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20 October 1986; accepted 12 February 1987

Cloning of Complementary DNA for GAP-43, a Neuronal Growth-Related Protein

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GAP-43 is one of a small subset of cellular proteins selectively transported by a neuron to its terminals. Its enrichment in growth cones and its increased levels in developing or regenerating neurons suggest that it has an important role in neurite growth. A complementary DNA (cDNA) that encodes rat GAP-43 has been isolated to study its structural characteristics and regulation. The predicted molecular size is 24 kilodaltons, although its migration in SDS-polyacrylamide gels is anomalously retarded. Expression of GAP-43 is limited to the nervous system, where its levels are highest during periods of neurite outgrowth. Nerve growth factor or adenosine 3′,5′-monophosphate induction of neurites from PC12 cells is accompanied by increased GAP-43 expression. GAP-43 RNA is easily detectable, although at diminished levels, in the adult rat nervous system. This regulation of GAP-43 is concordant with a role in growth-related processes of the neuron, processes that may continue in the mature animal.

THE TIP OF THE GROWING NEURITE is the growth cone. This motile structure, of a neuron otherwise fixed in space, is responsible for reaching and identifying appropriate postsynaptic targets. The molecular constituents of the growth cone are synthesized in the soma and transported down the axon, and constitute a limited subgroup of the total cellular proteins. Because growth-cone proteins presumably play an important role in generating patterns of specific connectivity in the nervous system, we (*1*) and others (*2*) have developed methods to enrich for such molecules and have identified several as candidates for involvement in either target recognition (*1*) or axon growth (*3*). GAP-43 is one of the latter group, which consists of proteins expressed or transported at higher rates in nerves of young animals or nerves undergoing regeneration (*3*). It is similar or identical to B-50, a phosphoprotein described by Gispen in both growing and adult nerves as a prominent substrate for protein kinase C, its phosphorylation being modulated by neurotransmitters and peptides (*4, 5*). Antibodies localize GAP-43 to the growth cone and B-50 to growing neurites and to the presynaptic membrane of adult nerves (*6, 7*). The regulation of GAP-43 and its cellular localization suggest that it has an important function in growth cones. We report here the complete sequence of GAP-43 obtained by complementary DNA (cDNA) cloning and present evidence for its regulation at the level of gene expression during neurite growth.

A cDNA library was generated from RNA of rat dorsal root ganglia from embryonic day 17 and cloned into the λgt11 expression vector (*8*). Three presumptive GAP-43 clones were identified with the antibody to GAP-43 described by Snipes *et al.* (*9*). The identity of the longest clone, GAP43-2, was confirmed by hybrid-selected translation (Fig. 1). GAP43-2 selected by hybridization a messenger RNA (mRNA) that directed the translation of a polypeptide that migrated in SDS-polyacrylamide gels with the expected mobility of native GAP-43, that is, a molecular size of about 43 kD. This in vitro translation product was selectively immunoprecipitated by antibody to GAP-43. The specificity of the immunoprecipitation was demonstrated by competition with unlabeled, purified GAP-43. For additional confirmation we sequenced a peptide prepared by cyanogen bromide cleavage of purified GAP-43. The sequence, Arg-X-Lys-Gln-Val-Glu-Lys-Asn-Asp-Glu-Asp-Gln-Lys-Ile, is completely included within the predicted open reading frame of GAP43-2. (The X represents a cycle of sequencing at which the identity of the amino acid could not be determined with certainty.)

The complete nucleotide sequence of GAP43-2 and the predicted amino acid sequence are shown in Fig. 2. The reading frame includes the peptide fragment that was sequenced and is in the same reading frame as the β-galactosidase gene of λgt11

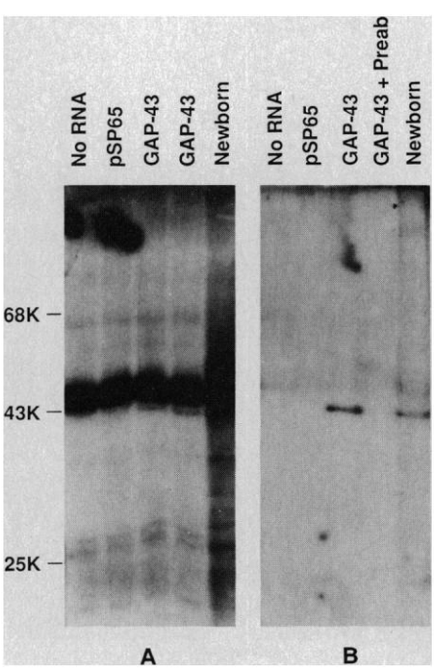

Fig. 1. Hybrid-selected translation of GAP-43 cDNA. The Eco RI insert, GAP43-2, was used to select mRNA by the procedure of Ricciardi *et al.* (*21*). In brief, 0.5 μg of the GAP43-2 insert, or equivalent amounts of nonspecific DNA, the bacterial plasmid pSP65, were spotted onto nitrocellulose and hybridized with 17.5 μg of newborn rat brain polyadenylated [poly(A)$^+$] RNA in a solution with 65% formamide, 400 m*M* NaCl, 10 m*M* 1,4-piperazine diethanesulfonic acid (Pipes) *p*H 6.4 at 42°C for 16 hours. After being washed in standard saline citrate (SSC) (X1), 0.5% SDS at 65°C, the filter was boiled, and the RNA was precipitated with ethanol and translated with rabbit reticulocyte lysate, and the proteins were labeled with [^{35}S]methionine (*22*). Translation products, or products immunoprecipitated with the antibody to GAP-43, were separated on a 12% SDS-polyacrylamide gel. (**A**) In vitro translation products with (i) no exogenous RNA, (ii) pSP65-selected RNA, (iii and iv) GAP43-2–selected RNA, and (v) poly(A)$^+$ newborn brain RNA (newborn). (**B**) Immunoprecipitations by antibody to GAP-43 of the translation products of (A), as described for (A) except for the fourth lane which shows immunoprecipitation of the translation product after having preabsorbed the GAP-43 antibody with GAP-43 protein, prepared as in (*9*).

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1    ggaggggggaataaggaagagaggaggaaaggagagaaggcaggaagaaggcaggggaa

          M  L  C  C  M  R  R  T  K  Q  V  E  K  N  D  E  D
61   gataccaccatgctgtgctgtatgagaagaaccaaacaggttgaaaagaatgatgaggac

     Q  K  I  E  Q  D  G  V  K  P  E  D  K  A  H  K  A  A  T  K
121  caaaagattgaacaagatggtgtcaaaccggaggataaggctcataaggctgcaaccaaa

     I  Q  A  S  F  R  G  H  I  T  R  K  K  L  K  D  E  K  K  G
181  attcaggctagcttccgtggacacataacaaggaaaaagctcaaagacgagaagaagggt

     D  A  P  A  A  E  A  E  A  K  E  K  D  D  A  P  V  A  D  G
241  gatgcaccagctgctgaggccgaggccaaggagaaggatgatgctcccgttgctgatggt

     V  E  K  K  E  G  D  G  S  A  T  T  D  A  A  P  A  T  S  P
301  gtggagaagaaggagggagatggctctgctactaccgatgcagccccagccaccagccct

     K  A  E  E  P  S  K  A  G  D  A  P  S  E  E  K  K  G  E  G
361  aaggctgaggagcccagcaaggcaggagatgcaccttctgaggagaagaagggcgaaggg

     D  A  A  P  S  E  E  K  A  G  S  A  E  T  E  S  A  A  K  A
421  gatgcggccccttcagaggaaaaggccggctcagctgagacagaaagtgctgctaaagct

     T  T  D  N  S  P  S  S  K  A  E  D  G  P  A  K  E  E  P  K
481  accactgataactcgccgtcctccaaggccgaagatggcccagccaaggaggagcctaaa

     Q  A  D  V  P  A  A  V  T  D  A  A  A  T  T  P  A  A  E  D
541  caagccgatgtgcctgctgctgtcactgatgctgctgccaccacccctgctgcagaggat

     A  A  K  A  A  Q  P  P  T  E  T  A  E  S  S  Q  A  E  E  E
601  gctgccaaggcagcgcagcctccaacggagactgcagaaagcagccaagctgaggaggag

     K  E  A  V  D  E  A  K  P  K  E  S  A  R  Q  D  E  G  K  E
661  aaagaagctgtagatgaagccaaacctaaggaaagtgcccgacaggatgagggtaaagaa

     D  P  E  A  D  Q  E  H  A  *
721  gaccccgaggctgaccaagaacatgcctgaactttaagaaacggctttccacgttgcccc
                                  Sau3A
781  cacctgaaccctgtctctcctgccctttctcagatccactctgaagtttcctctcctgtc

841  ctgctcacgtgtgtgagcctgtcctctcctacctatgagccctctctctctgtgtggcaa

901  acatttaaaaaaaaaaaaagcaggaaagatcccaagtccaacagtgtggcttaaacatttg

961  tttcttggtgttgttatggcgagtttttggtaatgatgatgcagtcatcttgggaaattc

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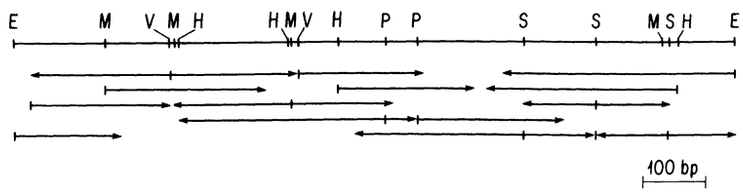

Fig. 2. Nucleotide sequence and predicted amino acid sequence of GAP-43. The cDNA library was generated with RNA from dorsal root ganglia from embryonic day 17–18 rats. Total cellular RNA was isolated by the method of Chirgwin *et al.* (*23*), and poly(A)$^+$ RNA was selected with oligo-dT cellulose. Double-stranded cDNA was generated by the ribonuclease H method described by Gubler and Hoffman (*24*), ligated to Eco RI linkers, and ligated into the Eco RI site of the lambda phage cloning vectors, λgt10 and λgt11. The longest clone identified, GAP43-2, and two phage with smaller inserts, were identified from about 5×10^4 plaques in the λgt11 library after induction with isopropyl β-D-thiogalactopyranoside (IPTG) by using the rabbit antibody to GAP-43 followed by alkaline phosphatase–conjugated antibody to rabbit immunoglobulin G (Promega Biotec). The cDNA inserts were subcloned into the Eco RI sites of M13 mp18. Initial DNA sequence analysis of the two shorter clones revealed that they were included within the longest. The insert, GAP43-2, was sequenced by using the series of overlapping restriction fragments shown below the sequence by the dideoxynucleotide chain–termination method (*25*). The 3′ end of this fragment is the Eco RI site common to the three independent λgt11 isolates, which is thought to be an Eco RI site that occurs naturally in the GAP-43 gene. Since none of the clones contained an insert with a polyadenylation sequence, it is likely the Eco RI sites within the cDNA were unsuccessfully methylated during the library construction. The predicted protein sequence for GAP-43 is shown above the DNA sequence. The first methionine in italics was chosen as the start of the coding region for the reasons described in the text. The only other methionine, shown here as amino acid 5, could alternatively serve as the initiation codon. The amino acid residues that were identified by direct protein sequencing from the arginine (R) at amino acid 7 to the isoleuine (I) at amino acid 20 are overlined. The first cycle of sequencing at which the amino acid could be determined with certainty was this arginine. The next amino acid could not be determined with certainty. Although we also attempted to sequence the unfragmented protein, our lack of success suggests that the amino terminus may be blocked. E, Eco RI; M, Msp I; V, Pvu II; H, Hae III; P, Pst I; S, Sau 3A.

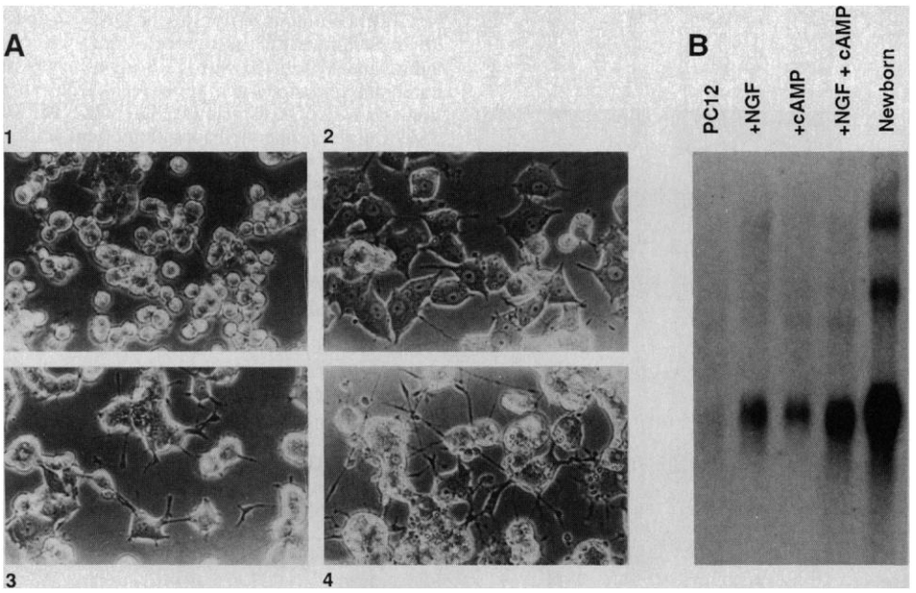

Fig. 3. Regulation of GAP-43 expression in PC12 cells. PC12 cells were passaged in RPMI medium containing 10% horse serum and 5% fetal bovine serum. Forty hours after plating the cells, the medium was changed to include the different additives. After 4 days, the cells were photographed (**A**), then RNA was isolated from each cell culture. RNA (10 μg per sample) was denatured and run on a 1.2% agarose-formaldehyde gel, transferred to a GeneScreen nylon filter, bound to the filter by ultraviolet cross-linking, and probed with ^{32}P-labeled GAP43-2 (**B**). The final wash was SSC (×0.2), 0.1% SDS at 65°C. The additives included were (i) none, (ii) 50 ng of NGF per milliliter, (iii) $10^{-3}M$ dibutyryl cAMP, and (iv) 50 ng of NGF per milliliter and $10^{-3}M$ dibutyryl cAMP. The last lane is 10 μg of RNA from newborn brain run as a positive control for the blotting and hybridization procedure.

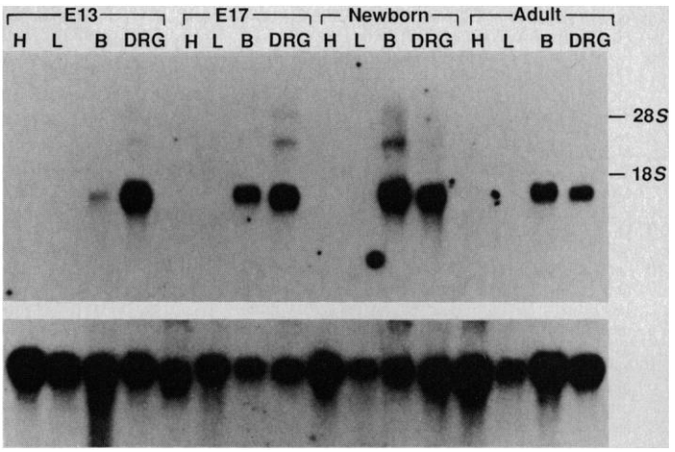

Fig. 4. Developmental regulation and tissue specificity of GAP-43 gene expression. Total cellular RNA was isolated from the designated rat tissues by a modification of the procedure of Chirgwin *et al.* (*23*). Each RNA (10 μg) was denatured, underwent electrophoresis in a 1.2% agarose-formaldehyde gel, and was transferred to nitrocellulose. The filter was hybridized overnight at 42°C with the Eco RI insert from λgt11 GAP43-2 labeled with deoxycytidine 5′-[α-^{32}P]triphosphate by nick translation. The final wash was done in SSC (×0.2), 0.1% SDS at 65°C. RNA samples: (i) embryonic day 13 (E13) heart (H), (ii) E13 liver (L), (iii) E13 brain (B), (iv) E13 dorsal root ganglion (DRG), (v) to (viii) embryonic day 17 heart, liver, brain, and dorsal root ganglion; (ix) to (xii) newborn heart, liver, brain, and dorsal root ganglion; (xiii) to (xvi) adult heart, liver, brain and dorsal root ganglion. The positions of the 18*S* and 28*S* ribosomal RNA are shown at the right. Below is hybridization of the same filter with a cDNA probe encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*26*).

(*10*). The methionine identified as the start of the open reading frame is the first methionine after the in-frame stop codon (TAA) at nucleotide position 13 and is surrounded by eight of the nine nucleotide consensus sequences suggested by Kozak (*11*) to be the most favorable context to initiate eukaryotic translation. This suggests that it is the first residue of the GAP-43 coding region. However, the information is insufficient to make this assignment unequivocally, and, therefore, the second methionine (amino acid 5) might play this role. The predicted composition of GAP-43 is highly polar, without evident transmembrane domains or potential N-linked glycosylation sites. This composition is compatible with the observations that GAP-43 is membrane-associated but inaccessible to antibody recognition in the absence of membrane permeabilization (*7*); thus it may be associated with the inner face of the membrane.

The predicted molecular size of the GAP-43 protein from the open reading frame is 24 kD, which is less than the 43 kD originally observed by Skene and Willard as the apparent molecular size of the molecule in SDS-polyacrylamide gels (*3*). The molecular size has been uncertain because the apparent molecular size of GAP-43 depend on polyacrylamide concentration (*4*), suggesting that this protein falls in the category of proteins that migrate anomalously on SDS-polyacrylamide gels (*12*). This property is unlikely to be due to posttranslational modification since the in vitro translation product has a mobility similar to that of native GAP-43 (Fig. 1).

To collect information concerning the SDS-polyacrylamide gel migration properties of the protein encoded within the putative open reading frame, we synthesized GAP-43 RNA from the cDNA in an in vitro transcription system with the use of the bacteriophage SP6 promoter, by the method of Melton *et al.* (*13*). An 800-base RNA was generated by transcribing the cDNA cut at the Sau 3A site, 65 bases 3′ of the end of the predicted open reading frame (Fig. 2), and a 1100-base RNA by truncating at the Hind III site in the polylinker region at the 3′ end of cDNA. Both the 800-base RNA and the 1100-base RNA directed the synthesis of a polypeptide with an apparent molecular size of 40 kD when translated in vitro with reticulocyte lysate and analyzed on a 15% SDS-polyacrylamide gel. The 40-kD translation product in both cases was immunoprecipitated with the antibody to GAP-43. GAP-43 synthesized in vitro from newborn rat brain RNA comigrated with these translation products.

Evidence for the belief that GAP-43 is important to the function of growth cones includes enrichment of the protein in growth-cone membranes (*7, 14*) and increased transport of the protein in developing and regenerating nerves (*3*). To investigate whether GAP-43 gene expression is regulated coordinately with extension of neurites, we examined its expression in PC12 cells in which neurite outgrowth was promoted by nerve growth factor (NGF) and to a lesser extent by adenosine 3′,5′-monophosphate (cAMP). These agents act by different mechanisms in inducing neurite outgrowth (*15*). Concomitant with the neurite growth induced by either agent is an increase in GAP-43 mRNA levels, with the largest increase in cells exposed to both agents (Fig. 3).

To determine the pattern of expression of the GAP-43 gene during normal development, we isolated total cellular RNA from brain, dorsal root ganglia, heart, and liver of embryonic day 13, embryonic day 17, newborn, and adult rats. GAP-43 was expressed in a neural-specific manner (Fig. 4). At all ages, the major hybridizing band of about 1500 nucleotides is visible only in the neuronal tissues. The faint, large-molecular-size bands may correspond to unspliced precursor molecules since the genomic GAP-43 gene contains intronic sequences (*16*). The GAP-43 mRNA in neuronal tissue is probably of neural rather than glial origin since GAP-43 is localized in neurons (*7*) and no GAP-43 RNA was detected in the glioma cell line C6.

In neural tissues, the amount of GAP-43 mRNA varies with developmental stage. Peak concentrations occur in the perinatal period, with some delay in the central nervous system relative to the peripheral nervous system. The timing of expression accords well with periods of axon growth (*17*). However, the significant amount of GAP-43 RNA in adult neural tissues is in agreement with observations that GAP-43 protein persists in adult rat cortex, albeit in significantly lower amounts than during the perinatal period (*4*). The persistence of GAP-43 expression suggests an ongoing role in the adult nervous system. The properties of B-50 and F1, phosphoproteins electrophoretically and antigenically indistinguishable from GAP-43, have been assessed in adult neuronal tissue. These proteins serve as substrates for a protein kinase C–like enzyme, and their phosphorylation is regulated by neuropeptides, neurotransmitters, and during the course of long-term potentiation (*4, 6, 18*). It is not known whether GAP-43 regulation in the adult also occurs by alterations in gene expression. One model for the function of GAP-43 in the mature animal would include an ongoing role in synaptic turnover (*19*) and in other "plastic" changes of the nervous system, such as learning, that are accompanied by structural growth at the nerve terminal (*20*).

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15 December 1986; accepted 2 March 1987

Primary Structure and Biochemical Properties of an M_2 Muscarinic Receptor

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A partial amino acid sequence obtained for porcine atrial muscarinic acetylcholine receptor was used to isolate complementary DNA clones containing the complete receptor coding region. The deduced 466–amino acid polypeptide exhibits extensive structural and sequence homology with other receptors coupled to guanine nucleotide binding (G) proteins (for example, the β-adrenergic receptor and rhodopsins); this similarity predicts a structure of seven membrane-spanning regions distinguished by the disposition of a large cytoplasmic domain. Stable transfection of the Chinese hamster ovary cell line with the atrial receptor complementary DNA leads to the binding of muscarinic antagonists in these cells with affinities characteristic of the M_2 receptor subtype. The atrial muscarinic receptor is encoded by a unique gene consisting of a single coding exon and multiple, alternatively spliced 5′ noncoding regions. The atrial receptor is distinct from the cerebral muscarinic receptor gene product, sharing only 38% overall amino acid homology and possessing a completely nonhomologous large cytoplasmic domain, suggesting a role for the latter region in differential effector coupling.

THE MUSCARINIC ACETYLCHOLINE receptor is the predominant cholinergic receptor of the central nervous system, where it is involved in both the excitation and inhibition of neurons, and of the parasympathetic nervous system, where it regulates autonomic responses such as the contraction of cardiac and smooth muscle and the secretory activity of exocrine glands (*1*). Muscarinic receptors belong to a class of integral membrane glycoproteins that includes visual rhodopsins as well as numerous hormone and neurotransmitter receptors, which transduce a light or agonist-binding stimulus to a specific effector through the activation of a guanine nucleotide–binding (G) protein (*2*). The diverse cellular effects elicited by muscarinic agonists via activation of G proteins include the inhibition of adenylyl cyclase activity, the stimulation of phosphoinositide (PI) breakdown (*3*), and the regulation of the inward K^+ current in myocardium (*4*). The distinct pharmacological and biochemical properties of muscarinic receptors in various tissues had led to the concept of different receptor subtypes, but the molecular basis of this diversity has remained obscure. The heterogeneous binding of muscarinic agonists suggests the existence of multiple binding states, and the ability to alter the proportion of each population with guanine nucleotides suggests that receptor subtypes may represent distinct conformations of a single protein regulated by effector coupling (*5*). Muscarinic subtypes have also been distinguished on the basis of tissue specific antagonists. The muscarinic antagonist pirenzepine is more effective in inhibiting gastric secretion than in regulating heart and smooth muscle contraction and thus discriminates between high affinity (M_1) receptors of cerebral cortex, hippocampus, and sympathetic ganglia and low affinity (M_2) receptors of myocardium, cerebellum, and medulla pons (*5*). Studies with purified M_1 and M_2 receptors from porcine cerebrum and myocardium (*6*) have revealed differences in their molecular mass and composition, suggesting that muscarinic subtypes may also differ in structure or posttranslational modification. To provide a structural basis for the study of muscarinic receptor function, receptor subtypes, and signal transduction coupling, we have utilized amino acid sequence peptides derived from the atrial receptor to isolate complementary DNA (cDNA) and genomic clones, and have expressed and characterized the cloned receptor in heterologous cells.

To obtain protein sequence data for the design of hybridization probes, we used the native atrial receptor purified by ligand affinity chromatography (*7*). Receptor preparations displayed high affinity for the muscarinic antagonist *L*-[^{3}H]quinuclidinyl benzilate (QNB), with a binding capacity of 12.4 nmol per milligram of protein. Analysis on SDS-polyacrylamide gels revealed a broad silver staining band with a peak mobility corresponding to an M_r of 80,000; this band had been previously identified as the muscarinic receptor by covalent affinity labeling with [^{3}H]propylbenzilylcholine mustard (Fig. 1A, lane 1) (*7*). Amino-terminal sequencing attempts suggested that the receptor was blocked to Edman degradation. Treatment of the native M_r 80,000 protein

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