UNCLASSIFIED

AD NUMBER
ADB123821

LIMITATION CHANGES

TO:

Approved for public release; distribution is unlimited.

FROM:

Distribution authorized to U.S. Gov't. agencies only; Contractor Performance Evaluation; Proprietary Information; 02 DEC 1987. Other requests shall be referred to U.S. Army Medical Research and Development Command, Attn: SGRD-RMI-S, Fort Detrick, Frederick, MD 21701-5012.

AUTHORITY

USAMRDC ltr, 4 May 1993

THIS PAGE IS UNCLASSIFIED

AD _____

AD-B123 821

PRIMARY STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTOR

ANNUAL REPORT

JAMES W. PATRICK

AUGUST 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5198

The Salk Institute
P.O. Box 85800
San Diego, California 92138

Distribution ~~limited~~ *auth* to U.S. Government agencies ~~and their contractors~~ *only* ~~(Administrative/Operational Use, November 30, 1984).~~ Other requests for this document shall be referred to the Commander, US Army Medical Research and Development Command (ATTN: SGRD-RMI-S), Fort Detrick, Frederick, Maryland 21701-5012. ~~PROPRIETARY INFORMATION~~, Contractor Performance Evaluation *2 Dec 87*

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

REPORT SECURITY CLASSIFICATION			1b. RESTRICTIVE MARKINGS			
Unclassified						
SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT			
DECLASSIFICATION/DOWNGRADING SCHEDULE			Distribution limited to US Government Agencies only; (Proprietary Information, Contractor Performance Evaluation, December 2, 1987).			
PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)			
NAME OF PERFORMING ORGANIZATION		6b. OFFICE SYMBOL *(If applicable)*	7a. NAME OF MONITORING ORGANIZATION			
The Salk Institute						
ADDRESS *(City, State, and ZIP Code)*			7b. ADDRESS *(City, State, and ZIP Code)*			
P.O. Box 85800 San Diego, California 92138						
NAME OF FUNDING/SPONSORING ORGANIZATION US Army Medical Research & Development Command		8b. OFFICE SYMBOL *(If applicable)* SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5198			
ADDRESS *(City, State, and ZIP Code)* Fort Detrick Frederick, Maryland 21701-5012			10. SOURCE OF FUNDING NUMBERS			
			PROGRAM ELEMENT NO. 61102A	PROJECT NO. 3M1 61102BS11	TASK NO. AA	WORK UNIT ACCESSION NO. 068

TITLE *(Include Security Classification)*

Primary Structure of Nicotinic Acetylcholine Receptor

PERSONAL AUTHOR(S)
James W. Patrick

TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT *(Year, Month, Day)*	15. PAGE COUNT
Annual	FROM 7/15/85 TO 7/14/86	1986 August	37

SUPPLEMENTARY NOTATION

COSATI CODES			18. SUBJECT TERMS *(Continue on reverse if necessary and identify by block number)*
FIELD	GROUP	SUB-GROUP	
06	13		
06	01		

ABSTRACT *(Continue on reverse if necessary and identify by block number)*

Cholinergic transmission occurs between nerve and muscle and between nerve and nerve. In both cases, acetylcholine is released by the nerve, bound by a nicotinic acetylcholine receptor, and destroyed by the enzyme acetylcholinesterase. Our goal has been to isolate cDNA clones encoding the nicotinic acetylcholine receptor at the neuromuscular junction and to use these clones to determine the structure of the receptor. In this report we present the amino acid sequence of the muscle nicotinic acetylcholine receptor and show that the clones we have isolated will direct the synthesis of the receptor. We have also used the clones to isolate related clones, which we argue encode the receptors found at synapses in the central nervous system. For one of these clones, we have determined its sequence and the distribution in the brain of the transcript.

DISTRIBUTION/AVAILABILITY OF ABSTRACT			21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS			Unclassified	
NAME OF RESPONSIBLE INDIVIDUAL			22b. TELEPHONE *(Include Area Code)* 301-663-7325	22c. OFFICE SYMBOL SGRD-RMI-S

AD _____

PRIMARY STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTOR

ANNUAL REPORT

JAMES W. PATRICK

AUGUST 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5198

The Salk Institute
P.O. Box 85800
San Diego, California 92138

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

2

Blank

SUMMARY

[handwritten: con't H]   *[handwritten: alpha-, Beta-, Gamma- delta]*

This study had two specific goals. The first was to obtain the primary structure for the subunits of a mammalian nicotinic acetylcholine receptor and the second was to isolate and sequence cDNA clones encoding the nicotinic acetylcholine receptors found in the central nervous system. Both of these goals have been realized. We have isolated cDNA clones encoding the α-, β-, γ- and δ-subunits of the mouse muscle nicotinic acetylcholine receptor and have determined the sequences *[handwritten: alpha-, Beta-, Gamma]* for the α-, β-, and γ-subunits. The sequence of the δ-subunit was completed in another laboratory. The clones were engineered to make them suitable for expression studies and inserted into plasmids downstream of a promotor. These plasmids have been used to synthesize RNA which will direct the synthesis of functional acetylcholine receptors in the *Xenopus* oocyte.

We used our clones encoding the muscle acetylcholine receptor α-subunit to screen by low stringency DNA/DNA hybridization a cDNA library prepared from a neuronal cell line. We isolated three clones out of one million examined and determined the sequence of the one with the longest insert. This sequence revealed that the clone encoded a protein with considerable structural homology to the *[handwritten: alpha]* muscle receptor α-subunits. We have been able to demonstrate by *in situ* hybridization that transcripts hybridizing to the clone are expressed in regions of the brain known to have cholinergic transmission. Based upon this evidence, we have *[handwritten: alpha]* proposed that the clone encodes the α-subunit of a neuronal nicotinic receptor.

4

Blank

TABLE OF CONTENTS

6

Blank

BACKGROUND

A major goal in the neurosciences is to understand how the communication between cells in the central nervous system determines behavior. To a large extent, we understand the general principles of the communication process. We know that neurotransmitters are released by the presynaptic cell and that receptors on the postsynaptic cell bind the neurotransmitters and generate an electrical event in the postsynaptic cell. We know that some of these electrical events are inhibitory and some are excitatory, and we know ionic mechanisms that are capable of generating both kinds of responses. We have also identified several different neurotransmitters and neurotransmitter receptors, but we know little about how this diversity might produce different responses to a single kind of neurotransmitter.

Our understanding of neurotransmission in the central nervous system is in part limited by the complexity of the system. The cells are small and may have thousands of synapses derived from neurons from many different parts of the brain. However, the abundance of synapses does not mean that the receptors are present in large quantities. In fact, the actual quantity is low and purification of receptors from the brain is difficult at best. Consequently, neither cellular nor biochemical approaches to studying neurotransmission within the brain is particularly promising. This is unfortunate because our best handle on the function of the brain may well be through modification of neurotransmission. Drugs that affect the activation of neurotransmitter receptors are potent modulators of behavior. However, testing drugs in the intact animal is difficult because so little is known about the various sites of actions of the test compounds. Ideally, one would identify all of the neurotransmitter receptors of a given type and determine their distribution throughout the known functional domains of the brain. One might then engineer the expression of these receptors in cell lines where their function could be assayed accurately and individually. One could then detect differential drug sensitivities and design drugs that differentially modulate receptor activity in specific functional regions of the brain.

As described in the present report, we have isolated cDNA clones encoding the four subunits of the mouse muscle nicotinic acetylcholine receptor and have engineered these clones to permit their expression in the *Xenopus* oocyte. In this system, we have explored the functional requirements for the individual subunits and have begun experiments to modify receptor function by mutation. These results demonstrate the feasibility of studying the properties of nicotinic acetylcholine receptors using RNA transcribed from cDNA clones and provide a foundation both for future studies of these receptors. They also establish the criteria to be applied in the analysis of neuronal nicotinic receptors. Clearly the ultimate criteria for a receptor is function and there are now established methodologies available for studying function of proteins encoded by recombinant DNA clones.

We have also used our clones encoding the muscle receptor to isolate clones encoding receptors from the central nervous system. We have found and sequenced two clones encoding α-subunits and have shown that the genes encoding these proteins are expressed in different parts of the rodent brain. It is not yet clear that these clones encode subunits of neuronal nicotinic acetylcholine receptors because we have not yet been able to apply the criteria of function discussed above. Clearly, this is an important next step. These results that we have obtained do, however, indicate that we have identified a family of genes encoding nicotinic acetylcholine receptors, that these genes are differentially expressed in the brain, and that we will be able to study the function and pharmacological properties of the gene products in a system free of the complexity of the central nervous system.

I. MOUSE MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR

We began our studies of the molecular biology of the nicotinic acetylcholine receptor using the electric organ of the ray *Torpedo* from which we could obtain large quantities of starting material (for reviews of receptor, see Popot and Changeux, 1984; Stroud and Finer-Moore, 1985). This work led to the isolation of cDNA clones encoding the α- and γ-subunits of the *Torpedo* receptor, determination

of their sequences, and construction of detailed models for the structure of the receptor molecule (Claudio et al, 1983; Patrick et al, 1983). This approach was limited in that the majority of the biophysical and developmental studies had been done in mammalian systems.

We decided to focus on the mammalian system and began by isolating a cDNA clone encoding the α-subunit of the mouse muscle nicotinic acetylcholine receptor (Boulter et al, 1985). The sequence of the α-subunit which we deduced from this clone led to new testable models of the ligand binding domain of the α-subunit. To test these models, we needed clones encoding all four subunits of the muscle acetylcholine receptor in a form suitable for expression in the *Xenopus* oocyte system. The isolation and characterization of the γ-subunit encoding clone were accomplished (Boulter et al, 1986). Dr. Norman Davidson's laboratory isolated a δ-subunit encoding clone which lacked the 5 prime untranslated sequences (LaPolla et al, 1985). We used this clone to isolate from our library a clone suitable for expression. In this report, we document the isolation and sequence of the β-subunit encoding cDNA clone and show the expression of all four clones in the *Xenopus* oocyte. We also report the isolation and sequence of two new clones which we propose encode the α-subunits of nicotinic acetylcholine receptors which mediate synaptic transmission between neurons in the peripheral and central nervous systems.

We screened a mouse muscle λgt10 cDNA library with a probe derived from a cDNA clone coding for the β-subunit of *Torpedo* acetylcholine receptor and isolated two clones which were found to encode the mouse muscle β-subunit. Figure 1 illustrates a partial restriction map and the sequencing strategy for clone BMB265. This clone lacks the initiator methionine and a portion of the leader sequence so a new library was constructed and screened to find clones suitable for expression. Clone BMB49 was isolated from this screening. The partial restriction map and sequencing strategy for this clone are also found in Figure 1.

The results in Figure 2 show the nucleotide and deduced amino acid sequence for the mouse muscle acetylcholine receptor β-subunit derived from clone BMB49. The total length of the cDNA is 2100 nucleotides. Alignment of translations from all

λBMB 265

λBMB 49

BASE PAIRS 0
5'
ECO RI

100

HPA II (145)

200

300

400

PVU II (462)

500

600

700

HINC II (763)

800

900

1000

1100

1200 HPA II (1191)

HPA II (1255)
SMA I (1286)

1300

1400

1500 HPA II (1528)

1600

1700

1800

1900 BGL II (1886)

BGL II (1946)

2000

2100 ECO RI
3'

AASEMB.FIG
5 DECEMBER 1985
16 DECEMBER 1985

FIGURE (2)
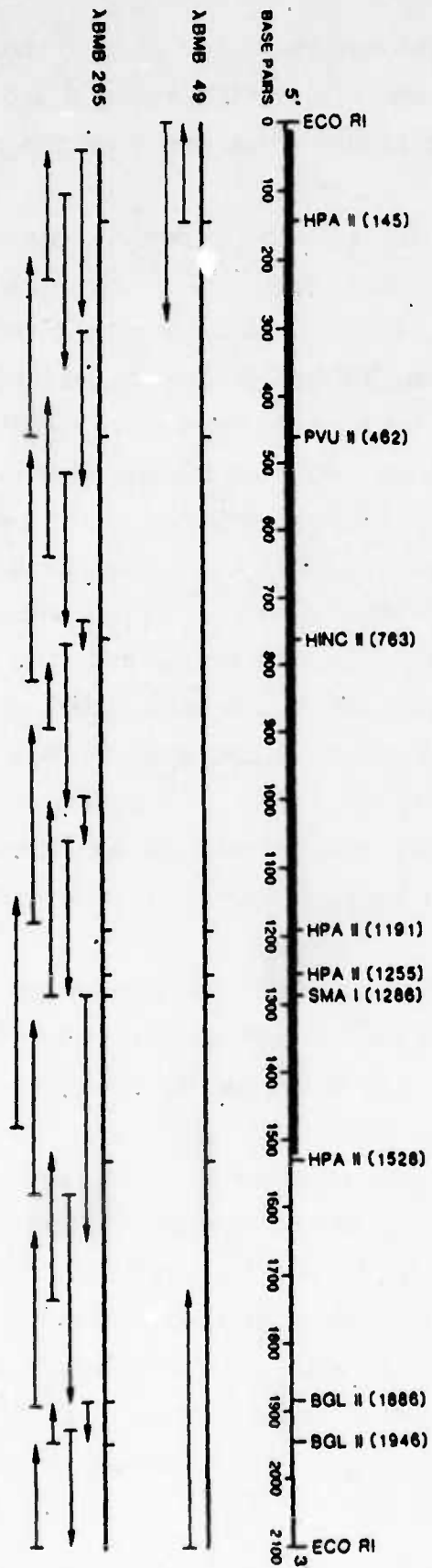
```
                                    5' ..... GAAAGAGGCAGGGCC
                                              -60
                                                              -20
                              Met Arg Leu Gly Ala Leu Leu Leu Leu Leu Gly Val
                              ATG CGC TTA GGG GCG CTG CTT CTG CTT CTA GGG GTA
                                         -50                            -40

        -10
Leu Gly Thr Pro Leu Ala Pro Gly Ala Arg Gly Ser Glu Ala Glu Gly Gln Leu Ile Lys Lys Leu Phe Ser Asn Tyr Asp Ser Ser Val
CTC GGG ACG CCC CTC GCC CCA GGC GCC CGC GGG TCG GAA GCC GAA GGC CAA CTG ATT AAG AAA CTT TTC TCC AAC TAT GAT AGC TCG GTG
           -20                                  -1 1                     20                            10

        20                                          30                                              40
Arg Pro Ala Arg Glu Val Gly Asp Arg Val Gly Val Ser Ile Gly Leu Thr Leu Ala Gln Leu Ile Ser Leu Asn Glu Lys Asp Glu Glu
AGG CCG GCG CGG GAG GTG GGA GAC CGC GTC GGG GTC AGC ATC GGC CTC ACC CTG GCG GAA CTC ATC AGC CTG AAC GAG AAG GAT GAA GAA
        60                                  80                                  120                            140

        50                                          60                                              70
Met Ser Thr Lys Val Tyr Leu Asp Leu Glu Trp Thr Asp Tyr Arg Leu Ser Trp Asp Pro Ala Glu His Asp Gly Ile Asp Ser Leu Arg
ATG AGC ACA AAG GTG TAC TTA GAC CTG GAG TGG ACC GAC TAC AGG TTA AGC TGG GAC CCC GCA GAA CAT GAT GGC ATC GAT TCT CTC CGC
              160                           180                           200                           220

        80                                          90                                              100
Ile Thr Ala Glu Ser Val Trp Leu Pro Asp Val Val Leu Leu Asn Asn Asn Asp Gly Asn Phe Asp Val Ala Leu Asp Ile Asn Val Val
ATC ACT GCT GAA TCC GTT TGG CTC CCT GAT GTG GTG CTG CTG AAC AAC AAT GAC GGA AAT TTC GAC GTT GCC CTG GAC ATC AAT GTT GTG
240                           260                           280                           300                           320

        110                                         120                                             130
Val Ser Phe Glu Gly Ser Val Arg Trp Gln Pro Pro Gly Leu Tyr Arg Ser Ser Cys Ser Ile Gln Val Thr Tyr Phe Pro Phe Asp Trp
GTA TCT TTC GAG GGC TCT GTG CGC TGG CAA CCC CCT GGC CTG TAC CGC AGC AGC TGC AGT ATC CAG GTC ACC TAC TTC CCC TTT GAC TGG
              340                           360                           380                           400

        140                                         150                                             160
Gln Asn Cys Thr Met Val Phe Ser Ser Tyr Ser Tyr Asp Ser Ser Glu Val Ser Leu Lys Thr Gly Leu Asp Pro Glu Gly Glu Glu Arg
CAG AAC TGC ACC ATG GTG TTT AGT TCC TAC AGC TAT GAC AGC TCT GAG GTC AGC CTG AAG ACG GGC CTG GAT CCT GAA GGA GAG GAG AGG
420                           440                           460                           480                           500

        170                                         180                                             190
Gln Glu Val Tyr Ile His Glu Gly Thr Phe Ile Glu Asn Gly Gln Trp Glu Ile Ile His Lys Pro Ser Arg Leu Ile Gln Leu Pro Gly
CAG GAA GTC TAC ATT CAT GAA GGG ACC TTC ATT GAG AAT GGC CAA TGG GAG ATT ATC CAC AAA CCT TCT AGG TTA ATC CAA CTT CCA GGG
              520                           540                           560                           580

        200                                         210                                             220
Asp Gln Arg Gly Gly Lys Glu Gly His His Glu Glu Val Ile Phe Tyr Leu Ile Ile Arg Arg Lys Pro Leu Phe Tyr Leu Val Asn Val
GAT CAG AGA GGT GGG AAG GAA GGG CAT CAT GAG GAA GTT ATC TTC TAT CTC ATT ATT CGG CGG AAG CCT CTC TTC TAC CTG GTC AAC GTC
600                           620                           640                           660                           680

        230                                         240                                             250
Ile Ala Pro Cys Ile Leu Ile Thr Leu Leu Ala Ile Phe Val Phe Tyr Leu Pro Gln Asp Ala Gly Glu Lys Met Gly Leu Ser Ile Phe
ATT GCC CCA TGC ATC CTC ATC ACG CTC CTG GCC ATC TTT GTC TTC TAC CTC CCA CAA GAT GCA GGA GAG AAG ATG GGG CTC TCC ATC TTT
              700                           720                           740                           760

        260                                         270                                             280
Ala Leu Leu Thr Leu Thr Val Phe Leu Leu Leu Leu Ala Asp Lys Val Pro Glu Thr Ser Leu Ala Val Pro Ile Ile Ile Lys Tyr Leu
GCC CTG CTG ACG CTC ACT GTG TTC TTG CTG CTG TTG GCC GAC AAA GTG CCT GAG ACC TCC CTG GCG GTG CCC ATC ATT ATC AAG TAC CTC
760                           800                           820                           840                           860

        290                                         300                                             310
Met Phe Thr Met Val Leu Val Thr Phe Ser Val Ile Leu Ser Val Val Val Leu Asn Leu His His Arg Ser Pro His Thr His Gln Met
ATG TTT ACC ATG GTC CTT GTC ACC TTC TCC GTT ATC CTT AGT GTT GTG GTC CTC AAC CTG CAT CAT CGC TCA CCC CAC ACC CAC CAA ATG
              880                           900                           920                           940

        320                                         330                                             340
Pro Phe Trp Val Arg Gln Ile Phe Ile His Lys Leu Pro Pro Tyr Leu Gly Leu Lys Arg Pro Lys Pro Glu Arg Asp Gln Leu Pro Glu
CCC TTT TGG GTC CGC CAG ATC TTC ATT CAC AAG CTC CCT CCA TAC CTG GGT CTG AAG AGG CCC AAA CCC GAG AGA GAC CAA CTC CCT GAA
960                           980                           1000                          1020                          1040

        350                                         360                                             370
Pro His His Ser Leu Ser Pro Arg Ser Gly Trp Gly Arg Gly Thr Asp Glu Tyr Phe Ile Arg Lys Pro Pro Ser Asp Phe Leu Phe Pro
CCA CAT CAC TCT CTT TCT CCA AGA AGT GGC TGG GGC AGA GGA ACT GAT GAA TAT TTC ATC CGG AAG CCT CCA AGT GAT TTT CTT TTC CCT
              1080                          1060                          1100                          1120

        380                                         390                                             400
Lys Leu Asn Arg Phe Gln Pro Glu Ser Ser Ala Pro Asp Leu Arg Arg Phe Ile Asp Gly Pro Thr Arg Ala Leu Gly Leu Pro Gln Glu
AAA CTT AAC AGG TTT CAG CCT GAA TCA TCT GCC CCG GAC CTG CGA CGA TTT ATC GAT GGT CCA ACC CGG GCT CTA GGT CTG CCT CAG GAG
100                           1160                          1160                          1200                          1220

        410                                         420                                             430
Leu Arg Glu Val Ile Ser Ser Ile Ser Tyr Met Ala Arg Gln Leu Gln Glu Gln Glu Asp His Asp Ala Leu Lys Glu Asp Trp Gln Phe
CTA CGA GAG GTC ATT TCC TCA ATC AGC TAC ATG GCC CGA CAG CTT CAG GAA CAG GAG GAC CAC GAC GCA CTG AAG GAG GAC TGG CAG TTT
              1240                          1260                          1260                          1300

        440                                         450                                             460
Val Ala Met Val Val Asp Arg Leu Phe Leu Trp Thr Phe Ile Val Phe Thr Ser Val Gly Thr Leu Val Ile Phe Leu Asp Ala Thr Tyr
GTG GCC ATG GTT GTG GAC CGT CTT TTT CTG TGG ACA TTC ATC GTT TTC ACA AGC GTC GGG ACC CTA GTC ATT TTC CTA GAT GCC ACG TAC
320                           1340                          1360                          1380                          1460

        470
His Leu Pro Pro Pro Glu Pro Phe Pro *
CAT TTG CCC CCT CCT GAA CCT TTC CCT TGA GGACCTCCGGTCATTTTTGCCAAATGCTGCAGGTACTCTCAAGCTCAATACTTGGCGCTTGTAAGAATCTTGTTTCTGG
              1420                          1440          1460                  1980                  1900

GAAGACGTTCAAGTGAGACTTGGAGGCTGAGCATGGTGCCTTGGACCCACCCGAAATGTCCTTCTACTTATTATTTAGAGTTTTGTTCTTCTGCGGTTTTGTTTTTGTTGTTTTTGAGA
1920                  1540                  1960                  1580                  1600                  1620

CAGAGTCTCACTCTGTGGCCAGGCTAGCCTGGAACTGTGGCGTCCAGGCTGGTCTCAAACTCCAACAATGTTACTTCCTTAGCCTAGCAATTGTTGGAATCACAGACCTGGTAAACCA
1640                  1660                  1660                  1700                  1720                  1760

GCACACTTGGAGTTTGTTTCTTGAAACTACATAGAATGTCACAGCTTAGATCTCTAGTGAAATAGAATTATAACCAAGTAGTAGACTGTAAGCCTTTTGAGGACAGGAGATCTGTTGGC
1760                  1760                  1800                  1620                  1640                  1660

TACTGAATTATCCCCAGCAATGTGTACAGGTCCTAGCCCCATAAGTGCTTAACATTTCTTGAAGAGAGGGGAAAATAAATCTGGAAGCAAATATAAATATCGCCTACAATTGCACGTATT
1860                  1900                  1920                  1940                  1960                  1980

AAAAATTAAAATGTAGAAAATGATT....... 3'
2000
```

reading frames with calf and *Torpedo* acetylcholine receptor β-subunits shows extensive amino acid homology for an open reading frame of 1506 nucleotides commencing at nucleotide -69 and ending with nucleotide 1437. This reading frame assignment predicts a primary structure for the mouse acetylcholine receptor β-subunit which consists of a 23-amino acid leader peptide and a mature protein of 478 amino acids. Clone BMB49 has 15 nucleotides preceding the initiator methionine and 578 nucleotides of 3' untranslated sequences.

We now have cDNA clones encoding all four subunits of the mouse muscle nicotinic acetylcholine receptor. The amino acid sequences of the four subunits of this receptor are shown in Figure 3. In this figure, the sequences have been aligned to maximize homologies. This emphasizes the conservation of proposed structural domains for the receptor. For example, the proposed membrane-spanning regions are quite conserved between the various subunits. In contrast, the proposed amphipathic helix is less conserved although the amphipathicity of the structure is still evident.

Several of the cDNA clones as originally isolated had extraneous sequences at their 5' ends. These sequences were presumably derived from errors made by the reverse transcriptase during the creation of the library. Since these sequences might have caused initiation of protein synthesis out of the proper reading frame, we engineered each clone to create a suitable 5' sequence and placed the clone downstream of the SP6 promotor. The arrangement of the clones thus created is found in Figure 4. These plasmids are now suitable for synthesis of RNA for injection into the *Xenopus* oocyte.

We synthesized RNA using the clones found in Figure 4 and injected the capped RNA into *Xenopus* oocytes using the procedures first described by Gurden et al., 1971. We assayed synthesis and assembly of a nicotinic acetylcholine receptor .electrophysiologically, by binding α-bungarotoxin, and by immunoprecipitation of the receptor peptides. The results in Figure 5 show a typical voltage trace recorded from an oocyte injected with RNA specific for each of the four subunits. The response is blocked by d-tubocurarine and is dependent upon the concentration of acetylcholine up to the reversal potential. In the future, we will be able to study the

FIGURE (3)

13

Mouse Nicotinic Acetylcholine Receptor

ALPHA
BETA
GAMMA
DELTA

SIGNAL PEPTIDE

MEMBRANE SPANNING I

MEMBRANE SPANNING II

MEMBRANE SPANNING III

AMPHIPATHIC HELIX

MEMBRANE SPANNING IV
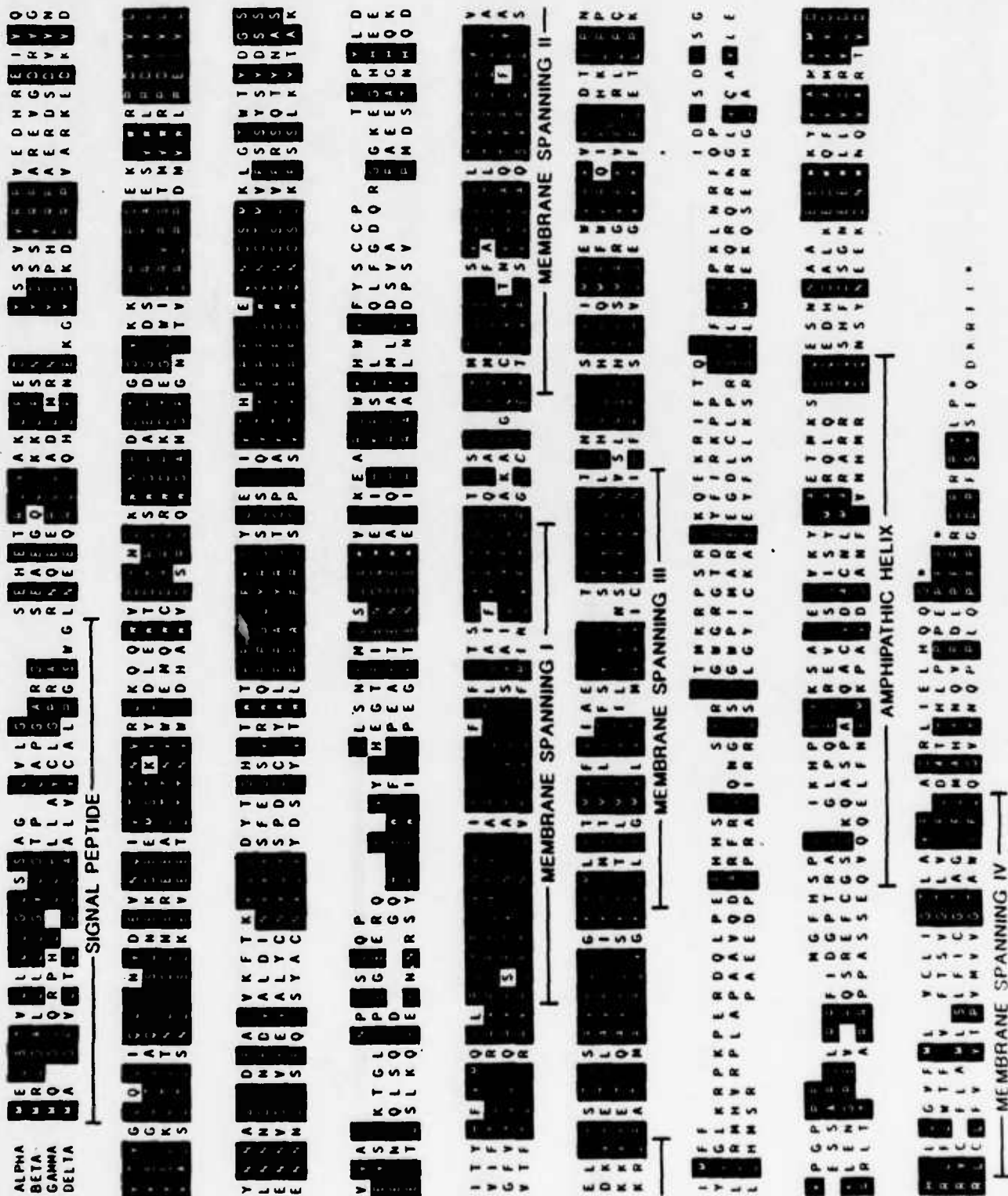
FIGURE (4)

FIGURE (5)
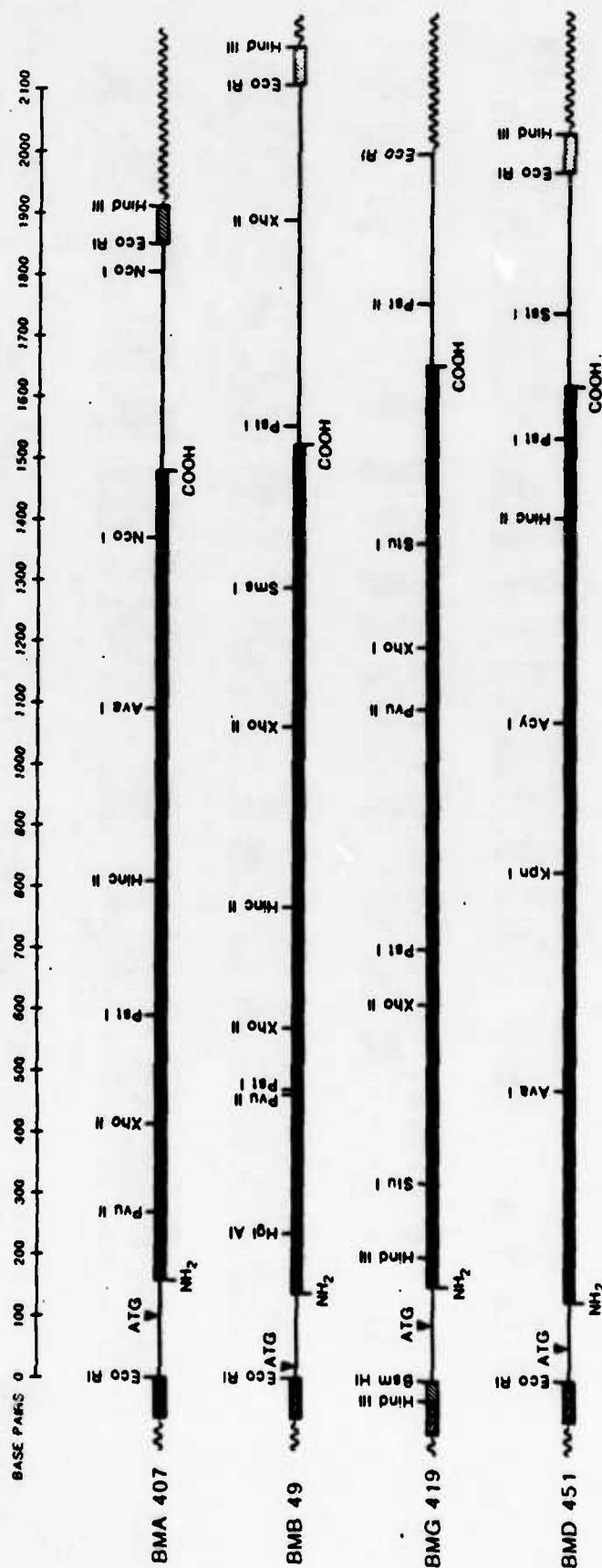
Voltage Responses of Oocyte Injected with mRNA for Mouse α-,β-,γ-,δ-, Subunits to Applied Agonists and Antagonists

(Control Solution: 115mM NaCl, 1.8mM CaCl$_2$, 2.5mM KCl, 10mM Hepes pH 7.2 and 1 μM Atropine).

A Response to 10^{-6} M ACH
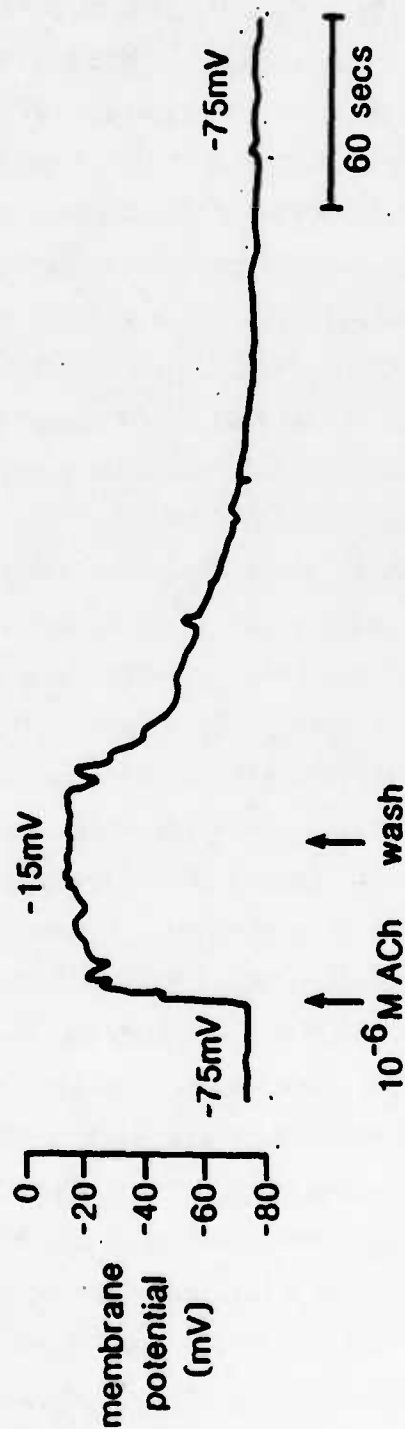
B Initial blocking of the response to 10^{-6} M ACH by 10^{-4} M d-tubocurarine; followed by washing and recovery of the response.
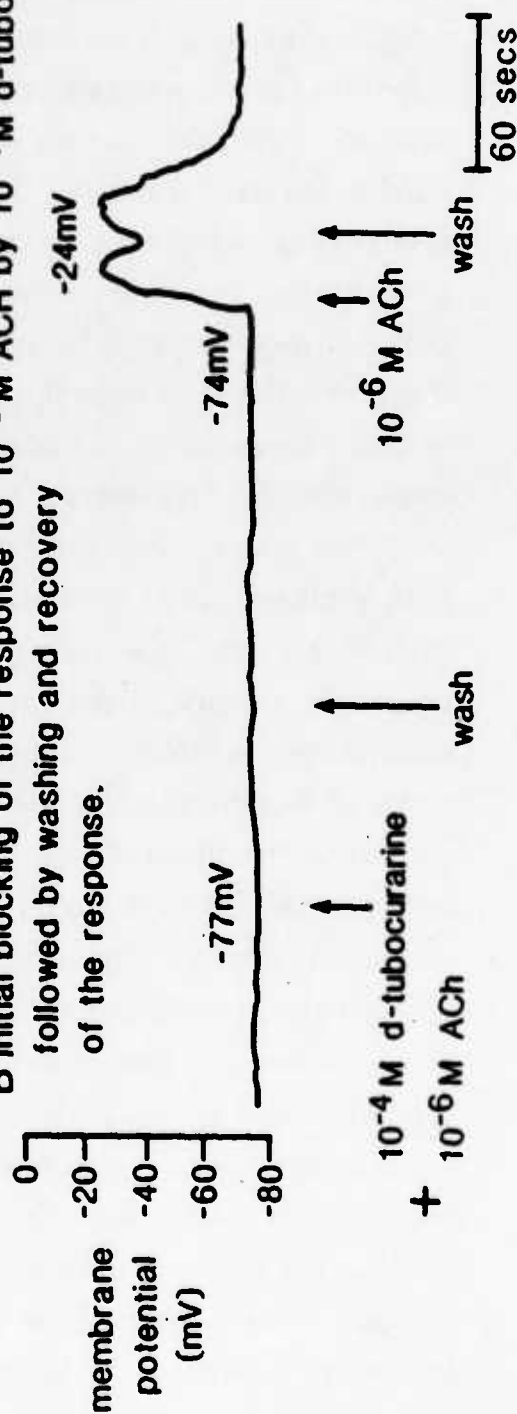
dose-response relationship under voltage-clamp conditions. The results in Figure 6A show sucrose gradient profiles of α-bungarotoxin-labeled detergent extracts of injected eggs. The α-bungarotoxin-binding component sediments with the α-bungarotoxin complexes prepared from the BC_3H-1 cell line used to prepare the cDNA clones. This figure also shows that only a fraction of the total toxin-binding activity is on the cell surface. In the BC_3H-1 cell line, the internal toxin-binding activity is a precursor to the cell surface receptor (Patrick et al., 1977a). It seems likely that this is also the case for the *Xenopus* oocyte, but it has not yet been shown. Finally, we have incubated the injected oocytes in ^{35}S-methionine and prepared detergent extracts from the labeled eggs. The extracts were sedimented on sucrose gradients and pooled fractions were precipitated with anti-receptor antibody. The peak of immunoprecipitable radioactivity sediments as the native receptor (Figure 7). Based on these three assays, we feel that we have successfully produced, in the *Xenopus* oocyte, a functional mouse muscle nicotinic acetylcholine receptor whose synthesis was directed by our subunit cDNA clones.

We are now in a position to modify the receptor by using as templates for RNA synthesis cDNA molecules that we have altered by *in vitro* mutagenesis. Our preliminary results suggest that this will be a powerful tool for studying and eventually modifying the functional receptor. As a start in this work, we have studied the requirements for individual subunits in the receptor oligomer. In a series of experiments we have injected oocytes with RNA molecules encoding various combinations of receptor subunits. Table 1 shows the combinations injected and the fraction of the oocytes giving a measurable response. We obtained functional receptors with all four subunits, with the $\alpha\beta\gamma$ combination as reported by Mishina et al. (1985), and with the $\alpha\beta\delta$ combination which has not previously been seen. A more quantitative analysis of the responses is found in Table 2. The wild-type response is the best, but clear responses are seen with the other two combinations. Figure 6B shows that toxin-binding activity can also be detected in these oocytes. We have not seen functional responses with any other combinations of subunits, nor have we been able to create a functional receptor with only the α-subunit. These observations are important because they place constraints upon models of receptor function and because they point out possible difficulties of *in*
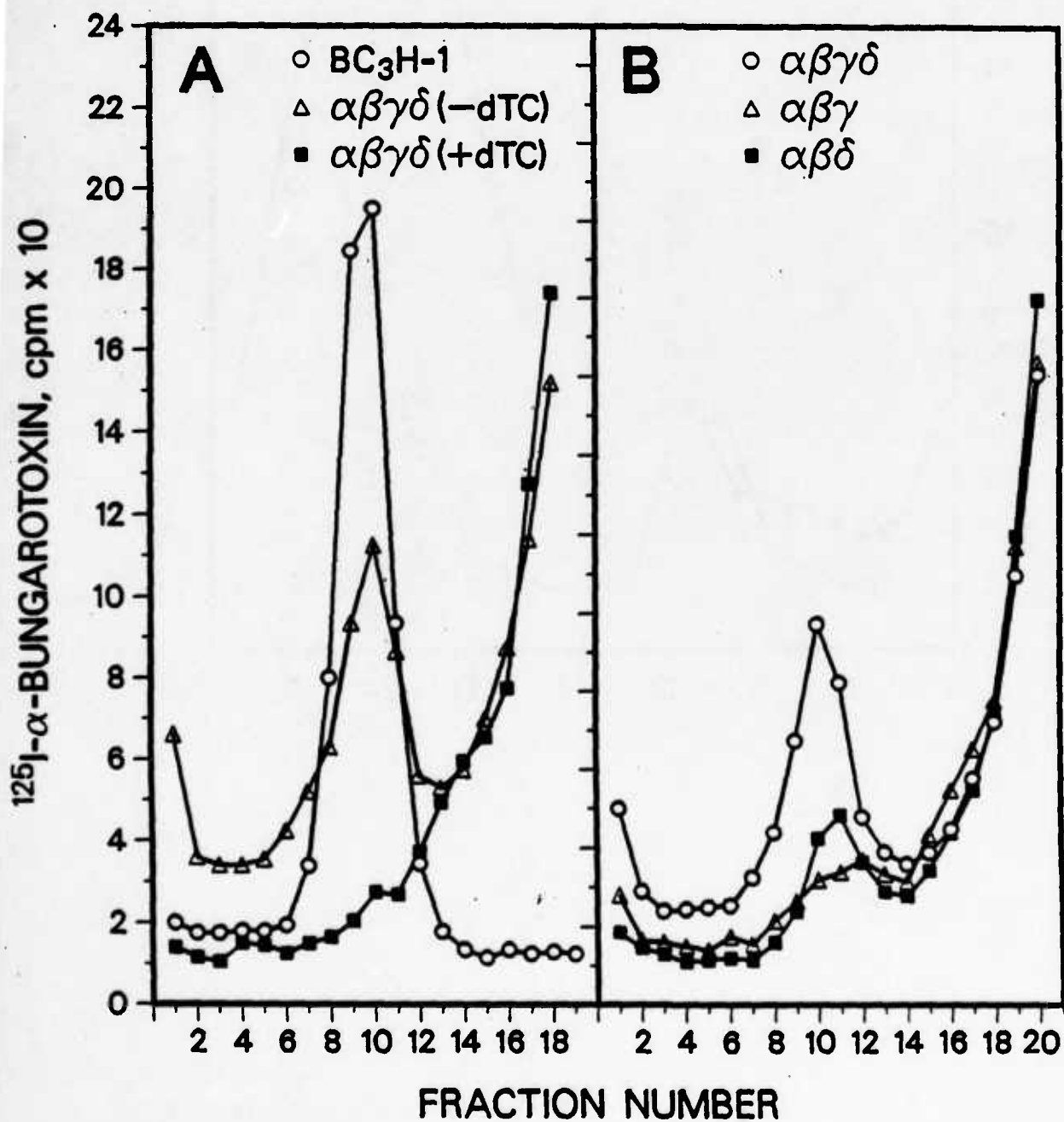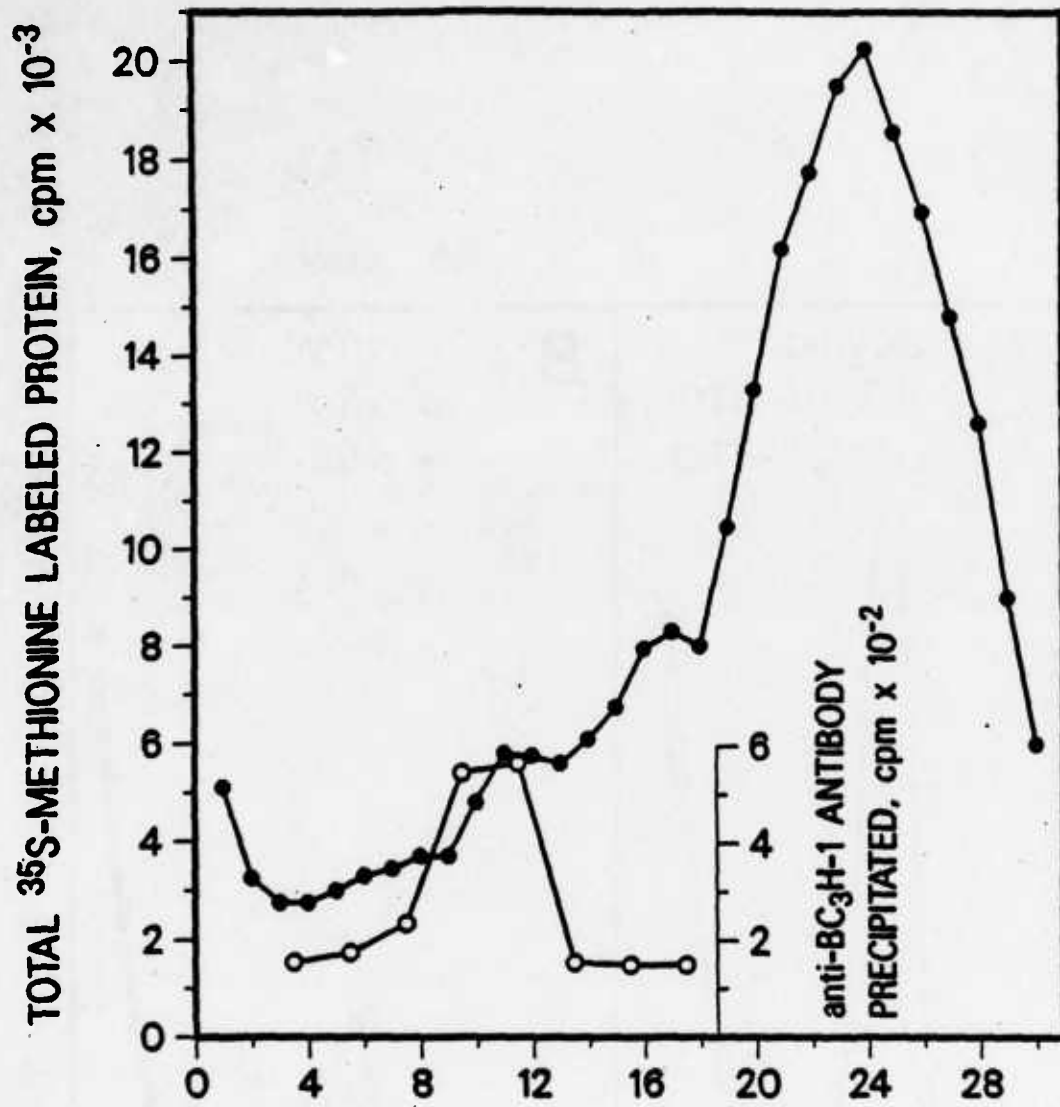
FIGURE (6)

FIGURE (7)

TABLE 1. EFFECT OF SUBUNIT COMPOSITION ON GENERATION OF A FUNCTIONAL RECEPTOR

Subunits	α	αβ	αβγ	αβδ	βγδ	αγδ	αβγδ	none	sham
Response	0/8	1/10	14/16	23/32	0/15	0/22	37/38	0/6	0/15

TABLE 2. SUMMARY OF SUBUNIT COMBINATION RESPONSES TO ACETYLCHOLINE.

Subunit	Day	Resting Potential	Response	Fraction Responsive	Acetylcholine	Surface Receptor	Total Receptor
αβγδ	1	46.8	32.4	28/29	10^{-6}		
αβγ	1	49.5	9.7	7/9	10^{-6}		
αβδ	1	48.7	2.7	5/13	10^{-4}		
αβγδ	2	57.5	43.8	9/9	10^{-6}	2.4	10.3
αβγ	2	39.1	14.3	5/5	10^{-6}	1.0	5.2
αβδ	2	45.0	12.6	7/12	10^{-4}	1.1`	5.2
αβδ	3	65.4	6.5	13/14	10^{-6}		

vitro mutagenesis studies. For example, if we place a mutation in the γ-subunit and find a functional receptor, we will have to prove that the response comes from the altered γ-subunit and not from formation of an αβδ oligomer.

The results outlined above constitute the completion of the first goal of our research program. We have obtained clones encoding all four subunits of a mammalian muscle nicotinic acetylcholine receptor, and the sequences of these clones have been determined. We have also engineered the clones to make them suitable for expression and have shown that they can direct the synthesis of a receptor in the *Xenopus* oocyte. We have also examined the properties of the expressed receptor. In addition, we have created the clones necessary for the creation of mutant receptor subunits and will use this system to test ideas about the structure and function of the mouse muscle nicotinic acetylcholine receptor.

II. NEURONAL NICONITIC ACETYLCHOLINE RECEPTORS

A major goal in obtaining clones coding for the mouse muscle nicotinic receptor was to use these clones to detect, by low stringency DNA/DNA hybridization, sequences coding for possible neuronal nicotinic acetylcholine receptors. We began this program using the PC12 cell line as a source of neural type nicotinic acetylcholine receptors (Greene and Teschler, 1976). On its surface, this cell line has both a neural type nicotinic receptor and an α-bungarotoxin-binding molecule. Both pharmacological (Patrick and Stallcup, 1977b) and immunological (Patrick and Stallcup, 1977a) studies have shown that these are distinct molecular species. The physiological role of the α-bungarotoxin-binding component on these cells is not known. We prepared poly(A)+ RNA from the PC12 cell line and found that a probe prepared from the mouse muscle α-subunit clone hybridizes to two size classes of RNA species, as seen in Figure 8 (Boulter et al., 1986a). The hybrids were not stable at high stringency, suggesting that the bands we detected represented partially but not completely homologous sequences.

3.5 Kb-

2.0 Kb-

PC12 RNA A(+)
PROBE: pMARα15

cDNA was prepared from the PC12 poly(A)+ RNA and cloned into the vector λgt10, and the resulting library was screened with a probe corresponding to a conserved region of the mouse α-subunit. This screening, which was done at low stringency, should have detected molecules which differed by as much as 30% in their sequences. Three clones were found and purified. Subsequent restriction site mapping suggested that the clones were similar if not identical and the longest, PCA48, was chosen for sequencing.

Clone PCA48 was sequenced and found to encode the precursor to a protein which we propose, for the following reasons, is an α-subunit of a neuronal nicotinic acetylcholine receptor. The sequence of this protein, aligned with sequences of other α-subunits, is shown in Figure 9. The encoded protein has the four hydrophobic sequences thought to form transmembrane segments in the muscle α-subunit and, in this respect, is structurally similar to the muscle receptor α-subunit. The protein encoded by PCA48 also contains a sequence homologous to the amphipathic helix found in the α-subunits of muscle receptor. Although the sequence homology is not as great as in the hydrophobic membrane-spanning regions, the distribution of charged and uncharged residues about the helix is similar. The glycosylation site found in the proposed extracellular domain of the muscle α-subunit is also found in the PCA48. There is an additional potential glycosylation site in the PCA48 protein at asparagine 24. Finally, the four cysteine residues in the proposed extracellular domain that are conserved in all muscle α-subunits sequenced to date are also found in the PCA48 protein. These observations are all consistent with the idea that the PCA48 protein is a neural nicotinic acetylcholine receptor α-subunit but do not rule out other alternatives.

For example, the protein encoded by PCA48 might be a rat muscle α-subunit. This was ruled out by the observation that a probe prepared from PCA48 does not hybridize at high stringency to RNA prepared from innervated or denervated rat muscle. Furthermore, rat muscle RNA does not protect PCA48 DNA from digestion by endonuclease S1. In contrast, a probe prepared from PCA48 recognizes RNA species in rat brain and in rat adrenal medulla but not in adrenal cortex.

We tried to gain insight into the function of the protein encoded by PCA48 by determining where the gene is expressed. Clone PCA48 was obtained from a library.

FIGURE (9)

Interspecies Comparison of Aligned Amino Acid Sequences for
the Alpha Subunit of Muscle and Nerve Acetylcholine Receptor

created from poly(A)$^+$ RNA prepared from the PC12 cell line which has both a nicotinic acetylcholine receptor and an α-bungarotoxin-binding component. Although the protein encoded by PCA48 is homologous to the mouse muscle nicotinic receptor α-subunit, it might in fact represent the α-bungarotoxin-binding component of this cell line. We tried to test this possibility using *in situ* hybridization to determine the distribution in mouse and rat brain of the RNA homologous to PCA48 (Goldman et al., 1986). Sections of rat and mouse brain were probed with radiolabeled single strand RNA derived from the rat neuronal clone PCA48 and the mouse muscle clone BMA407. Figure 10 shows autoradiograms of mouse brain hybridized with a mouse muscle probe (A) or the rat neuronal probe (B), and rat brain hybridized with the rat neuronal probe (C) or control probe consisting of the message sense strand of the rat neuronal clone.

The results in these figures show that probes prepared from the muscle and neuronal clones hybridize to different regions of the brain. The neuronal probe hybridizes most strongly to the medial habenula and to a clear but lesser extent to the ventral tegmental area, substantia nigra pars compacta, anteroventral nucleus of the thalamus, the medial geniculate nucleus and the neocortex. In contrast, the muscle α-subunit probe shows little hybridization to these areas. The dentate gyrus and hippocampus show a positive signal with both the neural and muscle probes but also show hybridization with the control probe, corresponding to a sense strand which should not hybridize to the mRNA. The hybridization seen in these areas might therefore represent nonspecific binding.

The hybridization results show that RNA species homologous to the neuronal clone are found in the brain but do not show that the RNA is identical. The question of identity was addressed by S1 analysis of poly(A)$^+$ RNA isolated from the habenula. This RNA, along with poly(A)$^+$ RNA isolated from the PC12 cell line, was size fractionated on denaturing formaldehyde agarose gels and transferred to Gene Screen Plus. Transcripts hybridizing to the neuronal probe were found in both RNA preparations. Heteroduplexes formed between the neural α-subunit and RNA isolated from either the PC12 cell line or the habenula afforded complete protection of the cDNA clone to S1 nuclease digestion, as seen in Figure 11. This shows that

FIGURE (10)

A

B

bases

~2000

-3.5Kb

-910

-2.0Kb

-659

-521

1   2

1   2   3

852362

FIGURE (11)

A

PROBE: λPCA48

B

PROBE: pMAR α15

C

PROBE: λPCA48

D

PROBE: λPCA48 Control "Sense" Strand
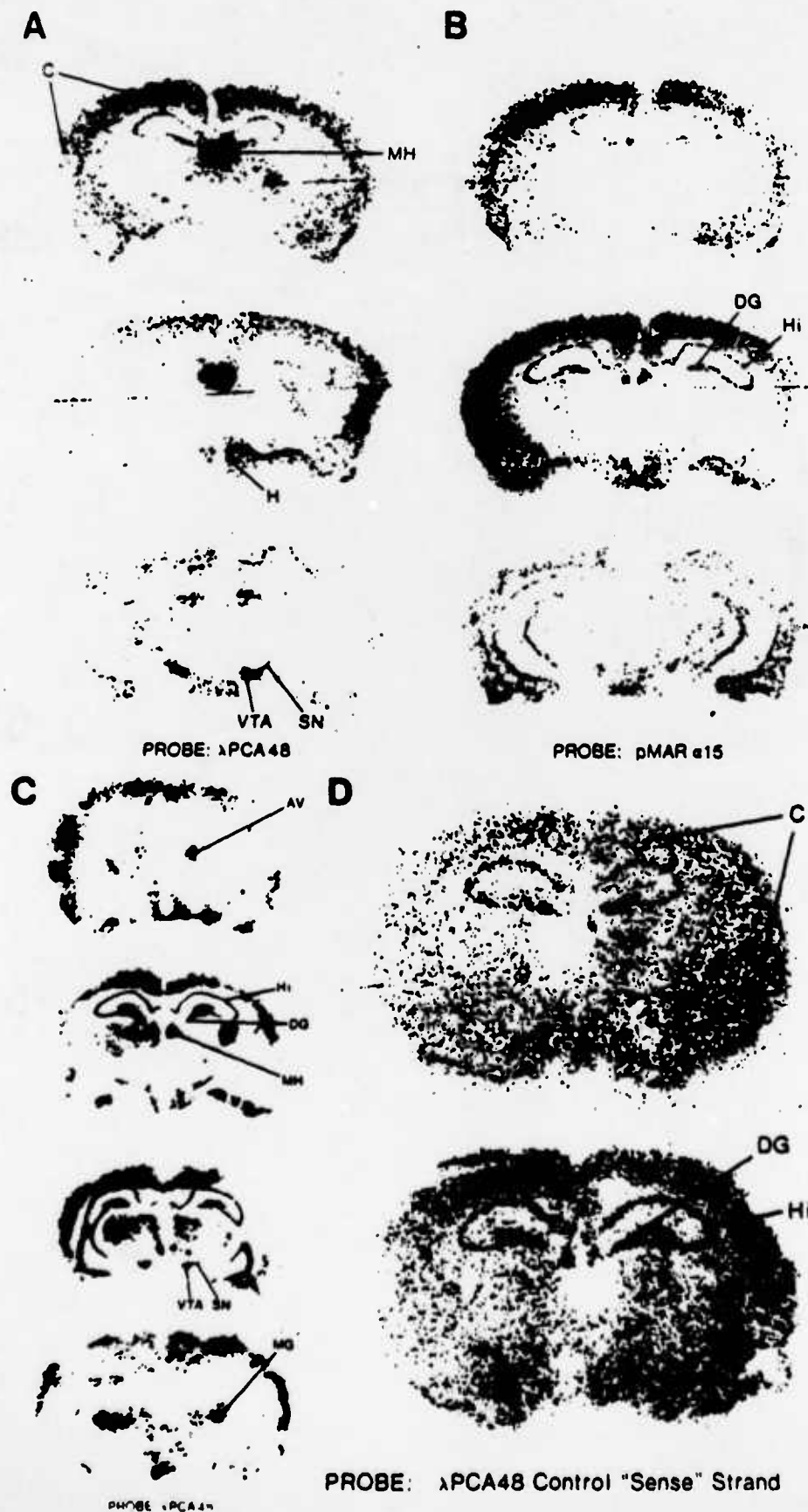
cells in the medial habenula express the same gene as is expressed in the PC12 cell line and suggests that the hybridization we see to this area of the brain is a consequence of this specific RNA species.

The fact that the medial habenula expresses the gene from which the PCA48 neuronal clone was derived suggests that this clone encodes an α-subunit of a nicotinic acetylcholine receptor rather than the α-bungarotoxin-binding component present on the PC12 cell line. Pharmacological studies show that the medial habenula binds cholinergic ligands but does not bind α-bungarotoxin (Hunt and Schmidt, 1978; Clarke et al., 1985). The argument, however, is slightly more complex because cholinergic neurons in the medial habenula project, via the fasciculous retroflexus, to the interpeduncular nucleus (Herkenham and Nauta, 1979; Gottesfeld and Jacobowitz, 1979) where the amount of α-bungarotoxin binding is controversial (Hunt and Schmidt, 1978; Clarke, 1985). Hence, the hybridizing RNA species seen in the medial habenula could code for the nicotinic receptor in the habenula or for the α-bungarotoxin-binding component thought to be in the interpeduncular nucleus. Antibodies directed against the protein encoded by the PCA48 clone should help distinguish between these possibilities.

The *in situ* hybridizations, Northern blots, and S1 protection experiments suggest that the PCA48 sequence is expressed in the medial habenula and that homologous or identical sequences are expressed in other brain regions. In addition, Southern blots of genomic DNA suggest that there are several different homologous sequences in the genome (Boulter et al., 1986). Since these sorts of analyses can be misleading, the only sure way to identify the hybridizing species and determine their relatedness is by cloning. Therefore, we purified poly(A)+ RNA from specific brain regions, made cDNA libraries, and isolated clones hybridizing to either our muscle or neuronal α-subunit clones. We found hybridizing, partially homologous but nonidentical clones in libraries prepared from the hippocampus, the thalamus, and the cerebellum. These clones define two and possibly four additional α-subunit-like molecules. The role that these molecules play in synaptic transmission in the central nervous system is unknown.

28

Blank

MATERIALS AND METHODS

RNA Isolation:

RNA was isolated as previously described (Goldman et al., 1985). Briefly, 1-2 grams of tissue were homogenized in buffered guanidine thiocyanate. After clarification, the homogenate was layered over a cushion of CsCl and centrifuged 15 hours at 35,000 rpm in a Beckman SW41 rotor. The RNA pellet was resuspended in water to which guanidine hydrochloride was added and then ethanol precipitated. The RNA precipitate was resuspended in water and ethanol precipitated again. Poly(A)$^+$ RNA was selected by chromatography over an oligo(dT)-cellulose column (Aviv and Leder, 1972).

Construction and Screening of cDNA Libraries:

Two cDNA libraries were constructed using poly(A)$^+$ RNA isolated from the hippocampus or a hypothalamic punch. The method of Gubler and Hoffman (1983) was used to prepare size-fractionated double-stranded cDNA. The cDNA was ligated to phosphorylated Eco R1 linkers and cloned into the Eco R1 site of bacteriophage λgt10 (Huynn et al., 1985). Approximately 5×10^5 plaques were screened from each library with a radiolabelled cDNA fragment coding for the mouse muscle acetylcholine receptor α-subunit (Boulter et al., 1985), as well as a probe made from the cDNA coding for the α3 gene product (Boulter et al., 1986).

DNA Sequence Determination:

DNA sequencing was performed using the dideoxynucleotide chain termination method of Sanger et al. (1977). cDNAs were subcloned into M13 bacteriophage vectors mp18 and mp19. Deletions were generated by the method of Dale et al. (1985).

RNA Blots:

RNA was denatured in formaldehyde at 65°C and electrophoresed in 2.2M formaldehyde, 1.4% agarose gels. The RNA was then transferred to a Gene Screen Plus membrane. Prehybridization and hybridization conditions were 5X SSPE

(0.75M NaCl, 57mM Na₂HPO₄, 5mM EDTA, pH 7.4), 1% SDS, 10% dextran sulfate, and 50% formamide at 42°C. After hybridization the blot was washed in 0.2X SSPE, 1% SDS at 65°C and was exposed to X-ray film with an intensify ing screen at -70°C.

S1 Nuclease Analysis:

Nuclease S1 digestions of heteroduplexes formed between poly(A)⁺ RNA and M13 subclones of the α4 cDNA clone were carried out as described (Goldman et al., 1985). The 3' 596 nucleotides of the α4 cDNA were subcloned into M13mp18 and the single-strand viral DNA was used to form heteroduplexes. Those hybrids surviving S1 nuclease digestion were analyzed by electrophoresis through a 3% polyacrylamide-8M urea gel, electroblotted to Gene Screen Plus and detected by hybridization to nick-translated radiolabelled α4 cDNA.

In Situ Hybridization:

In situ hybridization was performed as previously described (Cox et al., 1984; Goldman et al., 1986). Briefly, brain sections mounted on polylysine coated slides were treated with proteinase K, acetylated with acetic anhydride and dehydrated prior to hybridization. Sections were hybridized with single strand radiolabelled RNA probes prepared from an SP6 vector containing a cDNA insert encoding either the α3 or α4 gene product. Hybridization was performed at 42°C for 14-18 hours. Post-hybridization treatments included RNase A digestion and a final wash in 0.1X SSPE at 65°C. Slides were dehydrated and exposed to X-ray film at room temperature for 3-20 days.

Sequence Alignment and Homology Calculations:

Protein sequences were aligned using an INTELLIGENETICS software IFIND program that utilizes an algorithm developed by Wilbur and Lipman (1983). Parameters were set to default values. Alignments were adjusted by visual inspection. Homology percentages were calculated by dividing the number of identical residues by the number of residues in the shorter of the two sequences being compared.

REFERENCES

1. Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., and Patrick, J. (1986a) Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor alpha-subunit. Nature 319:368-374.

2. Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stengelin, S., Martin, G., Heinemann, S., and Patrick, J. (1985) Isolation of a clone coding for the alpha-subunit of a mouse acetylcholine receptor. J. Neurosci. 5:2545-2552.

3. Boulter, J.B., Goldman, D., Evans, K., Martin, G., Stengelin, S., Heinemann, S., and Patrick, J. (1986b) Isolation, sequence and preparation of a cDNA clone coding for the gamma subunit of mouse muscle nicotinic acetylcholine receptor. J. Neurosci. Res. 16:37-49.

4. Clarke, P.B.S., Schwartz, R.D., Paul S.M., Pert, C.B., and Pert, A. (1985) Nicotinic binding in rat brain: autoradiographic comparison of [3H] acetylcholine, [3H] alicotine, and [125I]-alpha-bungarotoxin. J. Neurosci. 5:1307-1315.

5. Claudio, T., Ballivet, M., Patrick, J., and Heinemann, S. (1983) Nucleotide and deduced amino acid sequences of *Torpedo californica* acetylcholine receptor gamma-subunit. Proc. Natl. Acad. Sci. U.S.A. 80:1111-1115.

6. Goldman, D., Simmons, D., Swanson, L., Patrick, J., and Heinemann, S. (1986) Mapping brain areas expressing RNA homologous to two different acetylcholine receptor alpha subunit cDNAs. Proc. Natl. Acad. Sci. U.S.A. 83:4076-4080.

7. Gottesfeld, Z. and Jacobowitz, D.M. (1979) Cholinergic projections from the septal-diagonal band area to the habenular nuclei. Brain Res. 176-391-396.

8. Greene, L.A. and Tischler, A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. U.S.A. 73:2424-2428.

9. Hunt, S.P. and Schmidt, J. (1978) The electron microscopic autoradiographic localization of alpha bungarotoxin binding sites within the central nervous system of the rat. Brain Res. 142:152-159.

10. La Polla, R.J., Mixter-Mayne, K., and Davidson, N. (1985) Isolation and characterization of a cDNA clone for the complete protein coding region of the delta-subunit of the mouse acetylcholine receptor. Proc. Natl. Acad. Sci. U.S.A. 81:7970-7974.

11. Mishina, M., Takahashi, T., Takai, T., Kurasaki, M., Fukuda, K., and Numa, S. (1985) Role of acetylcholine receptor subunits in gating of the channel. Nature 307:604-609.

12. Patrick, J. and Stallcup, W. (1977a) Immunological distinction between acetylcholine receptor and the alpha-bungarotoxin-binding component on sympathetic neurons. Proc. Natl. Acad. Sci. U.S.A. 74:4689.

13. Patrick, J. and Stallcup, W. (1977b) Alpha-bungarotoxin binding and cholinergic receptor function on a rat sympathetic nerve line. J. Biol. Chem. 252:8629-8633.

14. Patrick, J., Ballivet, M., Boas, L., Claudio, T., Forrest, J., Ingraham, H., Mason, P., Stengelin, S., Ueno, S., and Heinemann, S. (1983) Molecular Cloning of the Acetylcholine Receptor. Cold Spring Harbor Symp. on Quant. Biol. XLVIII: 71-78.

15. Popot, J-L. and Changeux, J-P. (1984) The nicotinic receptor of acetylcholine: structure of an oligomeric integral membrane protein. Physiol. Rev. 64:1162-1239.

16. Stroud, R.M. and Finer-Moore, J. (1985) Acetylcholine receptor structure, function and evolution. Annu. Rev. Cell Biol. 1:369-401.

17. Herkenham, M. and Nauta, W.J.H. (1979) J. Comp. Neurol. 187:19-48.

18. Gurden, J.B., Lane, C.D., and Woodland, H.R. (1971) Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. Nature 233:177-182.

19. Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography over oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci USA 69:1408-1412.

20. Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stengelin, S., Martin, G., Heinemann, S. and Patrick, J. (1985) Isolation of a clone coding for the alpha-subunit of a mouse acetylcholine receptor. J. Neurosci. 5:2545-2552.

21. Cox, K.H., DeLeon, D.V., Angerer, L.M. and Angerer, R.C. (1984) Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Developmental Biol. 101:485-502.

22. Dale, R.M.K., McClure, B.A. and Houchins, J.P. (1985) A rapid single-stranded cloning strategy for producing a sequential series of overlapping

clone for use in DNA sequencing: Application to sequencing the corn mitochondrial 18s rDNA. Plasmids 13:31-40.

23. Goldman, D., Boulter, J., Heinemann, S. and Patrick, J. (1985) Muscle denervation increases the levels of two mRNA coding for the acetylcholine receptor alpha-subunit. J. Neurosci. 5:2553-2558.

24. Gubler, U. and Hoffman, B.J. (1983) A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.

25. Huynh, T.V., Young, R.A. and Davis, R.W. (1985) In: DNA cloning: a practical approach, Chpt. 2. (eds. D. Rickwood, B.D. Hames) IRL Press, Vol. I, pp. 49-78.

26. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. USA 74:5463-5467.

27. Schiffer, M. and Edmundson, A.B. (1967) Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. Biophys. J. 7:121-135.

28. Wilbur, W.J. and Lipman, D.J. (1983) Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. USA 80:726-730.

34

Blank

DISTRIBUTION LIST

1 copy   Commander
                US Army Medical Research and Development Command
                ATTN: SGRD-RMI-S
                Fort Detrick, Frederick, Maryland   21701-5012

5 copies   Commander
                US Army Medical Research and Development Command
                ATTN: SGRD-PLE
                Fort Detrick, Frederick, Maryland   21701-5012

12 copies   Defense Technical Information Center (DTIC)
                ATTN: DTIC-DDAC
                Cameron Station
                Alexandria, VA   22304-6145

1 copy   Dean
                School of Medicine
                Uniformed Services University of the Health Sciences
                4301 Jones Bridge Road
                Bethesda, MD   20814-4799

1 copy   Commandant
                Academy of Health Sciences, US Army
                ATTN: AHS-CDM
                Fort Sam Houston, TX   78234-6100

THIS REPORT HAS BEEN DELIMITED
AND CLEARED FOR PUBLIC RELEASE
UNDER DOD DIRECTIVE 5200.20 AND
NO RESTRICTIONS ARE IMPOSED UPON
ITS USE AND DISCLOSURE.

DISTRIBUTION STATEMENT A

APPROVED FOR PUBLIC RELEASE;
DISTRIBUTION UNLIMITED.