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cDNA That Encodes Active Agrin

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Summary

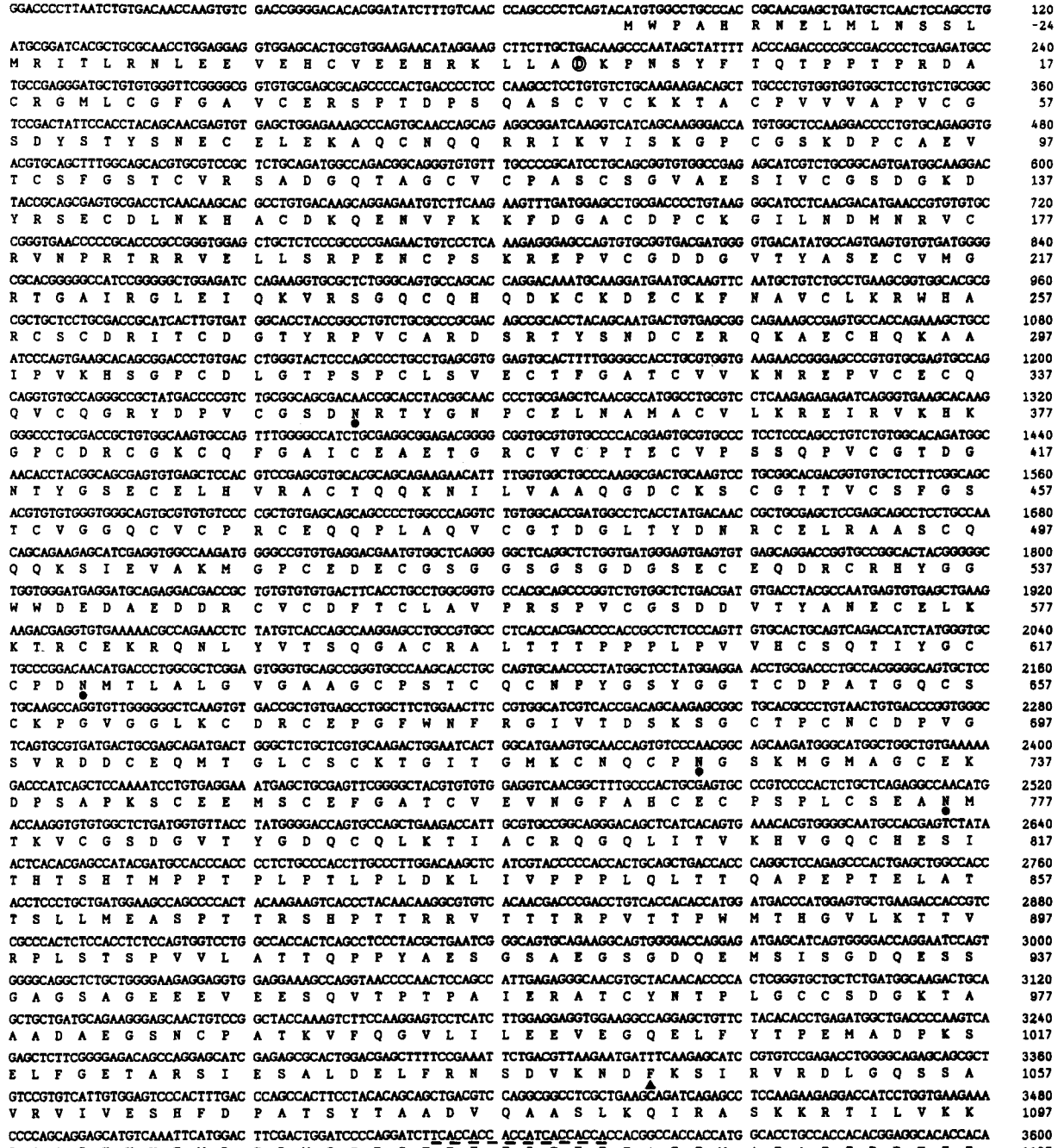
Agrin is thought to mediate the motor neuron–induced aggregation of AChRs and AChE on the surface of muscle fibers at neuromuscular junctions. We have isolated a cDNA from a chick brain library that, based on sequence homology and expression experiments, codes for active agrin. Examination of the sequence reveals considerable similarity to homologous cDNAs previously isolated from ray and rat libraries. A conspicuous difference is an insertion of 33 bp in chick agrin cDNA, which endows the encoded protein with AChR/AChE aggregating activity. Homologous transcripts having the 33 bp insertion were detected in the ray CNS, which indicates that an insertion of similar size is conserved in agrin in many, if not all, vertebrate species. Results of in situ hybridization studies and PCR experiments on mRNA isolated from motor neuron–enriched fractions of the spinal cord indicate that, consistent with the agrin hypothesis, motor neurons contain transcripts that code for active agrin.

Introduction

Several lines of evidence indicate that both the axon terminal- and the basal lamina–induced formation of the postsynaptic apparatus on developing and regenerating muscle fibers is mediated by the protein agrin (Reist et al., 1987; Nitkin et al., 1987; Magill-Solc and McMahan, 1988). According to the agrin hypothesis (Nitkin et al., 1987; McMahan, 1990), agrin is synthesized in the cell bodies of motor neurons in the brain and spinal cord and transported along their axons to muscle. Once at the muscle, it is externalized by the axon terminals to bind both to the basal lamina in the synaptic cleft and to agrin receptors on the muscle fibers, triggering the muscle fibers to form aggregates of acetylcholine receptors (AChRs), acetylcholinesterase (AChE), and other molecules constituting the postsynaptic apparatus. Agrin may also mediate the formation of postsynaptic protein aggregates at neuron–neuron synapses in the central nervous system (McMahan, 1990; M. A. Smith, C. Magill-Solc, F. Rupp, Y.-M. M. Yao, J. W. Schilling, P. Snow, and U. J. McMahan, submitted).

We have undertaken to make genetic probes that recognize agrin transcripts, an important step toward testing directly the agrin hypothesis and examining in detail the regulation of agrin's synthesis and its mechanism of action. Because agrin purified from extracts of the synapse-rich electric organ of the marine ray (Torpedo californica) has been best characterized, these studies began with the isolation of a partial marine ray cDNA (from a Discopyge ommata library) that codes for a protein both recognized by anti-agrin antibodies and having a stretch of sequence similar to the N-terminus of purified Torpedo agrin (Smith et al., submitted). In situ hybridizations revealed that the cell bodies of motor neurons contain mRNA which encodes similar proteins (Magill-Solc and McMahan, 1990). However, it was not determined whether the cDNA encoded a protein having AChR/AChE aggregating activity, if so whether the activity involved the same mechanisms as those used by agrin, and whether motor neurons contained transcripts for that specific protein. Such problems are of interest because AChR aggregation can be induced in myotubes by many different agents, some of which are clearly nonphysiological and act through mechanisms that differ from those used by agrin. Moreover, the possibility that agrin belongs to a family of proteins differing in activity and distribution has been repeatedly suggested because inactive proteins antigenically similar to agrin are immunoprecipitated from extracts of electric organ and other tissues (Nitkin et al., 1987; Godfrey, 1991) and because anti-agrin antibodies stain the basal lamina of structures in the central nervous system, muscle (Reist et al., 1987; Magill-Solc and McMahan, 1988; Fallon and Gelfman, 1989), and a variety of other tissues (Godfrey et al., 1988; Godfrey, 1991) that are not associated with synapses. Indeed, we present elsewhere (Ruegg et al., 1992) direct evidence for such a family. Recently, the ray cDNA has been used to isolate a cDNA that encodes a homologous protein in rat (Rupp et al., 1991), and expression experiments have demonstrated that the protein is active in aggregating AChRs in an in vitro assay (Campanelli et al., 1991). In rat, as in ray, it is clear that motor neurons contain agrin-like transcripts. However, the localization of the specific transcripts that encode the active protein and the nature of the protein's mechanism of action remain to be determined.

Here we describe a full-length chick cDNA that codes for a protein which is both homologous to the agrin-like proteins encoded by ray and rat cDNAs and recognized by anti-agrin antibodies. As shown for ray and rat, in situ hybridizations result in intense labeling of motor neurons. However, we demonstrate further by transfection experiments that the C-terminal half of the protein, which is encoded by the cDNA CBA-1 and is known to bear agrin's active domain, has AChR/AChE aggregating activity that is indistinguishable from that of authentic T. californica agrin. CBA-1 differs from the ray and rat cDNAs and from an agrin-related chick cDNA that codes for an inactive protein (Ruegg et al., 1992), by the presence of a 33 bp stretch that codes for 11 amino acids. This short stretch of amino acids is required for chick agrin's activity



(Ruegg et al., 1992). Polymerase chain reaction (PCR) experiments aimed at amplifying homologous transcripts having the 33 bp insert revealed that such transcripts are conserved across species and that motor neuron-enriched fractions of chick spinal cord are enriched in such transcripts, as predicted by the agrin hypothesis.

Results

Cloning and Functional Expression of CBA-1

An oligo(dT)-primed embryonic day 13 (E13) chick brain cDNA library, constructed in the phage λgt10, was screened by cross-hybridization with the ray (D. ommata) agrin-related cDNA, OL4 (Smith et al., submitted). Five clones among the 10^6 examined were labeled; CBA-1 was the longest.

CBA-1 is composed of 4733 bp having a single open reading frame of 2622 bp and a 3′ untranslated region of 2111 bp followed by a poly(A) tail (Figure 1). CBA-1 lacks a 5′ untranslated region, an initiator methionine, and an N-terminal signal sequence characteristic of membrane-spanning and secreted proteins, indicating that the cDNA is incomplete at its 5′ end. The open

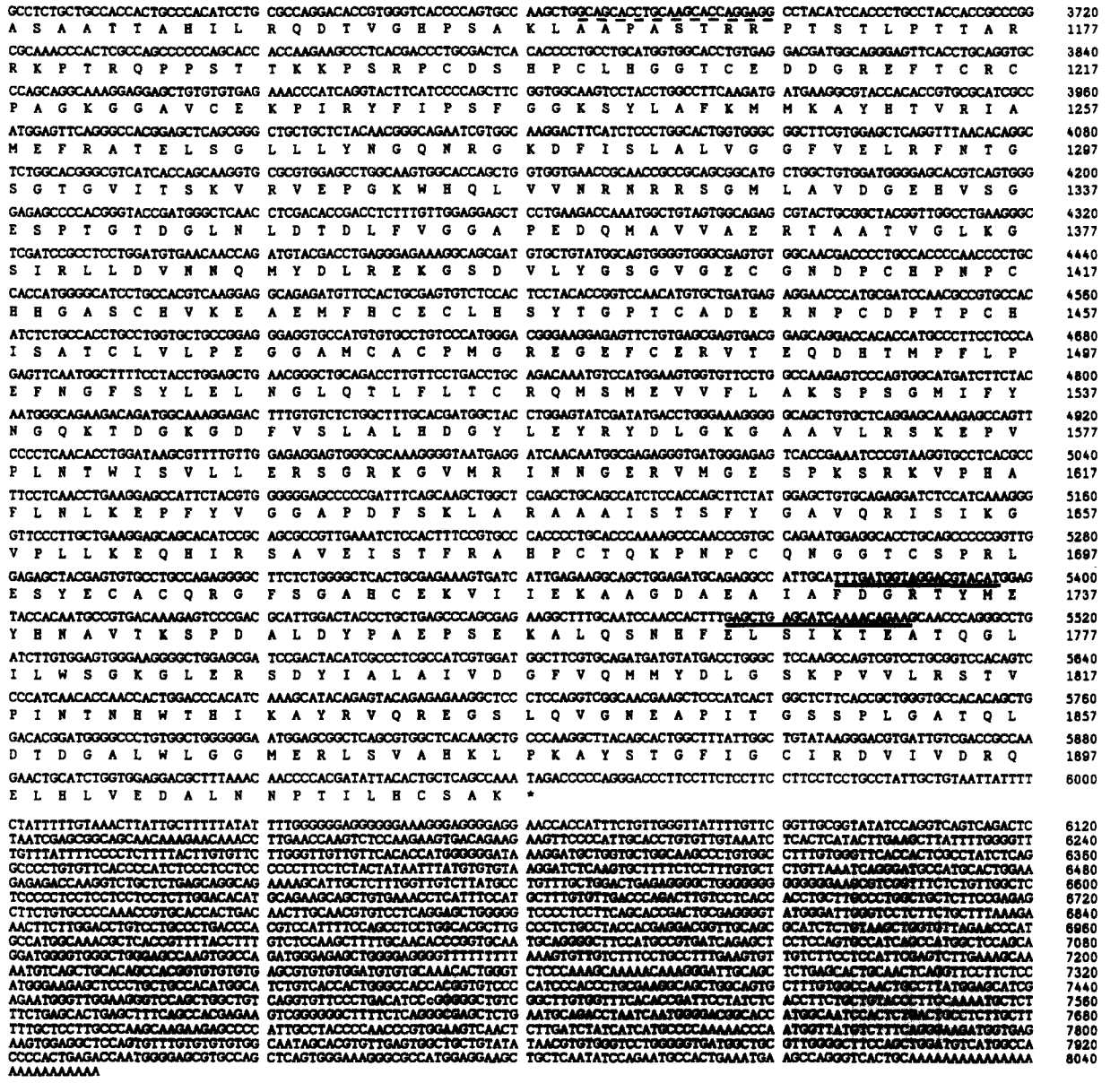


Figure 1. Nucleotide and Deduced Amino Acid Sequences of Chick Agrin

Positive numbering corresponds to the amino acids of the mature protein; the predicted N-terminus is at the circled D. Negative numbering of amino acids indicates the signal peptide. The amino acid sequence encoded by CBA-1 begins at Phe-1044 (arrowhead). Potential N-glycosylation sites (circles), oligonucleotides used for making the primer extension library (dashed line), and primers used for PCR (solid line) on the 11 amino acid stretch in motor neuron and non-motor neuron-enriched fractions are as indicated.

reading frame of CBA-1 encodes a polypeptide having 874 amino acids with a predicted M_r of 95,377. When aligned by the FASTA program (Pearson and Lipman, 1988) the amino acid sequence predicted by CBA-1 is 64% identical to that predicted by the corresponding length of the ray cDNA OL4 and 58% identical to that of the corresponding length of the agrin-related protein in rat.

CBA-1 has several structural motifs in common with ray and rat cDNAs; these are discussed in the section on the complete cDNA sequence (see below). A region of the nucleotide sequence in CBA-1 that is not present in the homologous ray and rat cDNAs described thus far is a 33 bp insert, spanning nucleotide 5424–5457 (Figure 2). This sequence codes for an 11 amino acid stretch of the protein that is required for AChR/AChE aggregating activity (Ruegg et al., 1992; see also Discussion). Examination of GenBank (Release 67.0) by the FASTA program revealed that the 11 amino acid sequence does not bear close identity to domains noted in other proteins. To learn whether this region is conserved in agrin across species, we sought to determine by PCR whether mRNA from the ray's brain contains transcripts for a homologous pro-

Chick/CBA-1$_{(1726\text{-}1777)}$	EAIAFDGRTYMEYHNAVTKSPDALDYPAEPSEKALQSNHFELSIKTEATQG
Ray/PCR$_{(17\text{-}68)}$	ESVAFNGRTFIEYHNTVTRSPEFMDYPMEQSEKAVQVNYFEMSIKTEATQG
Ray/OL4$_{(1147\text{-}1187)}$	ESVAFNGRTFIEYHNTVTRS EKAVQVNYFEMSIKTEATQG
Chick/ARP-1$_{cDNA}$	EAIAFDGRTYMEYHNAVTKS EKALQSNHFELSIKTEATQG
Rat$_{(1761\text{-}1801)}$	ETLAFDGRTYIEYLNAVIES EKALQSNHFELSLRTEATQG

Figure 2. Alignment of the Region of Chick Agrin Containing the Distinctive 11 Amino Acid Stretch with Corresponding Regions of Agrin Homologs

The 11 amino acid stretch encoded by CBA-1 is present in protein encoded by the PCR product from ray mRNA, but is not present in proteins encoded by agrin-related protein-1$_{cDNA}$ (ARP-1$_{cDNA}$), OL4, and a corresponding cDNA from rat. Identical residues are in bold type. Subscripts indicate amino acid numbers.

tein having such a sequence. Indeed, mRNA coding for the 11 amino acids was identified in D. ommata brain extracts (Figure 2). Six out of the 11 amino acids (54%) were identical to those in the 11 amino acid stretch in the chick protein; 3 were conservative substitutions. Transcripts are present in the chick brain that encode agrin-like proteins similar to those previously described for ray and rat, i.e., lacking the 11 amino acids. This has been demonstrated by examining the nucleotide sequence of another chick clone, agrin-related protein-1$_{cDNA}$ (Ruegg et al., 1992), which was identified among those in our initial screen with OL4 (see above). Agrin-related protein-1$_{cDNA}$ is nearly the same length as CBA-1, 4545 bp versus 4733 bp, and its sequence is identical to that of CBA-1 except for the absence of the 33 bp stretch (Figure 2).

The protein encoded by CBA-1 includes sequence homologous to the entire 95 kd form of active agrin in electric organ extracts (Smith et al., submitted), which suggested to us that it might be active in AChR/AChE aggregation assays. To determine whether CBA-1 encodes a protein functionally as well as structurally similar to agrin, we coupled its 5′ end to an initiation site of translation and a signal sequence. This construct was inserted into an expression vector and transfected into COS-7 cells (Gluzman, 1981). Protein secreted by the COS-7 cells was assayed in chick myotube cultures for AChR/AChE aggregating activity. As illustrated in Figure 3, when the COS-7 cells were cocultured with myotubes, they induced the myotubes to form aggregates of AChRs on their surface. Medium that had been conditioned by the transfected COS-7 cells also induced myotubes to form AChR aggregates (Figure 4). The active protein in the conditioned medium was immunoprecipitated (Figure 5) by monoclonal antibody (MAb) 5B1, which was raised against T. californica agrin and cross-reacts with chick agrin (Reist et al., 1987; Magill-Solc and McMahan, 1988). Medium conditioned by COS-7 cells transfected either with the vector alone (Figure 4) or with an out-of-frame construct was inactive.

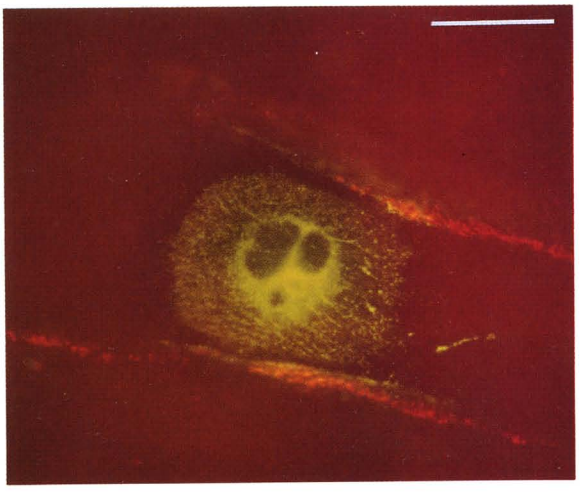


Figure 3. Protein Secreted by COS-7 Cells Transfected with a CBA-1 Construct Induces AChR Aggregation on Chick Myotubes in Cocultures

The COS-7 cell (green) was stained with anti-agrin MAb 5B1 followed by fluorescein-conjugated secondary antibodies. AChR aggregates (red) were stained with biotinylated α-bungarotoxin and rhodamine-labeled streptavidin. Bar, 50 μm.

The active protein in the medium conditioned by COS-7 cells transfected with CBA-1 induced the formation of AChR aggregates in a dose-dependent way, as does T. californica agrin (Godfrey et al., 1984). Moreover, the maximal number of aggregates induced by conditioned medium was not increased by the simultaneous addition of T. californica agrin to myotube cultures, indicating that the secreted protein acted by the same mechanisms as those used by agrin (conditioned medium, 16.3 ± 0.7 AChR aggregates per myotube segment; conditioned medium + 2 U of T. californica agrin, 17.2 ± 0.5 AChR aggregates per myotube segment; mean ± SEM, N = 3). The aggregates had the same size (on average ~15 mm^2), shape (ovoid), and distribution (primarily on the surface of the myotubes facing the substrate; Wallace, 1988) as those induced by T. californica agrin (Figures 4A and 4B). Like the aggregates induced by T. californica agrin (Godfrey et al., 1984), those induced by conditioned medium were formed by lateral migration of AChRs in the plasma membrane of the cultured myotubes; when AChRs were labeled with rhodamine-conjugated α-bungarotoxin prior to the addition of conditioned medium, there was a 3.5-fold increase in AChR aggregates over control. Conditioned medium that induced the aggregation of AChRs also induced the

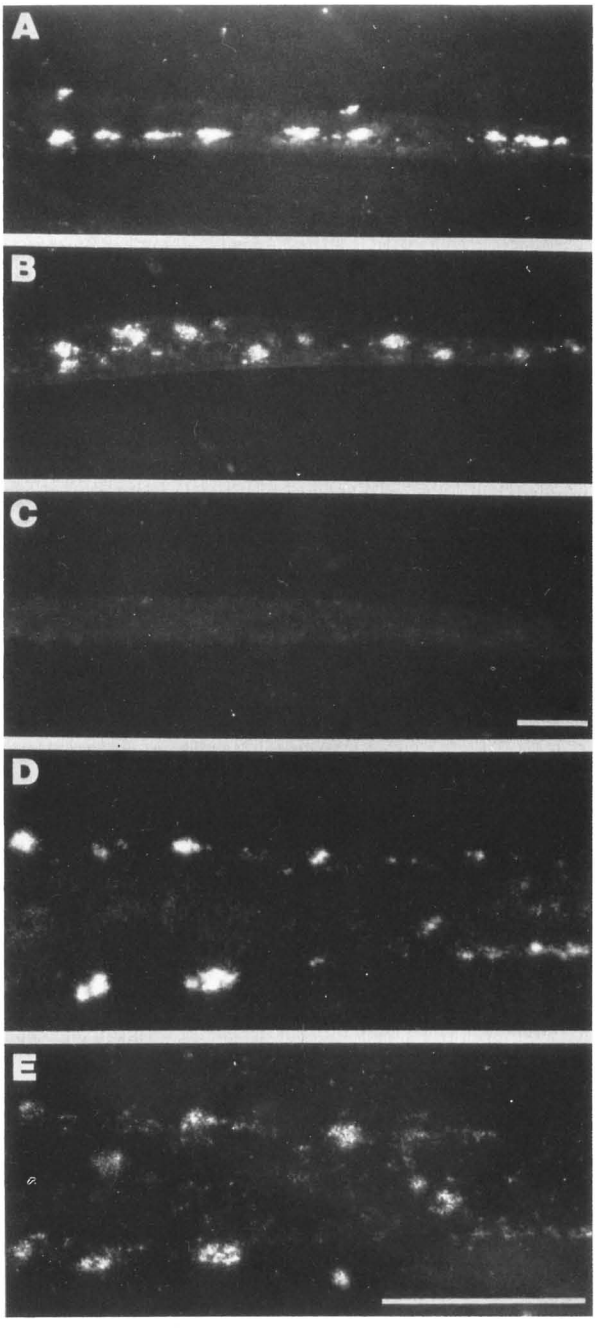


Figure 4. The Secreted Protein from COS-7 Cells Transfected with CBA-1 Induces Myotubes to Form Coextensive AChR and AChE Aggregates Identical to Those Induced by Agrin Purified from T. californica Electric Organ

Five-day-old cultures of chick myotubes were incubated overnight with test samples added to 1.5 ml of medium. AChR aggregates were labeled with biotinylated α-bungarotoxin and rhodamine-labeled streptavidin. AChE aggregates were labeled with an anti-AChE MAb and fluorescein-conjugated secondary antibodies. (A) Purified Torpedo agrin (4 U); (B) conditioned medium (0.2 ml) from COS-7 cells transfected with CBA-1; (C) conditioned medium (0.2 ml) from COS-7 cells transfected with expression vector alone; (D and E) photographs of a myotube that had been incubated with the conditioned medium from COS-7 cells transfected with CBA-1 and labeled with rhodamine-conjugated α-bungarotoxin (D) and anti-AChE MAb (E). (A, B, and C) Same magnification; (D and E) same magnification. Bars, 30 μm.

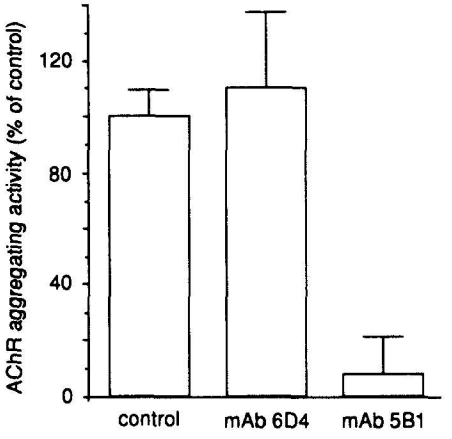


Figure 5. The Protein Secreted by COS-7 Cells Transfected with CBA-1 Is Immunoprecipitated by an Anti-Agrin MAb

Conditioned medium was incubated with two antibodies to T. californica agrin: MAb 6D4, which does not cross-react with chick agrin, or with MAb 5B1, which does cross-react with chick agrin (Reist et al., 1987; Magill-Solc and McMahan, 1988). Values are expressed as percentage of control (equal volume of conditioned medium without antibody treatment), mean ± SEM, N = 3.

myotubes to form aggregates of AChE; the AChE aggregates tended to be coextensive with the AChR aggregates (Figures 4D and 4E), as are those induced by agrin (Wallace, 1986). Thus transfection of COS-7 cells with CBA-1 resulted in the synthesis of a secreted protein that induced the formation of AChR and AChE aggregates indistinguishable from those induced by authentic T. californica agrin both in the manner of their formation and in their appearance.

Localization of Agrin-like Transcripts in Chick

Northern blot analysis using cDNA derived from CBA-1 (nucleotide 3322–4220; Figure 1) to probe for corresponding transcripts in different chick tissues provided results comparable to those obtained in similar studies on transcripts from ray (Smith et al., submitted) and rat (Rupp et al., 1991) tissues. Such transcripts were observed in poly(A)$^+$ RNA isolated from brain, spinal cord, and muscle of E16 chicks (Figure 6). The transcripts were less concentrated in mRNA isolated from muscle than in mRNA from brain and spinal cord. They were not detected in mRNA from liver; low levels of such transcripts have been observed in mRNA extracts from rat liver. The length of the corresponding transcripts in all cases was ~8 kb, nearly the same size as homologous transcripts in rat; ray tissues have two transcripts, 9.5 and 7.5 kb.

In situ hybridizations using antisense RNA generated from CBA-1 revealed intense labeling of the cell bodies of motor neurons in both brain and spinal cord (Figure 7). Such labeling was observed in the lumbosacral region of the spinal cord as early as E5–E6, the time at which motor neurons in this region begin to make neuromuscular synapses, and it was detected up to 1 month after hatching (the latest time

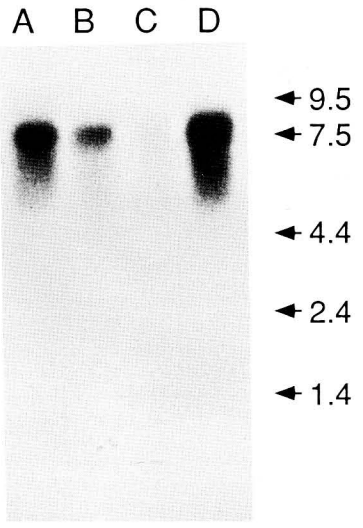


Figure 6. Northern Blot Analysis of Agrin and Agrin-Related Transcripts from E16 Chick Tissues

Spinal cord (lane A); muscle (lane B); liver (lane C); brain (lane D). Labeled transcripts are ~8 kb. They are most abundant in brain and spinal cord; none was detectable in liver. Size markers are in kilobases.

examined). Thus motor neurons contain agrin-like mRNA throughout the period of synapse formation (see, for example, Bourgeois and Toutant, 1982; Dahm and Landmesser, 1991). The intense staining of motor neurons by antisense probes that recognize agrin-like mRNA has also been observed in ray and rat (Magill-Solc and McMahan, 1990; Rupp et al., 1991; Smith et al., submitted). As noted for ray (Smith et al., submitted), other groups of neurons in chick CNS were also heavily labeled, and there was a low level of labeling in white matter of spinal cord, where the only mRNA is in glial and endothelial cells.

The probes used for Northern blots and in situ hybridizations did not distinguish between mRNAs with and without the 33 bp found in CBA-1. To determine whether motor neurons contain transcripts having the 33 bp insert, we made the following experiment. We first separated motor neurons from other cells of E6 chick spinal cords. The separation procedure resulted in two fractions: one that was enriched in motor neurons and one that was enriched in non-motor neurons (Dohrmann et al., 1986). cDNA was synthesized from poly(A)$^+$ RNA isolated from each fraction and subjected to PCR using oligonucleotides flanking the 33 bp insert in CBA-1. As illustrated in Figure 8, in the motor neuron-enriched fraction the amplification product (129 bp) containing the 33 bp insert was 4-fold enriched over the amplification product (96 bp) lacking the 33 bp. In the non-motor neuron-enriched fraction the PCR product lacking the 33 bp insert was 3-fold more abundant than the product having the 33 bp. The correlation of motor neuron enrichment with 33 bp enrichment in preparations from E6 chick indicates that motor neurons contain

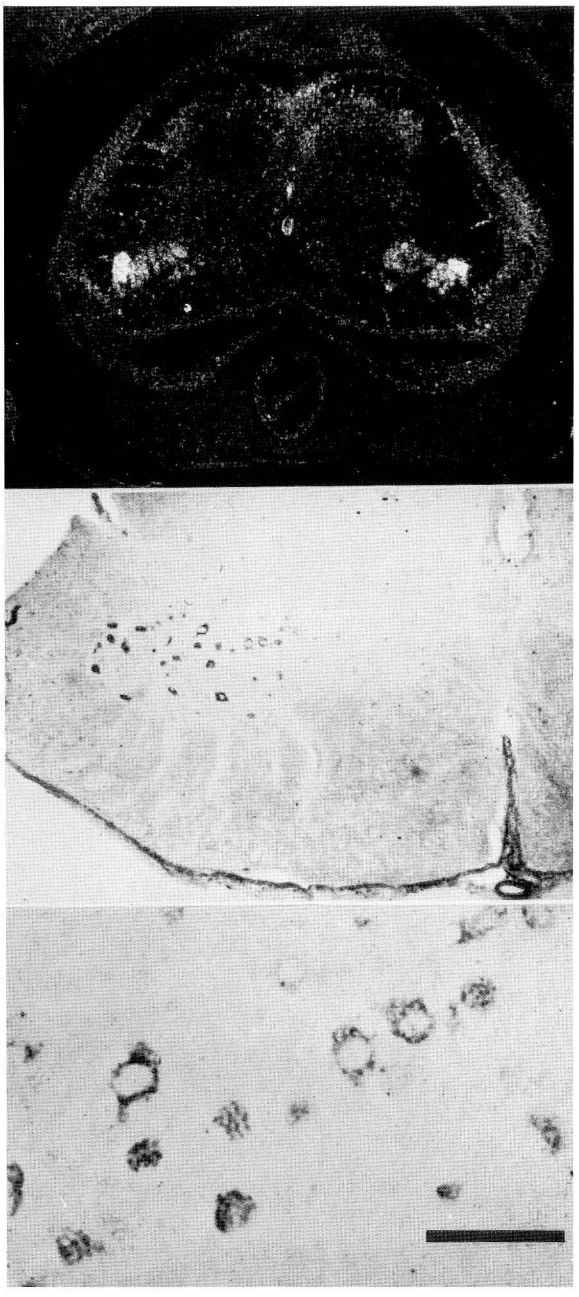


Figure 7. Localization of Agrin and Agrin-Related mRNA in Spinal Cord by In Situ Hybridization

(Top) Dark-field micrograph of an autoradiogram of a cross section from the lumbar region of the spinal cord of an E10 chick after hybridizing with ^{35}S-labeled agrin/agrin-related antisense RNA. The ventral horns of the spinal cord are heavily labeled. Less intense labeling was also observed in the ventral horn of E5-E6 spinal cords. (Middle) Bright-field micrograph of a cross section through a ventral quadrant of a chick spinal cord taken 1 month after hatching. Hybridization was done with digoxigenin-linked agrin antisense RNA, and the distribution of complementary transcripts was made visible by the anti-digoxigenin alkaline phosphatase immunohistochemical method. Cells in the ventral horn and at the spinal cord-pial interface are heavily labeled. (Bottom) Higher magnification of the section in the middle panel showing that the labeled cells in the ventral horn have the characteristic size and shape of motor neurons. Bar, 125 μm (top); 90 μm (middle); 20 μm (bottom).

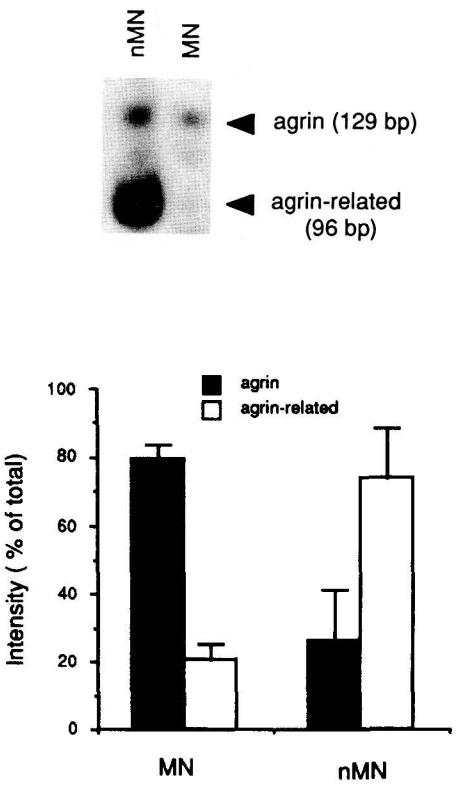


Figure 8. The Motor Neuron-Enriched Fraction of the Spinal Cord Is Enriched in mRNA Having the 33 bp Characteristic of Agrin Transcripts; the Non-Motor Neuron-Enriched Fraction Is Enriched in mRNA Lacking the 33 bp

(Top) The 96 bp product corresponds to agrin-related transcripts, lacking the 33 bp stretch, and the 129 bp product corresponds to agrin transcripts that have the 33 bp stretch. (Bottom) Relative amount of each PCR product is compared with the total amount of both products as determined by densitometry of autoradiograms from three different experiments. In the motor neuron-enriched fractions (MN), the agrin transcript is ~4-fold more abundant than agrin-related transcripts; while in non-motor neuron-enriched fractions (nMN), the agrin-related transcripts are ~3-fold more abundant than agrin transcripts. The amount of mRNA used differed between lanes. Values are mean ± SEM.

transcripts which encode active agrin and that such transcripts are present at the time when neuromuscular junctions are beginning to form.

A Complete cDNA Sequence

To obtain a full-length sequence for chick agrin and to compare chick agrin more fully with its homologs in rat and ray, a primer extension library was constructed in λgt10 using poly(A)$^+$ RNA isolated from E13 chick spinal cords and two oligonucleotides generated from the 5′ end of CBA-1 (Figure 1, dotted underlines). The library was then screened with a PCR product spanning nucleotides 3322–3555 in CBA-1. This resulted in the identification of a 3 kb cDNA (PE-1) that partially overlapped with CBA-1. The primer extension library was rescreened with PE-1, and two additional partially overlapping cDNAs, PE-2 and PE-3, were obtained (Figure 9).

The sequence constructed by aligning CBA-1, PE-1, PE-2, and PE-3 is 8051 nucleotides long and contains an open reading frame encoding a protein of 1917 amino acids, with a calculated M_r of 206,695 (Figure 1). The predicted initiation codon is the first ATG (at nucleotide 76) and is followed by a putative signal peptide of 38 amino acids at the N-terminus and a predicted cleavage site that conforms to empirical rules (von Heijne, 1986). As observed for that of other basal lamina molecules, the putative signal peptide does not show strong hydrophobicity (Pikkarainen et al., 1988). The upstream sequence of the first methionine contains an in-frame termination codon (TGA at nucleotide 16–19) which is strong evidence that the cDNA encodes a "full-length" protein. The cDNA has 75 bp of 5′ and 2111 bp of 3′ untranslated sequence. Moreover, the length of the complete cDNA is in good agreement with the ~8 kb length of complementary transcripts detected by Northern blot analysis of mRNA extracted from chick spinal cord as described above.

As observed for deduced amino acid sequences of agrin homologs in ray and rat, the amino acid sequence of the chick protein has several structural motifs common to other extracellular matrix and secreted proteins (Figure 9). These motifs are based on the number and spacing of cysteine residues, of which the N-terminal half of the protein is particularly rich. – There are nine domains, having 6 cysteine residues,

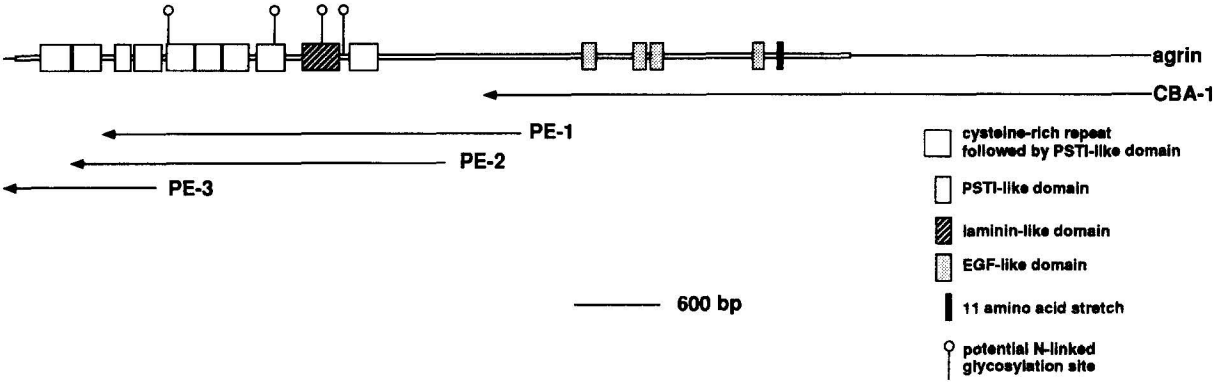


Figure 9. Structural Map of Chick Agrin and the Relationship of CBA-1 to Primer-Extended Clones PE-1, PE-2, and PE-3

In the structural map, the coding region is indicated by a double line; the untranslated regions, by a single line. The third PSTI-like domain can alternatively be viewed as an extension of the fourth PSTI-like domain.

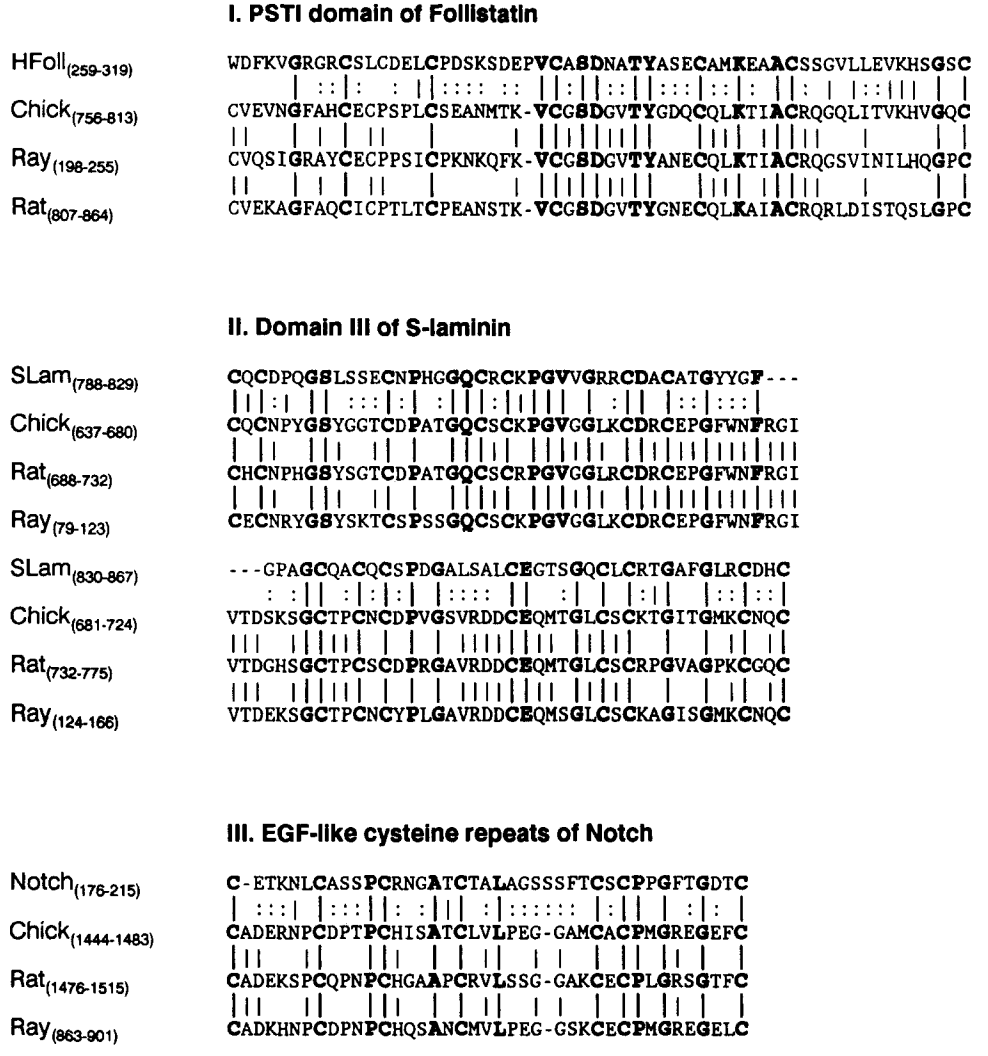


Figure 10. Alignment of Structural Domains in Agrin and Agrin Homologs with Domains in Follistatin, s-Laminin, and Notch Protein

Identical residues are in bold and are linked by bold lines; residues identical in agrin and agrin homologs are linked by light lines; conserved residues are linked by dots.

similar to the active domain of the pancreatic secretory trypsin inhibitors (PSTI) of the Kazal family (Figure 10). PSTI-like domains have also been identified in follistatin (Shima-Saki et al., 1988) and in another extracellular matrix protein, BM-40/osteonectin (Lankat-Buttgereit et al., 1988; Bolander et al., 1988). Eight of the nine PSTI-like domains follow cysteine-rich repeats having a consensus of Cys-X(4,5)-Cys-X(5)-Cys-X(6,9, 11)-Cys-X-Cys.

– There are four domains, having 6 cysteine residues, similar to domains in epidermal growth factor (EGF) that align best with the protein products of the Drosophila neurogenic loci *Notch*, *slit*, and *Delta* (Figure 10; Kidd et al., 1986; Knust et al., 1987; Rothberg et al., 1988).

– There is a region between amino acids 637 and 724 (Figure 10) having 15 cysteine residues that shares a high similarity with the domain III of laminin B1 and B2 chains and s-laminin (Pikkarainen et al., 1987; Chi and Hui, 1988; Hunter et al., 1989a). Within the 15 cysteines there are two groups of 6 cysteines arranged like those in the EGF-like domains. The position of the different structural domains is conserved between species, as illustrated in Figure 11 (Rupp et al., 1991; see also Smith et al., submitted). The roles the cysteine-rich domains might play in agrin's interaction with cellular and basal lamina components of the neuromuscular junction are discussed elsewhere (Rupp et al., 1991; Smith et al., submitted).

The chick protein, like the rat protein, has four potential N-linked glycosylation sites (Asn-352, Asn-621, Asn-726, and Asn-776). The chick sequence, also similar to the ray and rat sequences, contains the tripeptide LRE (amino acids 1391–1393) thought to be functionally significant in s-laminin (Hunter et al., 1989b). This tripeptide, however, varies markedly in position

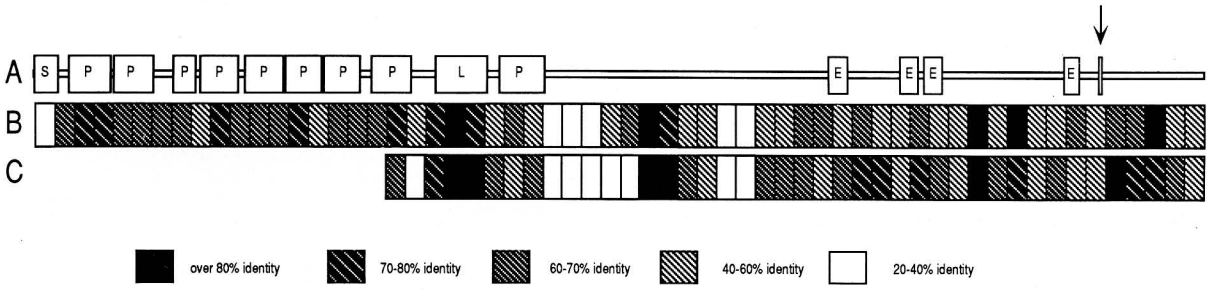


Figure 11. Comparison of Chick Agrin to Ray and Rat Homologs

(A) Domains of chick agrin. Signal sequence (S), protease inhibitor-like domains, and associated cysteine repeats (P), laminin-like domain (L), EGF-like domains (E). The arrow points to the 11 amino acids unique to agrin. The number of identical residues in the chick/rat alignment (B) and in the chick/ray alignment (C) was determined and expressed as a percentage for each stretch of 33 amino acids.

between the ray, rat, and chick proteins, suggesting that it may not have any functional significance in agrin and its related proteins.

Comparison of the predicted chick protein with the proteins predicted by the full-length rat cDNA and the ray partial cDNA revealed several other outstanding similarities (Figure 11). The chick protein is about the same length as the rat protein (including signal sequences: rat, 1940 amino acids; chick, 1955 amino acids). Moreover, the amino acid sequence of the chick protein is 60% identical overall to that of the rat (an additional 26% is conserved); the corresponding region of the partial ray agrin-like protein is 58% identical (21% conserved). As illustrated in Figure 11, some stretches have greater than 80% identity among the three species; others are not conserved at all. The regions least conserved are the signal sequence and two threonine-rich regions in the middle of the chick sequence. Better conserved are the protease inhibitor-like domains and the C-terminal portion of the molecule following the 11 amino acid stretch. Best conserved are the laminin-like region (66 of 88 amino acids identical in ray, rat, and chick), a region in the center of the molecule (39 of 49 amino acids), and a stretch situated between EGF-like domains 3 and 4 (31 of 38 amino acids).

The chick agrin sequence at Thr-183 to Pro-196 is highly similar to the N-terminal sequence of the 150 kd form of agrin (Smith et al., submitted) found in electric organ extracts of T. californica (8 of 13 amino acids are identical; 3 are conserved). The presence of this sequence similarity in the middle third of the predicted chick protein (overall size, 206 kd) is consistent with the suggestion (Smith et al., submitted) that the size of the active agrin proteins (150/95 kd) in electric organ extracts is likely to be due to proteolytic cleavage either during purification or during post-translational modification of a larger precursor. The size of the predicted chick protein is also larger than that of agrin-like proteins in immunoaffinity purified extracts from chick embryo (Godfrey, 1991), indicating that the extractable protein in this species is also the product of proteolytic cleavage. Indeed, the chemically determined N-terminal sequence (Earl Godfrey; personal communication) of 72 and 90 kd agrin-like proteins immunopurified from chick kidney aligns with the chick agrin sequence at residues Gln-1149 to Leu-1159.

Discussion

We demonstrate that the chick cDNA CBA-1 encodes a protein having an amino acid sequence very similar to the agrin-like protein predicted by ray cDNA OL4 (Smith et al., submitted) and a protein predicted by a homologous rat cDNA (Rupp et al., 1991). We also show that, like the fusion protein generated by OL4, the protein generated by transfecting CBA-1 into COS-7 cells is recognized by anti-agrin antibodies. We demonstrate further, by using the same assay conditions which led to the discovery of agrin, that the protein encoded by CBA-1 induces cultured chick myotubes to form AChR and AChE aggregates indistinguishable from those induced by agrin both in the manner of their formation and in their appearance. Altogether, the results leave little doubt that CBA-1 encodes chick agrin.

A striking difference between the protein encoded by CBA-1 and the proteins encoded by the homologous ray and rat cDNAs and two agrin-related chick cDNAs reported thus far is an 11 amino acid stretch absent from all but the CBA-1 protein (Ruegg et al., 1992). The 11 amino acid stretch is required for chick agrin's activity in standard AChR/AChE aggregation assays (Ruegg et al., 1992). Our PCR experiments indicate that ray has mRNA that codes for the 11 amino acid stretch, although 5 of the amino acids are different. Thus, agrin-like proteins with the 11 amino acid stretch are conserved across species, as are those without it, and the 11 amino acid stretch is likely to be characteristic of agrin in motor neurons in many, if not all, vertebrates. Roles the 11 amino acid stretch might play in endowing agrin with AChR/AChE aggregating activity are discussed elsewhere (Ruegg et al., 1992).

Based on Northern blot and sequence analyses, CBA-1 codes for the C-terminal half of agrin, the por-

tion of native T. californica agrin known to have AChR/AChE aggregating activity. By probing a primer extension library with oligonucleotides, we have pieced together cDNAs that code for a full-length protein. In general, the N-terminal half of this protein is very similar to the N-terminal half of the agrin homolog predicted by cDNA from rat. It contains several cysteine-rich domains having high similarity to domains in protease inhibitors, follistatin, and laminin. We do not know whether the primer extension cDNAs are derived from transcripts that code for agrin or agrin-related proteins. However, the fact that only one band can be detected in Northern blots of mRNA from chick CNS, which contains both agrin and agrin-related proteins, indicates that the N-terminal halves of agrin and agrin-related proteins, like the C-terminal halves, are very similar if not identical. The fact that the protease inhibitor and laminin-like domains are not encoded by CBA-1 is consistent with the conclusion from other studies (Smith et al., submitted) that their presence is not required for agrin's activity.

Extracts of motor neuron-enriched fractions of chick spinal cord are known to be enriched in proteins that are antigenically similar to agrin and have AChR/AChE aggregating activity in myotube cultures (Magill-Solc and McMahan, 1988). The correlation between the enrichment of motor neurons and the enrichment of this protein provided a crucial step in the development of the agrin hypothesis. Our findings from PCR experiments aimed at determining whether motor neurons contain transcripts that code for the 11 amino acid stretch characteristic of agrin are fully consistent with the hypothesis. Such transcripts were severalfold more abundant than homologous transcripts coding for protein lacking the 11 amino acids in motor neuron-enriched fractions. Thus we conclude that the protein extracted from the motor neuron-enriched fraction which both induces AChR/AChE aggregates on myotubes and is immunoprecipitated by anti-agrin antibodies is agrin.

The positive correlation of the enrichment of transcripts coding proteins lacking the 11 amino acids with enrichment of non-motor neurons in cell separation experiment is consistent with the likelihood that such transcripts are produced by cells other than motor neurons. We have no evidence to date that such proteins are produced by any neurons. However, the possibility that agrin-related proteins inactive in AChR aggregation are broadly distributed in the CNS and are produced by nonneural cells has been suggested based on the finding that the basal lamina which occupies the space between the capillary endothelium and astrocyte foot processes, neither of which have aggregates of AChRs, stains intensely with anti-agrin antibodies (Magill-Solc and McMahan, 1988; McMahan, 1990). Indeed, in the accompanying report (Ruegg et al., 1992) we provide direct evidence that agrin-related proteins lacking the 11 amino acids are expressed by nonneural cells in nerve and muscle.

Experimental Procedures

Construction and Screening of cDNA Libraries

An E13 chick brain cDNA library (Ranscht, 1988) was screened using procedures described by Sambrook et al. (1989). In brief, about 1×10^6 recombinant phage from the amplified λgt10 library were screened with random-primed (Feinberg and Vogelstein, 1983), ^{32}P-labeled ray cDNA OL4 (~4.3 kb; Smith et al., submitted) that had a specific activity of about 1×10^9 cpm per μg of DNA. Hybridization was carried out overnight at 42°C in 30% formamide, 5× SSC, 5× Denhardt's reagent, 0.1% SDS, 0.1 mg/ml salmon sperm DNA to which about 5×10^5 cpm/ml radioactive probe had been added. Filters were washed twice for 30 min in 2× SSC, 0.2% SDS at room temperature and then twice for 30 min at 50°C. Three rounds of screening were done to ensure the positive clones were from a single colony.

The primer-extended cDNA library in λgt10 was constructed according to Gubler and Hoffmann (1983) from poly(A)$^+$ RNA of chick E13 spinal cord using two specific oligonucleotides from the very 5′ end of CBA-1 (nucleotide 3533–3553 and nucleotide 3670–3690; dotted underlines in Figure 1). Screening of the primer-extended library was performed as described above except that filters were washed twice in 2× SSC, 0.2% SDS for 30 min at room temperature and twice in 1× SSC, 0.1% SDS for 30 min at 60°C. The probes used for screening were the 5′ end of CBA-1 (nucleotide 3322–3555) and the primer-extended clone PE-1.

All inserts of positive clones were excised by EcoRI and subcloned into Bluescript KS (Stratagene). ExoIII deletions (Henikoff, 1987) were performed in both directions, and the deleted clones were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (USB; Tabor and Richardson, 1987). The gaps of these clones were sequenced using synthetic oligonucleotides. Both strands of the cDNA clones were sequenced at least twice, and the DNA sequences were aligned by using the UWGCG software package (Devereux et al., 1984).

Polymerase Chain Reaction

Motor Neuron and Non-Motor Neuron Fractions

Motor neuron-enriched and non-motor neuron-enriched fractions of chick spinal cord were obtained according to the procedure of Dohrmann et al. (1986). Poly(A)$^+$ RNA was isolated from both cell fractions by using the Micro-Fast Track mRNA isolation kit (Invitrogen). First strand cDNA synthesis was performed using random hexamers as primers: the reaction mixture had a total volume of 50 μl and contained 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 50 pmol of the hexamer, ~0.2 μg of mRNA, and 0.6 mM dNTPs. After heating the reaction the mixture to 95°C for 2 min and then cooling it on ice, 10 U of RNAase inhibitor (Pharmacia) and 200 U of murine leukemia virus reverse transcriptase (BRL) were added, and mixture was incubated for 1 hr at 42°C. The mixture was heated to 65°C for 10 min, and the concentration of the dNTPs was lowered to 0.3 mM. Forty picomoles of a pair of oligonucleotides flanking the sequence of the 33 bp characteristic of agrin was added to the mixture: 5′-TTTGATGGTAGGACGTACAT-3′ (nucleotide 5377–5397 as forward primer) and 3′-CTCGACTCGTAGTTTTGTCTTC-5′ (nucleotide 5485–5507 as reverse primer). The primers are underlined in Figure 1. Radioactive forward primer (2×10^5 cpm) labeled by T4 kinase (Sambrook et al., 1989) and 2.5 U of Taq polymerase (Cetus) were added to each reaction; 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min were run on a Perkin Elmer Cetus DNA Thermal Cycler. The reaction products were separated on an 8% polyacrylamide gel and detected by autoradiography. The density of each band was measured by a densitometer (Pharmacia-LKB).

Identification of Agrin's 33 bp Stretch in D. ommata Brain

Poly(A)$^+$ RNA was isolated from total RNA (Sambrook et al., 1989) by using mRNA oligo(dT) spun columns (Pharmacia). First strand cDNA synthesis and PCR were as described above. The primers spanned nucleotide 3311–3337 and nucleotide 3646–3670 in

cDNA OL4 (Smith et al., submitted). The PCR products were reamplified by using another set of primers (nucleotide 3370–3394 and nucleotide 3561–3585), and the product was subcloned into PCR 1000 vector (Invitrogen) and sequenced.

Eukaryotic Expression of Agrin

To direct the protein encoded by CBA-1 into the secretory pathway of COS-7 cells, an artificial leader sequence and initiation site were introduced to CBA-1. Bluescript KS were cut with EcoRI, and protruding ends were filled with Klenow polymerase (Pharmacia). HindIII linkers (Pharmacia) of different length (8-mer, 10-mer, and 12-mer) were ligated to the vector according to Sambrook et al. (1989). The leader sequence from hemagglutinin of the avian influenza virus FPV was purified from the expression vector pM4-3 (generous gift of Dr. U. Suter, Department of Neurobiology, Stanford University School of Medicine), and this leader sequence was inserted into the modified KS vector. The correct orientation and insertion of the leader sequence was confirmed by sequencing. This signal sequence with its initiation site (excised from the modified KS) was subcloned into the SR α-based expression vector pJEF14 (Elliott et al., 1990). CBA-1 was then subcloned into this modified expression vector after EcoRI digestion. Open reading frame and orientation of CBA-1 were confirmed by restriction mapping and sequencing of the 5′ ends of the constructs. DNA used for the transfection of COS-7 cells was purified twice on a cesium chloride gradient. COS-7 cells were transiently transfected using a DEAE–dextran procedure as modified by Luthman and Magnusson (1983). After 3 days in culture, the conditioned medium was collected, cleared of cell debris by centrifugation, and assayed for AChR/AChE aggregating activity on cultured chick myotubes (Wallace, 1986). For COS-7 cell–chick muscle cell cultures, the transfected COS-7 cells were trypsinized 1 day after transfection and seeded onto chick myotubes that had been cultured for 4 days. After coculturing overnight, the cells were fixed and stained with antibody and rhodamine-conjugated α-bungarotoxin (see below).

Histochemistry

For staining agrin and AChR aggregates in COS-7 cell–chick myotube cultures, the cultures were washed twice with Dulbecco's modified Eagle's medium plus F12 medium (DMEM–F12; Sigma), 1 mg/ml bovine serum albumin, 1 mM $CaCl_2$ and incubated for 45 min at 37°C in 0.2 μg/ml biotinylated α-bungarotoxin (Molecular Probes) in DMEM–F12. Cultures were again washed twice and were treated for 1 hr at 37°C with rhodamine-labeled streptavidin (Molecular Probes), diluted 1:200 in DMEM–F12. After washing the cultures 3 times with phosphate-buffered saline (PBS), the cultures were fixed for 5 min at room temperature with 1% paraformaldehyde and 5% sucrose in sodium phosphate buffer (0.1 M, pH 7.2). They were rinsed twice with PBS, 0.1% Triton X-100 (PBST) and dehydrated. After rehydration, the cells were stained overnight at 4°C with hybridoma supernatant containing anti-agrin MAb 5B1, diluted 10-fold with 10% normal goat serum in PBST. Cultures were rinsed 5 times with PBST followed by an incubation for 2 hr at room temperature with fluorescein-coupled goat anti-mouse IgGs diluted 1:400 in 10% normal goat serum in PBST. Cultures were again washed 5 times with PBST and dehydrated. Coverslips were mounted with Citifluor (City University, London) mounting medium. The AChE aggregates were labeled by using an anti-AChE MAb (Tsim et al., 1988).

Immunoprecipitation

Immunoprecipitation was done as described in Magill-Solc and McMahan (1988). In brief, 5 μl of antibody-containing ascites fluid was mixed with 20 μl of COS-7 cell-conditioned medium for 1–2 hr at 37°C in a total volume of 200 μl. Fifty microliters of goat anti-mouse IgG-conjugated Sepharose beads was added, and the suspension was mixed at room temperature for 2–3 hr. The suspension was then spun at 1000 × g for 1 min to remove the beads from the supernatant. The supernatant was added to chick myotube cultures and assayed for AChR aggregating activity.

Northern Blot Analysis

Total RNA from different tissues of the E16 chick was prepared by the cesium chloride method described by Sambrook et al. (1989). Poly(A)$^+$ RNA was purified from total RNA using mRNA oligo(dT) spun columns (Pharmacia). Ten micrograms of poly(A)$^+$ RNA per lane was electrophoresed in a 1% formaldehyde–agarose gel and blotted onto a BA-S nitrocellulose membrane (Schleicher & Schuell). Prehybridization was done for 4 hr at 42°C in a buffer containing 50% formamide, 5× SSC, 5× Denhardt's reagent, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA. The hybridization buffer consisted of prehybridization buffer with 10% dextran sulfate, 0.1 mg/ml tRNA, and 5 × 10^6 cpm/ml ^{32}P-labeled DNA probe. The DNA probe was a restriction fragment from the 5′ end of CBA-1 (nucleotides 3322–4220), which had a specific activity of 10^9 cpm per μg of DNA. After overnight hybridization at 42°C, the blot was washed twice in 1× SSC, 0.1% SDS for 30 min at room temperature. The final two washes were for 30 min in 0.1× SSC, 0.1% SDS at 65°C. An RNA ladder (BRL) was used to standardize the RNA sizes.

In Situ Hybridization

^{35}S-labeled riboprobes were synthesized from a 516 bp stretch at the 5′ end of CBA-1 (nucleotides 3322–3838) using the Trans-T-probe kit (Pharmacia). The probes were hydrolyzed for 20 min and separated from unincorporated radioactive nucleotides on a NucTrap push column (Stratagene). Specific activity was ~10^9 dpm per μg of RNA. Thick (30 μm) frozen sections were mounted on 25 × 75 mm glass slides, silanized with Vectabond (Vektor Labs), and processed as delineated in Wilson and Higgins (1989); for a detailed protocol see Smith et al. (submitted). Briefly, probes were diluted to 10^6 cpm in 100 μl of hybridization mixture per slide and were allowed to hybridize overnight at 52°C. The sections were repeatedly washed; the highest stringency was 0.1× SSC, 50°C for 30 min. They were exposed to NBT-2 emulsion (Kodak) for 7–10 days at 4°C.

Digoxigenin riboprobes were synthesized using the Genius (Boehringer Mannheim) RNA synthesis kit. The hybridization and washes were performed as described above, except that dithiothreitol or 2-mercaptoethanol was omitted. The probes were diluted to 10 μg/ml, and 100 μl was used per slide. After the last wash, the sections were transferred to 0.15 M NaCl in 0.1 M Tris–HCl (pH 7.5), washed 3 times for 10 min, and then preincubated at room temperature in 2.5% normal sheep serum and 0.2% Triton X-100 in the same buffer for 2 hr. They were incubated overnight at 4°C with an alkaline phosphatase linked anti-digoxigenin antibody (diluted 1:500; Boehringer Mannheim) added to the preincubation buffer. The slides were washed in 0.1 M Tris–HCl (pH 9.5) 4 times for 15 min. The substrate (Vector Labs Kit III, SK 5300) was applied along with 2.4 mg/ml Levamisole (Sigma). The accumulation of reaction product in cells was monitored under the microscope so that it could be stopped before nonspecific background staining occurred. Coverslips were mounted with aqueous medium (Crystal Mount, Biomeda).

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