The EMBO Journal vol.9 no.7 pp.2101 – 2106, 1990

The human muscle nicotinic acetylcholine receptor α-subunit exists as two isoforms: a novel exon

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Communicated by E.Barnard

Analysis of acetylcholine receptor clones isolated from a human leg muscle cDNA library, revealed that the α-subunit existed as two isoforms. A novel exon, coding for 25 amino acids, was located in the human genomic DNA sequence; its insertion into the α-subunit gives the new isoform of 462 amino acids. In addition, mRNAs for the two isoforms were found in equal proportions in poly(A)$^+$ RNA obtained from three further sources including partially denervated and innervated human muscle and the rhabdomyosarcoma cell line TE671. Both protein isoforms can be expressed in *E.coli*. No evidence of a sequence related to that of the new exon was found in cDNA derived from poly(A)$^+$ RNA isolated from fetal calf or embryonic chick muscle or *Torpedo marmorata* electric organ.

Key words: acetylcholine receptor/α-subunit/expression/human/isoform

Introduction

The muscle nicotinic acetylcholine receptor (AChR) is a neurotransmitter-gated ion channel that mediates synaptic transmission at the vertebrate neuromuscular junction. The binding of acetylcholine to its receptor gives rise to the opening of a selective cation channel. The resulting ionic current depolarizes the muscle membrane, leading to muscle contraction. The AChR of fetal striated muscle is an integral membrane protein consisting of five subunits in the stoichiometry α_2, β, γ, δ. In mature muscle, the γ is replaced by an ϵ. The five subunits form a pentameric structure around a central ion channel, with each subunit containing several transmembrane domains (reviewed in Maelicke, 1986). The human AChR is of particular interest in that it is the target for autoimmune attack in myasthenia gravis (Vincent, 1980), a disease characterized by fatiguable muscle weakness. The α-subunit contains both the site for ACh binding and the main focus for antibody binding in myasthenia gravis (Tzartos *et al.*, 1982; Heidenreich *et al.*, 1988).

The genes that code for AChR are the best characterized of the gene superfamily of ligand-gated ion channels, which also includes receptors for glycine and γ-aminobutyric acid. cDNA cloning shows that each subunit is encoded by a distinct mRNA, but that there is extensive homology between subunits and across species (Raftery *et al.*, 1980; Numa *et al.*, 1983; Kubo *et al.*, 1985; Boulter *et al.*, 1985), suggesting that the genes evolved from a common ancestor. Conservation of sequence across species is greatest for the α-subunit, in particular the transmembrane segments, implying strong evolutionary constraints.

Genomic clones for the human AChR α-subunit have previously been described (Noda *et al.*, 1983) in which the gene is divided into eight introns and nine exons, with each of the exons corresponding to an analogous region of a calf AChR α-subunit cDNA clone. The nine exons are termed P1–P9. In accordance with this, a cDNA clone derived from rhabdomyosarcoma cell line TE671 shows a sequence identical to that predicted from the genomic clone (Schoepfer *et al.*, 1988).

Buonanno *et al.*, 1989, noted that the organization of exons 1–4 is conserved in all known muscle and neuronal AChR genes, with the exception of the *Drosophila ARD* gene. In obtaining α-subunit clones from a human leg muscle cDNA library for expression in *Escherichia coli* (Beeson *et al.*, 1989), we found two forms, one as previously predicted and a second with an additional 75 bp of coding sequence. We also isolated genomic clones for the α-subunit, which showed that the 75 bp region constitutes a novel exon located between exons P3 and P4. We were unable to detect an analogous sequence corresponding to the novel exon in cDNA synthesized from calf or chick muscle or *Torpedo* electric organ poly(A)$^+$ RNA.

Results

Isolation and characterization of cDNA and genomic clones for the human muscle AChR α-subunit

We are interested in identifying B and T cell epitopes on the AChR involved in the autoimmune disease myasthenia gravis. To overcome difficulties caused by the low levels of AChR found in human muscle we aimed to isolate cDNA clones coding for the human subunits and then insert them into either prokaryotic or eukaryotic expression vectors. Clones were isolated from a cDNA library constructed in λgt10 using poly(A)$^+$ RNA derived from partially denervated human leg muscle (Beeson *et al.*, 1989). Fragments from restriction digests of an α-subunit clone were ligated into plasmid vectors pRex or pUC8 for expression in *E.coli*.

DNA sequencing of these constructs revealed a previously unreported 75 bp region coding for a 25 amino acid insert found between amino acids 58 and 59 of the predicted protein sequence (Noda *et al.*, 1983); this would correspond to the border between exons P3 and P4. The full length cDNA clone that was used in making our expression constructs was sequenced completely and shown to be identical to the predicted sequence (Noda *et al.*, 1983), except for the 75 bp insert and a single base pair change of a G residue to an A residue at position 1132. This alters the amino acid at position 378 from glycine to serine. Since previous reports for muscle α-subunits (Boulter *et al.*, 1985) have a glycine at this position, we believe this change may have occurred

during cDNA synthesis.

DNA sequence analyses of two other full length α-subunit cDNA clones isolated from this library showed that a second clone contained the insert, whereas a third did not. Restriction digests of a fourth and fifth clone, which were not full length, again revealed one with and one without the 75 bp insert. Thus, of the five α-subunit cDNA clones we characterized, three contained the extra 75 bp insert and two did not.

To confirm the presence of the 75 bp sequence in the α-subunit gene, we isolated clones from a human genomic library constructed from an unrelated individual in the vector EMBL3, and screened with a 138 bp *Bgl*II fragment containing 63 bp from the 3′ end of the new insert and 75 bp from P4. The analysis of one of these clones, λαGHAChR15 and derived subclones, is shown in Figure 1. The 75 bp insert found in the cDNA is also found in this genomic clone, has appropriate splice sites (Breathnach *et al.*, 1978) and may constitute a previously unreported exon for the α-subunit located between P3 and P4. We have called this exon P3A.

Levels of mRNA coding for the α-subunit isoforms

To ascertain whether the mRNA coding for the two isoforms is present in both innervated and denervated muscle, and to establish the relative amounts of each form, we carried out PCR studies combined with RNase A protection assays.

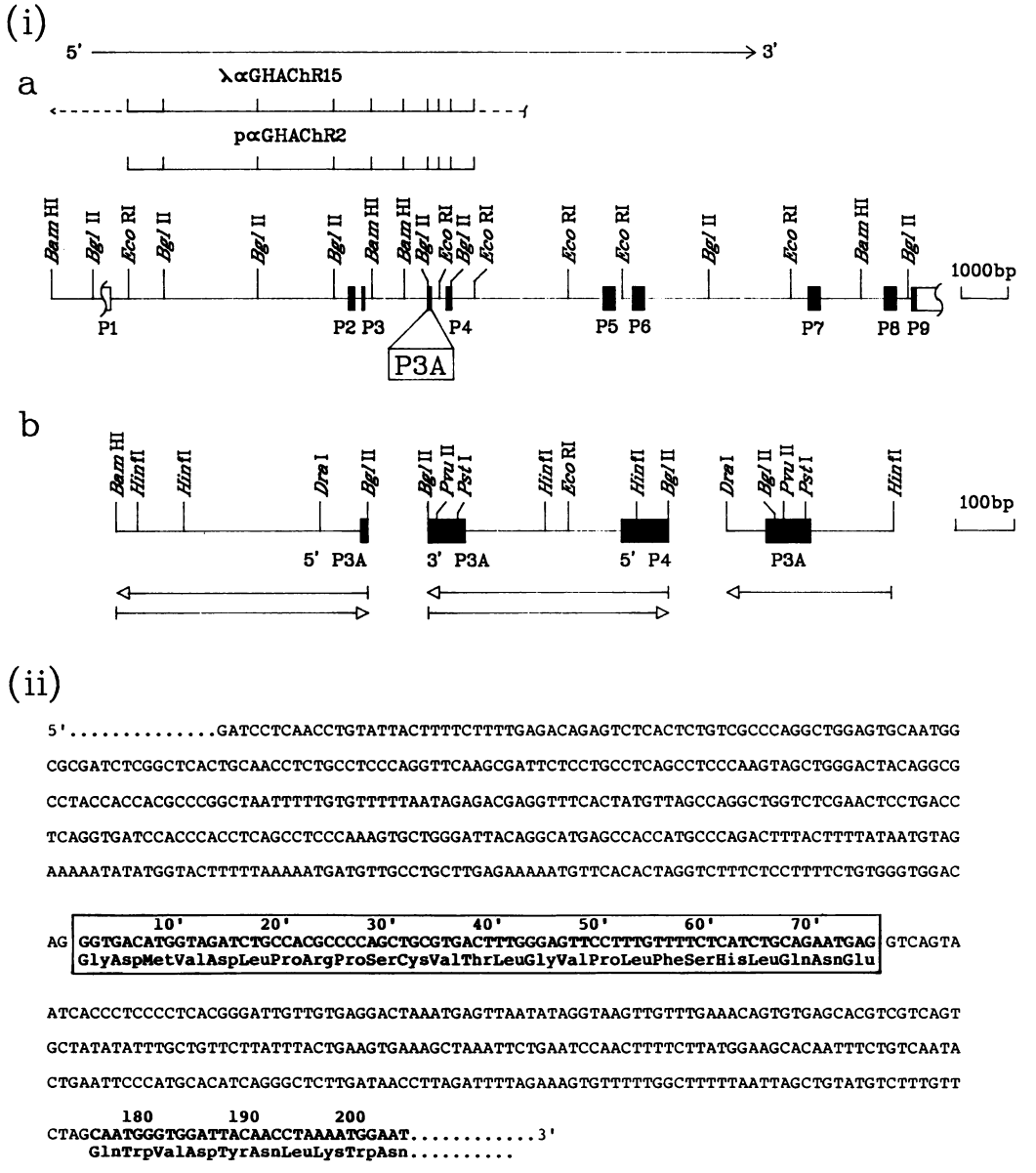

Fig. 1. (**i**): the arrangement of introns and exons in the human AChR α-subunit gene (Noda *et al.*, 1983), including the novel P3A exon. Also shown are the clones, subclones and sequencing strategy used to determine this arrangement. **a**: The EMBL3 clone, λαGHAChR15, shown above the gene extends ~10–12 kb upstream, as indicated by the arrow, and ends at an undetermined point between the third and fourth *Eco*RI sites. From this lambda clone, the pUC8 subclone pαGHAChR2 was constructed, which contains the region bounded by the first and third *Eco*RI sites. **b**: the three further subclones, made from pαGHAChR2, that were sequenced to determine the presence and position of the P3A exon in the gene. The *Bam*HI–*Bgl*II and *Bgl*II fragments were sequenced in both directions in M13mp18. The *Dra*I–*Hin*fI fragment was sequenced in pUC8 (in one direction only) to confirm that there was only one *Bgl*II site in this region and that the sequence read through as anticipated. (**ii**): the nucleotide sequence determined from the subclones illustrated in (i)b. The P3A exon and its deduced amino acid sequence are shown boxed. Also shown, in bold type, are the first 30 nucleotides of the P4 exon and corresponding amino acid sequence. The nucleotide sequence of P3A has been given the EMBL database accession number X17104.

cDNA was synthesized from poly(A)$^+$ RNA prepared from innervated and denervated human leg muscle samples from different individuals (see Materials and methods), and from the cell line TE671. TE671 has been shown to express AChRs similar to those found in human denervated muscle (Walker *et al.*, 1988; Luther *et al.*, 1989). Oligonucleotides homologous to the 5′ end of P3 and complementary to the 3′ end of P4 were used for PCR amplification of the cDNAs (Figure 2). The two bands of 155 and 230 bp derived from denervated muscle and the cell line TE671 were subcloned into pGEM-3Z, and subjected to DNA sequencing. The smaller band (155 bp) consisted of exons P3 and P4; the larger band (230 bp) contained P3, P3A and P4. One in four of the subclones for the 230 bp fragments had the T residue at position 56 of P3A changed to a C residue; this probably arose during PCR amplification.

The pGEM-3Z subclones were used to generate radiolabelled antisense RNA, to P3/P4 and P3/P3A/P4 respectively, for the RNase A protection assay (Figure 3). The results show that when hybridizing to poly(A)$^+$ RNA with the shorter probe, a band of 155 bp is protected, whereas bands of 230, 110 and 45 bp are found with the longer probe. Poly(U:A) stretches may be sensitive to RNase A digestion (Little and Jackson, 1987). It is likely, therefore, that the fainter, smudged bands seen approximately 50 bp below the protected fragments in both the poly(A)$^+$ RNA and control RNA samples [Figure 3(i) and (ii)] are caused by digestion at the run of seven A residues found 55 bp from the 3′ end of P4. The interpretation of these results [Figure 3(iii)] for the tissues so far analysed points to a mixed α-subunit mRNA population with the two forms being present in a ratio of approximately 1:1. In denervated muscle and TE671 cells, both α-subunit mRNA forms are present at a higher level than in innervated muscle.

Is a P3A-like sequence present in mRNA from other species?

To address this question, we isolated poly(A)$^+$ RNA from *Torpedo* electric organ, embryonic chick and fetal calf muscle, synthesized cDNA and carried out PCR amplification using oligonucleotide primers specific for the appropriate species (Figure 4). The primers were chosen in each case to amplify a sequence containing the P3/P4 exon boundary, yet it is only in the human PCR products that two bands are found. Thus, so far, we have detected the P3A exon only in the human α-subunit gene.

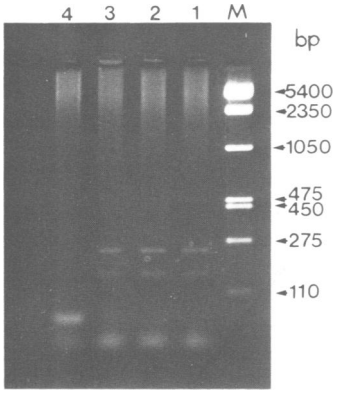

Fig. 2. PCR amplification of cDNA regions P3/P4 and P3/P3A/P4. cDNA was synthesized from poly(A)$^+$ RNA and used in PCR amplification with the oligonucleotide primers 5′ GATGAAGTAAATCAGATCGTGAC 3′ and 5′ TTGTTATAGAGAACAAGGTCTGG 3′. PCR products were run on a 1.4% agarose gel and visualized under UV after staining with ethidium bromide. poly(A)$^+$ RNA was from (**1**) Innervated muscle, (**2**) Denervated muscle, (**3**) TE 671 cell line, (**4**) Marek's virus transformed chicken T lymphocytes. (**M**) Size markers: *Hin*dIII cut PM2 DNA (Boehringer Mannheim).

Expression of the two human α-subunit isoforms in E.coli

We have constructed expression vectors that allow us to synthesize almost full length versions of the two isoforms from *E.coli* (Figure 5). The constructs used express amino acids 1−429 (+ or −P3A) of the mature protein, plus some additional residues at the C terminus derived from the plasmid sequence prior to termination. Binding of both

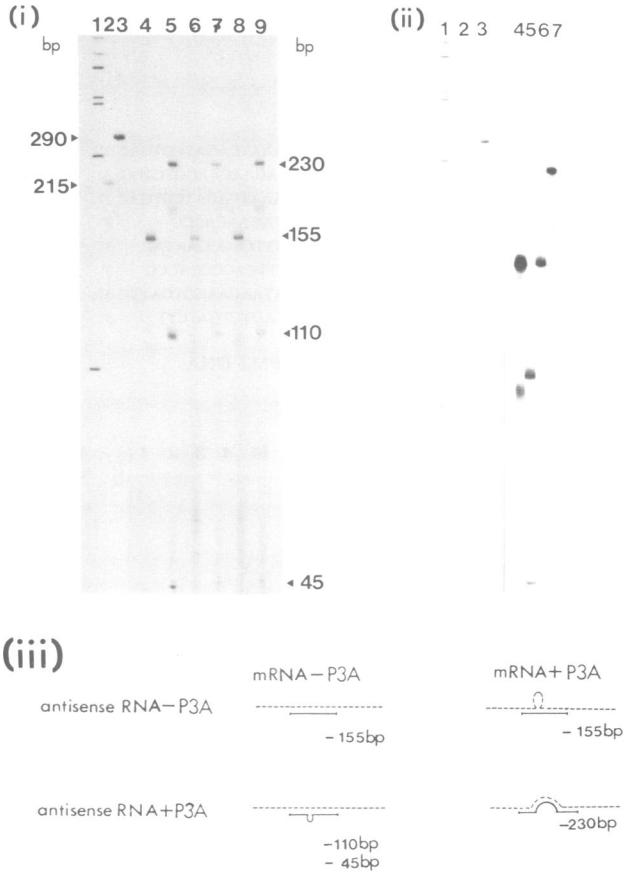

Fig. 3. RNase A protection assay. (**i**): ^{32}P-labelled antisense RNA to P3/P4 and P3/P3A/P4 was synthesized by run-off transcription (**lanes 2** and **3**). Hybrids were formed between each of these two transcripts and poly(A)$^+$ RNA from TE 671 cells (**lanes 4** and **5**), innervated muscle (**lanes 6** and **7**) and denervated muscle (**lanes 8** and **9**). Samples 4−9 were digested with RNase and fractionated on a standard sequencing gel. The gel was dried and exposed to X-ray film for 8 h at −70°C. In order to give even band intensity, loading on the gel was in the ratio 1:15:2, TE 671 cell:innervated muscle:denervated muscle, poly(A)$^+$ RNAs. Controls in which no poly(A)$^+$ RNA was added to the hybridization gave complete digestion of the antisense RNA (data not shown). Marker: *Hin*dIII-cut PM2 DNA (lane 1). (**ii**): Control RNase digests. The protocol was as described in (i), except that synthetic sense RNA containing either the full α-subunit coding sequence without P3A (**lanes 4** and **5**) or with P3A (**lanes 6** and **7**), made using SP6 RNA polymerase, was used in place of poly(A)$^+$ RNA. Antisense marker transcripts (**lanes 2** and **3**) and markers (**lane 1**) were as in (i). (**iii**): Diagram showing proposed hybridizations giving rise to the bands seen in (ii).

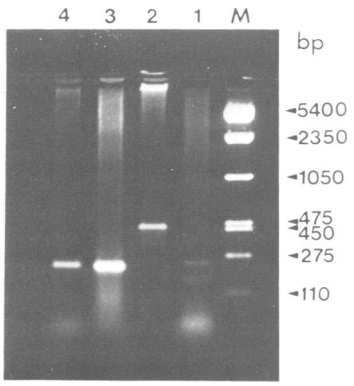

Fig. 4. PCR products, synthesized using species-specific oligonucleotide primers chosen to amplify across the P3/P4 exon boundary (Sumikawa *et al.*, 1982b; Noda *et al.*, 1983; Nef *et al.*, 1988), run on a 1.4% agarose gel and visualized under UV after staining with ethidium bromide.

Lane	Poly(A)$^+$ RNA source	Oligonucleotides	Fragment size (bp)
1.	Human denervated muscle	5'GATGAAGTAAATCAGATCGTGAC 3'	
		5'TTGTTATAGAGAACAAGGTCTGG 3'	155 230
2.	Fetal calf muscle	5'GATGAAGTAAATCAGATCGTGAC 3'	
		5'AGCACGCATAGAACACCC 3'	447
3.	Chick embryo muscle	5'GATGACCTGTTCCGGGAATAC 3'	
	(15 day)	5'CGGATTTGTTTCACGCCACCG 3'	211
4.	*Torpedo* electric organ	5'AGAAAATTATAACAAGGTGATTC 3'	
		ATCAGAAGGCAGTCTGATCTT 3'	213

The marker lane (M) shows *Hin*dIII-cut PM2 DNA.

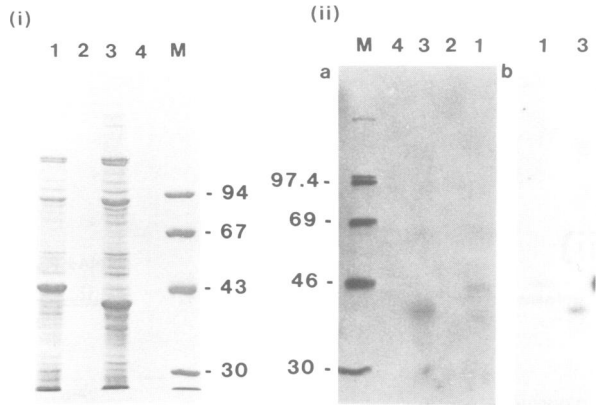

Fig. 5. (i) Polyacrylamide gel stained with Coomassie Brilliant blue, showing inclusion body protein extracts from induced and non-induced cell cultures containing +P3A or −P3A expression vectors (see Materials and methods). (**1**) +P3A, induced; (**2**) +P3A, non-induced; (**3**) −P3A, induced; (**4**) −P3A, non-induced; (**M**) protein markers (molecular weights in kd). (**ii**) Western blots using the protein extracts shown in 5(i) overlaid with: (**a**) 1 nM [^{125}I] α-bungarotoxin (10^6 c.p.m./ml). (**b**) antiserum raised against a synthetic α-subunit peptide (amino acids 125−143).

α-bungarotoxin and antisera raised against an α-subunit peptide (amino acids 125−143) identifies the major protein bands at 42 or 44 kd as AChR α-subunit.

Discussion

Our data show that the human muscle AChR α-subunit can exist as two isoforms with calculated molecular weights (M_r) of 49 694 (Noda *et al.*, 1983) and 52 851. The presence of mRNA coding for the two isoforms has been demonstrated in muscle poly(A)$^+$ RNA isolated from three individuals and from the cell line TE671. Comparison of the published restriction map for human AChR α-subunit genomic clones (Noda *et al.*, 1983) with our own reveal that the maps are identical. This indicates the presence of the P3A exon in the human AChR α-subunit gene in both cases. Extensive Southern blot analysis of human genomic DNA (Lobos *et al.*, 1989; J.Harrison and D.Beeson, unpublished data) has not shown the type of RFLP patterns that would be expected if the two α-subunit mRNA forms were encoded by separate alleles. We therefore conclude that the two isoforms derive from alternative splicing of an exon, P3A, located between P3 and P4, coding for 25 amino acids.

Two forms of AChR mRNA have been reported for the mouse AChR α-subunit (Goldman *et al.*, 1985) and the rat β-subunit (Goldman and Tamai, 1989). In the former case, the difference was due to the use of alternative polyadenylation sites and in the latter, to alternative splice junctions at the same exon. Alternative splicing from a single primary transcript has been proposed to account for certain cDNAs coding for neuronal AChRs (Goldman *et al.*, 1987); however, our report is the first example of alternative splicing generating two mRNA species, one with and one without an additional exon sequence, for muscle AChRs.

The presence of the P3A protein isoform has not yet been demonstrated in human muscle. However, both isoforms are translated with comparable efficiency in *E.coli*, and microinjection of *Xenopus* oocytes with synthetic mRNA for the P3A α-subunit isoform and human β-, γ- and δ-subunits (generated using the SP6 transcription system) results in the expression of an AChR ion channel with a conductance of 30 picoSiemens (N.Byrne *et al.*, unpublished observations). Since studies by several groups (Goldman *et al.*, 1985; Klarsfeld and Changeux, 1985; Buonanno and Merlie, 1986; Moss *et al.*, 1987) have shown that the major control of AChR synthesis is at the transcriptional level, it is most unlikely that the P3A isoform mRNA is transcribed but not translated in muscle. Indirect evidence that the isoform containing P3A is translated in human muscle comes from the response of T cell lines and clones, derived from a patient with myasthenia gravis, that were raised against the recombinant product of a cDNA clone (r37-429A) containing P3A (Harcourt *et al.*, unpublished data). Assaying with synthetic peptides has shown that the T cell epitope spans the P3A/P4 boundary. The T cells failed to respond to r37-429B, derived from a clone that did not contain P3A.

As expected, the levels of α-subunit mRNA were higher in TE671 and denervated muscle than in innervated muscle (Figure 3). In contrast to the situation found in rat, where one of the two mRNA species for the AChR β-subunit predominates (Goldman and Tamai, 1989), RNase A protection assays showed that the two species of human α-subunit mRNA are present in approximately equal amounts. This was true for innervated and denervated muscle and for the cell line TE671. This finding is supported by the analysis of our cDNA library where three out of five α-subunit clones characterized contained the P3A sequence.

The only other species for which α-subunit genomic clones have been fully characterized is chicken, in which the gene structure was reported to be the same as that of the human (Nef *et al.*, 1988). To address the question of whether the P3A exon is present in other species, we undertook PCR

amplification of cDNA from *Torpedo*, chick and calf using species-specific oligonucleotide primers (Figure 4), but we were not able to detect an analogous sequence in any of these. It is worth noting that the RNase A protection assay with the short probe gave only a single protected band, indicating that if the equivalent assay was performed for other species or subunits, it would not necessarily reveal the presence of a longer isoform. However, this would not apply to the S1 nuclease analysis used by Goldman *et al.*, 1985, which did not reveal the presence of a sequence related to the P3A exon in mouse muscle mRNA.

The predicted M_r for the human α-subunit (Noda *et al.*, 1983) and the new isoform are 49 694 and 52 851 respectively. The expressed polypeptides migrate in sodium dodecylsulphate polyacrylamide gels with M_r values of ~42 kd and ~44 kd, similar values to those previously described for the α-subunit of AChRs purified from human muscle (Stephenson *et al.*, 1981; Momoi and Lennon, 1982) or other mammalian species (Nathanson and Hall, 1979; Gotti *et al.*, 1982; Einarson *et al.*, 1982). The anomalous migration of the α-subunit when subjected to SDS−PAGE is seen both when it has been synthesized in *E.coli* and when purified in native form from muscle.

Problems experienced isolating AChR from muscle make the results obtained from SDS−PAGE of purified AChR difficult to interpret. The α-subunit of AChR contains the agonist binding site and can be covalently labelled with the ACh analogue 4-(*N*-maleimido)benzyltri[^{3}H]methyl-ammonium iodide ([^{3}H]MBTA), but SDS−PAGE has not revealed two affinity labelled bands for the human AChR (Momoi and Lennon, 1982). The 44 kd M_r band, frequently seen to co-purify with muscle AChR, has been shown to be actin (Gotti *et al.*, 1982); however this band is where, from expression in *E.coli*, we would predict the P3A isoform to migrate, and may, therefore, mask the presence of the larger isoform. Interestingly, Nathanson and Hall (1979) reported affinity labelling with [^{3}H]MBTA of two subunits with M_r of 45 and 49 kd in both junctional and extrajunctional rat AChR. Moreover, in certain other species, the two α-subunits in the AChR pentamer have been shown to differ in glycosylation, bromoacetylcholine and curare affinity and in the size of fragments generated by V8 protease (Gullick *et al.*, 1981; Sumikawa *et al.*, 1982a; Ratnam *et al.*, 1986). However, at present, we have no direct evidence that the P3A exon is expressed in any species other than man. It will be of interest to look for the presence of a related sequence in genomic clones of the α-subunit from other species and so determine its evolutionary origin.

When translated, P3A adds an extra 25 amino acids to the extracellular domain of the α-subunit. A large proportion of the antibodies in myasthenia gravis bind to a region on the α-subunit, termed the 'main immunogenic region' (MIR), that includes amino acids 61−76 (Barkas *et al.*, 1988). The P3A insert, located between amino acids 58 and 59, could therefore modify antibody binding to the MIR or form additional B cell epitopes. Since we have already shown that the P3A sequence can form part of a T cell epitope (see above), the existence of this isoform may have a role in the pathogenicity of myasthenia gravis.

A further question relates to the functional role of P3A. Muscle α-subunits have adjacent cysteine residues at positions 192 and 193, which are thought to be essential for ligand binding. They also have the cysteines found at positions 128 and 142 of all AChR subunits (Kubo *et al.*, 1985); these are thought to form a disulphide bridge essential for maintaining the AChR structure. The presence of a cysteine residue at position 11 of P3A suggests it may have a structural role, possibly in the interaction with another subunit. The observation that there is an equal ratio of the isoform mRNAs makes it tempting to speculate that in man, each of the two α-subunit isoforms is represented in the AChR pentamer.

Materials and methods

Human muscle was obtained from an intercostal muscle biopsy, taken at thoracotomy, and two leg amputations (Vincent and Newsom-Davis, 1985), one of which was from a diabetic with peripheral neuropathy. This latter muscle was characterized as denervated by the relatively high level of α-bungarotoxin binding and the presence of γ-subunit mRNA (as shown on a Northern blot) and γ-subunit clones in the muscle cDNA library (D.Beeson, unpublished data). TE671 cells were grown at 37°C in 95% air/5% CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 1000 mg/l glucose and 10% fetal calf serum.

mRNA preparation and cDNA synthesis

Total RNA was prepared by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979) and the poly(A)$^+$ RNA isolated by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). cDNA was synthesized as described by Watson and Jackson (1985).

Screening of libraries

cDNA clones were isolated from a human muscle cDNA library constructed in λgt10 as previously reported (Beeson *et al.*, 1989). Genomic clones were isolated from a library constructed in EMBL3. Approximately 3×10^4 phages were plated out onto each 132 mm plate and screened according to the procedure of Benton and Davis (1977). Hybridization was in 5 × SSPE, 5 × Denhardt's, 0.1% SDS, and 100 μg/ml sonicated and heat-denatured herring sperm DNA, for 18 h at 60°C. Filters were washed in 0.2 × SSC, 0.1% SDS at 60°C and exposed to autoradiography film. Probes were labelled by the oligonucleotide-primed method of Feinberg and Vogelstein (1982) and added to the hybridization solution at 5×10^5 d.p.m./ml. The genomic library was initially screened with a full length α-subunit clone containing the P3A exon; in subsequent rounds of screening, a 138 bp *Bgl*II fragment derived from this clone was used.

DNA sequencing

Appropriate subclones of genomic DNA or cDNA were ligated into either pUC8 (Vieira and Messing, 1982) or M13mp18 (Messing and Vieira, 1982). Sequencing was carried out using T7 DNA polymerase sequencing kits obtained from either Pharmacia or USB.

PCR

PCR amplification was carried out according to the Perkin Elmer Cetus instructions, using 50 ng of cDNA from each amplification.

RNase A protection assay

PCR products of P3/P4 and P3/P3A/P4 were ligated into pGEM-3Z to give the plasmids pHA155 and pHA230 respectively. pHA155 and pHA230 were linearized and ^{32}P-labelled antisense RNA probes were prepared by run-off transcription using T7 RNA polymerase. Two cDNA clones pHA54 and pHA8, with and without the P3A sequence respectively, were used to generate control sense RNA containing the full coding region. Template DNA was removed by digestion with RNase free DNase for 20 min at 37°C. Hybridization to poly(A)$^+$ RNA or control RNA was in 0.03 ml of 80% formamide, 40 mM PIPES pH 6.7, 400 mM NaCl, and 1 mM EDTA pH 8, for 8 h at 40°C. RNase digestion was carried out in 0.3 ml of solution containing 0.01 mg/ml RNase A, and 0.5 μg/ml, RNase T1, in 10 mM Tris−HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA, pH 8, at 30°C for 1 h. The digests were terminated by the addition of 0.03 ml 10% SDS and incubation with 0.01 mg proteinase K for 10 min at 30°C. Samples were phenol/chloroform extracted and ethanol precipitated before being run on DNA sequencing gels. Protected fragments were visualized by autoradiography.

Expression in E.coli

Expression plasmids containing an α-subunit sequence coding either for amino acids 1−P3A−429 (pR[1−429A]) or for amino acids 1−429

(pR[1−429B]) of the mature protein, i.e. +P3A and −P3A respectively, were constructed in expression vector pRex (Beeson *et al.*, 1989), a modification of pKK233-2 (Pharmacia). To achieve translation from amino acid 1, the *Nla*IV fragment (1−259) was ligated to the initiating ATG codon contained in the expression vector. Appropriate restriction digestions and ligations were then carried out to obtain pR[1−429A] and pR[1−429B], which were subsequently transformed into *E.coli* strain RB 791 for expression. The expressed products included 20 amino acids at the carboxy-terminus derived from vector DNA. Induction of expression and isolation of inclusion bodies were as described in Beeson *et al.* (1989). Inclusion body preparations were dissolved in SDS loading buffer and subjected to SDS−PAGE (Laemmli, 1970). Proteins were visualized by Coomassie blue staining or Western blotting with either [^{125}I]α-bungarotoxin (Barkas *et al.*, 1987) or rabbit polyclonal serum raised against a synthetic peptide (amino acids 125−143 of the α-subunit). Bound antibody was detected by the overlaying with ^{125}I-labelled protein A, followed by autoradiography.

Acknowledgements

We would like to thank Dr K.Johnson, St. Mary's Hospital Medical School, London, for the generous gift of the human genomic library, and Dr A.Jermy for the rabbit polyclonal antiserum. This work was supported by the Muscular Dystrophy Group and the Medical Research Council.

References

Aviv,H. and Leder,P. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 1408−1412.
Barkas,T., Mauron,A., Roth,B., Alliod,C., Tzartos,S. and Ballivet,M. (1987) *Science*, **235**, 77−80.
Barkas,T., Gabriel,J.-M., Mauron,A., Hughes,J., Roth,B., Alliod,C., Tzartos,S. and Ballivet,M. (1988) *J. Biol. Chem.*, **263**, 5916−5920.
Beeson,D., Brydson,M., Wood,H., Vincent,A. and Newsom-Davis,J. (1989) *Biochem. Soc. Trans.*, **17**, 219−220.
Benton,W. and Davis,R. (1977) *Science*, **196**, 180−182.
Buonanno,A. and Merlie,J. (1986) *J. Biol. Chem.*, **261**, 11452−11455.
Buonanno,A., Mudd,J. and Merlie,J. (1989) *J. Biol. Chem.*, **264**, 7611−7616.
Boulter,J., Lutyen,W., Evans,K., Mason,P., Ballivet,M., Goldman,D., Stengelin,S., Martn,G., Heinemann,S. and Patrick,J. (1985) *J. Neurosci.*, **5**, 2545−2552.
Breathnach,R., Benoist,C., O'Hare,K., Gannon,F. and Chambon,P. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4853−4857.
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294−5299.
Einarson,B., Gullick,W., Conti-Tronconi,B. and Lindstrom,J. (1982) *Biochemistry*, **21**, 5295−5302.
Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6−13.
Goldman,D. and Tamai,K. (1989) *Nucleic Acids Res.*, **17**, 3049−3056.
Goldman,D., Boulter,J., Heinemann,S. and Patrick,J. (1985) *J. Neurosci.*, **5**, 2553−2558.
Goldman,D., Deneris,E., Lutyen,W., Kochar,A., Patrick,J. and Heinemann,S. (1987) *Cell*, **48**, 965−973.
Gotti,C., Conti-Tronconi,B. and Raftery,M.A. (1982) *Biochemistry*, **21**, 3148−3154.
Gullick,W.J., Tzartos,S. and Lindstrom,J. (1981) *Biochemistry*, **20**, 2173−2180.
Heidenreich,F., Vincent,A., Roberts,A. and Newsom-Davis,J. (1988) *Autoimmunity*, **1**, 285−297.
Klarsfeld,A. and Changeux,J.-P. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4558−4562.
Kubo,T., Noda,M., Takai,T., Tanabe,T., Kayano,T., Shimizu,S., Tanaka,K., Takahashi,H., Hirose,T., Inayama,S., Kikuno,R., Miyata,T. and Numa,S. (1985) *Eur. J. Biochem.*, **149**, 5−13.
Laemmli,U. (1970) *Nature*, **227**, 680−685.
Lobos,E., Rudnick,C., Watson,M. and Isenberg,K. (1989) *Am. J. Genet.*, **44**, 522−533.
Luther,M.A., Schoepfer,R., Whiting,P., Casey,B., Blatt,Y., Montal,M.S., Montal,M. and Lindstrom,J. (1989) *J. Neurosci.*, **9**, 1082−1096.
Maelicke,A. (ed.) (1986) *Nicotinic Acetylcholine Receptor Structure and Function*. Springer-Verlag, Berlin, NATO ASI Series, Vol. 3.
Messing,J. and Vieira,J. (1982) *Gene*, **19**, 269−276.
Momoi,M.Y. and Lennon,V.A. (1982) *J. Biol. Chem.*, **257**, 12757−12764.
Moss,S.J., Beeson,D.M.W., Jackson,J.F., Darlison,M.G. and Barnard,E.A. (1987) *EMBO J.*, **6**, 3917−3921.
Nathanson,N.M. and Hall,Z.W. (1979) *Biochemistry*, **18**, 3392−3401.
Nef,P., Oneyser,C., Alliod,C., Couturier,G. and Ballivet,M. (1988) *EMBO J.*, **7**, 595−601.
Noda,M., Furutani,Y., Takahashi,H., Toyosato,M., Tanabe,T., Schimizu,S., Kikyotani,S., Kayano,T., Hirose,T., Inayama,S. and Numa,S. (1983) *Nature*, **305**, 818−823.
Numa,S., Noda,M., Takahashi,H., Tanabe,T., Toyosato,M., Furutani,Y. and Kikyotani,S. (1983) *Cold Spring Harbor Symp. Quant. Biol.*, **48**, 57−70.
Raftery,M.A., Hunkapiller,M.W., Strader,C.D. and Hood,L.E. (1980) *Science*, **208**, 1454−1456.
Ratnam,M., Gullick,W., Spiess,J., Wan,K., Criado,M. and Lindstrom,J. (1986) *Biochemistry*, **25**, 4268−4279.
Schoepfer,R., Luther,M. and Lindstrom,J. (1988) *FEBS Lett.*, **226**, 235−240.
Stephenson,F.A., Harrison,R. and Lunt,G.G. (1981) *Eur. J. Biochem.*, **115**, 91−97.
Sumikawa,K., Barnard,E.A. and Dolly,J.O. (1982a) *Eur. J. Biochem.*, **126**, 473−479.
Sumikawa,K., Houghton,M., Smith,J.C., Bell,L., Richards,B.M. and Barnard,E.A. (1982b) *Nucleic Acids Res.*, **10**, 5809−5822.
Tzartos,S., Seybold,M. and Lindstrom,J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 188−192.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259−268.
Vincent,A. (1980) *Phys. Rev.*, **60**, 756−824.
Vincent,A. and Newsom-Davis,J. (1985) *J. Neurol. Neurosurg. Psychiatry.*, **48**, 1246−1252.
Walker,R., Vincent,A. and Newsom-Davis,J. (1988) *J. Neuroimmunol.*, **19**, 149−157.
Watson,C.J. and Jackson,J.F. (1985) In Glover,D.M. (ed.), *DNA Cloning—A Practical Approach*. IRL Press, Oxford, Vol. I, pp. 79−88.

Received on January 3, 1990; revised on March 30, 1990