Cell-Free Synthesis of Acetylcholine Receptor Polypeptides

Abstract. *Messenger RNA coding for acetylcholine receptor peptides has been identified. This polyadenylate [poly(A)$^+$] RNA from* Torpedo californica *directs, in a cell-free system, the synthesis of peptides 60,000, 51,000, 49,000 41,000, and 35,000 daltons which account for approximately 2 percent of the total synthesized proteins. The results suggest that several different messenger RNA's code for the receptor subunits. These proteins react specifically to antiserum to native acteylcholine receptor, suggesting that the primary translational product has conformational features similar to the native receptor. Further, the results support the idea that there is post-translational modification of receptor subunits as the molecular weights of the cell-free synthesized proteins differ from those of purified receptor subunits.*

Receptors for neurotransmitters and hormones are responsibile for mediating intracellular communication in higher organisms. Although these proteins have been the focus of intensive study, little information is available about the mechanisms of receptor synthesis, post-translational modifications, assembly of subunits or (in the case of cell-surface receptors) insertion of the receptor into the membrane.

The acetylcholine receptor (AChR) is an oligomeric protein which mediates synaptic transmission on binding acetylcholine released from presynaptic terminals. The interaction between acetylcholine and its receptor causes a transient increase of membrane permeability to cations resulting in a change of electrical potential across the postsynaptic membrane (*1*). Much of the recent work on membrane-bound receptors has been devoted to elucidating the AChR structure and has relied mainly on its isolation and purification, particularly from electric organs of *Electrophorus* and *Torpedo*, which constitute a rich source of receptor (*2*). In *T. californica*, the AChR has been obtained as a highly purified, detergent-solubilized form which has a molecular size of 250,000 daltons and contains four different polypeptide chains of 40,000, 50,000, 60,000, and 65,000 daltons (*3, 4*). In addition, antibodies to the purified AChR from the electric organ tissue of several mammals have been obtained; these antibodies bind to and affect the function of the receptor in situ (*5*).

Thus, the AChR appears to be ideal for initial efforts at studying a receptor (messenger RNA) mRNA. We report here that an mRNA preparation isolated from *T. californica* electric organ directs the synthesis of AChR polypeptides in a reticulocyte lysate. Analysis of these peptides gives some information about the nature of the primary translation products of receptor mRNA.

RNA was prepared from electric organs of *T. californica* (Biomarine Laboratories, Venice, California) with the use of guanidine thiocyanate, as described (*6*). RNA enriched in polyadenylated species [poly(A)$^+$] was isolated by affinity chromatography on oligodeoxythymidylate [oligo(dT)]-cellulose (Type T2, Collaborative Research, Waltham, Massachusetts) (*7*). This RNA was assayed for its capacity to direct protein synthesis in a reticulocyte lysate (*8*). The translation products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. This poly(A)$^+$-RNA directed the synthesis of several polypeptides not observed in the control lysate, which had not received poly(A)$^+$RNA (Fig. 1A). In the presence of *T. californica* RNA at 25 to 50 μg/ml, approximately 90 to 95 percent of the total [^{35}S]methionine was incorporated in electric organ polypeptides.

Polypeptides synthesized in the reticulocyte lysate were analyzed by immunoprecipitation with rabbit antiserums against (i) purified native or (ii) denatured (SDS-treated) *T. californica* AChR (*9*) Affinity-purified AChR [100 μg, prepared according to Froehner and Rafto (*10*), without *N*-ethylmaleimide] in 1 ml of phosphate-buffered saline was emulsified with 1 ml of complete Freund's adjuvant and injected into two rabbits at 15 to 20 spots along their spines. A second injection with incomplete Freund's adjuvant was made 25 days later; the rabbits were bled about 1 week later. For production of antiserum against the denatured AChR, receptor was boiled in 2 percent SDS, then administered to two rabbits as above. The activities of the antiserums to denatured and native AChR were 56, 34, 28 and 16 pmole of ^{125}I-labeled AChR bound per microliter of serum, respectively (*9*). Antigen-antibody complexes were collected by adsorption to formaldehyde-treated bacteria from the Cowan I strain of *Staphylococcus aureus* (*11*) as described (*12*). Five distinct bands were detected after specific immunoprecipitation with either of the rabbit antiserums to AChR (Fig. 1B). These bands were not observed when normal rabbit serum were used as a control. The molecular weights of these bands, determined relative to the mobilities of ^{14}C-labeled proteins from bacteriophage T4 (*13*) are 60,000, 51,000, 49,000, 41,000 and 35,000. Similar results (not shown) were obtained with goat antiserum to native AChR (*14*). Other bands corresponding to proteins with molecular weights of 63,000, 46,000, 43,000, and approximately 23,000 were also seen after immunoprecipitation with normal rabbit serum or antiserums to AChR (Fig. 1B); these bands are considered as nonspecific immunoprecipitates. As a control, cell-free products of poly(A)$^+$ RNA from *T. californica* liver (a tissue that probably does not synthesize AChR) were immunoprecipitated with antiserums to AChR and normal rabbit serum. Under these conditions, only bands corresponding to nonspecific immunoprecipitates and none of the putative AChR peptides were observed.

Table 1. Radioactivity incorporated into *T. californica* polypeptides and the percentage immunoprecipitated by antiserum to native or SDS-treated AChR.

Experiment	Total *T. californica* peptides (count/min*)	Precipitation with antiserum to			
		Native AChR		SDS-treated AChR	
		Counts per minute†	Percent‡	Counts per minute†	Percent‡
1	174,870	3,280	1.8	1,800	1.0
2	233,170	11,200	4.8	9,660	4.1
3	175,880	2,870	1.6	4,870	2.7
4	363,990			6,725	1.8
5	274,575			2,950	1.0

*The total radioactivity incorporated into protein was measured by precipitating 5-μl portions of the cell-free translation mixture with 10 percent trichloroacetic acid. The samples were spotted on Whatman 3MM filter paper disks, boiled in 10 percent trichloroacetic acid, rinsed, dried, and counted. The radioactivity incorporated into *T. californica* peptides was calculated by subtracting the counts obtained without exogenous RNA from the counts obtained with poly(A)$^+$ RNA. Incorporation depending on poly(A)$^+$ RNA amounted to 90 to 95 percent of the total radioactivity precipitable by trichloroacetic acid. †Immunoprecipitated counts per minute were obtained by spotting, on 3MM filter paper disks, 5-μl portions of samples of immunoprecipitated peptides taken in SDS sample buffer (Fig. 1B). Specific immunoprecipitated radioactivity was calculated by subtracting the value obtained with normal rabbit serum to the counts obtained with antiserum to either native or SDS-treated AChR. ‡Specific immunoprecipitated radioactivity is expressed as a percentage of radioactivity incorporated in *T. californica* polypeptides; the mean is 2.4 ± 1.4 (standard deviation) percent.

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The specific immunoprecipitated radioactivity, presumably corresponding to AChR peptides, accounts for approximately 2.4 ± 1.4 percent of the [^{35}S]-methionine incorporated into electric organ polypeptides (Table 1). If all mRNA's are translated with the same efficiency and the proportion of methionine to AChR is similar to that of the average content in the other proteins, then the data of Table 1 would indicate that 2.4 percent of the poly(A)$^+$ RNA is AChR mRNA (or mRNA's). This percentage is in reasonable agreement with the calculation, based on data of α-bungarotoxin binding by Green *et al.* (*15*), that a minimum of 0.6 percent of the total protein of *T. californica* electric organ is AChR protein.

As a further test for the specificity of immunoprecipitation of the AChR-related peptides, purified AChR from *T. californica* was added with the antiserum in the binding reaction. This competitor significantly decreased the radioactivity of the bands corresponding to specifically immunoprecipitated peptides (60,000, 51,000, 49,000, 41,000, and 35,000 daltons) but did not decrease the radioactivity in those bands corresponding to unspecific immunoprecipitates (63,000, 46,000 and 43,000 daltons) (Fig. 1C). As determined by scanning of the radioautographs, 3 μg of purified AChR (Fig. 1C. lane 4) decreased the amount of radioactivity in the putative receptor bands by 84 to 94 percent for the 35,000, 41,000, 49,000, and 60,000-dalton peptides and by 60 percent for the 51,000-dalton peptide. These data suggest that there is competition between purified AChR and the five polypeptides during the reaction with antiserum to AChR. The data, taken together, suggest that poly(A)$^+$ RNA from *T. californica* can direct the synthesis of AChR polypeptides of 60,000, 51,000, 49,000, 41,000 and 35,000 daltons. The complete absence of immunoprecipitated larger peptides suggests that these proteins reflect the primary translation products of individual AChR mRNA's.

When analyzed in the same gel, the molecular size of the immunoprecipitated peptides (see above) did not correspond exactly to those of the purified AChR subunits (~ 65,000, 60,000, 50,000 and 40,000 daltons). There is an "extra" immunoprecipitated protein made in the cell-free system and a tendency for some of the cell-free products to be smaller than the purified subunits of *T. californica*. Some of the differences in molecular size may be due to the fact that the AChR subunits undergo post-translational processing, such as glycosylation, which would result in larger sizes than those of the primary products. It is conceivable that some of the differences could be explained, if the poly(A)$^+$ RNA directs the synthesis of all the AChR subunits except that of 65,000 daltons, even though several larger proteins are synthesized (Fig. 1A, lane 2). Since the 65,000-dalton subunit appears to be in some manner protected from both the outside surface medium and the intrinsic membrane lipid components (*16–18*), there could be special requirements for its synthesis. The synthesis of incompleted polypeptide chains (*19*) as well as proteolytic degradation (*3*) should also be considered in explaining some of the molecular weight discrepancies.

Purified native receptor reacts with

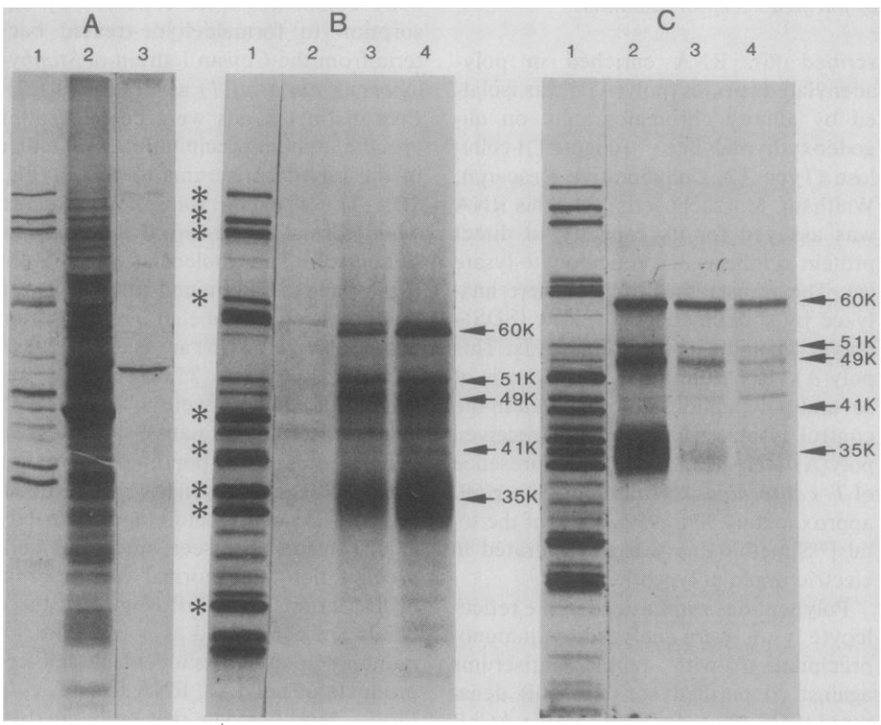

Fig. 1. (A) Autoradiographs of *T. californica* polypeptides synthesized in the reticulocyte lysate and chromatographed on polyacrylamide gels. Cell-free translations were performed in a micrococcal nuclease-digested lysate from rabbit reticulocytes (*8*). Reactions contained 10 m*M* Hepes buffer (*p*H 7.6), 100 m*M* KCl, 1 m*M* magnesium acetate, 2 m*M* guanosine triphosphate, 15 m*M* creatine phosphate, 150 μ*M* of each amino acid except methionine, 60 μg of creatine phosphokinase per milliliter, 0.9 to 1 mCi of [^{35}S]methionine (800 Ci/mmole) per milliliter, and *T. californica* poly(A)$^+$ RNA (25 to 50 μg/ml) prepared as described in the text (*6*). After being held for 60 minutes at 25° to 30°C, 2-ml samples were taken to determine trichloroacetic acid-precipitable radioactivity, and 2- to 5-μl samples were appplied to a 10 percent acrylamide-SDS gel. After electrophoresis (20 mA, 3 to 4 hours) gels were fixed, dried, and exposed to x-ray film for 3 to 10 days. (Lane 1) ^{14}C-Labeled T4 proteins used as molecular weight standards (*13*); (lane 2) polypeptides synthesized in a reticulocyte lysate containing *T. californica* poly(A)$^+$ RNA at 27 μg/ml; (lane 3) polypeptides synthesized in a reticulocyte lysate without exogenous poly(A)$^+$ RNA. (B) Autoradiographs of immunoprecipitations with antiserum to AChR. For immunoprecipitation assays, 15- to 25-μl portions of the translation mixture were diluted with phosphate buffered saline [0.025*M* sodium phosphate (*p*H 7.6), 0.1*M* sodium chloride] and Nonidet P-40 (0.5 percent; Particle Data Laboratory) was added. Antigen-antibody complexes were precipitated by absorption to formaldehyde-treated bacteria from the Cowan strain of *Staphylococcus aureus* (SAC) (*11*) following a general procedure described previously (*12*). The SAC complexes were disrupted in 50 μl of SDS sample buffer and SAC was removed by centrifugation. Portions (5 μl) of supernatant were used to count immunoprecipitated radioactivity, and the remaining sample was placed on a 10 percent acrylamide-SDS gel. (Lane 1) Molecular weight standards, as in lane 1 of (A). The molecular weights of bands marked with an asterisk are, from top to bottom: 112,000, 103,000, 95,000, 71,000, 45,000, 40,500, 36,000, 33,000, and 25,000; (lane 2) immunoprecipitates obtained with normal rabbit serum; (lane 3) immunoprecipitates obtained with serum against native AChR; and (lane 4) immunoprecipitates obtained with serum against SDS-treated AChR. (C) Autoradiographs of gels of immunoprecipitates obtained with antiserum to SDS-treated AChR after competition with nonradioactive *T. californica* AChR. Since the precipitation with antiserum to native or SDS-treated AChR gave similar results, antiserum to SDS-treated AChR was used in these experiments. Three samples, prepared as described in (B), were incubated overnight with antiserum to AChR and 0, 1.5, or 3 μg of purified *T. californica* AChR, respectively. (Lane 1) Molecular weight standards, as in lane 1 of (A); (lane 2) immunoprecipitates obtained with serum against SDS-treated AChR, without the addition of cold AChR; (lane 3) same as lane 2, but with 1.5 μg of AChR in the assay; and (lane 4) same as lane 2, but with 3 μg of AChR in the assay.

antibodies against both native AChR or SDS-treated AChR, whereas denatured purified subunits react only with antibodies against SDS-treated AChR (*20, 21*). Our results indicate that the putative AChR peptides synthesized in this system are immunoprecipitated by both antiserums. This would indicate that the conformation of the cell-free synthesized peptides is similar in terms of antigenic determinants to those present in the native AChR. However, experiments with ^{125}I-labeled α-bungarotoxin have failed to show binding of the toxin to the in vitro synthesized peptides.

The above system constitutes a simple assay for the detection of mRNA coding for AChR peptides and provides a method for the study of the mechanisms of AChR biosynthesis. It may eventually allow the purification of any AChR mRNA's that would made the synthesis of complementary corresponding DNA's feasible; the latter can be used as (i) genetic probes in studies of AChR genes, transcription, and mRNA processing and degradation and (ii) for detailed analysis of the primary structure of these peptides by DNA sequencing.

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Viviparity and Intrauterine Feeding in a New Holocephalan Fish from the Lower Carboniferous of Montana

Abstract. *A new species of Lower Carboniferous holocephalan chondrichthyan,* Delphyodontos dacriformes, *is described from two fetal specimens. The well-developed slashing and piercing dentition, enlargement of the abdominal region, and fecal material indicate the probable evolution of intrauterine oophagy and viviparity in Paleozoic Chondrichthyes.*

Viviparity has been extensively documented among members of the chondrichthyan Elasmobranchii (*1*), osteichthyan Teleostei (*2*), modern Coelacanthiformes (*3*), and urodele and caecilian amphibians (*4*), but all known modern chondrichthyan holocephalan fish are reported to be oviparous (*1*). A new fetal holocephalan from the Lower Carboniferous of Montana has slashing and piercing dentition and evidence of a greatly enlarged, functioning gut, indicating that viviparity was a significant adaptive feature among the Paleozoic chondrichthyans, irrespective of their phylogenetic status.

The Bear Gulch Limestone assemblage contains 69 known species of fish, of which 35 species are chondrichthyans. Preservation of whole bodies is excellent in the fine-grained matrix, and growth series are known for several chondrichthyan species (*5*). Two fetal specimens of a chondrichthyan from this assemblage are provisionally assigned to the Holocephali on the basis of cranial morphology, tooth plate numbers, body form, and incipient cranial ornamentation. The large size of the fetuses, specialized dentition, and lack of developed fins show that they are unrelated to any of the other five holocephalans in the Bear Gulch assemblage with similar features.

Class: Chondrichthyes

Subclass: Bradyodonti

Infraclass: Holocephali

Delphyodontos, n. gen.

Type species: Delphyodontos dacriformes, n. sp.

Diagnosis: A holocephalan with a blunt, large head, a small, ventroterminal mouth, and a body with a horizontal ventral outline and a dorsal outline sloping evenly down from its highest point immediately behind the head. The squamation is complete and of placoid denticles. There are two tooth plates in each upper jaw and one tooth plate in each lower jaw. Upper tooth plates are sharply bladed and composed of high, coarsely serrated cusps; lower tooth plates each contain three compressed, simple recurved cusps on a convex basal lamina. Cusps on the posterior upper and lower plates increase in size in an anteroposterior direction corresponding to the order of their addition to the plates. Enlarged denticles are present along the postorbital arcade, and there is evidence of incipient paired occipital and mandibular spines. Differentiation of hypochordal caudal radials is seen, but no other fins are differentiated. Size at birth is believed to exceed 35 mm.

Delphyodontos dacriformes, n. sp.

Type specimen: University of Montana 6148.

Referred specimen: Carnegie Museum of Natural History 35455.

Horizon and locality: Bear Gulch Limestone, Heath Formation, Namurian E_2B, south of Becket, Fergus County (University of Montana vertebrate locality 7106).

Diagnosis: The same as for the genus.

Derivation of name: Delphyodontos (womb tooth) *dacriformes* (teardrop-shaped body).

Morphology: The holotype is 35 mm long, preserved outstretched in lateral view, and shows only a slight ventral expansion in the abdominal region (Fig. 1). Outlines of calcified cartilage on the head indicate a short rostrum, large, dorsally unrestricted orbits, and a mouth of relatively short gape and holostylic suspension (Fig. 2), as in the Chimaeriformes. The postorbital region of the braincase is relatively longer than that of the Chimaeriformes (*5*); chimaeroids are also not known to possess paired occipital, postorbital, or mandibular spines, although these are known in related extinct groups (*5, 6*).

The second specimen is 29 mm long, fully squamated, and preserved in lateral view curved around a fully squamated, rounded protrusion of the abdominal region resembling a yolk sac (Figs. 3 and 4). Eye and branchial pigments are preserved, as is a bolus of material, at the rear of the abdominal region, that

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