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A single gene codes for the nicotinic acetylcholine receptor α-subunit in *Torpedo marmorata*: structural and developmental implications

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We have used Southern blot hybridization to analyze the genomic structure encoding the α-subunit of the acetylcholine receptor (AChR) in *Torpedo marmorata*, with cDNA probes isolated from the electric organ. Four different radiolabelled probes, corresponding to various parts of the α-subunit mRNA, hybridized to several genomic fragments of *T. marmorata* DNA generated by digestion with the restriction enzymes *Sst*I, *Pvu*II and *Pst*I. The same hybridization pattern was observed after washing the blots under low- or high-stringency conditions. As a check for detection sensitivity of heterologous sequences, the same probes were hybridized to *Pvu*II-digested chicken DNA, revealing bands at low stringency which disappeared at higher stringencies. Unambiguously, two of our probes (one of them entirely within the coding region) hybridized to a single genomic fragment from *T. marmorata* DNA. This feature, as well as the results of an extensive study of the whole hybridization pattern, points towards the uniqueness of α-subunit-specific sequences in the genome of *T. marmorata*. Since overall more bands were found than expected from the cDNA sequence, this α-subunit gene must be split by several introns (at least four, possibly more). The length of this gene is at least 20 kb. The existence of a single α-subunit gene is consistent with the absence of chemical heterogeneity in the NH_2-terminal sequence of the purified α-chain, and supports the view that the two α-chains belonging to one AChR oligomer have an identical primary structure. It also suggests that localization and stabilization of the AChR in well-defined post-synaptic areas of *T. marmorata* electric organ basically relies, during development, on 'epigenetic' mechanisms.

Key words: acetylcholine receptor/Southern blot/synaptogenesis/neurobiology

Introduction

Transmission of the nerve impulse at the neuromuscular junction or the electromotor synapse involves a membrane-bound regulatory protein – a receptor – which converts a rapid change of neurotransmitter concentration – acetylcholine – into a transient increase of permeability to ions. Since its initial characterization in fish electric organs (Changeux *et al.*, 1970; Miledi *et al.*, 1971), the acetylcholine receptor (AChR) remains the best known member of this important family of allosteric proteins (Changeux, 1981; Conti-Tronconi and Raftery, 1982). Its basic unit, or light form, consists of four different polypeptide chains of exact mol. wt. (in *Torpedo californica*): α = 50 116, β = 53 681, γ = 56 279, δ = 57 565 (Noda *et al.*, 1982, 1983a, 1983b) in a 2.1.1.1. stoichiometry (Reynolds and Karlin, 1978; Lindstrøm *et al.*, 1979a; Raftery *et al.*, 1980; Saitoh *et al.*, 1980) and this heterologous pentamer carries both the acetylcholine binding sites and the ion channel.

As a consequence of the chemical determination of the partial amino acid sequence of the chains (Devillers-Thiéry *et al.*, 1979; Hunkapiller *et al.*, 1979; Raftery *et al.*, 1980) and of the subsequent isolation of cDNA clones coding for the α-chain in *T. marmorata* (Giraudat *et al.*, 1982; Sumikawa *et al.*, 1982) and for the four chains in *T. californica* (Ballivet *et al.*, 1982; Noda *et al.*, 1982, 1983a, 1983b), studies on this protein have entered a new era. The knowledge of the complete amino acid sequence of the four chains (Noda *et al.*, 1982, 1983a, 1983b; Claudio *et al.*, 1983; Devillers-Thiéry *et al.*, 1983) provides new perspectives for investigations of the transmembrane organization of the molecule (see Devillers-Thiéry *et al.*, 1983; Claudio *et al.*, 1983; Noda *et al.*, 1983b; Kosower, 1983a, 1983b). Also, the availability of cDNA probes makes it possible to analyze the structure of the genes coding for the four chains and to investigate the regulation of their expression during development.

The presence of two α-subunits in the light form pentamer inevitably raises the question of the number of α-subunit genes required to construct the receptor protein in the subsynaptic membrane, especially in view of the distinct binding properties of the receptor sites associated with each of the two subunits (reviewed in Karlin, 1980; Changeux, 1981).

A knowledge of the number of genes involved is also necessary for the understanding of the mechanisms of localization and metabolic stabilization of the receptor during synapse development. For instance, at the neuromuscular junction, the embryonic extrasynaptic and the adult subsynaptic receptors have been shown to differ (reviewed in Fambrough, 1979; Changeux, 1981) by their state of aggregation and translational motion, their metabolic turnover, the mean open time of their ionic channel (in rat and frog), and their immunological reactivity. An additional issue is the extent of similarity between adult muscle and electric organ AChR. In both instances, the observed differences might result from the expression of different genes and/or from post-transcriptional events.

The results of the hybridization experiments presented in this paper are consistent with the presence of a single gene coding for the α-subunit in *T. marmorata* genome.

Results

Probing T. marmorata genomic DNA with fragments of cDNA clones specific for the α-subunit

The inserts of the two overlapping cDNA clones specific for the α-subunit, previously isolated and sequenced (Giraudat *et al.*, 1982; Devillers-Thiéry *et al.*, 1983) were cut into four fragments by restriction endonucleases (see Figure 1) and the fragments were radiolabelled by nick-translation. These

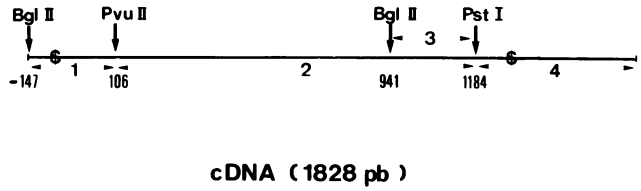

Fig. 1. cDNA probes used in this study. The 1828 bp of α-subunit cDNA were derived from the two overlapping clones described by Giraudat *et al.* (1982) and Devillers-Thiéry *et al.* (1983). Restriction sites used to obtain the various probes (see text) are indicated by vertical arrows. The extent of probes is shown by horizontal arrowheads, with their numbers in between (nucleotide numbering as in Devillers-Thiéry *et al.*, 1983). The $ signs mark the ends of the coding region.

labelled fragments were used as probes. Following the nomenclature and model for the transmembrane organization of the α-subunit from Devillers-Thiéry *et al.* (1983), the four probes contain the following cDNA sequences, starting from the 5′ end: no. 1, a *Bgl*II-*Pvu*II restriction fragment without GC tails (nucleotides −147 to 106): 75 nucleotides of the 5′-untranslated region, codons of the entire signal sequence and the sequence of the mature α-subunit up to amino acid residue 35; no. 2, a *Pvu*II-*Pst*I fragment (nucleotides 106–1184): the N-terminal synaptic domain of the α-subunit, three of the putative transmembrane domains (α-helices I, II, III) and most of the cytoplasmic domain, up to residue 394; no. 3, the *Bgl*II-*Pst*I fragment of probe no. 2 (nucleotides 941–1184): the cytoplasmic domain from amino acid 313 to amino acid 394; no. 4, generated by digestion with *Pst*I: the transmembrane α-helix IV and 369 nucleotides of the 3′-non-translated region (for this probe, no effort was made to eliminate the GC tails).

High mol. wt. DNA, prepared from the liver of *T. marmorata*, was digested to completion with two restriction enzymes, *Pst*I and *Pvu*II, for which a unique site is present in the sequence of the α-subunit cDNA and with a third enzyme, *Sst*I, for which no site is present in the cDNA sequence. The digested fragments were run on agarose gels and transferred to nitrocellulose filters after denaturation. The blots were hybridized to one of the four radioactively labelled probes and washed at 40°, 58° or 65°C, depending on the desired stringency (see Materials and methods).

Figure 2 shows the Southern hybridization pattern obtained with the three restriction enzymes and the four radioactively labelled probes under low-stringency conditions (40°C). When the blots were washed under higher stringency conditions, no qualitative differences became apparent in this pattern (one example is shown in Figure 4A for a *Pvu*II-*T. marmorata* DNA digest hybridized with probe no. 3). Moreover, probes no. 3 and 4 systematically revealed only one complementary genomic fragment for all three enzymes. This fragment was at least 11 kb long for probe no. 4, which implies that the corresponding 3′-most site is located some distance from the gene coding for the α-subunit. This simple hybridization pattern is consistent with the presence of a single gene coding for the α-subunit in *T. marmorata* genome (see Discussion).

On the other hand, probes no. 1 and 2 hybridized to multiple genomic DNA fragments (see Figure 2A, B, C, lanes 1 and 2), but with different intensities. The multiple band pattern persisted under higher stringency conditions and thus did not reflect hybridization with genomic DNA sequences exhibiting a lower degree of homology with the probes. Rather, it is interpreted as resulting from the splitting of an α-subunit gene at restriction sites absent from the cDNA sequences, but located in intervening non-coding sequences, i.e., in introns. The restriction pattern presented in Figure 2 is consistent with the presence of at least four, two and four restriction sites for *Sst*I, *Pvu*II and *Pst*I, respectively, in correspondingly as many introns of the α-subunit gene.

Two contiguous and non-overlapping probes generally produce hybridization patterns which have one, and only one, band in common (see for instance the 9.3-kb band in lanes 1 and 2 of Figure 2A or the 15-kb band in lanes 3 and 4 of Figure 2B). Indeed, the restriction site which separates two such probes (*Pvu*II in the first case, *Pst*I in the second) necessarily lies somewhere between the two cleavage sites of another enzyme which themselves define one genomic fragment of Figure 2. Both probes therefore contain regions complementary to that fragment, either on the 5′ or on the 3′ side of the cDNA restriction site considered. The absence of an additional common band in any such case is consistent with the completeness of the restriction reactions and, again, with the uniqueness of the detected sequences in the genome of *T. marmorata*. We did not observe bands common to lanes 1 and 2 of Figure 2B, lanes 3 and 4 of Figure 2C and lanes 1 and 2 of Figure 2C. In the first two cases (the last one is discussed below), this is expected since the same enzyme was used to generate the two contiguous cDNA probes and to cleave the genomic DNA.

The differences in intensity noticed between the several hybridizing bands at a given stringency appeared to be related to the proportion of the probe length that hybridized to the genomic fragments. A comparison between lanes 2 and 3 of Figure 2C illustrates this point. The 2.7-kb band strongly hybridized to probe no. 3, but gave a much weaker signal with probe no. 2, although the latter included the former. Also, small fragments (below ~1 kb) diffused more during gel migration and tended to give broader bands with lower signal density. Lane 1 of Figure 2B and 2C contained bands at 350 bp and 700 bp, respectively, which were much more difficult to detect than the sharper ones present at high mol. wts. and indeed are not visible on photographs of Figure 2. Fragments smaller than this might even migrate out of the gel. This plausibly explains the absence of a band common to lanes 1 and 2 of Figure 2C (see above), which should exist since there must be a *Pst*I genomic restriction fragment including the *Pvu*II site of the cDNA (nucleotide position 106). Another possible explanation is that this cDNA site (*Pvu*II) is close to an exon-intron border, leaving exonic sequences on either probe no. 1 or 2 too short to hybridize efficiently to the *Pst*I genomic fragment that contains this *Pvu*II site. Finally, one may recall that the cDNA exonic probes used in this work cannot hybridize with restriction fragments that contain exclusively intronic sequences. Therefore, the estimates of the number of intronic restriction sites and of the total gene length presented in this work are minimal values.

Cross-hybridization of T. marmorata probes with chicken genomic sequences

N-terminal sequences of electric eel (Conti-Tronconi *et al.*, 1982b) and calf muscle AChR (Conti-Tronconi *et al.*, 1982a) display higher homology between corresponding chains in different species than between the four chains of the receptor from a given species. Although our cDNA probes did not react with any other *T. marmorata* subunit, it nevertheless appeared of interest to check for a possible hybridization to

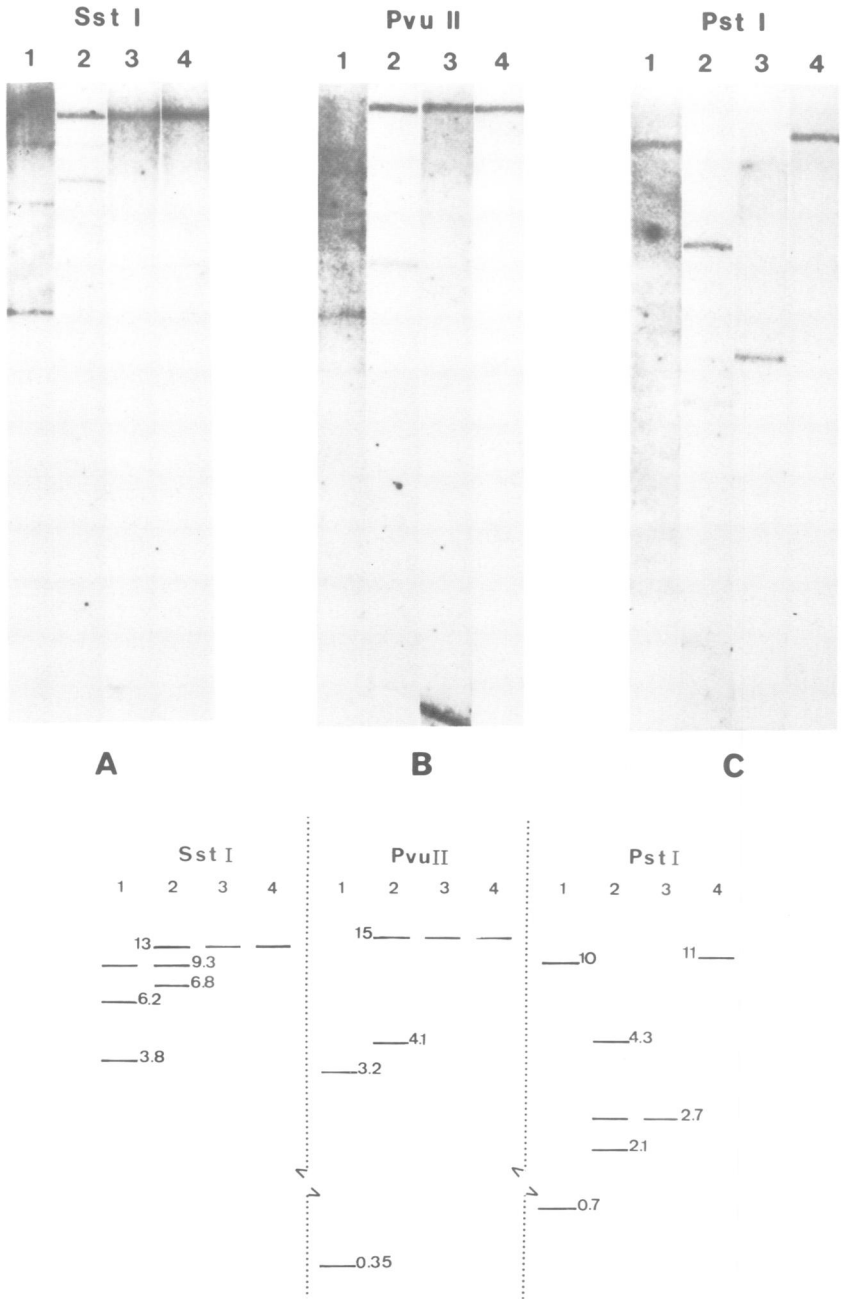

Fig. 2. Autoradiograms of Southern blots of *T. marmorata* DNA. **Upper part:** 25 μg *T. marmorata* DNA were digested to completion with *Sst*I **(A)**, *Pvu*II **(B)** and *Pst*I **(C)**. DNA fragments were separated on 0.7% agarose gels, transferred onto nitrocellulose filters and hybridized to one of the four radioactively-labelled probes described in Figure 1. Blots were washed under low stringency conditions (see Materials and methods). In each panel, numbers on top of each lane indicate the probe which was used (see Figure 1). Fragment sizes were estimated by parallel migration of marker DNA (λ-*Hind*III fragments and φX174-*Hae*III fragments). **Lower part:** schematic representation of the autoradiograms. Because gel migration was different from one blot to the other, care was taken to use the proper calibration curves in order to compute the fragment sizes given in the text, and displayed on this drawing.

heterologous sequences by following their cross-hybridization to genomic sequences from another species.

Chicken liver DNA was digested with *Pvu*II enzyme and hybridized to the four *T. marmorata* probes as above. The results obtained under low-stringency conditions are shown in Figure 3. Interestingly, probes no. 1 and 4, which both correspond in part to non-coding regions of the mRNA, gave complex hybridization patterns, whereas purely coding regions, such as probes no. 2 and 3, hybridized to a few well-defined bands. One should also notice that only one of the three bands in Figure 3 lane 3 appeared in lane 2, although probe no. 3 is a subfragment of probe no. 2. This is in line with our above comment on the relationship between hybridization intensity and total probe length.

Approximate limits can be set on the homology between chicken genomic DNA sequences and the *T. marmorata* probes from the disappearance of the corresponding bands when the filters were washed at progressively increasing stringencies. Figure 4 shows that hybridization of probe no. 3 with *T. marmorata* DNA was not abolished by washing at 58°C (intermediate stringency) or even 65°C (high stringency), whereas cross-hybridization with chicken DNA was already barely visible at intermediate stringency (58°C). Using the reasoning of Heidmann and Rougeon (1982), one can

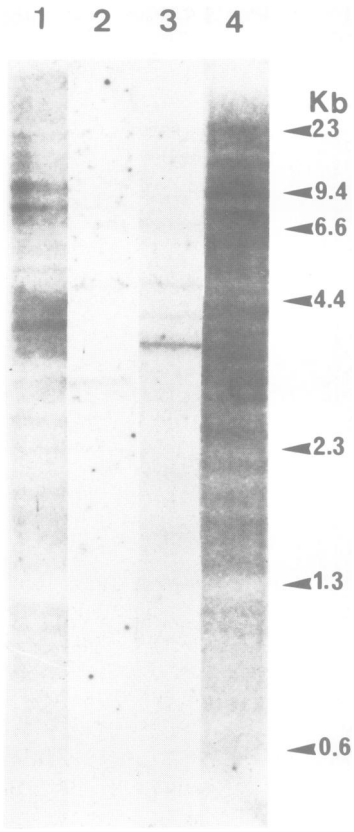

Fig. 3. Autoradiograms of Southern blots of chicken liver DNA. 25 μg chicken liver DNA were digested to completion with *Pvu*II. DNA fragments were separated on 0.7% agarose gels, transferred onto nitrocellulose filters and hybridized to the four radioactively-labelled probes of Figure 1. Blots were washed under low stringency conditions. Numbers on top of each lane indicate the probe used for hybridization. Lane numbers correspond to the probe numbers of Figure 1.

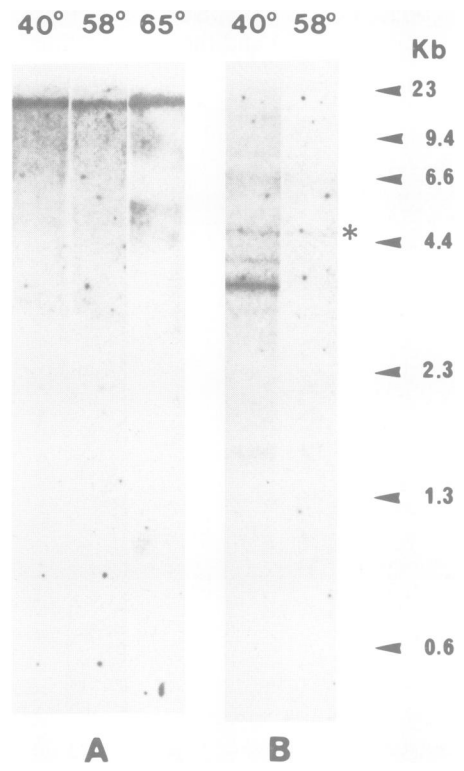

Fig. 4. Effect of different stringency conditions on the hybridization pattern of *T. marmorata* **(panel A)** and chicken DNA **(panel B)**. *T. marmorata* and chicken genomic DNAs were digested with *Pvu*II. Restriction fragments were blotted on nitrocellulose filters. Blots were hybridized with probe no. 3 and washed under three conditions as indicated on top of each lane: low (40°C), intermediate (58°C) and high (65°C) stringency for *T. marmorata* DNA **(panel A)**. Only two conditions are shown for chicken DNA **(panel B)**, since all the bands had disappeared at high stringency. * points to one band which remained at intermediate stringency. Mol. wt. markers are as in Figure 2. Exposure times were 4 – 14 days with an intensifying screen at −70°C.

estimate the overall homology between the cross-hybridizing sequences to be at least 70%. Since a 66% homology was reported between the N-terminal amino acid sequences of *T. marmorata* electric organ and calf muscle α-subunits, it is reasonable to assume that at least some of the detected chicken DNA fragments correspond to the chicken α-subunit gene(s).

Discussion

How many genes code for the α-subunits of T. marmorata acetylcholine receptor?

The AChR light form comprises one β, one γ and one δ subunit, but two α-subunits (Reynolds and Karlin, 1978; Lindstrom *et al.*, 1979a; Raftery *et al.*, 1980; Saitoh *et al.*, 1980). Moreover, in the adult electroplaque (and of course at the neuromuscular junction), the receptor protein is not evenly distributed on the cell surface, but localized at a very high density on the innervated face of the electroplaque under the nerve terminals. The occurrence of multiple structural genes for the α-subunit might, at first sight, seem a plausible hypothesis. One could argue for instance that: (i) the stoichiometry of the encoding genes should match that of the subunits; (ii) different structural genes should account for the observed functional and structural differences between the embryonic, 'distributed' and labile, and the adult, 'localized' and stable, forms of the receptors (in particular in the case of the neuromuscular junction) (reviewed in Fambrough, 1979; Changeux, 1981). The data presented here support an alternative model in the case of *T. marmorata* electromotor synapse.

Hybridization of four cDNA probes specific for the α-subunit to *T. marmorata* genomic DNA gives only one or a few bands which can be attributed to the gene(s) coding for the α-subunits. Since an important sequence homology exists between the different subunits of *T. californica* receptor (Raftery *et al.*, 1980; Noda *et al.*, 1983b), cross-hybridization could have occurred between α-subunit cDNA probes and genomic fragments specific for the other three subunits. According to Marmur and Doty (1962), Bonner *et al.* (1973) and Heidmann and Rougeon (1982), the low stringency conditions used in this work allowed detection of sequences exhibiting more than ~70% overall homology with the probe. Computer sequence comparison of the α and δ subunits of *T. californica* yields a 60% homology over a 200 nucleotide stretch, with even a 75% homologous, but only 44 residue long, domain, located 5′ of the *Bgl*II site shown in Figure 1. Similar figures are obtained for α-β and α-γ comparisons. Since none of the bands observed with *T. marmorata* under low stringency conditions disappeared at higher stringency, such limited similarities are, in agreement with the values quoted above, below detection level. Thus, under our ex-

perimental conditions, no cross-hybridization takes place between the α-subunit gene(s) and the genes coding for the β, γ and δ chains.

The possible presence of several different but homologous genes coding for the α-subunit in *T. marmorata* genome was then investigated. As discussed above, we were able to detect heterologous genomic sequences (which probably exhibit at least ~70% homology with the cDNA probes). If several genes with different coding sequences were present, the Southern blots would give complex patterns of multiple bands, which would vary with the stringency conditions used. On the other hand, probes no. 3 and 4 hybridized to single genomic fragments under all stringency conditions, and for all restriction enzymes.

It was still conceivable that these fragments were generated from different genes with identical restriction patterns. Although in multigene families coding regions are in general highly conserved, or even identical (for the actin and tubulin genes: Firtel, 1981; Minty *et al.*, 1982; for globin genes: Liebhaber *et al.*, 1981), the 3′- and 5′-non-translated regions often diverge (Firtel, 1981), the homology between the genes ending abruptly near the limits of the coding region (Michelson and Orkin, 1980). Pseudogenes, which often closely resemble the 'original' functional genes, even in non-translated regions (except sometimes for the presence of introns) (Proudfoot *et al.*, 1982; Sharp, 1983) also share this characteristic rupture of homology. Therefore, some of the restriction sites in introns or other non-coding regions of the DNA would not be conserved within members of a multigene family. However, the 3′-most site for all restriction enzymes used must be located far from any coding (or even transcribed) region specific for the α-subunit, since the unique 3′-terminal fragments hybridizing with probe no. 4 are all >11 kb long. It thus appears unlikely that several different genes could have generated these unique fragments. We conclude that, within 70% homology, the *T. marmorata* genome contains a single copy of α-subunit-specific sequences. This result is consistent with the observation that all the individual cDNA clones isolated up to now for the α-subunit have, for a given *Torpedo* species, the same sequence (Sumikawa *et al.*, 1982; Devillers-Thiéry *et al.*, 1983; Noda *et al.*, 1982).

Although in our conditions the α-subunit probes did not detect the genes coding for the other *T. marmorata* subunits, they did hybridize to homologous sequences in chicken DNA. We would reasonably expect that at least some of the few bands obtained with exclusively coding probes (Figure 3, lanes 2 and 3) are α-subunit specific, in contrast to the much lower specificity exhibited by non-coding probes (Figure 3, lane 1). Also, this would be consistent with immunological (Lindstrøm *et al.*, 1979b) and primary structure (Conti-Tronconi, 1982a) data which disclose a higher degree of homology between the same subunit from different species than between the different subunits within the same species.

Some structural properties of the α-subunit gene from T. marmorata

As noted in the Results section, the observed restriction patterns imply that at least four (for *Pst*I and *Sst*I) and two (for *Pvu*II) additional sites are located in as many introns. The α-subunit gene therefore contains a minimum of four introns. Perhaps of particular interest is the pattern of lane 1 of Figure 2A, which reveals at least two introns 5′ of the *Pvu*II site in the cDNA, for only 252 bp of exonic sequences. Several authors have suggested a possible relationship between exons and structural motifs of the encoded protein (Gilbert, 1978; Inana *et al.*, 1983; Gô, 1983). Within that framework, it would be reasonable to expect an intron following the 5′-non-coding region (or part of it) and another one after the 72 bp encoding the signal peptide, accounting for the three bands of lane 1, Figure 2A.

The minimum length of the region spanned by α-subunit genomic sequences can be deduced from Figure 2A. Excluding the 3′-specific fragment and the larger of the two 5′-specific fragments (6.2 kb in lane 1 of Figure 2A) because they might end far from the gene itself, a minimum of 9.3 kb + 6.8 kb (lane 2, Figure 2A) + 3.8 kb (lane 1, Figure 2A) is still needed to account for 75% of the coding and part of the 5′-non-translated regions of the mRNA. This gives a length of at least 20 kb for the gene of *T. marmorata* encoding the α-subunit mRNA, in striking contrast to the 2 kb to which it is trimmed after maturation.

Structural and developmental implications of a single gene coding for the α-subunit

The major implication of this finding is that the same gene codes for the two α-subunits which belong to the light form of the receptor. No heterogeneity in the NH_2-terminal amino acid sequence determined for purified α-subunit from *T. marmorata* (Devillers-Thiéry *et al.*, 1979), *T. californica* (Hunkapiller *et al.*, 1979; Raftery *et al.*, 1980) and higher vertebrates (Conti-Tronconi *et al.*, 1982a) and in the α-subunit cDNA sequences (Noda *et al.*, 1982; Sumikawa *et al.*, 1982; Devillers-Thiéry *et al.*, 1983) has ever been noted. Thus, most likely, this unique α-subunit gene produces a single mature mRNA species (at least in a given organ), and therefore a single polypeptide chain, rather than several ones via alternative splicing (King and Piatigorsky, 1983). On the other hand, even though each α-subunit carries at least part of one acetylcholine (and α-toxin) site, the two sites present per light form show marked differences in their affinity and/or reactivity towards reversible or irreversible cholinergic ligands (including α-toxins) (reviewed in Karlin, 1980; Changeux, 1981). These differences thus may result from the overlap of each site with a neighboring subunit which, according to the known pentameric organization of the molecule, must differ from one α-chain to the other. They may also originate from a differential post-translational modification of the two α-subunits (see Roisin *et al.*, 1983).

Another implication of the single gene organization of the α-subunit concerns the tissue specificity of the AChR. Binding studies with α-bungarotoxin revealed nicotinic AChR in skeletal muscle from *T. marmorata* (unpublished results) as expected from its common embryonic origin with the electric organ. Most likely the same gene codes for the α-subunit present in these two different tissues. Indeed, considerable homology exists between *T. marmorata* electric organ α-subunit and its counterpart in muscle over a wide evolutionary range (Merlie and Sebbane, 1981; Conti-Tronconi *et al.*, 1982a, 1982b; Momoi and Lennon, 1982; see also below). In the simplest phylogenetic picture, this implies an even closer similarity within a single species and indeed makes plausible a complete identity of the two receptors. The existence of a single gene raises the question of the nature of the tissue-specific regulations(s), (which might even include alternative splicing events), involved in the expression of the α-subunit at different levels and different localizations in muscle and electric organ.

Finally, this single gene organization has important

developmental implications. It means that, at least as far as the α-subunit is concerned, the selective localization and stabilization of the receptor protein in the subsynaptic membrane of the adult electromotor synapse do not require the differential expression of several genes, but rather result from the regulation of the expression of a single gene and thus are 'epigenetic' in nature (Changeux and Danchin, 1976). Accordingly, the differences noticed between embryonic and adult receptors (reviewed in Fambrough, 1979; Changeux, 1981) in *T. marmorata* electric organ might result from post-translational modifications of the polypeptide chains such as glycosylation (Roisin *et al.*, 1983), phosphorylation (Teichberg *et al.*, 1977; Gordon *et al.*, 1977; Saitoh and Changeux, 1981; Davis *et al.*, 1982; Huganir and Greengard, 1983), disulfide bonding between light forms (Holton *et al.*, in preparation) and from the interaction of the receptor with its membrane environment (Brenner and Sakmann, 1983), with cytoplasmic proteins such as the 43 K (ν_1) protein (Sobel *et al.*, 1978; Saitoh *et al.*, 1979; Gysin *et al.*, 1981; Cartaud *et al.*, 1981) and/or components of the basal lamina (Burden *et al.*, 1979).

Materials and methods

Materials

Adult *T. marmorata* were obtained live from the Station Marine d'Arcachon (France). Restriction endonucleases and DNA mol. wt. markers were from New England Biolabs or from Bethesda Research Laboratories. *Escherichia coli* DNA polymerase I used in nick-translation was from Boehringer (Mannheim), proteinase K was from Merck (Darmstadt), polyvinylpyrrolidone, DNase, RNase, bovine serum albumin (BSA, fraction V) and lysozyme were from Sigma. Ficoll 400 was from Pharmacia. Nitrocellulose filters (BA 85) for DNA transfer were from Schleicher and Schüll. [α-^{32}P]deoxynucleotides (400 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, UK).

Preparation of high mol. wt. genomic DNA

DNA was extracted from *T. marmorata* and chicken livers by the method of Blin and Stafford (1976), slightly modified as described by Heidmann and Rougeon (1982). After a purification step on a CsCl gradient (without ethidium bromide), DNAs were extensively dialyzed against 10 mM Tris HCl pH 7.8, 5 mM NaCl, 0.5 mM EDTA. On a 0.7% agarose gel, the genomic DNA migrated as a broad band slower than λ DNA.

Restriction enzyme digestion

50–100 μg of DNA were digested in the appropriate buffer with 4 enzyme units/μg DNA for 3 h at 37°C, 2 units/μg were then added and incubation was continued for 3 h at 37°C. Completeness of the reactions was systematically tested in three parallel experiments: (i) λ-*Hind*III restriction fragments were digested under the conditions described above; (ii) equal amounts of λ-*Hind*III fragments and genomic DNA were digested together under these same conditions; (iii) addition to the genomic DNA digest of 2 enzyme units/μg DNA for 4–12 h did not change the hybridization patterns.

Genomic DNA electrophoresis and transfer to nitrocellulose paper

Restriction reaction mixtures were ethanol-precipitated, and resuspended at ~1 μg DNA/μl. They were then electrophoresed on 0.7% agarose gels in 100 mM Tris borate pH 8.3, 2 mM EDTA, overnight at 50 V, 15 mA. DNA fragments were denatured in the gels and transferred to nitrocellulose paper by blotting with 20 x SSC (Southern, 1975). λ-*Hind*III fragments and φX174-*Hae*III fragments were used as mol. wt. markers.

Hybridization of the filters

Treatment of the filters and hybridization were performed as described by Heidmann and Rougeon (1982). Washing conditions were as detailed below (20–30 min per wash).

Low stringency. Filters were washed 3–4 times in 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na citrate), 0.5% SDS, 1 x Denhardt's (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA) solution at 65°C, then once in 0.2 x SSC, 0.1% SDS at 50°C, and finally once in 0.1 x SSC, 0.1% SDS at 40°C.

Intermediate stringency. Filters were washed 3–4 times in 2 x SSC, 0.5% SDS, 1 x Denhardt's solution at 65°C, then once in 0.2 x SSC, 0.1% SDS at 50°C, and finally once in 0.1 x SSC, 0.1% SDS at 58°C.

High stringency. Filters were washed 3–4 times in 2 x SSC, 0.5% SDS, 1 x Denhardt's solution at 65°C, then once in 0.2 x SSC, 0.1% SDS at 65°C and finally once in 0.1 x SSC, 0.1% SDS at 65°C.

The same filter was used up to three times. Dehybridization was performed in 0.05 x SSC, 0.05% SDS at 70°C for 30–40 min. Then the filter was prehybridized again, and treated as above. Autoradiography of the hybridized filters was done at −70°C under an intensifying screen for 2–20 days.

Probe preparation and labeling

DNA from the two recombinant plasmids, pα1 and pα2, was prepared by SDS lysis (Godson and Vapnek, 1973) followed by a CsCl centrifugation. DNA was digested for 2 h at 37°C, with 2 enzyme units/μg DNA, with three restriction enzymes, to yield the cDNA insert fragments described in the Results section. The resulting fragments were separated on 5% acrylamide gels in 50 mM Tris borate pH 8.3, 1 mM EDTA. Fragments 1, 2, 3, 4 (see Figure 1) were electroeluted at 100 V for 2–3 h, and ethanol-precipitated. To improve the signal intensity in hybridization experiments, fragment 4 (see Figure 1) was self-ligated with T4 DNA ligase, at a concentration of 50–100 ng DNA/μl with 1 unit ligase in 20 μl, prior to nick-translation experiment.

The cDNA fragments were nick-translated (Rigby *et al.*, 1977) using [α-^{32}P]dATP and dTTP (sp. act. ~400 Ci/mmol), resulting in 2–4 x 10^8 c.p.m./μg DNA.

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