Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7255–7259, September 1989
Neurobiology

Snake acetylcholine receptor: Cloning of the domain containing the four extracellular cysteines of the α subunit

(ligand-binding site/α-bungarotoxin/polymerase chain reaction)

DRORIT NEUMANN*, DORA BARCHAN*, MIA HOROWITZ*, ELAZAR KOCHVA†, AND SARA FUCHS*

*Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel; and †Department of Zoology, Tel Aviv University, Tel Aviv 69978, Israel

Communicated by Michael Sela, June 19, 1989 (received for review March 22, 1989)

ABSTRACT **The acetylcholine receptor (AcChoR) at the neuromuscular junction of elapid snakes binds cholinergic ligands but unlike other muscle AcChoRs does not bind α-bungarotoxin. Numerous studies indicate that the ligand-binding site of the AcChoR includes cysteine residues at positions 192 and 193 of the α subunit. We have previously shown that a synthetic dodecapeptide corresponding to residues 185–196 of the *Torpedo* AcChoR α subunit contains the essential elements of the ligand-binding site. In an attempt to elucidate the structural basis for the precise binding properties of snake AcChoR, we sequenced a portion of the snake AcChoR α subunit. First, a mouse AcChoR α-subunit cDNA probe was used to screen a size-selected snake (*Natrix tessellata*) genomic library. A genomic clone was isolated and was found to contain sequences homologous to the exon including the first two cysteines (Cys-128 and -142) of AcChoR α subunit. The domain of the α subunit from *Natrix* and cobra AcChoR (amino acid residues 119–222), which contains the four extracellular cysteines (128, 142, 192, and 193), was amplified by reverse transcription of mRNA and the polymerase chain reaction and then sequenced. The deduced amino acid sequence showed that the snake α subunit contains the two tandem cysteines at positions 192 and 193, resembling all other AcChoR α subunits. Sequence comparison revealed that the cloned region of the snake α subunit is highly homologous (75–80%) to other muscle AcChoRs and not to neuronal AcChoR, which also does not bind α-bungarotoxin. In the presumed ligand-binding site, in the vicinity of Cys-192 and Cys-193, four major substitutions occur in the snake sequence—at positions 184 (Trp → Phe), 185 (Lys → Trp), 187 (Trp → Ser), and 194 (Pro → Leu). In addition, Asn-189 is a putative N-glycosylation site, present only in the snake. These changes, or part of them, may explain the lack of α-bungarotoxin-binding to snake AcChoR.**

The nicotonic acetylcholine receptor (AcChoR) is the ligand-gated ionic channel that binds the neurotransmitter acetylcholine and mediates synaptic transmission between nerve and muscle (for review, see ref. 1). Among the four subunits of the receptor, present in a stoichiometry of $\alpha_2\beta\gamma\delta$, the α subunit has been shown to contain the cholinergic binding site (2, 3). Affinity-labeling experiments have previously indicated that within the α subunit the binding site is in close proximity to a sulfhydryl group (4), thus focusing attention to the four cysteines (residues 128, 142, 192, and 193) in the extracellular portion of the α subunit. The first two cysteines (128 and 142) are common to all AcChoR subunits, whereas the other two (192 and 193) are present exclusively in the α subunit (5).

By using synthetic peptides, we had previously shown that residues 185–196 of the α subunit contain the essential components of the ligand-binding site of AcChoR (6, 7). This segment contains the two tandem cysteine residues, at positions 192 and 193, which are unique to the α subunit of AcChoR and are present at this position in *all* α subunits of muscle AcChoR thus far sequenced. We have shown that these two cysteines as well as tryptophan at position 187, which is also conserved in muscle AcChoR, are crucial for retaining toxin binding (7). Studies by other groups using CNBr-derived fragments of affinity-labeled α subunit (8), proteolytic fragmentation (9), synthetic peptides (10–12), and genetic constructs (13–15) have also localized the cholinergic binding site in close proximity or contiguous to the two tandem cysteine residues 192 and 193. Interestingly, the α subunit of the brain nicotinic AcChoR, which does not bind α-bungarotoxin (α-BTX), also contains the two tandem cysteines at positions 192 and 193 (16), although it differs in other amino acid residues from muscle AcChoRs.

Snakes, both venomous and nonvenomous, are resistant to venoms and contain in their blood neutralizing substances against certain toxins, but apparently not against α-neurotoxins (for review, see ref. 17). Their resistance to these latter toxins may be explained by the finding that muscle AcChoR of elapid and other snakes does not bind α-neurotoxins, although this AcChoR responds to other cholinergic ligands (18). It is therefore of special interest to dissect the structure of snake AcChoR and, in particular, its cholinergic binding site. By comparing the sequence and binding properties of snake AcChoR with that of muscle AcChoRs from other species, as well as with the neuronal AcChoR, we may gain insight into the molecular origin of the specific pharmacological profile of the snake receptor and a general knowledge of the structure–function relationship of the AcChoR–ligand binding site. This study describes the cloning and sequence of a fragment from snake AcChoR α subunit,‡ which encodes the extracellular domain containing the four cysteines at positions 128, 142, 192, and 193. Several amino acid substitutions in the presumed ligand-binding region may explain the lack of α-BTX binding to snake AcChoR.

MATERIALS AND METHODS

Animals. Cobras (*Naja naja atra*) were obtained from Latoxan (Rosans, France). Water snakes (*Natrix tessellata*) were collected from the fish ponds of Kibutz Maagan Michael (Israel). Lizards (*Agama stellio*) were obtained from the Canadian Centre of Ecological Zoology (Tel-Aviv University). Mice were of the BALB/c strain.

DNA Preparation and Southern Blot Analysis. *Natrix* liver tissue was chopped into a solution (1 g of tissue per 3 ml) containing 0.1 M EDTA, 0.5% SDS, 50 mM Tris (pH 8), and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AcChoR, acetylcholine receptor; α-BTX, α-bungarotoxin; PCR, polymerase chain reaction; nt, nucleotide(s).

‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M26388 (cobra) and M26389 (*Natrix*)].

proteinase K (250 μg/ml) and shaken overnight at 55°C. Nucleic acid extraction and Southern blot analysis (19) were done as described (20).

Construction of *N. tessellata* Genomic Library. Snake genomic DNA digested with *Eco*RI was size fractionated on 0.7% agarose gel. DNA ranging between 3 and 6 kilobases (kb) was isolated by electrophoresis onto DEAE-cellulose paper. DNA (50 ng) was ligated to *Eco*RI-digested λgt10 arms, packaged *in vitro* (Gigapack; Stratagene), and the phages were plated on the HflA150 strain of *Escherichia coli* (21). The library (5×10^5 phages) was screened with a 900-base pair (bp), *Nco* I fragment, coding for the 280 N-terminal amino acids of mouse AcChoR α subunit. This probe was derived from the cDNA clone pMAR-15 (22), provided by S. Heinemann (Salk Institute, San Diego). The probe was labeled by the multiprime method (23) to a specific activity of $1–3 \times 10^8$ cpm/μg. DNA inserts from the plaque-purified recombinant phage were subcloned into pUC18 and M13mp18 or -19 (24).

RNA Preparation and Northern (RNA) Blot Analysis. Snakes were decapitated, and trunk muscles were excised. RNA preparation and Northern blot analysis were done as described (25).

Preparation and Amplification of cDNA. Five micrograms of poly(A^+) RNA were resuspended in 100 μl of H_2O and treated with methyl mercury at a final concentration of 1 mM for 10 min at room temperature. One microliter of 2-mercaptoethanol was added, and the RNA was precipitated in ethanol in the presence of 0.3 M sodium acetate. The RNA was washed in 70% ethanol, and the first cDNA strand was prepared as described (26). The cDNA (300 ng) was subjected to amplification by the polymerase chain reaction (PCR; ref. 27) in 100-μl reaction mixture containing 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris buffer (pH 8.8), 6.71 mM $MgCl_2$, 67 mM EDTA (pH 8.0), 10% (vol/vol) dimethyl sulfoxide, 10 mM dithiothreitol, bovine serum albumin (180 μg/ml), 300 mM of each dNTP (dATP, dCTP, dTTP, and dGTP), and 50 pmol of each primer. Samples were boiled (7 min), cooled on ice (2 min), and 2.5 units of *Thermus aquaticus* (*Taq*) polymerase (New England Biolabs) were added before incubation at 56°C for 5 min and at 72°C for 2 min. The next 35 cycles were each as follows: 1 min at 94°C, 2 min at 56°C, and 3 min at 72°C. Thermal cycling was performed in a home-made PCR apparatus. Fragments obtained by PCR amplification were purified on agarose gel and subcloned into M13 bacteriophage vectors mp18 and mp19.

Nucleotide Sequence Determination. DNA was sequenced by using the dideoxynucleotide chain-termination technique of Sanger *et al.* (28).

RESULTS

Resistance of *N. tessellata* to α-BTX. Several elapid snakes were reported to be resistant to the antagonistic effects of α-BTX, whereas their AcChoR was shown to be sensitive to agonist activation (18). As a first step toward understanding the molecular basis of this phenomenon, we examined whether the nonpoisonous water snake *N. tessellata* is also resistant to α-BTX. The effect of i.p. injection of α-BTX was examined in the snake and compared with that in lizard (*A. stellio*) and mouse. Intraperitoneal administration of α-BTX into water snakes in concentrations several orders of magnitude higher than the LD_{50} for other species did kill the snakes (Table 1). Similar weight-equivalent doses were lethal in lizards, which are phylogenetically related to the snake and have been reported sensitive to α-BTX (18). We ruled out the possibility that resistance of the snake to α-BTX was due to neutralizing factors in the serum, because preincubation of snake serum with α-BTX (10 μg) did not abolish its lethal effect upon subsequent injection into mice (Table 1).

Table 1. Lethality of α-BTX in snake, lizard, and mouse

Species	Dose,* μg of α-BTX/g of body weight	Death, min after injection
Snake (*N. tessellata*)	10	Survived
	100	Survived
Lizard (*A. stellio*)	10	30
	100	5
Mouse	0.1	10
	0.1†	10

*α-BTX (in 1 ml of phosphate-buffered saline) was injected i.p. into snakes and lizards and i.v. into mice.
†α-BTX was incubated with snake serum (1 ml) for 30 min before injection into mice.

Isolation and Characterization of a Snake Genomic Clone. In search of the snake AcChoR α subunit, we digested *Natrix* genomic DNA with several restriction enzymes and analyzed the fragments by Southern blots with the mouse α-subunit cDNA probe (data not shown). *Eco*RI-digested DNA showed one 4.3-kb hybridizing fragment. On the basis of this hybridization, a library in λg10 vector was prepared from DNA fragments in the range of 3–6 kb obtained by *Eco*RI digestion of *Natrix* DNA and screened with the mouse α-subunit cDNA as probe. A positive clone, designated 15-2, was isolated and found to contain a 4.3-kb insert. Digestion of clone 15-2 with *Bgl* II yielded a 3.5-kb fragment that was

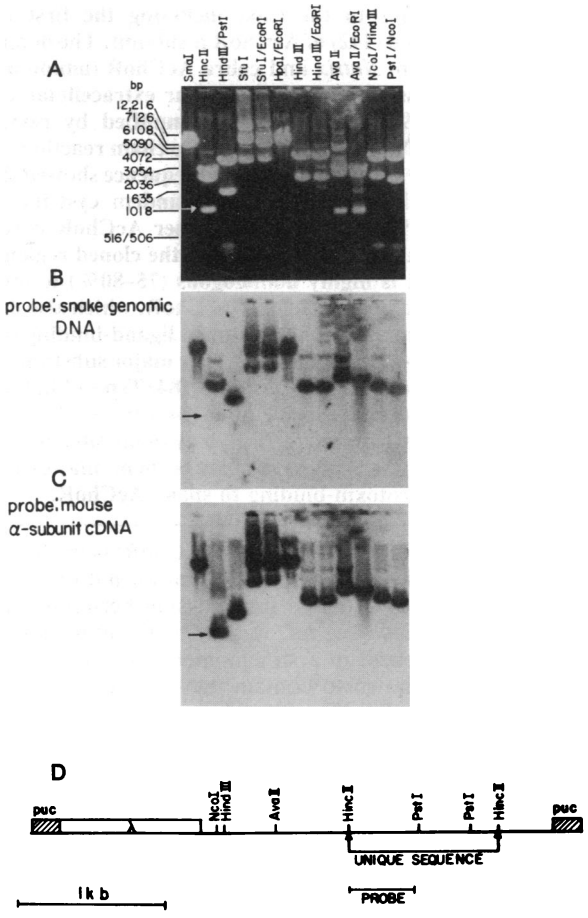

FIG. 1. Identification of unique sequences homologous to mouse α-subunit cDNA in pBgl. (*A*) Ethidium bromide staining. (*B*) Hybridization to ^{32}P-labeled sheared snake DNA. (*C*) Hybridization to ^{32}P-labeled mouse AcChoR α-subunit. (*D*) Restriction map of the clone. Note that the 1-kb *Hinc*II fragment hybridizes selectively to ^{32}P-labeled mouse α-subunit cDNA and not to ^{32}P-labeled total sheared snake DNA.

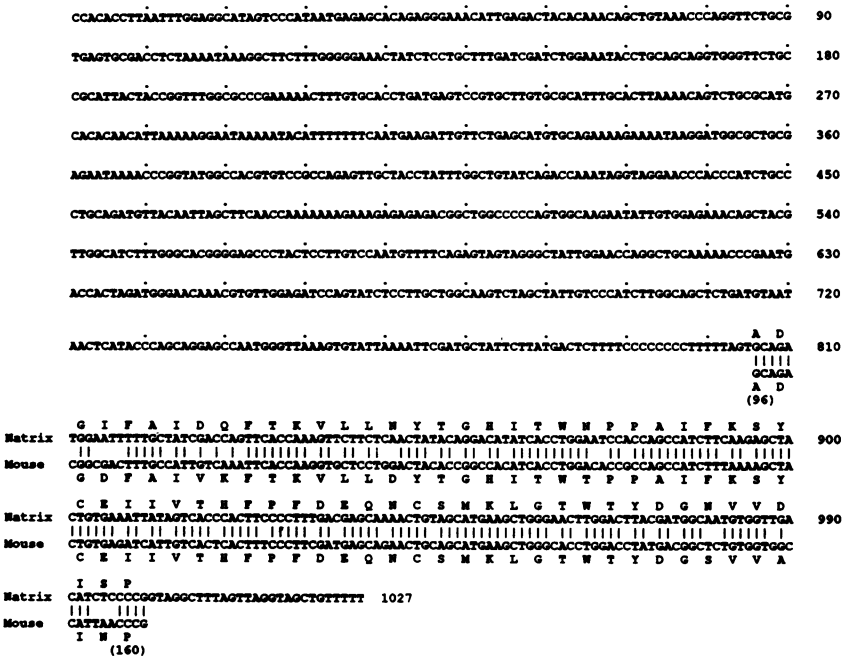

FIG. 2. Nucleotide sequence of *Natrix Hin*cII genomic fragment. Homology to mouse AcChoR α-subunit cDNA and deduced amino acid sequences are depicted.

subcloned into pUC19 and designated pBgl. It should be noted that pBgl contains 1 kb derived from λgt10, because in the cloning process one *Eco*RI site of clone 15-2 was lost (Fig. 1*D*). Restriction enzyme mapping of pBgl identified a 1-kb *Hin*cII fragment that hybridized only to the mouse α-subunit cDNA and not to total sheared snake DNA—therefore, being devoid of repetitive sequences (Fig. 1). Sequence analysis of this 1-kb fragment revealed significant homology (75%) to mouse α-subunit cDNA on a stretch of 200 nucleotides (nt) (Fig. 2). According to the homology, the sequence AGTG at nt 803–806 and CGGT at nt 999–1002 are candidates for acceptor and donor splice sites, respectively (29). Comparison of sequences of the α-subunit gene at a parallel region from chick, *Torpedo* (30), and human (31) also reveals that amino acid residues 96–160 compose an exon. The deduced amino acid sequence between these two putative splice sites revealed considerable homology with residues 96–160 of the mouse AcChoR α subunit. This segment includes the two cysteine residues at positions 128 and 142 of the α subunit, which are present in all four subunits of AcChoR (32). The homology was much higher with the α subunit than with the other subunits (data not shown).

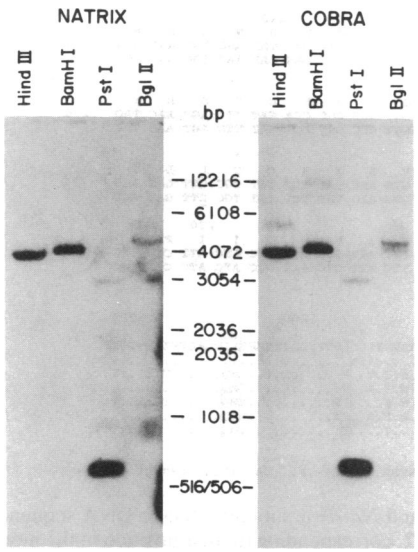

FIG. 3. Southern blot analysis of α-subunit sequences in cobra and *Natrix*. Genomic DNA (15 μg) from *Natrix* and cobra were digested with several restriction enzymes and separated electrophoretically on a 0.7% agarose gel. The blot of this gel was hybridized to the *Natrix* genomic 530-bp *Pst* I–*Hin*cII probe.

Southern and Northern Blot Analyses of Cobra and *Natrix*. Our initial studies were performed on the nonpoisonous water snake *N. tessellata*. It was thus of interest to compare genomic DNA digests of *Natrix* with those of cobra, which contains α-neutrotoxin in its venom. Hence, DNA from *Natrix* and cobra was digested with several restriction enzymes. Samples were electrophoresed through 0.7% agarose gel, blotted, and hybridized with a 530-bp *Hin*cII–*Pst* I fragment derived from pBgl (designated PROBE in Fig. 1*D*). As depicted in Fig. 3, the hybridization pattern obtained with both DNAs was virtually identical.

To identify snake AcChoR α-subunit transcripts, Northern blot analysis was performed. Cobra muscle poly(A)-containing RNA was electrophoresed through a 1.5% formaldehyde–agarose gel and blotted onto nylon (GeneScreen *Plus*) membrane filters. The blots were hybridized with the *Natrix* 530-bp probe and with a mouse AcChoR α-subunit cDNA probe. As shown in Fig. 4, a 3.7-kb transcript hybridized specifically to both probes. Similar results were obtained with *Natrix* poly(A^+) RNA (data not shown).

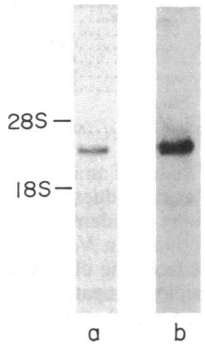

FIG. 4. Northern blot analysis of cobra RNA. Poly(A^+) RNA from cobra was electrophoresed on a 1.5% formaldehyde–agarose gel and blotted onto a nylon (GeneScreen*Plus*) membrane filter. The blot was probed with mouse AcChoR α-subunit cDNA (lane *a*), and after probe removal the DNA was subsequently hybridized to the snake genomic 530-bp *Hin*cII–*Pst* I probe (lane *b*).

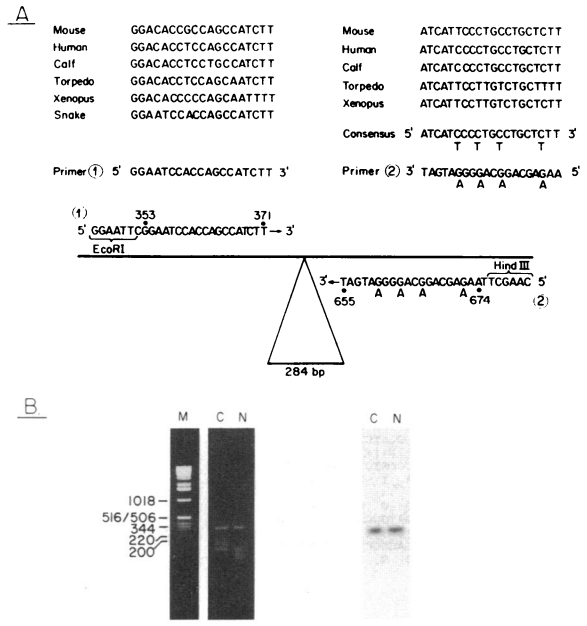

FIG. 5. Amplification of cobra and *Natrix* single-stranded cDNA. (*A*) Schematic presentation of primer selection. Note that primer 1 was from the snake genomic sequence, and primer 2 was from the consensus sequence of the first transmembranal segment of the α subunit. Primers 1 and 2 had *Eco*RI and *Pst* I sites respectively attached at their 5′ ends. (*B*) Aliquots (5 μl) of the amplified products were electrophoresed in a 1% agarose gel and analyzed by ethidium bromide staining (*Left*) and by hybridization to ^{32}P-labeled mouse α-subunit cDNA (*Right*). M, marker proteins; C, cobra; N, *Natrix*.

Cloning of a Snake cDNA Fragment by PCR. It was of special interest to identify, in the snake, the region that in other species, such as *Torpedo* and mouse, contained essential components of the α-BTX binding site [i.e., the segment containing the tandem cysteines 192 and 193 (5, 21)]. We used the PCR to specifically amplify the cDNA encompassing this region. The primer at the 5′ end (GGAATCCACCAGCCATCTT, nt 873–891; Fig. 2) accorded to the sequence obtained from the snake genomic clone and corresponded to a highly conserved region preceding Cys-128 (Fig. 5*A*). At the 3′ end the primer (AARAGCAGRCARGGRATGAT, where R = G or A) was a mixed oligonucleotide presenting a consensus sequence for the first transmembranal region of the α subunit in different species (Fig. 5*A*). The primers had either *Eco*RI or *Hin*dIII sites at their 5′ end (Fig. 5*A*). Cobra and *Natrix* single-stranded cDNA were subjected to 35 consecutive PCR cycles. This procedure yielded a 300-bp fragment, which hybridized specifically to the mouse AcChoR α-subunit cDNA (Fig. 5*B*). Sequence analysis of the amplified 300-bp fragments of *Natrix* and cobra revealed 72% homology to the mouse α-subunit cDNA on the level of nucleotides and 78% homology at the deduced amino acids (Fig. 6*A*). The presence of two cysteines at positions 192 and 193 verifies that this fragment corresponds to the snake AcChoR α subunit. Most differences between snake and mouse sequences concentrate in the vicinity of these tandem cysteines and might contribute to the specific pharmacological properties of the snake receptor.

DISCUSSION

We have cloned the extracellular fragment of the snake AcChoR α subunit, which encompasses the four cysteines at positions 128, 142, 192, and 193. By using a size-selected snake (*Natrix*) genomic library we first isolated sequences coding for the exon containing the cysteine loop between residues 128 and 142. These cysteines were shown by site-directed mutagenesis to be crucial for receptor function (35). The sequence of the snake clone reflected considerable homology to the mouse α subunit (Fig. 2), indicating the evolutionary conservation of this region.

A 3.7-kb AcChoR α-subunit transcript was identified by using either cloned genomic snake sequences or the mouse α-subunit cDNA as probes (Fig. 4). This analysis confirmed that the genomic clone isolated codes for portions of the snake AcChoR α subunit. The size of the snake α-subunit mRNA (3.7 kb) is higher than that reported for the *Torpedo* (5), mouse (22), and human (33) α-subunit mRNAs. Interestingly, a mRNA of a size similar to that of snake has been detected for the calf α subunit and was found to contain a long 3′ untranslated region (31).

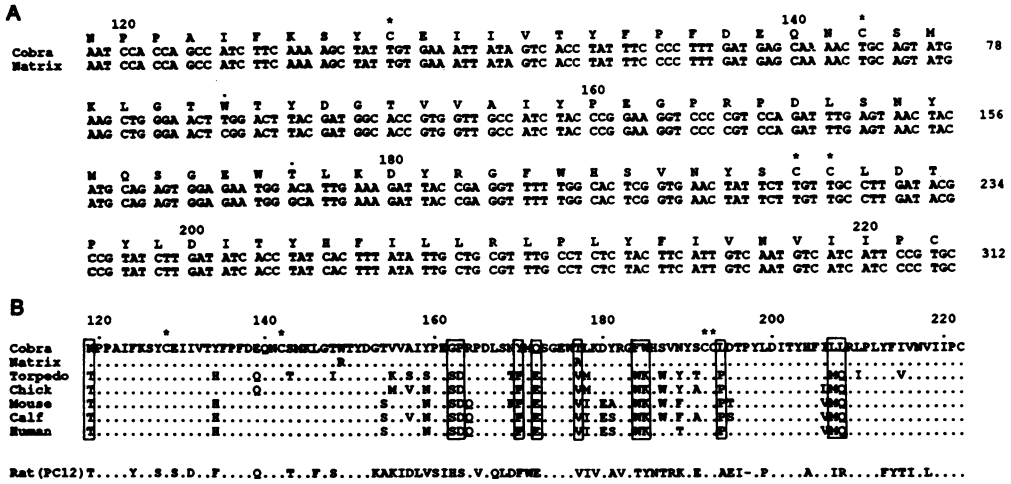

FIG. 6. (*A*) Alignment of nucleotides and deduced amino acid sequences of cobra and *Natrix* α subunits. Snake DNA sequence was from clones obtained by PCR (see *Results*). Amino acid residues are numbered from 119 to 222, corresponding to their position in the mouse AcChoR α subunit. Note that cobra and *Natrix* fragments are identical at all positions except for tryptophan and threonine at positions 149 and 177 of cobra, which are arginine and alanine, respectively, in *Natrix*. Cysteine residues are marked with an asterisk. Note that the sequence of the genomic clone (Fig. 2) differs at four codons from the cDNA sequence given above. We cannot account for these differences, unless they represent different genes or alleles. (*B*) Interspecies comparison of amino acid residues 119–222 of nicotinic AcChoR α subunit. Sequence sources are as referenced: *Torpedo* (5), chick and mouse (22), calf and human (31), and rat neuronal (16). All sequences were compared to cobra. Sequences common to chick, mouse, calf, and human AcChoR but different from snake AcChoR are boxed.

On the basis of sequence data from the snake genomic clone and the fact that the first transmembranal segment of the α subunit is conserved among species, we constructed two nucleotide primers. These primers were used to specifically amplify, by means of the PCR method, an extracellular region of the snake α subunit that includes the four cysteines (126, 142, 192, and 193) of special interest. It is noteworthy that the same two primers were also suitable for amplifying the counterpart domain of the α subunit from mouse cDNA (data not shown), in spite of three mismatches in the first primer that represents the snake sequence. This fact may indicate the potential of the PCR method for analyzing other cholinergic binding sites of special interest.

Analysis of the snake α-subunit sequence obtained via PCR amplification reveals that cysteines 192 and 193, which are characteristic of all AcChoR α subunits and participate in the cholinergic binding site, are also conserved in the snake (Fig. 6).

Alignment of the cobra and *Natrix* PCR-derived sequences and those of the respective segments of muscle AcChoR α subunits of other species, as well as with neuronal AcChoR, is depicted in Fig. 6*B*. The snake sequence is highly homologous to other muscle AcChoRs and not to neuronal AcChoR, which also does not bind α-BTX. Among the various muscle AcChoRs the snake receptor exhibits some significant differences, most of which occur in the vicinity of cysteines 192 and 193. Several amino acids that are identical in distant species, such as *Torpedo*, chick, mouse, calf, and human, are different in snake (boxed in Fig. 6*B*). Of these, substitution of Trp-184 for phenylalanine, Lys-185 for tryptophan, and Pro-194 for leucine are in close proximity to the two tandem cysteines and might be relevant for binding properties. In addition, of particular interest is Trp-187, which is conserved in *Torpedo*, chick, mouse, and calf but substituted for serine in the snake as well as in human. We have previously shown that chemical modification of this tryptophan in the synthetic peptide 185–196 of the *Torpedo* α subunit abolished its binding to α-BTX (7). Moreover, the synthetic dodecapeptide corresponding to residues 185–196 of the human α subunit, which does not contain Trp-187, did not bind α-BTX (7); this substitution may contribute to the lower affinity of human AcChoR to α-BTX (34).

Finally, both cobra and *Natrix* PCR-derived fragments contain three putative N-glycosylation sites (Asn-111, -141, and -189), whereas all other known α subunits contain only Asn-141 as the N-glycosylation site. Hence, it is possible that the snake AcChoR α subunit might be more glycosylated than are the other AcChoRs.

In conclusion, we have shown that the ligand-binding domain of snake AcChoR differs in several crucial amino acid residues from other muscle AcChoRs that bind α-BTX. Whether all the changes in primary structure or only part of them contribute to the specific pharmacological profile of the snake receptor and explain the resistance of snakes to α-neurotoxins remains to be determined. The changes in the AcChoR ligand-binding domain in the muscle of snakes might have occurred early in their evolution, but after their separation from the lizards, from which they presumably originated (17).

We thank Prof. David Givol for fruitful discussions and for critically reviewing this manuscript, Prof. S. Heinemann for the gift of plasmid pMAR-15, and Irit Orr from the Computer Service Unit for her help in the preparation of sequence figures. This research was supported, in part, by grants from the Muscular Dystrophy Association of America, the Association Francaise Contre les Myopathies, the Los Angeles Chapter of the Myasthenia Gravis Foundation, the United States–Israel Binational Science Foundation, and the Leo and Julia Forschheimer Center for Molecular Genetics at the Weizmann Institute.

1. McCarthy, M. P., Earnest, J. P., Young, E. F., Choe, S. & Stroud, R. M. (1986) *Annu. Rev. Neurosci.* **9**, 383–413.
2. Haggerty, J. G. & Froehner, S. C. (1981) *J. Biol. Chem.* **256**, 8294–8297.
3. Gershoni, J. M., Hawrot, E. & Lentz, T. L. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4973–4977.
4. Karlin, A. (1980) in *The Cell Surface and Neuronal Functions*, eds. Cotman, G. W., Poste, G. & Nicolson, G. L. (Elsevier, Amsterdam), pp. 191–260.
5. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. & Numa, S. (1982) *Nature (London)* **299**, 793–797.
6. Neumann, D., Barchan, D., Safran, A., Gershoni, J. M. & Fuchs, S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3008–3011.
7. Neumann, D., Barchan, D., Fridkin, M. & Fuchs, S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9250–9253.
8. Kao, P. N. & Karlin, A. (1986) *J. Biol. Chem.* **261**, 8085–8088.
9. Pederson, S. E., Dreyer, E. B. & Cohen, J. B. (1986) *J. Biol. Chem.* **261**, 13735–13743.
10. Wilson, P. T., Lentz, T. L. & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8790–8794.
11. Mulac-Jericevic, B. & Atassi, M. Z. (1986) *FEBS Lett.* **199**, 68–74.
12. Gotti, C., Mazzola, G., Longhi, R., Fornasari, D. & Clementi, F. (1987) *Neurosci. Lett.* **82**, 113–119.
13. Barkas, T., Mauron, A., Roth, B., Alliod, C., Tzartos, S. J. & Ballivet, M. (1987) *Science* **235**, 77–80.
14. Gershoni, J. M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4318–4321.
15. Aronheim, A., Eshel, Y., Mosckovitz, R. & Gershoni, J. M. (1988) *J. Biol. Chem.* **263**, 9933–9937.
16. Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S. & Patrick, J. (1986) *Nature (London)* **319**, 368–374.
17. Kochva, E. (1987) *Toxicon* **25**, 65–106.
18. Burden, S. Y., Hartzell, M. C. & Yoshikami, D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3245–3249.
19. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
20. Reiner, O., Wigderson, M. & Horowitz, M. (1988) *DNA* **7**, 107–116.
21. Huynh, T. V., Young, R. A. & Davis, R. W. (1984) in *DNA Cloning Techniques: A Practical Approach*, ed. Glover, D. (IRL, Oxford), Vol. 1, pp. 49–78.
22. Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stengelin, S., Martin, G., Heinemann, S. & Patrick, J. (1985) *J. Neurosci.* **5**, 2545–2552.
23. Feinberg, P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
24. Messing, J., Crea, R. & Seeburg, P. M. (1981) *Nucleic Acids Res.* **9**, 309–321.
25. Asher, D., Neumann, D. & Fuchs, S. (1988) *FEBS Lett.* **233**, 277–281.
26. Watson, C. J. & Jackson, J. F. (1985) in *DNA Cloning: A Practical Approach*, ed. Glover, D. M. (IRL, Oxford), Vol. 1, pp. 79–88.
27. Saiki, R. K., Scharf, S., Falloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230**, 1350–1354.
28. Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. (1980) *J. Mol. Biol.* **143**, 161–178.
29. Sharp, P. A. (1981) *Cell* **23**, 643–646.
30. Ballivet, M., Nef, P., Stalder, R. & Fulpius, B. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **48**, 83–87.
31. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S. & Numa, S. (1983) *Nature (London)* **305**, 818–823.
32. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. & Numa, S. (1983) *Nature (London)* **302**, 528–532.
33. Schoepfer, R., Luther, M. & Lindstrom, J. (1988) *FEBS Lett.* **226**, 235–240.
34. Lecky, B. & Morgan-Hughes, J. (1981) *Ann. N.Y. Acad. Sci.* **377**, 61–76.
35. Mishina, M., Tobimatsu, T., Imoto, K., Tanaka, K., Fujita, Y., Fukuda, K., Kurasaki, M., Takahashi, H., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, T., Kuno, M. & Numa, S. (1985) *Nature (London)* **313**, 364–369.