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Identification of a cDNA clone coding for the acetylcholine binding subunit of *Torpedo marmorata* acetylcholine receptor

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A recombinant DNA plasmid has been constructed that contains sequences of the gene coding for the acetylcholine binding subunit (α-subunit, 40 000 daltons) of *Torpedo marmorata* acetylcholine receptor protein (AChR). Polyadenylated RNA purified from *Torpedo* electric organ was used to construct a cDNA library. The AChR α-subunit cDNA clone was then identified by a two-step screening of 700 recombinant clones. As AChR is present in *Torpedo* electric organ but not in *Torpedo* liver or spleen, differential screening led to the selection of 12 clones specific for the electric organ. We then tested the ability of cDNA inserts to hybridize α-subunit mRNA specifically, as judged by cell-free translation and immunoprecipitation. The insert from one clone, pα-1, selectively hybridized with a mRNA species which elicited the synthesis of a 38 000 mol. wt. polypeptide. This polypeptide was precipitated by: (1) a rabbit serum raised against purified denatured α-subunit (the pure α-subunit displaced the complex); and (2) a rat monoclonal antibody specific for the denatured α-subunit. It was thus identified as a precursor of the α chain. Blot hybridization analysis of polyadenylated RNA from *Torpedo* electric organ with the pα-1 probe revealed a major species of 2.0 kb, which thus contains ~800 non-coding nucleotides.

Key words: acetylcholine receptor protein/cDNA cloning/differential screening/hybrid mRNA selection/neurobiology

Introduction

Acetylcholine (ACh) mediates neurotransmission at the neuromuscular junction and at the electromotor synapse of fish electric organs (see Stjärne *et al.*, 1981). During the past decade, its pharmacological receptor, the acetylcholine receptor (AChR), has been purified as a well-defined protein entity which remains one of the best characterized membrane receptors for a neurotransmitter (reviewed in Changeux, 1981).

The light form of the AChR is an asymmetric pentamer of mol. wt. ~250 000 composed of four different transmembrane polypeptides of apparent mol. wts. 40 (α), 50 (β), 60 (γ), and 66 (δ) K in an $\alpha_2\beta\gamma\delta$ stoichiometry (Reynolds and Karlin, 1978; Lindstrøm *et al.*, 1979; Saitoh *et al.*, 1980; Raftery *et al.*, 1980; Kistler *et al.*, 1982). Affinity reagents for the ACh receptor site selectively label the α-subunit, which thus carries at least part of this site. The N-terminal amino acid sequences of the α-subunit (Devillers-Thiery *et al.*, 1979) and of the α, β, γ, and δ chains (Raftery *et al.*, 1980) have been established in the case of *Torpedo marmorata* and *T. californica* AChR, respectively.

Here we report the construction and identification of a recombinant plasmid, designated pα-1, which carries a DNA sequence complementary to the mRNA coding for the α-subunit from *T. marmorata* AChR. *Torpedo* electric organ was selected as starting material because of its higher content in AChR mRNA compared to mammalian muscle (Merlie *et al.*, 1978). The cDNA clone was identified by differential screening followed by analysis of the *in vitro* translation product elicited by plasmid-selected mRNA. AChR α-subunit synthesized *in vitro* was characterized by immunoprecipitation using, independently, a serum raised in rabbit against purified denatured α-subunit from *T. marmorata* AChR and a monoclonal antibody, specific for the denatured α-subunit, generously donated by S. Tzartos (Tzartos and Lindstrøm, 1980).

Results

Characterization of the anti-denatured AChR α-subunit antibodies

The *in vitro* synthesized AChR α-subunit was detected by immunoprecipitation; this was routinely achieved using an antiserum raised by injection of purified denatured α-subunit into rabbit. The purified α-subunit fraction used for immunization and later for competition experiments migrated as a single band on SDS gel electrophoresis and gave a unique N-terminal amino acid sequence (Devillers-Thiery *et al.*, 1979). The specificity of this anti-α-subunit serum was checked by immunoblotting as described by Towbin *et al.* (1979). A single band corresponding to the position of the α-subunit was stained in AChR-enriched membranes (Figure 1, lane 2). This staining was completely abolished by preincubation of the serum with a 10-fold excess of pure α-subunit (Figure 1, lane 3). Monoclonal antibody #5, known to bind specifically to denatured α-subunit (Tzartos and Lindstrøm, 1980 and Figure 1, lane 4), was used as another independent means of final characterization of the translation products.

RNA extraction and immunoprecipitation of the AChR α-subunit synthesized in vitro

Poly(A)-containing RNA was extracted from adult *T. marmorata* electric organs. Since this organ contains rather low amounts of mRNA (800 μg/kg organ), purified *Escherichia coli* RNA (~30 mg RNA/l of electric organ homogenate) was added as a carrier during the first steps of RNA extraction. *E. coli* RNA, not being polyadenylated, was subsequently eliminated during oligo-dT chromatography. This procedure yielded an mRNA preparation which elicited translation in the rabbit reticulocyte lysate system.

Poly(A)-containing RNAs isolated from electric organ were translated *in vitro* and the translation products were analysed by SDS gel electrophoresis. *In vitro* synthesized AChR α-subunit was identified among them by immunoprecipitation and subsequent gel electrophoresis. Monoclonal antibody #5 specifically precipitated a major band of apparent mol. wt. 38 K (Figure 2, lane 2) which does not appear

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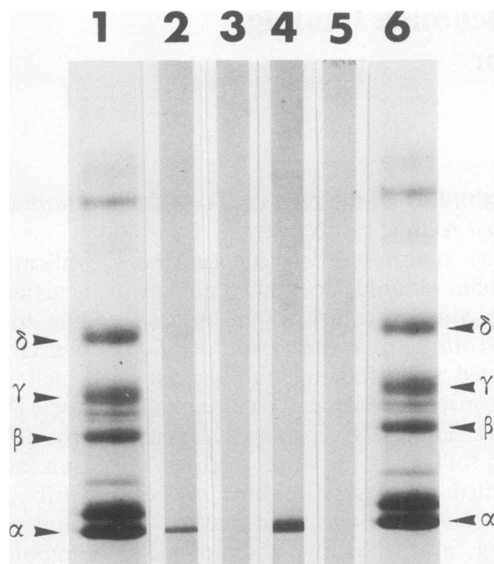

Fig. 1. Characterization of the anti-AChR α-subunit antiserum. **Lanes 1** and **6**: Coomassie blue staining of proteins separated on a SDS 10% acrylamide: 0.27% bisacrylamide gel. Loading was 50 μg of AChR enriched membrane fragments from *Torpedo* electric organ. **Lanes 2–5**: Specificity of anti-α-subunit antibodies tested by immunoblotting. AChR enriched membrane fragments (7 μg) were electrophoresed as in **lane 1** and transferred to nitrocellulose paper. Blots were incubated with antibodies and immune complexes then revealed by a second and a third antibody coupled to horseradish peroxidase. Tested antibodies were: **(lanes 2–3)**: anti-purified α-subunit rabbit antiserum, 1/100 dilution. Staining of the α-subunit indicated by an arrow was abolished **(lane 3)** when competing pure α-subunit (10 μg) was added with the first antibodies. **(Lane 4)**: monoclonal antibody # 5 (Tzartos and Lindstrøm, 1980) 1/2000 dilution. **(Lane 5)**: non-immune serum, 1/100 dilution.

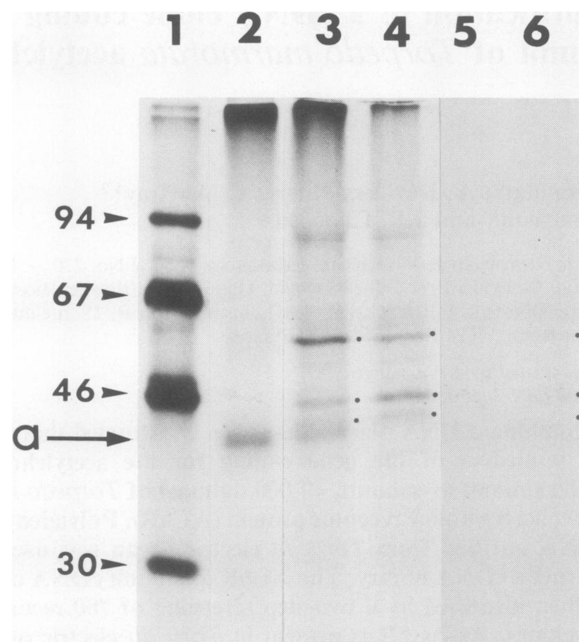

Fig. 2. Identification of *in vitro* synthesized AChR α-subunit. 0.5 μg electric organ mRNA **(lanes 2–5)** or no RNA **(lane 6)** were added to 25 μl of *in vitro* translation mixture and incubated for 1 h at 30°C. Translation products were analysed by immunoprecipitation and electrophoresis on a 10% acrylamide gel. The gel was exposed for 3 days to a preflashed Kodak X-O Mat film. Antibodies used were **(lane 2)** 0.2 μl of # 5 monoclonal antibody; **(lanes 3–4, 6)** 5 μl of anti-α-subunit rabbit antiserum; 10 μg of competing pure α-subunit were added in **lane 4**; **(lane 5)** 5 μl of non-immune serum. Mol. wts. were estimated by comparison with radioactive standards; **(lane 1)**: phosphorylase B (94 K), bovine serum albumin (67 K), ovalbumin, (46 K), and carbonic anhydrase (30 K). Black dots indicate contaminating bands.

as a major band in the pattern of total translation products. AChR α-subunit indeed represents only 0.5% of the synthesized polypeptides. The anti-α-subunit serum precipitated several bands, including the same 38-K band (Figure 2, lane 3). Simultaneous addition with the antibodies of 10 μg pure α chain specifically inhibited the precipitation of this 38-K band (Figure 2, lane 4), which can thus be identified as an α chain precursor. The difference in apparent mobility between this *in vitro* translation product and the authentic α-subunit most likely results from the absence of signal peptide cleavage and protein glycosylation in our *in vitro* translation system (Anderson and Blobel, 1981). Some of the contaminating bands present on the gel were also immunoprecipitated from a translation assay where no exogenous mRNA was added (shown in Figure 2, lane 6).

Under our experimental conditions, radioactive material was systematically observed at the top of the gels but disappeared in the presence of competing α-subunit (compare Figure 2, lanes 3 and 4) indicating that an aggregation of the *in vitro* synthesized α-subunit takes place. It is likely that this aggregation results from incomplete denaturation of the polypeptide, since, to avoid the artefactual presence of several bands for the α chain observed by Anderson and Blobel (1981), heating of the translation product in the presence of SDS was routinely omitted.

Construction and identification of cDNA clones

Electric organ mRNA was copied into double-stranded cDNA using reverse transcriptase and DNA polymerase I (Auffray *et al.*, 1980). After fractionation on a 5–20% sucrose gradient, cDNA molecules longer than 300 bp were pooled and used subsequently. cDNA was inserted into the *Pst*I site of the pBR322 plasmid using the conventional dG/dC tailing method. *E. coli* C 600 strain was transformed with the hybrid plasmids, giving rise to 700 tetracycline-resistant, ampicillin-sensitive clones.

These clones were first screened for their ability to hybridize with [^{32}P]cDNA complementary to electric organ mRNA but not with [^{32}P]cDNA complementary to *Torpedo* spleen and/or *Torpedo* liver mRNAs. These last two organs contain at least 1000 times less AChR than the electric organ, as revealed by conventional α-toxin binding site titration (Weber and Changeux, 1974). Forty-six colonies gave a signal with the electric organ probe and no signal with spleen or liver probes. These clones were then hybridized with [^{32}P]cDNA complementary to an electric organ mRNA fraction enriched in AChR RNAs. Enrichment was obtained by centrifugation of electric organ mRNA on a 5–20% sucrose gradient. Aliquots of the various fractions were translated *in vitro* and those containing AChR mRNA, as revealed by immunoprecipitation and SDS gel electrophoresis, were pooled (data not shown). The enrichment factor was estimated to be 4–5. Twelve clones gave a strong signal with that [^{32}P]cDNA probe and were further characterized. Figure 3 illustrates the results of such positive (Figure 3, A and D) and negative (B and C) hybridizations for pα-1 clone.

The plasmids corresponding to these 12 clones were individually purified and screened by the positive mRNA hybridization selection method (Ricciardi *et al.*, 1979; Ploegh *et al.*, 1980). Purified DNA of each clone immobilized on

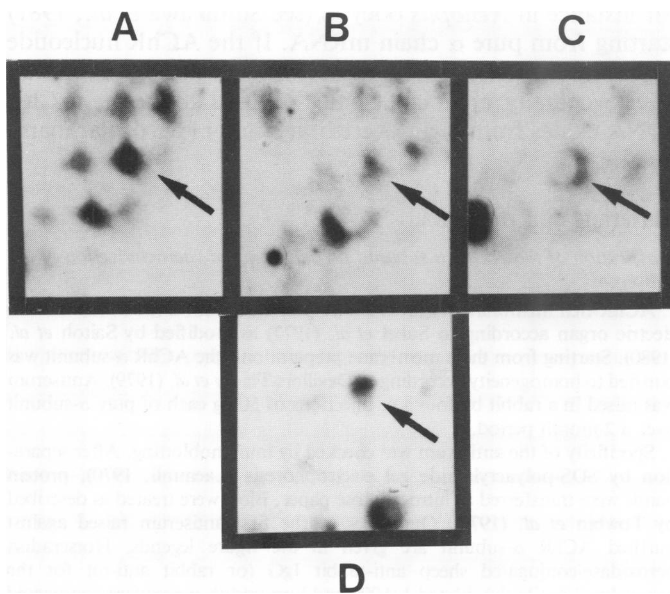

Fig. 3. Differential *in situ* colony hybridization to pα-1 clone. Colonies were grown on nitrocellulose filters on agar plates. *In situ* hybridizations were performed with ^{32}P-labelled cDNA probes complementary to *Torpedo* **(A)** total electric organ mRNA, **(B)** liver mRNA, **(C)** spleen mRNA, **(D)** electric organ mRNA fraction enriched in AChR-coding RNA. The pα-1 clone (arrows) shows a strong and selective signal with the electric organ probes. The geometrical arrangement of the clones in **D** is different from that in **A – C**.

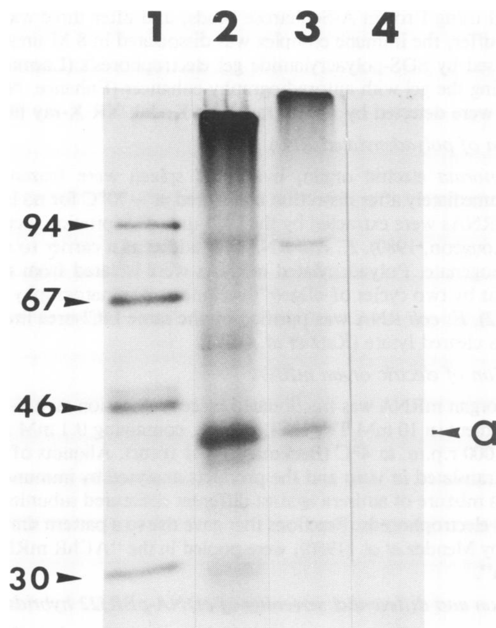

Fig. 4. Characterization of the *in vitro* translation product of plasmid pα-1 selected mRNA. pα1-plasmid DNA, immobilized on 10 nitrocellulose filters, was hybridized to 30 μg electric organ mRNA. Selected mRNA was added to 25 μl translation mixture. Aliquots of the translated products were analysed as in Figure 2: **(lane 2)** 0.2 μl of monoclonal antibody #5; **(lane 3)** 5 μl of rabbit anti-α-serum; **(lane 4)** 5 μl of non-immune serum. Exposure time was 8 days.

nitrocellulose filters was hybridized with electric organ mRNA. The dehybridized RNA was translated *in vitro* and the presence of synthesized AChR α chain detected by immunoprecipitation and SDS gel electrophoresis. One clone

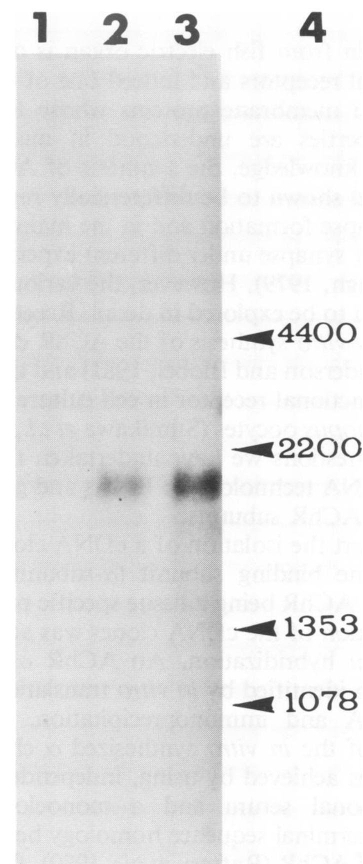

Fig. 5. Blot hybridization of pα-1 DNA to polyadenylated RNA. 0.5 μg of each RNA was denatured with glyoxal and electrophoresed on a 1% agarose gel: **(lane 1)** spleen mRNA; **(lane 2)** total electric organ mRNA; **(lane 3)** mRNA fraction enriched in AChR coding RNAs. Position of size standards: λ DNA-*Hind*III fragments and φX174 RF DNA-*Hae*III fragments are indicated in **lane 4**. Transfer and hybridization were carried out as described in Materials and methods using: 5 x 10^6 c.p.m. of pα-1 labeled DNA.

yielded a major 38-K band precipitated by both the polyclonal antiserum and monoclonal antibody #5 which ran on the gel at the same position as AChR α chain synthesized from electric organ mRNA (Figure 4, lanes 2 and 3). Some aggregated material was also observed at the top of the gel. This clone was considered specific for the AChR α-subunit and designated pα-1. The cDNA insert was excised by *Pst*I restriction enzyme and found to contain ~750 bp (data not shown).

Characterization of the mRNA coding for AChR α-subunit

Polyadenylated RNAs were denatured by glyoxal, electrophoresed on 1% agarose gels, transferred to nitrocellulose paper, and hybridized to ^{32}P-labeled pα-1 DNA. Autoradiography revealed a major RNA species present in total electric organ mRNA and in the AChR mRNA enriched fraction (Figure 5, lanes 2 and 3). This signal was due to pα-1 cDNA insert as it was not observed in parallel experiments carried out with other recombinant plasmids (data not shown). This AChR α-subunit mRNA was estimated by comparison with DNA standards to be ~2000 nucleotides long (Figure 5, lane 4). No signal could be detected with spleen mRNA (Figure 5, lane 1), thus confirming the tissue specificity of α-subunit mRNA.

Discussion

AChR protein from fish electric organ is one of the few pharmacological receptors and indeed one of the few multi-subunit integral membrane proteins whose functional and structural properties are understood in molecular terms. Thanks to this knowledge, the synthesis of AChR has been investigated and shown to be differentially regulated during embryonic synapse formation and in the maintenance of the functional adult synapse under different experimental conditions (Fambrough, 1979). However, the various mechanisms involved remain to be explored in detail. Recent reports have described the *in vitro* synthesis of the AChR chains (Mendez *et al.*, 1980; Anderson and Blobel, 1981) and the sequence of assembly of functional receptor in cell culture (Merlie *et al.*, 1981) or in *Xenopus* oocytes (Sumikawa *et al.*, 1981). To approach these questions we have undertaken the analysis by recombinant DNA technology of RNAs and genomic DNAs coding for the AChR subunits.

Here we report the isolation of a cDNA clone specific for the acetylcholine binding subunit (α-subunit) of *T. marmorata* AChR. AChR being a tissue specific protein, an efficient first selection of the cDNA clones was achieved by differential *in situ* hybridization. An AChR α-subunit clone (pα-1) was then identified by *in vitro* translation of plasmid-selected mRNA and immunoprecipitation. Unambiguous identification of the *in vitro* synthesized α chain as a 38-K polypeptide was achieved by using, independently, a mono-specific polyclonal serum and a monoclonal antibody. Despite the N-terminal sequence homology between the four polypeptides of AChR (Raftery *et al.*, 1980), the monoclonal antibody used (#5) gave a satisfactory test of specificity since, as shown by Tzartos and Lindstrøm (1980), it recognizes iodinated denatured α-subunit but not denatured β, γ, or δ chains.

Hybridization of pα-1 probe to total electric organ mRNA revealed a 2-kb species. Authentic α-subunit contains ~365 amino acid residues (Vandlen *et al.*, 1979). If one assumes the presence of a signal peptide of 25 residues on the early synthesized α chain, ~1200 nucleotides would be sufficient to code for this polypeptide. The identified mRNA species thus contains ~800 non-coding nucleotides. Two thousand nucleotides would be sufficient to code for a 50, 60, or 66 K protein. On the basis of immunological data (Tzartos and Lindstrøm, 1980) and of protein sequence data (Raftery *et al.*, 1980) the hypothesis has been raised that the four AChR subunit genes have evolved from a single ancestral DNA sequence by gene duplications. Thus, when the nucleotide sequence of the four AChR subunit mRNAs becomes available it will be interesting to determine whether these mRNAs have the same size but differ in the length of their translated region.

The pα-1 cDNA, which is 750 bp long, may contain only a small fraction of the coding sequence and possibly none of the nucleotides coding for the known 55 N-terminal amino acid residues. We thus could not use DNA sequence analysis to establish pα-1 clone specificity. Nevertheless, besides being useful for identifying longer cDNA clones and genomic DNA clones of the α chain, pα-1 might serve as an appropriate tool to investigate the regulation of the α chain biosynthesis at transcriptional and post-transcriptional levels in adult and developing *Torpedo* electric organ. Such a probe could also be used to define the intrinsic functional properties of the α chain synthesized in the absence of the other AChR subunits, for instance in *Xenopus* oocytes (see Sumikawa *et al.*, 1981) starting from pure α chain mRNA. If the AChR nucleotide sequences have been sufficiently conserved through evolution then eventually pα-1 clone may be used to isolate AChR cDNA clones from higher vertebrates and in particular mammals.

Materials and methods

Purification of the AChR α-subunit: preparation and characterization of the antiserum

AChR-rich membrane fragments were prepared from fresh *T. marmorata* electric organ according to Sobel *et al.* (1977) as modified by Saitoh *et al.* (1980). Starting from these membrane preparations, the AChR α-subunit was purified to homogeneity according to Devillers-Thiery *et al.* (1979). Antiserum was raised in a rabbit by four s.c. injections of 50 μg each of pure α-subunit over a 2-month period.

Specificity of the antiserum was checked by immunoblotting. After separation by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), protein bands were transferred to nitrocellulose paper. Blots were treated as described by Towbin *et al.* (1979). Quantities of the first antiserum raised against purified AChR α-subunit are given in the figure legends. Horseradish peroxidase-conjugated sheep anti-rabbit IgG (or rabbit anti-rat for the monoclonal antibody) diluted 1/5000 and horseradish peroxidase-conjugated rabbit anti-sheep (or sheep anti-rabbit for the monoclonal antibody) IgG diluted 1/5000 were added successively. Horseradish peroxidase reaction was revealed using 3,3′-diaminobenzidine as a substrate.

In vitro protein synthesis and immunoprecipitation

In vitro translation of mRNAs was performed using the reticulocyte lysate translation system (Pelham and Jackson, 1976) in the presence of [^{35}S]methionine. For immunoprecipitation, 25 μl translation mixture was diluted to a final volume of 75 μl containing 10 mM Tris HCl, pH 7.5, 0.15 M NaCl, 0.1% SDS, 0.5% NP40, 5 mM EDTA, 100 U/ml aprotinin, and incubated overnight at 4°C with the antibodies. Immunoglobulins were then precipitated using Protein A-Sepharose beads, and after three washes using the same buffer, the immune complex was dissociated in 8 M urea. Proteins were analysed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). After treating the gel with autoradiography enhancer (Enhance, NEN), protein bands were detected by fluorography on Kodak XR X-ray films.

Purification of polyadenylated RNA

T. marmorata electric organ, liver, and spleen were frozen in liquid nitrogen immediately after dissection and stored at −70°C for no longer than 2 months. RNAs were extracted by the LiCl-urea precipitation method (Auffray and Rougeon, 1980). *E. coli* RNA was added as a carrier to the electric organ homogenate. Polyadenylated mRNAs were isolated from total RNA preparations by two cycles of oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). *E. coli* RNA was purified by the same LiCl-urea method starting from a cleared lysate (Katz *et al.*, 1973).

Fractionation of electric organ mRNA

Electric organ mRNA was fractionated by centrifugation on a 5−20% w/v sucrose gradient in 10 mM Tris HCl, pH 7.6, containing 0.1 mM EDTA for 16 h at 40 000 r.p.m. at 4°C (Beckman SW41 rotor). Aliquots of each fraction were translated *in vitro* and the products analysed by immunoprecipitation using a mixture of antisera against different denatured subunits, followed by SDS gel electrophoresis. Fractions that gave rise to a pattern similar to that described by Mendez *et al.* (1980), were pooled in the "AChR mRNA enriched fraction".

Construction and differential screening of cDNA-pBR322 hybrids

Electric organ mRNA was transcribed into single-stranded cDNA using avian myeloblastosis virus reverse transcriptase (a kind gift of W. Beard) and oligo(dT) as a primer. The cDNA was rendered double-stranded by using *E. coli* DNA polymerase I (Rougeon and Mach, 1976). After treatment with S1 nuclease (Rougeon *et al.*, 1975), the cDNA was fractionated on a 5−20% (w/v) sucrose gradient in 10 mM Tris HCl pH 7.6, containing 1 mM EDTA, 1 M NaCl for 5 h at 50 000 r.p.m. at 20°C (Beckman SW50 rotor). The length of double-stranded cDNA was inferred by comparison with the position of φX174 RF DNA-*Hae*III fragments run in a parallel gradient. Molecules longer than 300 bp were tailed with deoxycytidine by terminal deoxynucleotidyl transferase in the presence of Co^{2+} (Rougeon and Mach, 1977). The pBR322 vector was digested with restriction endonuclease *Pst*I and tailed with deoxyguanosine in the presence of Mg^{2+} (Rougeon and Mach, 1977). The tailed molecules were hybridized and used to transform *E. coli* C 600 ($r_k^- m_k^-$). Ampicillin-sensitive, tetracycline-resistant transformants

were selected and transferred to nitrocellulose filters on agar plates containing 10 μg/ml tetracycline. The *in situ* hybridizations were performed with ^{32}P-labelled cDNA probes synthesized by reverse transcription (specific activity $0.5 - 1 \times 10^6$ c.p.m./μg) (Grunstein and Hogness, 1975). These experiments were carried out in compliance with the French guidelines on recombinant DNA research.

RNA selection on plasmid DNA immobilized on nitrocellulose filters

DNA from individual recombinant plasmids was prepared according to Katz *et al.* (1973). Supercoiled plasmid DNA was further purified on a CsCl gradient (Pays *et al.*, 1980) followed by a 5–20% (w/v) sucrose gradient in 10 mM Tris HCl pH 7.6, containing 1 mM EDTA, 0.1 M NaCl for 4 h at 40 000 r.p.m. and 20°C (Beckman SW 41 rotor). The method of Ricciardi *et al.* (1979) as modified by Ploegh *et al.* (1980) was essentially used. Plasmid DNA was digested with *Pst*I, denatured by heating for 10 min at 100°C, quickly chilled on ice, and 4 μg DNA was spotted on a 10 mm² square of nitrocellulose filter. Hybridization was for 16 h at 42°C with 30–80 μg electric organ mRNA in 300 μl of 50% formamide, 10 mM piperazine N-N′ bis(2-ethanosulfanic acid) pH 6.8, 0.4 M NaCl. The filters were then rinsed in 2 x SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate), 0.1% SDS, 8 x 5 min at 42°C, twice at 50°C and once in 2 mM EDTA pH 7.9 at room temperature. Bound RNAs were recovered by boiling the filters for 1.5 min in distilled water, and chilled on ice. 0.2 M Na acetate pH 4.5 and 5 μg yeast tRNA were added; RNA was precipitated with 2.5 volumes of ethanol at −20°C. Dried RNA was resuspended in water and used to elicit *in vitro* translation. Translation and immunoprecipitation were carried out as described above.

RNA electrophoresis, transfer, and hybridization

0.5 μg polyadenylated RNA or 0.2 μg λ DNA-*Hind*III fragments plus 0.2 μg of φX174 RF DNA-*Hae*III fragments were denatured with glyoxal (McMaster and Carmichael, 1977). Each sample was electrophoresed on 1% agarose gels, transferred to nitrocellulose paper, and hybridized to DNA probes according to Thomas (1980). Double-stranded recombinant plasmid DNA was labelled by nick-translation to a specific activity of 50 x 10^6 c.p.m./μg by using [α-^{32}P]dCTP (Rigby *et al.*, 1977). λ DNA fragments and φX174 DNA fragments labelled by nick-translation to a specific activity of 30 x 10^6 c.p.m./μg were mixed with the recombinant plasmid DNA probe during hybridization and served to estimate RNA size.

Materials

Adult *T. marmorata* were obtained live from the Station Marine d'Arcachon (France). Terminal deoxynucleotidyl transferase, DNase, DNA polymerase I, and oligo(dT)-cellulose (T7) were from PL Biochemicals (Milwaukee, WI). φX174 RF DNA-*Hae* fragments, λ DNA-*Hind*III fragments, and *Pst*I restriction endonuclease were from BRL, Caithersburg, MD. Nitrocellulose paper used for protein transfer was from Millipore, nitrocellulose (BA 85) used for DNA or RNA binding were from Schleicher and Schuell. [α-^{32}P]Deoxynucleotides (≥400 Ci/mmol) and [^{35}S]methionine (1000 Ci/mmol) were from Radiochemical Centre (Amersham, UK), 3,3′-diaminobenzidine from Sigma, horseradish peroxidase-conjugated IgG from Institut Pasteur and protein A-Sepharose was from Pharmindustrie (France). All other chemicals were from Merck.

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