DSGS.NE..VLIGKVI.KA.FW
TORPEDO δ ...NI....E.....VP .V.P RIGFG NN N.NIAASDQ.HD.I.SGI.ST.Y..KQIKEK.A.D..VG...L.GQ.I...SM.
TORPEDO α MFFST.KRASK.K QE NKIFADDIDISDISGKQVTGEV IFQTPLIKNPDV.S.IE.VKY.AE..KSDEESSNAAEE.KY..MVI.HIL.C
CHICK α MFFST.KRPSRDK PD KKIFAEDIDISEISGKQGPVPV NFYSPLTKNPDV.N.IE.IKY.AET.KSDQESSNAA.E.KF..MVL.H.L.V
MOUSE α MFFST.KRPSRDK QE K.IF.EDIDISDISGKPGPPPM GFHSPLIKHP.V.S.IE.VKY.AET.KSDQES.NAAEE.KY..MVM.HIL.G
TORPEDO β ...IR.PAG.FVCPVDNA.VAVQP E..FS EMKWH LNGLTQPVT.PQDL.E..EAIKY.AEQLESASEFDDL.KD.QY..MVA...F.Y
MOUSE γ .GDLCLP..E.L.R .RQ.N..VQAVLEKL ENG.EVRQ. Q.FCGSLKQASPAIQAC..AC.LMARAR.Q.SHFDSGNEE.LL.G.VL..V.FL
M4
MOUSE δ 476 VVTPVMVVGTAWIFLQGVYNQPPLQPFPGDPFSYSEQDKRFI 517
TORPEDO γ IALLLFSI..LA...T.HF..V.EF.....RK.VP
TORPEDO δ II....L..IF..VM.NF.H..AK..E....D..SDHP.CA
TORPEDO α .FMLICII..VSV.AGRLIELSQEG
CHICK α IFML.CII..LAV.AGRLIELNQQG
MOUSE α .FML.CLI..LAV.AGRLIELHQQG
TORPEDO β .FFVICSI..FS...DASH.V..DN..A
MOUSE γ AMLSLFIC...G...MAH...V.DL.....RP.LPLPD

Figure 1

Figure 2. Schematic representation of chimeric subunit constructions. Each subunit's amino acid sequence is represented by a distinctive line pattern as shown in the key. Breakpoints are represented by the gaps in the horizontal lines, emphasized by two diagonal lines. The M1, M2, M3, MA, and M4 regions are represented by solid blocks and are included as reference points.

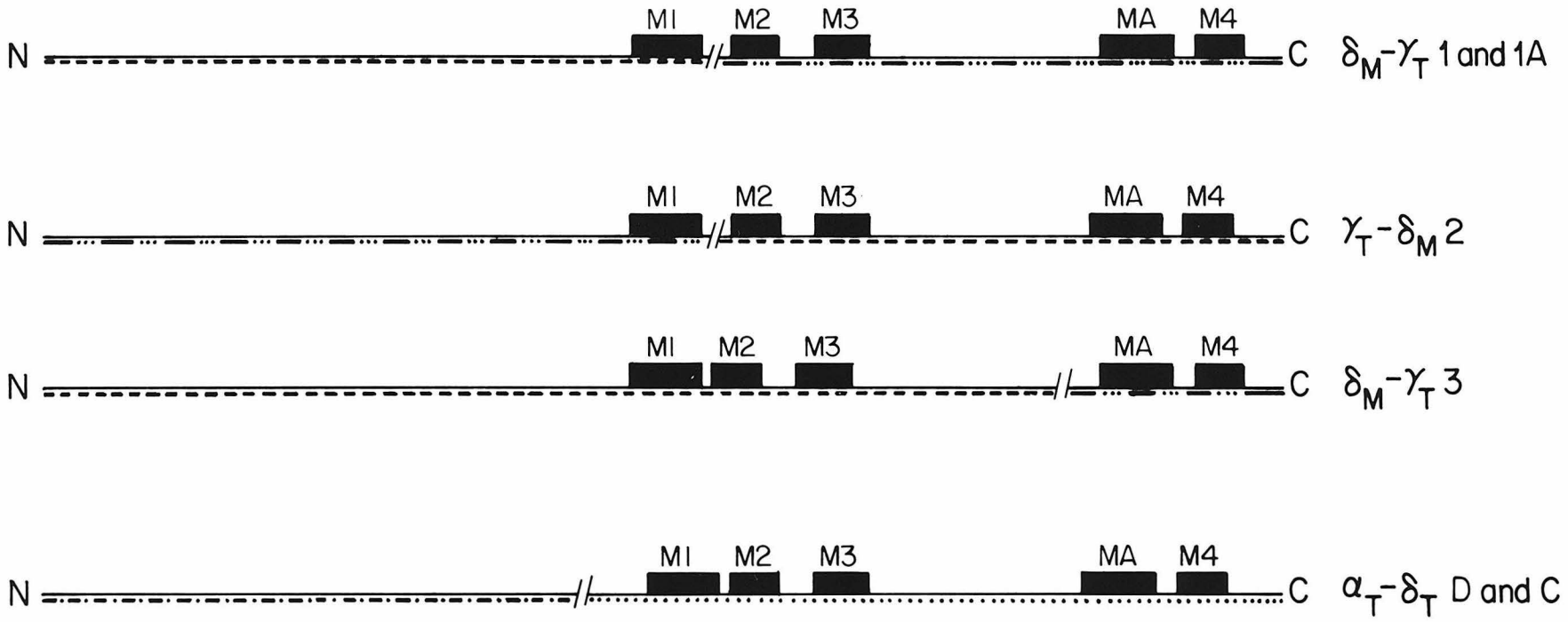
M1
M2
M3
MA
M4
N
C
δ_M-γ_T 1 and 1A
γ_T-δ_M 2
δ_M-γ_T 3
α_T-δ_T D and C
δ_M
γ_T
α_T
δ_T

Figure 2

sequence. The alignment of the whole subunits is retained across the breakpoints so that no functional regions are deleted, as shown in detail in Figure 3.

We created four chimeras between the δ_M and γ_T subunits, as well as one chimera between the α_T and δ_T subunits. The first two, δ_M-γ_T 1 and δ_M-γ_T 1A are identical except for a single amino acid at 256. δ_M-γ_T 1A was constructed as the exact reciprocal of γ_T-δ_M 2; each have gly-258 from γ_T as the first (or last) residue from the γ_T half of the chimera. Chimera δ_M-γ_T 1 was constructed to test the effect of deleting cys-256 from δ_M-γ_T 1A (see Figure 3). As indicated by their names, δ_M-γ_T 1 and 1A have their N-terminal portions from δ_M while their C-terminal residues are from γ_T. The breakpoints for this group of chimeras lie between M1 and M2, which is roughly in the middle of the protein sequences. The chimera δ_M-γ_T 3 consists of the N-terminal 412 amino acids from δ_M and the remaining 100 amino acids from the C-terminus of γ_T. This breakpoint falls just before the MA helix. The breakpoint in the α_T-δ_T chimera lies on the N-terminal side of M1; all the membrane spanning helices of this chimera are derived from δ_T.

The ligation reactions used to generate each of the chimeras are diagramed in Figure 4. The general scheme followed is that the 5' end of one cDNA molecule is ligated onto the 3' end of the other cDNA, with a double stranded oligonucleotide "adaptor" in the middle. The two ends of this recombinant cDNA must then be ligated into the vector in proper orientation with respect to the SP6 promotor. The oligonucleotide portion serves several functions. They were each designed to bring the two halves of the protein sequence together in frame, as well as to encode 6 - 25 amino acids. The specific overhanging ends of the oligonucleotides hybridized to and ligated to the sticky ends left by the restriction enzymes used to generate the cDNA fragments. All the oligonucleotides used in this work, their lengths, and their sequences are listed in Figure 5. The protruding ends of the vector, cDNA, and oligonucleotide components

Figure 3. Sequences of the cDNAs surrounding, and oligonucleotides involved in the formation of each breakpoint. Double stranded DNA sequences are used to diagram the position of each restriction endonuclease site and each oligonucleotide. Each oligonucleotide is labeled above the sequence for the top strand and below for the bottom sequence. Endonuclease recognition sequences are underlined and labeled and their cutting specificities are indicated by the (usually bent) vertical lines that also signify the ends of the oligonucleotides. Each DNA sequence is translated above using the single letter amino acid code. The numbers on the left correspond to the first amino acid shown here in these segments and use the same numbering system as Figure 1. The amino acid sequence of each chimera is underlined for clarity. The different sequences of δ_M-γ_T 1 and 1A are distinguished by a dashed line in the δ_M-γ_T 1A specific region. The sequence of α_T-δ_T D is marked by a dashed line, though the desired clone, α_T-δ_T C, marked by a solid line, was not actually isolated (see text). A dotted line in the γ_T-δ_M 2 diagram delineates the ends of the four oligonucleotides and resulting 5' overhangs.

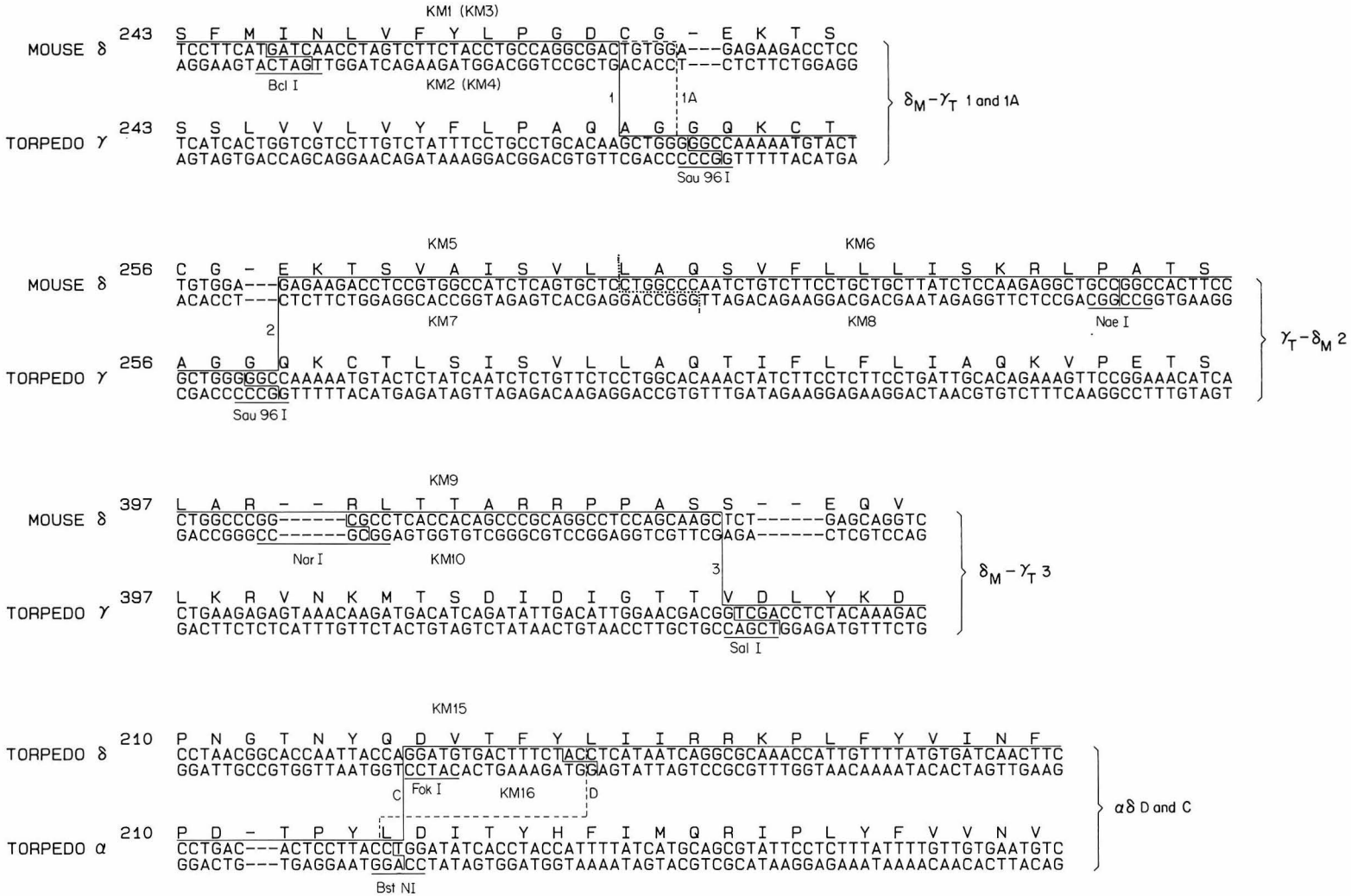
KM1 (KM3)
MOUSE δ 243 S F M I N L V F Y L P G D C G - E K T S
TCCTTCATGATCAACCTAGTCTTCTACCTGCCAGGCGACTGTGGA---GAGAAGACCTCC
AGGAAGTACTAGTTGGATCAGAAGATGGACGGTCCGCTGACACCT---CTCTTCTGGAGG
BclI
KM2 (KM4)
1
1A
TORPEDO γ 243 S S L V V L V Y F L P A Q A G G Q K C T
TCATCACTGGTCGTCCTTGTCTATTTCCTGCCTGCACAAGCTGGGGGGCCAAAAATGTACT
AGTAGTGACCAGCAGGAACAGATAAAGGACGGACGTGTTCGACCCCCGGGTTTTTACATGA
Sau 96I
δM−γT 1 and 1A
KM5
KM6
MOUSE δ 256 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 T S
TGTGGA---GAGAAGACCTCCGTGGCCATCTCAGTGCTCCTGGCCCAATCTGTCTTCCTGCTGCTTATCTCCAAGAGGCTGCCGGCCACTTCC
ACACCT---CTCTTCTGGAGGCACCGGTAGAGTCACGAGGACCGGGTTAGACAGAAGGACGACGAATAGAGGTTCTCCGACGGCCGGTGAAGG
2
KM7
KM8
NaeI
TORPEDO γ 256 A G G Q K C T L S I S V L L A Q T I F L F L I A Q K V P E T S
GCTGGGGGCCAAAAATGTACTCTATCAATCTCTGTTCTCCTGGCACAAACTATCTTCCTCTTCCTGATTGCACAGAAAGTTCCGGAAACATCA
CGACCCCCGGTTTTTACATGAGATAGTTAGAGACAAGAGGACCGTGTTTGATAGAAGGAGAAGGACTAACGTGTCTTTCAAGGCCTTTGTAGT
Sau 96I
γT−δM 2
KM9
MOUSE δ 397 L A R - - R L T T A R R P P A S S - - E Q V
CTGGCCCGG------CGCCTCACCACAGCCCGCAGGCCTCCAGCAAGCTCT------GAGCAGGTC
GACCGGGCC------GCGGAGTGGTGTCGGGCGTCCGGAGGTCGTTCGAGA------CTGTCCAG
NarI
KM10
3
TORPEDO γ 397 L K R V N K M T S D I D I G T T V D L Y K D
CTGAAGAGAGTAAACAAGATGACATCAGATATTGACATTGGAACGACGGTCGACCTCTACAAAGAC
GACTTCTCTCATTTGTTCTACTGTAGTCTATAACTGTAACCTTGCTGCCAGCTGGAGATGTTTCTG
SalI
δM−γT 3
KM15
TORPEDO δ 210 P N G T N Y Q D V T F Y L I I R R K P L F Y V I N F
CCTAACGGCACCAATTACCAGGATGTGACTTTCTACCTCATAATCAGGCGCAAACCATTGTTTTATGTGATCAACTTC
GGATTGCCGTGGTTAATGGTCCTACACTGAAAGATGGAGTATTAGTCCGCGTTTGGTAACAAAATACACTAGTTGAAG
C
FokI
KM16
D
TORPEDO α 210 P D - T P Y L D I T Y H F I M Q R I P L Y F V V N V
CCTGAC---ACTCCTTACCTGGATATCACCTACCATTTTATCATGCAGCGTATTCCTCTTTATTTTGTTGTGAATGTC
GGACTG---TGAGGAATGGACCTATAGTGGATGGTAAAATAGTACGTCGCATAAGGAGAAATAAAACAACACTTACAG
BstNI
αδ D and C

Figure 3

Figure 4. Diagramatic representation of plasmid constructions showing each ligation event. Simple ligations at restriction endonuclease sites are depicted as gaps between vertical lines. The overhanging ends of each ligation event involving oligonucleotides are denoted by bent vertical lines (except for the NaeI site which is blunt) through the relevant sequences. Each subunit's cDNA is represented by a distinctive line pattern shown in the key (similar to those in Figure 2). Open boxes represent SV40 sequences derived from the original cloning vectors (Okayama and Berg, 1983). Hatched boxes represent either 3' or 5' untranslated regions (as marked) from *Xenopus* globin cDNA. These are derived from the pSP64T vector that δ_M was subcloned into (Krieg and Melton, 1984). The relative locations and orientations of the SP6 promoter (SP6), plasmid origin of replication (Ori), and ampicillin resistance gene (Amp^R) are marked by arrows. None of the components are drawn to scale.

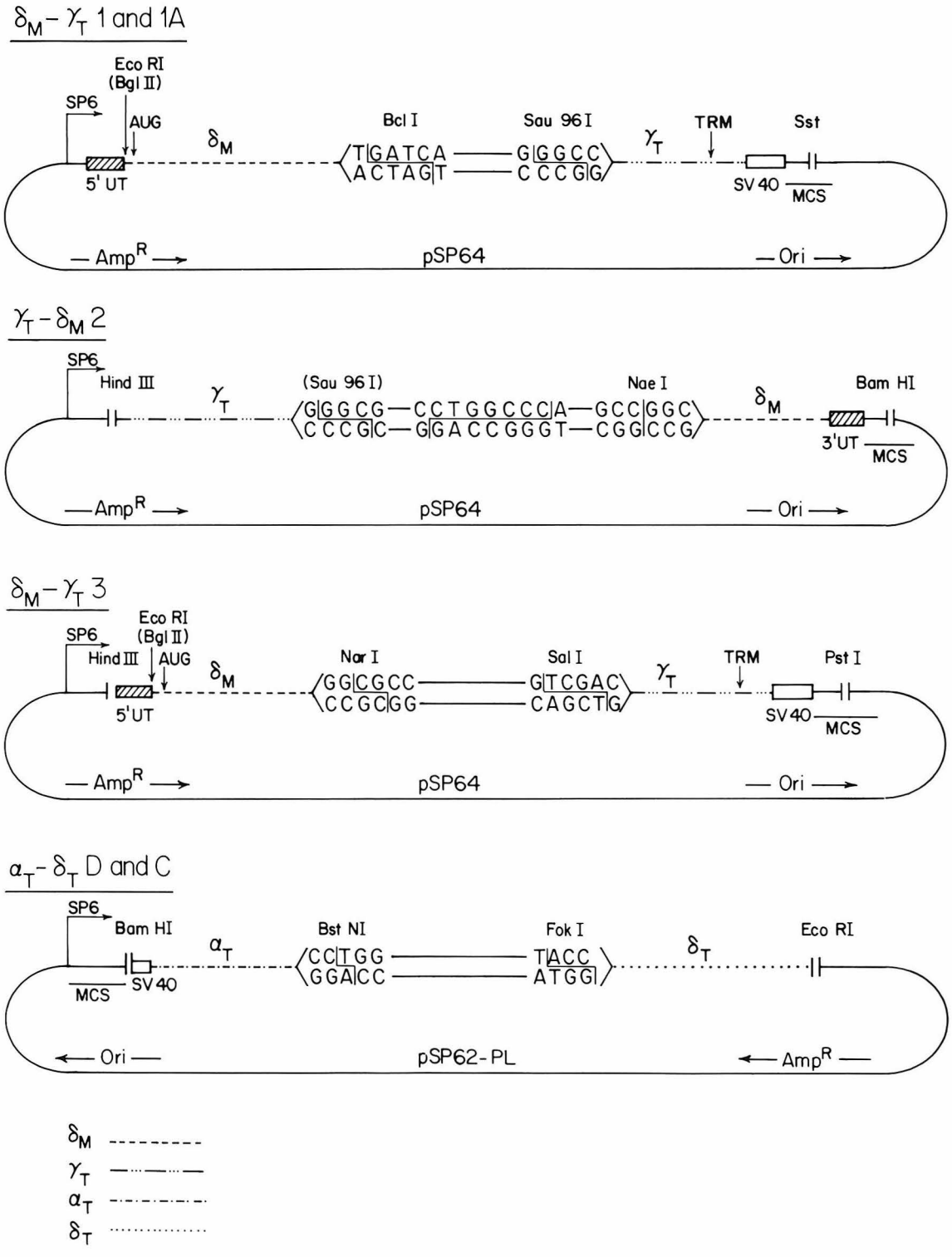
δM – γT 1 and 1A
Eco RI
(Bgl II)
SP6
AUG
δM
Bcl I
Sau 96 I
γT
TRM
Sst
5' UT
SV 40
MCS
— AmpR →
pSP64
— Ori →
γT – δM 2
SP6
Hind III
γT
(Sau 96 I)
Nae I
δM
Bam HI
3'UT
MCS
—AmpR →
pSP64
— Ori →
δM – γT 3
Eco RI
(Bgl II)
SP6
Hind III
AUG
δM
Nar I
Sal I
γT
TRM
Pst I
5'UT
SV40
MCS
— AmpR →
pSP64
— Ori →
αT – δT D and C
SP6
Bam HI
αT
Bst NI
Fok I
δT
Eco RI
MCS
SV40
← Ori —
pSP62-PL
← AmpR —
δM
γT
αT
δT

Figure 4

Figure 5. Sequences and lengths of oligonucleotides. Each oligonucleotide used in this work is listed by name in the first column, followed by length in nucleotides, and sequence beginning with the 5' end.

KM1	37	GATCAACCTAGTCTTCTACCTGCCAGGCGACGCTGGG
KM2	36	GCCCCCAGCGTCGCCTGGCAGGTAGAAGACTAGGTT
KM3	37	GATCAACCTAGTCTTCTACCTGCCAGGCGACTGTGGG
KM4	36	GCCCCCACAGTCGCCTGGCAGGTAGAAGACTAGGTT
KM5	40	GGCGAGAAGACCTCCGTGGCCATCTCAGTGCTCCTGGCCC
KM6	37	AATCTGTCTTCCTGCTGCTTATCTCCAAGAGGCTGCC
KM7	30	GAGCACTGAGATGGCCACGGAGGTCTTCTC
KM8	44	GGCAGCCTCTTGGAGATAAGCAGCAGGAAGACAGATTGGGCCAG
KM9	34	CGCCTCACCACAGCCCGCAGGCCTCCAGCAAGCG
KM10	36	TCGACGCTTGCTGGAGGCCTGCGGGCTGTGGTGAGG
KM15	15	TGGATGTGACTTTCT
KM16	17	GGTAGAAAGTCACATCC
KM17	19	GTCACATCCAGGTAAGGAG

Figure 5

specify exactly one theoretically possible ligation event in each ligation reaction, as shown in Figure 4.

The ligations used to construct the δ_M-γ_T 1 and 1A clones were identical except for exchanging pairs of oligonucleotides as shown in Figure 3. KM1 and KM2 were used in δ_M-γ_T 1, while KM3 and KM4 formed the adaptor for δ_M-γ_T 1A. These oligonucleotides only differ at two base pairs. This pair of ligations were further simplified because the cDNA encoding the 5' end of δ_M was not cleaved from its original vector sequences prior to gel isolation.

The ligation leading to the γ_T-δ_M2 clone was the most complex, since four oligonucleotides were required. The gap of 77 nucleotides was too large to be reliably synthesized as a single pair of oligonucleotides. A set of four oligonucleotides (KM5, 6, 7, and 8) was used to span this gap, with a seven base pair overlap in the middle.

The ligation to construct the α_T-δ_T chimera proved to be the most difficult. Repeated attempts yielded only forty colonies for hybridization screening, through the other ligations resulted in hundreds of colonies. Only two colonies of the forty hybridized to the α_T and δ_T cDNA probes. The first, α_T-δ_T D, did not hybridize to the oligonucleotide probe, KM16. Sequence analysis demonstrated that α_T-δ_T D is a fusion of the two cDNA fragments, in frame, without the oligonucleotide adapter. The chimeric subunit encoded, therefore, has a six amino acid deletion, as diagramed in Figure 3. α_T-δ_T C, the second clone that hybridized to α_T and δ_T cDNA probes, also hybridized to KM16. The sequence of this clone reveals a single base pair deletion of the T in codon 216, which creates a frame shift. Attemps to reinsert this T by oligonucleotide-directed mutagesis (Zoller and Smith, 1983) with KM17 proved unsuccessful, apparently because KM17 fails to hybridize to α_T-δ_T C. A stem-loop structure is predicted by computer in this region, which would prevent hybridization with KM17 (data not shown).

In vitro transcription. Linearized plasmid DNAs were used as templates for *in vitro* transcription of RNA by phage SP6 or T7 polymerase. Figure 6 shows the RNA products of *in vitro* transcription reactions, as displayed on an agarose gel after glyoxal treatment (McMaster and Carmichael, 1977). The α_M RNA is transcribed by T7 polymerase, while all the others are SP6 transcripts. We note that several of the templates give more than one discrete transcript. The largest bands (relatively fainter than most bands,and well above the size markers) for α_T, β_T, γ_T, δ_T, and α_C are thought to be artifactual transcripts arising from initiation at the 3' protruding ends produced by Aat II and Pvu I. Schenborn and Mierendorf (1985) have documented this phenomenon, where SP6 and T7 polymerases transcribe the entire fragment length if that fragment has a 3' protruding end, but not if the fragment has blunt or 5' protruding ends. These largest bands in Figure 6 disappear if the protruding ends of the template DNA are removed by treatment with the Klenow fragment of DNA polymerase I (data not shown). The largest intense band in each lane is the expected transcript, initiated at the SP6 (or T7) promotor and extended to the end of the template fragment. In most cases this transcript includes a substantial length of RNA on the 3' end that is transcribed from the bacterial vector. The second intense band, transcribed in all except the α_M, δ_M, and α_T-δ_T D reactions, appears to be the result of a premature termination of the coding transcript. These transcripts' lengths were measured and compared to the individual plasmid maps. When the 5' end was assumed to be the phage promotor, all the 3' ends aligned in the same place on the plasmid maps, within the limits of error of both the measurements and the maps. This common 3' termination point falls very close to the plasmid origin of replication. This phenomenon of SP6 transcription termination at the origin of replication has been observed by others working with SP6 transcripts (C. Rice, personal communication). The three lanes that lack these bands do not transcribe through the origin of replication.

Figure 6. Phage SP6 or T7 transcription products of AchR subunits and subunit chimeras. An aliquot of each transcription reaction was treated with glyoxal and electrophoresed through 1% agarose as described by McMaster and Carmichael (1977). Bands on the dried gel were visualized by autoradiography. The plasmids were linearized by restriction endonuclease digestion: α_T, γ_T, δ_T, α_C (Aat II); β_T, γ_M, α_M, and all 5 chimeras (Pvu I); δ_M (Bam HI). α_M was transcribed by T7, all the others by SP6 polymerase.

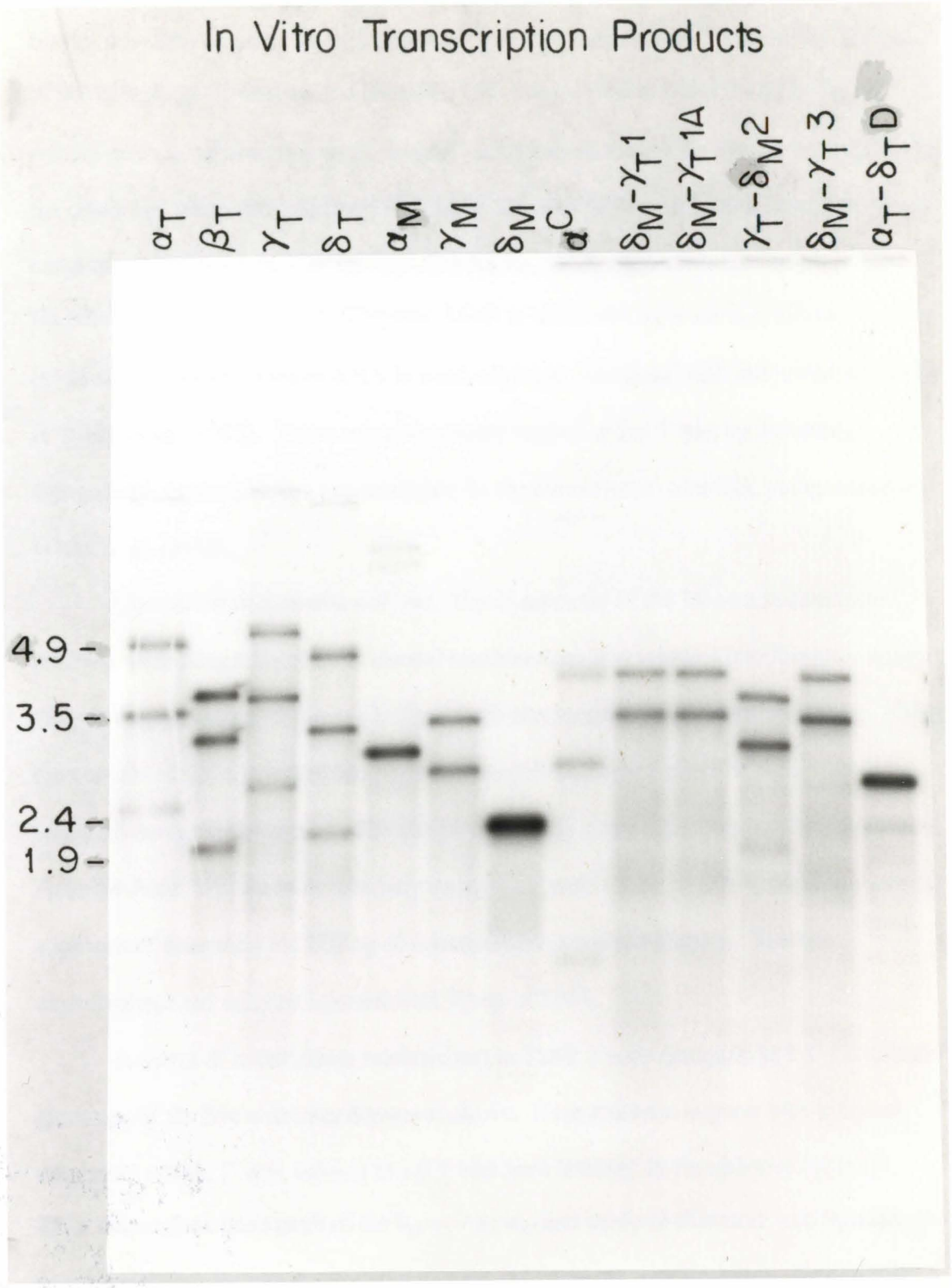
In Vitro Transcription Products
αT
βT
γT
δT
αM
γM
δM
αC
δM-γT1
δM-γT1A
γT-δM2
δM-γT3
αT-δTD
4.9
3.5
2.4
1.9

Figure 6

The four *Torpedo* subunit lanes each exhibit a third intense band ~0.4 kb longer than the size of the cDNA insert. These transcripts apparently terminate within the 0.5 kb region of bacteriophage f1 fragment in the pSPS4.f1$^-$ vector. The smallest band in the α_C transcription is apparently a weak internal terminator in the cDNA for this subunit. While the origins of these multiple transcripts have not been proven, it is clear that they do not present a problem when injected into oocytes for AchR expression (see below). The magnitude of response for the *Torpedo* AchR is increased, as is the δ_M replaced version, (even though fewer moles of RNA is injected) when compared with the previous results of White *et al.* (1985). These transcripts were capped at the 5' end by including diguanosine triphosphate, a cap analogue, in the transcription reaction, as described by White *et al.* (1985).

Expression in Xenopus oocytes. Equal amounts of the *in vitro* synthesized mRNAs were mixed together in several combinations and injected into *Xenopus* oocytes. Oocyte responsiveness to acetylcholine (Ach) was measured under voltage clamp. When the amount of RNA injected was varied, a roughly sigmoidal curve of Ach responsiveness was observed. The minimum readily detectable Ach response for the all *Torpedo* AchR was measured in oocytes injected with 1.5 ng of RNA, and the response approached saturation for 100 ng of injected RNA (data not shown). Routine experiments used oocytes injected with 50 ng of RNA.

A series of experiments summarized in Table 1 were designed to test the subunit identities of the five chimeras discussed above. Each chimeric subunit was tested to determine which, if any, subunit identity had been retained in the chimera molecule. RNA transcribed from each of the δ_M-γ_T / γ_T-δ_M and α_T-δ_T D chimeras was injected into oocytes with α_T, β_T, and γ_T RNAs to test its ability to act as a δ subunit. Conversely, each δ_M-γ_T /γ_T-δ_M chimeric RNA was tested for its ability to act as a γ subunit by

Table 1. Mean responses are given in namperes ± s.d. for oocytes tested at each Ach concentration. A minimum of 3 oocytes were tested by voltage clamp. Surface α-BTX binding is given in fmole per oocyte. The mean binding ± s.d. is given, and the number of individual oocytes is shown in parentheses. The ratio of these values is calculated and expressed in namperes per fmole. 50 ng RNA per oocyte was injected. The resting voltage ranged from -31 to -41 mV. The chimeras are abbreviated by their number or letter (e.g., δ_M-γ_T 3 is "3"). Subunits α, β, and γ are from *Torpedo*. δ 's are identified by subscript: "T," *Torpedo* ; "M," mouse. "--" means not tested, a blank space is left where the calculated number is insignificant. Uninjected oocytes bind 0.24 fmol/oocyte ± 0.02 (13) which has been subtracted; of this, 0.2 fmol/oocyte is contributed by the empty tube. The response of the $\alpha,\beta,\gamma,\delta_M$ complex to 0.2 mM Ach was 268 namperes ± 132.

TABLE 1

Acetylcholine sensitivity and surface α-BTX binding of Xenopus oocytes injected with RNA from chimeras in various combinations

RNAs	1 μM Ach	5 μM Ach nanoamps	20 μM Ach	α-BTX fmole	1 μM Ach	5 μM Ach nanoamps per fmole	20 μM Ach
α,β,γ	17 ± 25	173 ± 197	535 ± 578	1.44 ± 0.4 (9)	11.8	120	371
α,β,γ,1A	38 ± 41	159 ± 132	816 ± 375	2.53 ± 0.8 (13)	15.2	63.6	326
α,β,γ,2	28 ± 25	150 ± 109	457 ± 316	2.25 ± 1.0(16)	12.4	66.7	203
α,β,γ,3	40 ± 9	217 ± 135	917 ± 535	3.01 ± 1.4 (7)	13.3	72.1	305
α,β,δ_M	13 ± 14	--	430 ± 410	0.07 ± 0.04 (7)		--	
$\alpha,\beta,1A,\delta_M$	3 ± 3	--	101 ± 67	0.52 ± 0.24 (9)	5.8	--	194
$\alpha,\beta,2,\delta_M$	7 ± 6	--	40 ± 26	0.05 ± 0.01 (8)		--	
$\alpha,\beta,3,\delta_M$	0 ± 0	--	2 ± 3	0.05 ± 0.02 (9)		--	
β,γ,δ_T	0 ± 0	--	3 ± 6	0.07 ± 0.08 (10)		--	
D,β,γ,δ_T	0 ± 0	--	0 ± 0	0.07 ± 0.02 (11)		--	
α,β,γ,D	13 ± 10	--	467 ± 208	1.29 ± 0.64 (9)	10	--	362
$\alpha,\beta,\gamma,\delta_T$	605 ± 95	--	--	8.29 ± 2.3 (9)	73	--	--
$\alpha,\beta,\gamma,\delta_M$	3633 ± 1789	--	--	4.86 ± 3.2 (7)	747	--	--

injecting oocytes with the chimera, plus α_T, β_T, and δ_M RNAs. The α_T-δ_T D chimera was tested for α subunit character by injecting its RNA in combination with *Torpedo* β, γ, and δ subunit RNAs. These results are summarized in Table 1. The high levels of Ach used in these experiments are not physiologically significant, and would, in fact, cause desensitization. We use them here and in Table 2 to try to detect very low responses.

When any one of the δ_M-γ_T /γ_T-δ_M chimeras is used in place of δ, both the Ach response and the α-BTX binding are increased slightly over that observed for α, β, γ alone. However, the ratio of response per fmole α-BTX (shown in the last three columns) does not change significantly as compared to α, β, γ alone. Thus the small increase in response and α-BTX binding are due to the presence of more AchRs on the oocyte surface. The flux per receptor in all these combinations is much lower than that of the holo-*Torpedo* receptor. Thus, the chimeras apparently are incorporated into AchRs on the surface but they do not respond to Ach more strongly than the weakly responding α, β, γ complexes. We have no direct evidence that the chimeric polypeptides actually are on the oocyte surface, so more non-specific effects on the stability of the α, β, γ subunits or RNAs cannot be ruled out.

The δ_M-γ_T /γ_T-δ_M chimeras are not effective substitutes for γ either as shown in the fifth through eighth lines of Table 1. Ach responsiveness is actually lowered when the chimeric RNAs are added to the α, β, δ_M RNA combination. α-BTX binding is, in most cases, unaffected. Chimera δ_M-γ_T 1A gives increased α-BTX binding in this combination, though lowered Ach sensitivity. Thus, δ_M-γ_T 1A may actually form complexes with α, β, δ_M on the surface that are insensitive to Ach.

The α_T-δ_T D chimera was not capable of acting as either an α or δ subunit.When α_T-δ_T D RNA was used to replace the α RNA, all Ach sensitivity and α-BTX binding

were abolished. When used as a δ subunit, the α_T-δ_T D chimera had no effect on the response normally detected with α, β, γ RNAs alone.

We conclude that none of the chimera constructions retained enough subunit characteristics from either half to function fully in the place of either of its parent subunits to form an AchR that is responsive to Ach.

A set of experiments reported in Table 2 addresses the ability of the *Torpedo* subunits to form functional AchRs in combination with mouse and chick subunits. The α_C, α_M, γ_M, and δ_M subunits are each tested individually as replacements of the analogous *Torpedo* subunit, and also in every possible combination with each other. The simple replacement of δ_M for δ_T has been discussed previously (White *et al.*, 1985).

The first four lines of Table 2 address the ability of α_C to form functional AchRs with other subunits When the α_C subunit is used in place of α_T, a definite, but reduced, response is detected to Ach. The response is only slightly increased at 1μM Ach if δ_M is used as the δ subunit in place of δ_T, in contrast to the large difference detected in the last two lines of Table 2. The substitution of γ_M for γ_T in these combinations abolishes nearly all activity, regardless of the other subunits present. In Table 1 and in Mishina *et al.* (1984), a small response is measured when no γ subunit RNA is included at all. Apparently γ_M RNA is exhibiting a "poisoning" effect that actually inhibits formation of AchRs. Alternatively, γ_M could incorporate into AchRs with the other subunits, but these complexes are completely unresponsive to Ach. The four combinations utilizing α_M demonstrate that this subunit can form functional AchRs with β_T, γ_T and either of the two δ's, though not with γ_M. The amplitude of the Ach response is, however, substantially reduced. We have no independent method for measuring the stability of the chick and mouse subunits at either the RNA or protein levels. Thus, the low responsiveness may

TABLE 2

Acetylcholine sensitivity of Xenopus oocytes injected with RNA in various combinations of Torpedo, mouse, and chick AchR subunits

RNAs	0.2 μM Ach	1 μM Ach	5 μM Ach	20 μM Ach
$\alpha_C,\beta_T,\gamma_T,\delta_T$	6 ± 2	127 ± 45	1217 ± 293	--
$\alpha_C,\beta_T,\gamma_T,\delta_M$	13 ± 3	148 ± 20	700 ± 180	--
$\alpha_C,\beta_T,\gamma_M,\delta_T$	--	--	3 ± 1	--
$\alpha_C,\beta_T,\gamma_M,\delta_M$	0	0	16 ± 7	25 ± 0
$\alpha_M,\beta_T,\gamma_T,\delta_T$	0	28 ± 8	227 ± 64	--
$\alpha_M,\beta_T,\gamma_T,\delta_M$	0	43 ± 6	70 ± 20	--
$\alpha_M,\beta_T,\gamma_M,\delta_T$	0	0	0	0
$\alpha_M,\beta_T,\gamma_M,\delta_M$	--	--	0	0
$\alpha_T,\beta_T,\gamma_M,\delta_T$	0	0	0	0
$\alpha_T,\beta_T,\gamma_M,\delta_M$	0	0	0	0
$\alpha_T,\beta_T,\gamma_T,\delta_T$	57 ± 6	617 ± 38	> 5000	--
$\alpha_T,\beta_T,\gamma_T,\delta_M$	700 ± 50	~ 4700		

Responses are given in nanoamperes, the average of three oocytes tested. Oocytes were injected with 75 ng of RNA for each combination. Resting voltages were lower than -40 mV. Subscripts indicate species: "T," *Torpedo*; "M," mouse; and "C," chick.

reflect instability of these components rather than inability to associate with subunits of the other species.

Data similar to that reported in Table 2 are often plotted on double-logarithmic coodinates (response vs Ach concentration) to determine the Hill coefficient for a given channel. The Hill coefficient, equal to the slope of the line from such a plot, indicates the number of ligand molecules that must bind to open the channel. The data reported in Figure 2 are not very useful for such analysis because 1) there are too few points to be reliable, and 2) the relationship is nonlinear in this concentration range because of desensitization. With these limitations in mind, an interesting effect is shown in the 1 μM vs. 5μM Ach data for $\alpha_C,\beta_T,\gamma_T,\delta_T$ and $\alpha_C,\beta_T,\gamma_T,\delta_M$ combinations. At 1 μM Ach the complex with δ_M gives a larger response than the δ_T complex, as expected from our previous work, confirmed in the last two lines of Table 2. However, at 5 μM Ach the relationship is reversed: the δ_T complex response is higher than that of the δ_M complex. It is possible that δ_M causes the complex to be more sensitive to desensitization, at least when α_C is used. A similar phenomenon is observed in the analogous experiment using α_M.

Discussion

Our previous attempts to locate a "deltaness" determining region by sequence comparisons have focused on the membrane spanning helices since dimensions of the overall AchR require that the α-helices in the membrane be in close contact. Our selection of the types of chimeric subunits to construct reflects this viewpoint, but asks more general questions as well. Is there, in fact, one or more discrete subunit determining regions for the AchR subunits? If so, are these regions in the same location for the various subunits? Chimeras δ_M-γ_T 1A and γ_T-δ_M 2 ask simply if either half of a subunit

contains such a region for the γ or δ. Chimera δ_M-γ_T 3 tests the proposal in White *et al.* (1985) that MA and M4 contain the subunit determinant. This construction also acts as a control for the δ_M-γ_T 1 and γ_T-δ_M 2 pair in case the determinant spans their common breakpoint between M1 and M2. The results from these three chimeras indicate that there are regions critical for γ and δ subunit function at least as far apart as M1 and MA.

Data from our α_T-δ_T D chimera indicates that α_T and δ_T specific domains are included in both the N-terminal extracellular and the membrane imbedded halves of the molecule. The effect of the six amino acid deletion at the junction point of this molecule cannot be estimated. The α subunit has previously been studied by deletion studies (Mishina *et al.*, 1985). This work demonstrates that the intracellular portion between M3 and MA does not have a critical function for the α, while each of the proposed membrane spanning regions is absolutely required.

Models based on the sequences available for so many AchR subunits emphasize the homology seen in the overall proposed topologies of the subunits. This overall homology of form encourages us to suppose that subunit determining regions are in the same relative positions in all the AchR subunits. If this is valid, then we have demonstrated a minimum of two subunit-determining regions. These are the N-terminal extracellular portion and the region beginning with MA and extending to the C-terminus (since these two regions were never derived from the same parental subunit in any of the chimeras). Our data would also be consistent with the conclusion that there are no discrete regions that determine subunit identity, but instead that this information is rather evenly distributed along subunit length. We can also conclude that there are regions needed for the efficient function of these subunits that are not necessary for the formation of surface complexes.

Our subunit substitution experiments extend the number of subunits and species that have been studied by this methodology. Clearly the α_C and α_M subunits form functional channels with β_T, γ_T, and either δ_T or δ_M. Oocytes injected with these combinations show a smaller amplitude of response that the α_T controls. When calf subunits are substituted in a similar series of experiments, the calf α, β_T, γ_T, δ_T complex demonstrates a larger response than the *Torpedo* AchR (Sakmann *et al.*, 1985). The α_C and α_M subunits are highly homologous to the *Torpedo* α, as is the calf α (Beeson *et al.*, 1986; Noda *et al.*, 1983; Boulter *et al.*, 1985). The functional differences, reflected in the different results obtained in this work and that of Sakmann *et al.* (1985), between these α subunits have not been identified at the sequence level. Another possibility that cannot be ruled out is that the α_C, α_M and γ_M subunits are unstable at either the RNA or protein level in the present constructions.

The γ_M subunit clearly does not form channels in any of the combinations presented in Table 2. The low response detected in combinations with α_C probably reflects channels that are formed without a γ subunit at all, analogous to Table 1, line 5. Sakmann *et al.* (1985) reported similar results with the calf γ. The same result was obtained when these workers attempted to combine the calf β subunit with α_T, γ_T, and δ_T: virtually no response. Our α_M, β_T, γ_M, δ_M combination also was unresponsive to Ach. These results may mean that the γ and β subunits are the most sensitive to alterations in neighboring subunits, as we have attempted. If γ and β subunits continue to resist formation of AchRs with unusual neighboring subunits, identification of subunit-specific regions by the method of chimera construction may not be feasible.

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APPENDIX

The actin genes of *Drosophila*: protein coding regions are conserved but intron positions are not

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The Actin Genes of Drosophila: Protein Coding Regions Are Highly Conserved but Intron Positions Are Not

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Summary

The entire set of six closely related Drosophila actin genes was isolated using recombinant DNA methodology, and the structures of the respective coding regions were characterized by gene mapping techniques and by nucleotide sequencing of selected portions. Structural comparisons of these genes have resulted in several unexpected findings. Most striking is the nonconservation of the positions of intervening sequences within the protein-encoding regions of these genes. One of the Drosophila actin genes, DmA4, is split within a glycine codon at position 13; none of the remaining five genes is interrupted in the analogous position. Another gene, DmA6, is split within a glycine codon at position 307; at least two of the Drosophila actin genes are not split in the analogous position. Additionally, none of the Drosophila actin genes is split within codon four, where the yeast actin gene is interrupted. The six Drosophila actin genes encode several different proteins, but the amino acid sequence of each is similar to that of vertebrate cytoplasmic actins. None of the genes encodes a protein comparable in primary sequence to vertebrate skeletal muscle actin. Surprisingly, in each of these derived actin amino acid sequences the initiator methionine is directly followed by a cysteine residue, which in turn precedes the string of three acidic amino acids characteristic of the amino termini of mature vertebrate cytoplasmic actins. We discuss these findings in the context of actin gene evolution and function.

Introduction

Much remains to be learned about the expression of eucaryotic genes, and in particular how the activities of thousands of such genes are coordinated to mediate the ordered sequence of events leading to metazoan ontogeny. Increasingly, eucaryotic structural genes, or portions thereof, are being found not to be uniquely represented in animal genomes, but rather to be members of small gene families (reviewed by Long and Dawid, 1980). In some cases the members of such families are expressed differentially, either in temporal sequence or tissue specific distribution (Maniatis, et al., 1980; Hagenbüchle et al., 1980). Continued study of such gene families will increase our understanding of gene evolution and of the regulatory elements that control gene expression.

The actin genes are well suited for such a study. Because actin is a highly conserved and ubiquitous protein (Pollard and Weihing, 1974), the structure of these genes can be examined across very large evolutionary distances. Furthermore, actin synthesis is highly regulated during metazoan development. As various types of muscle cells differentiate they begin synthesis of specific actin isoforms (Whalen et al., 1976; Garrels and Gibson, 1976; Saborio et al., 1979), each of which is encoded by one or more members of a small family of closely related genes (Vandekerckhove and Weber, 1978a, 1978c). Further investigations of structural and functional aspects of these genes will help elucidate the mechanisms by which genes are selectively expressed.

We have compared the structural features of the actin genes of Drosophila melanogaster. We report several unexpected results which are relevant to the evolution and functioning of the actin gene family.

Results

Isolation of All Members of the Drosophila Actin Gene Family

To isolate all members of the Drosophila actin gene family we screened a library of Drosophila genomic DNA using the technique of Benton and Davis (1977). The probe for this screen was derived from λDmA2, a recombinant phage that contains a Drosophila actin gene, and that we have characterized previously (Fyrberg et al., 1980). Of 40,000 plaques screened (approximately four Drosophila genome equivalents), 30 phages that hybridized strongly were selected. The Eco RI restriction patterns of the purified DNA from these phages indicated that they were members of six distinct classes. Furthermore, blot hybridization experiments have demonstrated that DNA pooled from representatives of these six phage classes contains all of the actin-gene-containing fragments observed in digests of Drosophila genomic DNA (data not shown). We therefore conclude that these six phages contain all of the Drosophila actin genes.

We have denoted the six actin-gene-containing phages as λDmA1–λDmA6 in order of the decreasing sizes of their actin-gene-containing Eco RI fragments. By in situ hybridization to polytene chromosomes we have determined the chromosomal location of the Drosophila DNA segment inserted within each phage genome: λDmA1, 88F; λDmA2, 5C; λDmA3, 42A; λDmA4, 57A; λDmA5, 87E; λDmA6, 79B (data not shown). These results confirm and extend those of Tobin et al. (1980) and Fyrberg et al. (1980) who reported that actin gene probes hybridized to these six locations.

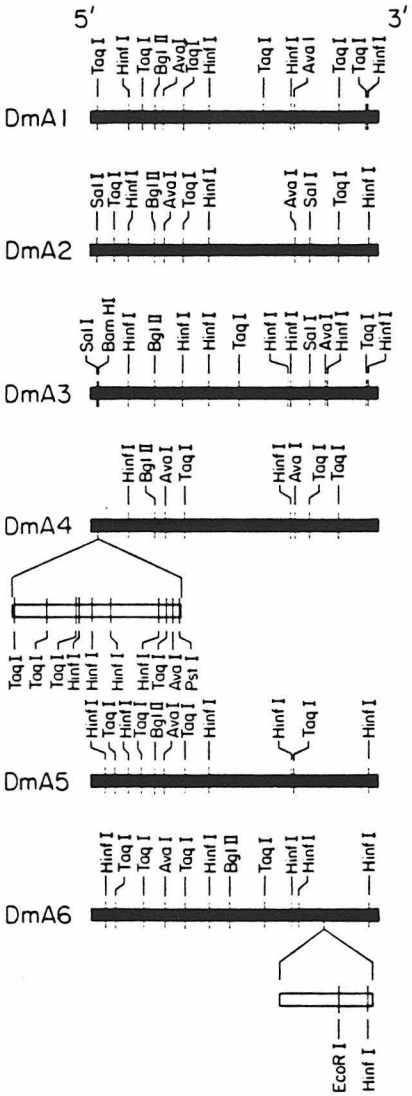


Figure 1. High Resolution Restriction Maps of the Protein Coding Regions of DmA1–DmA6

Solid blocks represent the 1.1 kb protein coding regions of the actin genes, which are aligned to facilitate comparison of restriction sites. The 5′ to 3′ orientation is left to right as indicated. Open blocks represent intervening sequences, which are inserted at the positions indicated. The intervening sequence of DmA4 measures approximately 630 nucleotides while that of DmA6 is 357 nucleotides in length. A small intervening sequence within codon 307 of DmA1 which has recently been discovered by F. Sánchez, S. L. Tobin and B. J. McCarthy (personal communication) is not shown. Intervening sequences within untranslated regions are not shown, since these regions have been analyzed only in DmA2 and DmA4. DmA2 is known to have an intervening sequence in its 5′ untranslated region, however (see text). There is an Ava I site at codon 333 of DmA1 which is not shown in the figures.

High Resolution Restriction Mapping of the Protein-Coding Region of Each Actin Gene Reveals Many Conserved Sites

Low resolution restriction mapping and blot hybridization experiments using the chimeric phage DNAs revealed that each contained a single actin structural gene (data not shown; however, restriction maps of λDmA1—DmA6 are available on request). After localizing each gene within a reasonably small (2–6 kb) restriction fragment, subclones were prepared by inserting fragments into the plasmid vector pBR322. Subsequently the protein coding region of each of the structural genes was localized within the subcloned fragments and mapped precisely with frequently cutting restriction enzymes. Appropriate fragments were mapped with Ava I, Bgl II, Hinf I and Taq I using the technique of Smith and Birnstiel (1976). These results are presented in Figure 1.

As expected for a family of closely related genes, these maps reveal considerable conservation of restriction sites. The conserved sites allow maps of the Drosophila actin genes to be aligned with those of Dictyostelium (Firtel et al., 1979) and yeast (Ng and Abelson, 1980). This alignment revealed the tentative positions of the 5′ and 3′ ends of the protein-coding portion of each Drosophila actin gene and thus determined their transcriptional polarities. Interestingly there are four well conserved Hinf I sites within codons 51, 154, 259 and 363 of the coding regions. Hinf I recognizes the nucleotide sequence GANTC. In each of these four cases this sequence is generated by consecutive (Asp or Glu)–Ser codons. The extremely regular spacing of these (Asp or Glu)–Ser sequences in the actin proteins (104 ± 1 amino acids) may be of functional significance.

These restriction maps allowed us to identify tentatively an intervening sequence within DmA6. Alignment of restriction enzyme sites, some of which are not shown in Figure 1 (Ava II, Alu I, Hpa II, Hae III) revealed that an approximately 400 nucleotide insertion split this gene into segments of 900 and 225 nucleotides. No such insertion is indicated by maps of the other five genes. A larger intervening sequence within DmA4 was overlooked in this analysis, however, because it lies very close to the 5′ end of the coding region (refer to Figure 1).

Direct Visualization of Nonconserved Interruptions within DmA4 and DmA6

To map more precisely the sequence relationships among the Drosophila actin genes, we prepared het-

eroduplexes with pairs of genes and examined resulting structures in the electron microscope. A logical starting point for this analysis was to compare the structure of DmA2 to that of DmA4. These two genes were known to be related very closely on the basis of previous cross-hybridization experiments (Fyrberg et al., 1980). Additionally, both of these genes were known to be split by intervening sequences near their 5′ ends. For DmA2, this was known from previously described experiments (Fyrberg et al., 1980). In the case of DmA4 the intervening sequence was identified by an experiment in which λDmA4 DNA was hybridized to total cellular poly(A)$^+$ RNA from Drosophila embryos under R-loop conditions (Kaback et al., 1979), and the resulting molecules were examined in the electron microscope. Observation of these molecules revealed that a 630 ± 70 nucleotide intervening sequence separates an approximately 100 nucleotide 5′ proximal segment from the major portion of the gene, which measures 1.45 kb (data not shown).

To visualize homologous regions of DmA2 and DmA4, we formed heteroduplexes between an 8.7 kb Eco RI fragment of DmA2 and a 4.2 kb Bam HI–Eco RI fragment of DmA4. In both cases these fragments contain the entire actin protein-coding region, 5′ and 3′ untranslated regions, plus substantial flanking sequences. Equimolar amounts of chimeric plasmids containing these fragments were linearized by cleaving with Bam HI, then denatured and allowed to reanneal. The resulting molecules were then spread for electron microscopy.

An electron micrograph of a representative molecule is shown in Figure 2. Homology of these two genes is limited to a 1.1 ± 0.1 kb region. Within experimental error, this segment has a length and position indicating that it consists entirely of the protein-coding regions. No homology can be seen in the 5′ or 3′ untranslated or flanking regions. At the 5′ end of this duplex region we observe a single-stranded loop of length 630 nucleotides in 60% of the molecules. The presence of a loop implies that a region of homology too short to be recognized as duplex by electron microscopy exists 5′ to the loop. Because the size of this loop is the same size as the intervening sequence of DmA4, a short region of DmA4 5′ to the loop must be homologous to the contiguous coding region of DmA2. Thus DmA2 and DmA4 must be split by intervening sequences in different positions. The observed heteroduplex structures were consistent with DmA2 being split at a position upstream from its coding region in the 5′ untranslated region, and with DmA4 being split within the protein-coding region extremely close to the 5′ end. This interpretation is fully confirmed by the sequence data presented below.

Heteroduplexes of DmA1 with DmA5, DmA5 with DmA6, and DmA3 with DmA6 have also been prepared. In all cases a duplex segment corresponding

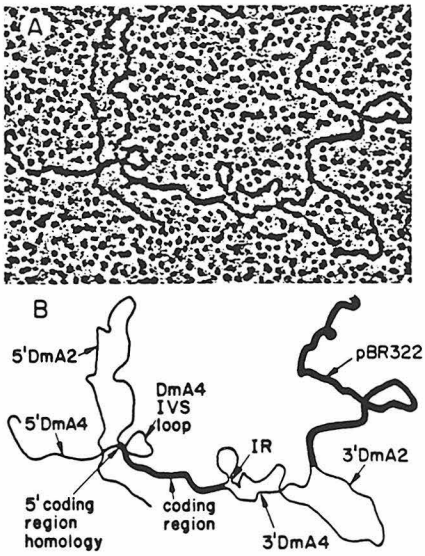


Figure 2. Heteroduplex of the DmA2 and DmA4 Actin Genes

Two chimeric plasmids, one containing an 8.7 kb Eco RI fragment of DmA2 and one a 4.2 kb Bam HI–EcoRI fragment of DmA4 were linearized with Bam HI and used to prepare heteroduplexes. A representative molecule is shown in (A) and depicted schematically in (B). The long duplex region on the right is the pBR322 vector sequence. The shorter duplex region in the lower center corresponds in size and position to the protein coding regions of the two genes. The 3′ untranslated and flanking regions are nonhomologous. Short inverted repeat segments separated by approximately 600 nucleotides form a stem-loop structure immediately 3′ to the DmA2 coding region. At the 5′ end of the coding regions a loop of the same size as the DmA4 intervening sequence (630 nucleotides) can be seen. The loop forms because a short segment 5′ to the DmA4 intervening sequence hybridizes to the 5′ end of the DmA2 coding region. The 5′ untranslated and flanking regions of the two genes are completely nonhomologous.

to the protein-coding regions terminates in forked single strands. The interpretation again is that the protein-coding regions of the several genes are homologous, but that the untranslated and flanking sequences are not. Figure 3A shows a heteroduplex of Drosophila actin genes DmA3 and DmA6. A 360 ± 40 nucleotide loop of single-stranded DNA can be seen near the 3′ end of the DmA6 coding region. This sequence splits the gene into segments of approximately 910 and 210 nucleotides in agreement with the restriction mapping data presented in Figure 1. As shown below, the nonhomologous segment within the coding region of DmA6 is an intervening sequence.

Heteroduplex analysis of DmA1 and DmA5 (Figure 3B) has failed to reveal intervening sequences within either coding region.

In summary, these experiments allowed tentative identification of a 630 nucleotide intervening sequence near the 5′ end of the DmA4 protein coding region and one of 360 nucleotides near codon 300 of DmA6. No other introns were seen within the coding regions of any of the remaining actin genes. These experiments would not allow visualization of introns less than 100 nucleotides in length. Finally, the results of the DmA2/DmA4 heteroduplex experiment indicated that the previously identified intervening sequence of DmA2 is located upstream from the protein-coding region and lies within the 5′ untranslated region.

Precise Mapping of the Introns of DmA2, DmA4 and DmA6

The precise positions of the introns of DmA2, A4, and A6 deduced from the DNA sequence data are shown in Figure 4. In panel A we show nucleotide sequence data for the regions that correspond to the beginning of translation in DmA2 and DmA4. In the former, the ATG initiator codon is followed by a cysteine codon, then by a sequence which by comparison with known actin amino acid sequences positively identifies it as an actin gene. The sequence of DmA4 is nearly identical to that of DmA2 from the amino terminus up to the glycine codon at amino acid position 13. The sequence beyond that point does not encode amino acids of actin; however there is an acceptable exon–intron splice junction sequence at this point (Lerner et al., 1980). By sequencing the appropriate region, approximately 600 nucleotides downstream we find an acceptable intron–exon junction followed by a continuation of the characteristic actin gene sequence. The sequencing data therefore confirm the presence of an intervening sequence close to the 5′ end of the protein-coding region of DmA4 and define its position precisely.

The coding region of DmA2 appears to be continuous, yet R-loop experiments have revealed that it is split near the 5′ end of the coding region by a 1.65 kb

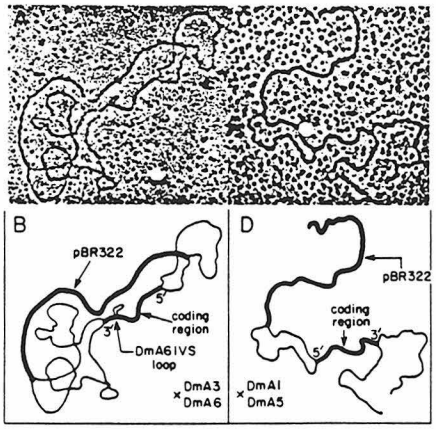


Figure 3. DmA3/DmA6 and DmA1/DmA5 Heteroduplexes

The DmA3/DmA6 heteroduplex was prepared by hybridizing a plasmid containing a 6.0 kb Eco RI fragment of DmA3 with one containing a 5.0 kb Bam HI fragment of DmA6. Plasmids were separately digested with Bgl II, which cleaves the plasmids in their respective actin coding regions, but in different positions (refer to Figure 1). Resulting heteroduplexes have long staggered ends which anneal to form circular structures. (A) shows such a molecule and (B) its schematic representation. One of the actin structural genes is interrupted by a 360 nucleotide intervening sequence, which appears as a small single-stranded loop. Since the same loop is apparent in DmA5/DmA6 heteroduplexes, we were able to assign it unambiguously to DmA6. The DmA1/DmA5 heteroduplex shown in (C) and (D) was prepared by hybridizing a plasmid containing a 3.6 kb Bam HI fragment of DmA1 to one containing a 5.2 kb Eco RI fragment of DmA5. This heteroduplex failed to reveal intervening sequences within either actin coding region.

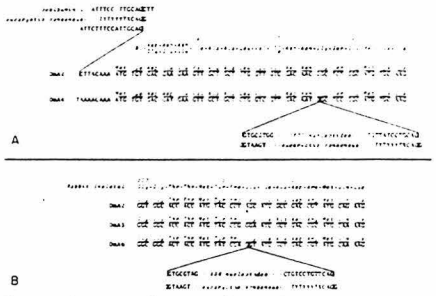


Figure 4. Nucleotide Sequences Surrounding Interruptions within Drosophila Actin Genes

(A) shows the nucleotide sequences of the 5′ ends of DmA2 and DmA4. The sequence of the amino terminal tryptic peptide encoded by each gene is aligned with that of vertebrate cytoplasmic actin (Bl represents the acetyl group which blocks the N terminus). Eight nucleotides upstream from the ATG initiation codon of DmA2 is a sequence which appears to be a functional intron–exon junction sequence based on previous R-loop experiments and on the homology of this sequence to the eucaryotic consensus sequence and to the ovalbumin G intron–exon junction sequence. (Y denotes a position where either pyrimidine can occur and X a position where either a purine or a pyrimidine is acceptable.) The lower portion of (A) illustrates that DmA4 is interrupted within the glycine codon at position 13 by an approximately 630 nucleotide intron. Inspection of the sequences of the 5′ and 3′ ends of this interruption reveals that they share homology with the eucaryotic consensus exon–intron and intron–exon sequences. (B) shows the sequence of codons 300–314 for DmA2, DmA3 and DmA6. The sequence of each encoded protein is identical to the corresponding sequences of vertebrate actins except for the serine at position 307 in DmA2 (however, we are unsure of this amino acid assignment due to the sequencing ambiguity denoted by the asterisk). Comparison of the sequences of the three Drosophila genes reveals that DmA6 is interrupted within the glycine codon at position 307. Inspection of the sequence of the 5′ and 3′ ends of this interruption again reveals that they share homology with the eucaryotic consensus exon–intron and intron–exon sequences.

intron (Fyrberg et al., 1980). From results of the heteroduplex experiment shown in Figure 5 we suspected that the gene was split in the 5′ untranslated region. Figure 4 shows that 8 nucleotides upstream from the ATG initiation codon of DmA2 there is a sequence homologous to an intron–exon junction. Fortuitously, we presume, the proposed DmA2 intron–exon junction almost exactly matches that of ovalbumin G (Breathnach et al., 1978). No other reasonable intron–exon splice junction was found for the 200 nucleotides upstream from this position. We therefore are reasonably certain that this junction is the intron–exon junction of DmA2.

The nonconservation of the intron within DmA6 is illustrated in Figure 4B. Comparison of the nucleotide sequence through the region near codon 300 of DmA6 with that of other actin genes shows that the interruption is within a glycine codon at position 307, and that an acceptable exon–intron junction exists at this point. Exactly 357 nucleotides downstream we find an intron–exon junction and the continuation of the actin gene sequence. Sequencing of DmA2 and DmA3 through the corresponding region shows that neither of these genes has an interruption in the same position. Although we have not yet sequenced DmA1, DmA4 and DmA5 through this region, none of these genes displayed evidence for an intron at this position in heteroduplex mapping experiments, and any intervening sequences which interrupt these genes at codon 307 must therefore be extremely short. However, F. Sánchez, S. L. Tobin and B. J. McCarthy (personal communication) have recently found that DmA1 is split by a 60 nucleotide intron within codon 307, demonstrating that at least one other Drosophila actin gene is split in the analogous position.

Since we had not yet examined R-loops of DmA6 it was reasonable to question whether this insert actually represents an intervening sequence. One could argue that DmA6 encodes an actinlike protein of molecular weight 55,000–60,000, and that the presumed insertion actually encodes amino acids present in the mature protein. Nucleotide sequence data have revealed, however, that the insert of DmA6 is A+T rich and encodes stop codons in all three reading frames (data not shown). This evidence argues very strongly that the insert of DmA6 is an intervening sequence.

The Amino Terminal Sequences of All Drosophila Actins Are Similar to Those of Vertebrate Cytoplasmic Actins

The amino terminal tryptic peptides of several vertebrate actins have been sequenced by Vandekerckhove and Weber (1978c). This analysis has revealed that a relatively high number of amino acid replacements occurs within these peptides and that sequence divergences tend to be tissue-specific, rather than species-specific. Thus, skeletal muscle actins from several vertebrate species are identical in amino acid sequence, but differ in sequence from the cytoplasmic actins (Vandekerckhove and Weber, 1978a, 1978c). In mammals these muscle specific versus cytoplasmic actin amino acid replacements involve charged amino acids; and, as a result, the isoelectric point of skeletal muscle actin differs from that of the cytoplasmic species (Vandekerckhove and Weber, 1978a). Because these charged amino acid exchanges are limited to the amino terminal tryptic peptide, determination of its primary sequence should allow a reasonably accurate prediction of the isoelectric points of respective actin proteins and thereby facilitate assignment of biological functions to each. We therefore derived the Drosophila actin amino terminal sequences from the nucleotide sequence of the corresponding region of each gene.

A comparison of the amino terminal tryptic peptides of each Drosophila actin with those of vertebrate skeletal muscle and cytoplasmic actin isoforms is shown in Figure 5. Within the boxes are the amino acid replacements characteristic of vertebrate skeletal and cytoplasmic actins. The sarcomeric actin sequence is Asp^{1}Glu^{2}Asp^{3}Glu^{4}(Ser or Thr)^{5}Thr^{6} . . . Cys^{10} . . . Leu^{16}Val^{17} While that of its cytoplasmic counterpart is (Asp or Glu)^{2}(Asp or Glu)^{3}(Asp or

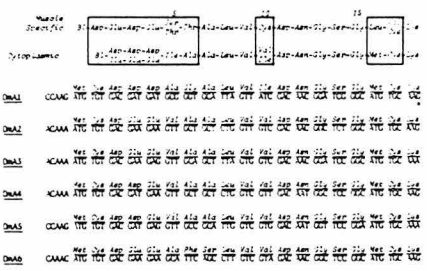


Figure 5. Sequences of the Amino Terminal Tryptic Peptides Encoded by Each Drosophila Actin Gene

The sequence of each encoded protein is aligned with the composite sequence of vertebrate muscle-specific and cytoplasmic actins. Within the boxed regions are the replacements which distinguish the vertebrate-muscle-specific actins from their cytoplasmic counterparts (Vandekerckhove and Weber, 1978a, 1978b, 1978c). Each of the Drosophila sequences resembles the vertebrate cytoplasmic actin sequences, partial exceptions being DmA1 and DmA6. Each of the derived Drosophila sequences encodes a cysteine that immediately follows the initiator methionine; however, cysteine does not occur at this position in the mature vertebrate actins. The sequence CAAPu precedes each initiator codon, except in DmA6 where the sequence is CAAAC. The asterisk in the DmA1 sequence denotes an ambiguous nucleotide. This base may be A; however, this would not alter our amino acid assignment. The unusual phenylalanine-serine sequence occurring at positions 6 and 7 in DmA6 would become serine-alanine if either T of the phenylalanine codon were inserted as the result of a sequencing error. However, then to return the sequence to the proper reading frame a nucleotide must be inserted in the leucine or valine codon at positions 8 and 9. We have failed to detect any evidence for such a nucleotide in our sequence determination through this region and we therefore believe that the sequence as shown is correct.

Glu)[4]Ile[5]Ala[6] . . . (Val or Ile)[10] . . . Met[16]Cys[17]. Inspection of the Drosophila actin gene sequences reveals first that only DmA4 is split within the glycine codon at position 13, second, that none of the Drosophila genes is split within codon four, as is the yeast actin gene, and third, that each of the derived actin amino acid sequences closely resembles the vertebrate cytoplasmic sequences, partial exceptions being DmA1 and DmA6. Examination of other portions of the Drosophila actin amino acid sequences shows that for the most part they also conform to the vertebrate cytoplasmic actin sequences (refer to Vandekerckhove and Weber, 1978a, 1978b, 1978c and our Appendix). Therefore, Drosophila apparently does not synthesize actin forms comparable in amino acid sequence to that of vertebrate skeletal muscle.

Figure 5 reveals another surprising result, namely the presence of a cysteine codon following the initiator methionine codons of each of the Drosophila actin genes. All vertebrate actins thus far characterized begin with an aspartic or glutamic acid residue that is acetylated. If only the methionine is cleaved from the Drosophila actins each would begin with cysteine followed by the three acidic residues characteristic of vertebrate cytoplasmic actins. Neither the Dictyostelium nor the yeast actin genes encode a cysteine at this position (Firtel et al., 1979; Gallwitz and Sures, 1980; Ng and Abelson, 1980). It is of course possible that the cysteine residue is cleaved by in vivo processing, and that the mature Drosophila actins begin with the string of acidic residues as do those of vertebrates.

A conserved sequence in the 5′ untranslated region of each Drosophila actin gene is apparent in Figure 5. In DmA1–DmA5 the sequence CAAPu precedes each ATG initiation codon. In DmA6 a cytidine has been inserted to give the sequence CAAAC. Immediately upstream from this region the sequences of the genes become completely divergent (data not shown). Thus selective pressure may preserve this four nucleotide sequence.

The Six Drosophila Actin Genes Encode Several Different Polypeptides

An interesting question is whether the six Drosophila actin genes encode proteins that differ in their primary amino acid sequences. Knowledge of such sequence differences may lead to a better understanding of the relationship of the structure of actin isoforms to their particular developmental roles. In the course of our studies we have obtained some, but not all, of the data needed for a decisive answer. These incomplete data are presented in the Appendix, in which we show Drosophila actin gene sequences not presented in Figures 4 or 5. Comparison of all of the available sequence information reveals that the six genes encode at least five different proteins. Only the comparison of DmA4 with DmA5 has as yet failed to reveal a difference in the sequence of the encoded proteins.

Far more striking than the differences in the sequences of these proteins are their similarities. Sequences of Drosophila actins are apparently extremely conserved. Most of the amino acid replacements are very conservative, such as replacement of valine with isoleucine, or threonine with serine. Therefore the observed amino acid replacements may not be functionally significant. Rather, we think it equally probable that for the most part they represent neutral mutations which have accumulated and resulted in slight divergences of the respective sequences.

Discussion

In this communication we have reported the isolation and structural characterization of the six Drosophila actin genes. We have shown that the protein-coding regions of these genes are highly homologous and that each encodes an actin protein with an amino acid sequence closely resembling those of the vertebrate cytoplasmic isoforms. We find no homology of untranslated or flanking regions of these genes. Additionally, we have discovered two introns within protein-coding regions (one in DmA4, one in DmA6) which are either in a position where no other Drosophila actin genes have an intron (as is the case for the DmA4 intron), or are in a position where some, but not all, other actin genes have introns (as is the case for the DmA6 intron). Additional introns in the Drosophila actin genes may be discovered when the characterization of the genes is completed.

The probe used to isolate the complete set of Drosophila actin genes was derived from λDmA2. Since this probe encodes an actin protein that resembles the vertebrate cytoplasmic isoforms one could argue that our screen succeeded in isolating the genes encoding cytoplasmic actin isoforms but failed to isolate those encoding muscle-specific isoforms. We think this improbable. Mammalian skeletal muscle actins differ from the cytoplasmic isoforms in approximately 25 of 375 amino acid residues (Vandekerckhove and Weber, 1978a, 1978b). Analysis of these amino acid replacements shows that they would increase nucleotide mismatch of these two types of actin genes by at most 3% above the value due to codon degeneracy. The moderately stringent hybridization conditions employed in our library screening and blot hybridization protocols do not distinguish such a small increase in nucleotide mismatch. Therefore unless the amino acid sequences of Drosophila muscle-specific actins are considerably more divergent from their cytoplasmic counterparts than is the case for vertebrates, our hybridization experiments would have detected them.

The variability in the positions of the introns in the Drosophila actin genes is in sharp contrast to the situation observed in globin genes (Maniatis et al.,

1980), vitellogenin genes (Wahli et al., 1980) and the ovalbumin-X-Y gene series (Royal et al., 1979). In each of these cases the placement of intervening sequences is exactly the same for all members of the gene family. For the globins this property extends across rather large evolutionary distances; for example, globin genes of Xenopus and humans are interrupted in exactly the same positions (Patient et al., 1980) despite the fact that their ancestral lines diverged approximately 350 million years ago (Young, 1962). In fact, there are only two cases where members of vertebrate multigene families differ in placement of introns, the first being one of the nonallelic rat insulin genes, which has lost an intron (Lomedico, et al., 1979), and the second being a nonfunctional mouse α-globin-like gene which has lost both introns (Nishioka et al., 1980).

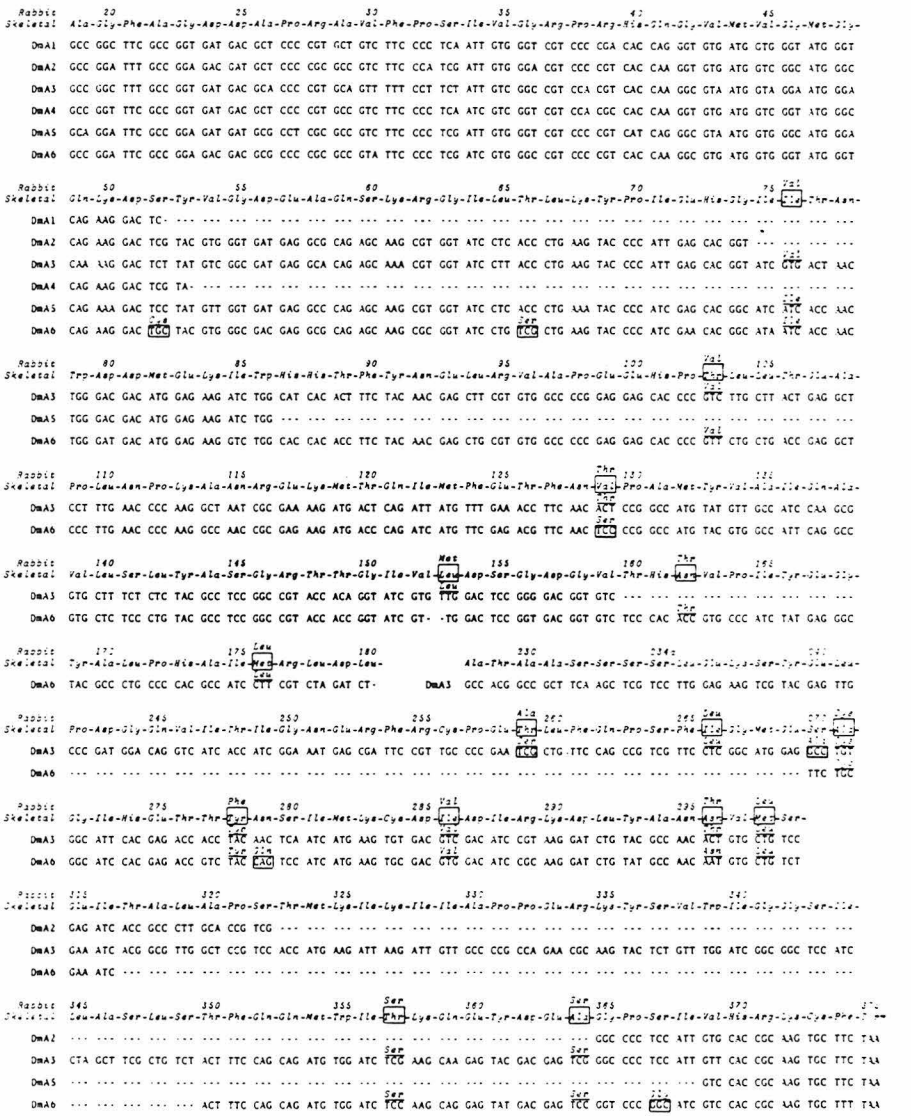
Placement of intervening sequences is much more divergent in the Drosophila actin gene family than in the multigene families discussed above. At least three interruptions occur within the protein-coding regions of Drosophila actin genes, one within codon 13 of DmA4, one within codon 307 of DmA6, and a shorter intron at the same position of DmA1 (F. Sánchez, S. L. Tobin and B. J. McCarthy, personal communication). The interruption within codon 13 is not present in any other Drosophila actin genes, while that within codon 307 is not present in at least two other genes. Furthermore, none of the Drosophila actin genes is interrupted within codon 4 where the yeast actin gene is split (Gallwitz and Sures, 1980; Ng and Abelson, 1980) and at least two (DmA3 and DmA6) are not split between codons 121 and 122 where one of the sea urchin actin genes is split (refer to Durica et al., 1980; Schuler and Keller, 1981; and our Appendix). Finally, none of the several Dictyostelium actin genes sequenced by Firtel et al. (1979) appears to have interruptions within its protein-coding regions.

These results may help to elucidate the functional and/or evolutionary roles of intervening sequences. Gilbert (1979) has proposed that the primary function of introns is to facilitate gene evolution and as a corollary that the older form of a gene will have more introns. According to a strict form of this hypothesis, the primordial metazoan actin gene had at least four introns: one in the 5′ untranslated region (as does DmA2), one in codon 13 (as does DmA4), one after codon 121 (as does sea urchin) and one in codon 307 (as do DmA6 and DmA1). If this were the case, then many introns have been deleted from individual Drosophila actin genes during evolution. This could be for any or all of several reasons. First, because the actin genes are probably of ancient evolutionary origin there has been a long period in which to excise introns. Second, there may be strong selective pressure to delete introns from genes encoding proteins which evolve slowly, such as actin. Third, Drosophila may have more rapidly deleted introns during its evolution than have typical eucaryotes. Consistent with this notion is the observation that Drosophila has one of the smallest eucaryotic genomes.

Further comparisons of the positions of introns within the actin genes of a variety of metazoans will be most informative. If positions of introns are conserved even between protostomes and deuterostomes, this will support the hypothesis that the primordial metazoan actin gene contained many introns, several of which have been deleted from Drosophila actin genes. Conversely, if no conservation of intron positions within actin genes is found, then perhaps at least some introns are the vestiges of transposonlike elements which have inserted into genes, become fixed, and subsequently diverged in nucleotide sequence (see Doolittle and Sapienza, 1980; Orgel and Crick, 1980).

The observation that all of the Drosophila actins are similar in sequence to the vertebrate cytoplasmic isoforms has implications for both the evolution and functioning of actin proteins. Lower eucaryotes such as Physarum and Dictyostelium synthesize only actins with amino acid sequences closely resembling the vertebrate cytoplasmic isoforms (Vandekerckhove and Weber, 1978d, 1980), suggesting that the vertebrate cytoplasmic actins predate the vertebrate muscle-specific isoforms. Our results indicate that vertebrate muscle-specific actin isoforms evolved after insects separated from the phylogenetic line that gave rise to the vertebrates. Furthermore, our data suggest that the amino acid replacements found in vertebrate muscle-specific actin isoforms are not required for striated muscle function per se. Many types of insect muscle bear an anatomical resemblance to vertebrate striated muscle (Smith, 1968), and Drosophila muscle-specific actin isoforms have been documented (Storti et al., 1978; Fyrberg and Donady, 1979; Horovitch et al., 1979). However, our data clearly show that the amino acid sequences of Drosophila muscle-specific isoforms most closely resemble those of vertebrate cytoplasmic actins. In accordance with this conclusion, Lubit and Schwartz (1980) have found that rabbit antibodies elicited by actin extracted from the body wall musculature of the marine mollusc Aplysia will crossreact with vertebrate cytoplasmic actins but not with vertebrate skeletal muscle actin. Myofibrillar actin in Aplysia may therefore also have an amino acid sequence like vertebrate cytoplasmic actins.

Our results and those of Tobin et al. (1980) and Fyrberg et al. (1980) have revealed that Drosophila actin genes comprise a highly conserved family that encodes a nearly identical set of proteins. Examination of the constellation of tissue types and developmental intervals in which each gene is expressed should enhance our knowledge of the factors that regulate eucaryotic gene expression and additionally those factors that determine the cellular roles of newly synthesized proteins.



Appendix. Available Sequences of the Six Drosophila Actin Genes Not Presented in Figures 4 and 5

Codons of the Drosophila genes are aligned with the amino acid sequence of rabbit skeletal muscle actin. Amino acid residues of the skeletal muscle actin sequence that are replaced in vertebrate cytoplasmic actin isoforms are boxed, and their replacements appear directly above (replacements within the amino terminal tryptic peptides of actins are presented in Figure 5). In most cases the sequences of the encoded Drosophila actins conform to those of the vertebrate cytoplasmic isoforms. Amino acid replacements among the Drosophila actins occur at residues 3, 4, 5, 6, 7, 10, 52, 66, 76, 129, 270, 279, 296, 307 and 367 (refer also to Figures 4 and 5), accounting for five slightly divergent proteins. The amino acids encoded at these positions by the Drosophila genes appear above the appropriate codons. Codons that are not assigned an amino acid specify the same residue as is found in the rabbit skeletal muscle actin sequence. Codons that specify amino acids not expected on the basis of the vertebrate sequence data are boxed, and the amino acid assignments are written directly above. Codon 277 of DmA6 encodes valine, but this is not indicated in the figure.

Experimental Procedures

Isolation of Genomic Actin Clones

A library of Drosophila genomic DNA (Canton S strain) constructed by ligation of Eco RI partial digestion products to Charon 4 arms (Yen et al., 1979; Davidson et al., 1980) was screened using the in situ plaque hybridization technique (Benton and Davis, 1977). A 1.8 kb Hind III fragment derived from a previously characterized Drosophila actin gene (Fyrberg et al., 1980) was ^{32}P-labeled by nick translation (Schachat and Hogness, 1973; Maniatis et al., 1975) and used as a probe. This particular DNA fragment is known to contain the entire actin protein-coding sequence (E. A. Fyrberg and N. D. Hershey, unpublished data).

From a screen of 40,000 plaques, 30 which gave strong signals were selected. After purification, each of these phages was grown up individually as a plate lysate and DNA was prepared according to the method of Maniatis et al. (1978). The phage DNAs were digested with various restriction enzymes, and the fragments that hybridized to the 1.8 kb Hind III probe were identified by blotting (Southern, 1975).

Subcloning of Genomic Actin Sequences into Plasmid Vectors

Chimeric plasmids were constructed by ligation of gel isolated Eco RI, Hind III or Bam HI-cut fragments of lambda clones to pBR322 DNA that had been cut by the same enzyme(s) in order to generate complementary ends (Mertz and Davis, 1972; Cohen et al., 1973). DNA prepared in this fashion was used for transformation of E. coli K-12 strain HB 101 (Cohen et al., 1972). Selection of clones containing the desired fragments and their subsequent DNA preparation were as previously described (Fyrberg et al., 1980).

R-Loop and Heteroduplex Mapping

To form R-loops we used a protocol essentially as described by Kaback et al. (1979). Hybridizations were carried out in 20 μl reactions which contained 70% formamide (3X recrystallized), 0.5 M NaCl, 0.1 M PIPES (pH 7.2), 0.01 M EDTA, 50 ng DNA and 10 μg of total cellular poly(A)$^+$ RNA from 18 hr embryos. Hybridizations were for 24 hr and during this period temperature was lowered from 55°C to 47.5°C.

Heteroduplexes were formed essentially according to the method of Davis et al. (1971). Chimeric plasmid DNAs were linearized with the appropriate restriction enzymes and equimolar amounts of each (approximately ½ μg of each fragment) were mixed in 25 μl of DNA buffer (0.010 M NaCl, 0.01 M Tris–Cl (pH 7.4) and 0.001 M EDTA). The DNA was denatured by addition of 2.5 μl of 3 M NaOH. After 10 min at room temperature the solution was neutralized by the addition of 10 μl of 2.5 M Trizma–HCl (Sigma). An equal volume of 3X recrystallized formamide was then added, and the single strands were allowed to renature at room temperature. Aliquots of the reaction were removed at 10, 20 and 30 min and quenched by the addition of 2 vol of ice-cold double-distilled water.

Both R-loops and heteroduplexes were spread from a hyperphase that contained 45% formamide, 0.10 M Tris–HCl (pH 8.5), 0.01 M EDTA and both single- and double-stranded φX DNA as length markers. The hypophase contained 17% formamide, 0.01 M Tris–HCl (pH 8.5) and 0.001 M EDTA (Davis et al., 1971). Grids were strained in uranyl acetate, shadowed in Pt-Pd and examined in a Philips 300 electron microscope.

Restriction Mapping and Sequencing of DNA Fragments

Actin-gene-containing phage DNAs were mapped using the conventional complete single and double restriction-enzyme digests. Subcloned fragments were mapped in finer detail by the method of Smith and Birnstiel (1976). DNA to be mapped was digested using enzymes that produce 3′ recessed ends, and these were filled using appropriate α–^{32}P nucleotides and the Klenow fragment of E. coli DNA polymerase (Klenow and Henningsen, 1970). DNA was resuspended in 50 μl of 0.0066 M NaCl, 0.0066 M Tris–HCl (pH 7.5), 0.0066 M $MgCl_2$, and 0.0066 M DTT. Of each labeled nucleotide 50 μC was then added (400 Ci/mmole Amersham-Searle), and the reaction was initiated by addition of 1 U of enzyme. After 15 min at room temperature the reaction was stopped by addition of EDTA, and the labeled fragment was separated from unincorporated nucleotides by passage over a small G-50 Sephadex column. The double end-labeled fragment was cleaved with a restriction enzyme and the two ends were purified by gel electrophoresis and elution. Partial digestion products of end-labeled fragment were electrophoresed on 5% acrylamide gels and autoradiogrammed after drying on a Hoefer gel dryer.

Fragments to be sequenced were prepared in an identical fashion, except that they were labeled with nucleotides of higher specific activity (2000–3000 Ci/mmole; Amersham-Searle, Inc.). Additionally, we routinely "chased" the labeled fragments with excess cold nucleotides to ensure that the staggered ends were completely filled in by the polymerase. End-labeled fragments were sequenced by the method of Maxam and Gilbert (1980).

Most of the presented data were obtained by sequencing only one strand of DNA. We have taken several measures to ensure that these determinations were very accurate. Six separate base cleavage reactions (G, G > A, G + A, A > C, T + C, C) were carried out to reduce ambiguous base assignments. Intensifying screens were not used during autoradiography, thus maintaining better resolution of cleavage products. Gels were read only in regions where bands were clearly resolved. All sequences from which interesting conclusions were drawn were read independently by three observers. Since all of the sequences presented lie within actin protein-coding regions, we eliminated the majority of clerical errors by deriving the encoded protein sequence and comparing it with other actin amino acid sequences.

From the cases where overlapping sequences were determined, we judged our error rate to be approximately 1%. This error rate would not influence any of the major conclusions drawn from the sequence data.

Biosafety

This research was carried out in accordance with NIH Guidelines, using P2/EK1 containment.

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