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Human nicotinic acetylcholine receptor α-subunit isoforms: origins and expression

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ABSTRACT

A majority of the autoantibodies in the disease myasthenia gravis (MG) are directed against the α-subunit of the muscle nicotinic acetylcholine receptor (AChR). Unlike AChR α-subunits previously characterised from other species, the human α-subunit exists as two isoforms. The isoforms are generated by alternate splicing of an additional exon located between exons P3 and P4, termed P3A. The 25 amino acids encoded by the P3A exon are incorporated into the extracellular region of the α-subunit, and so may be relevant to the pathogenesis of MG. Genomic sequences from rhesus monkey, and from dog and cat, which are susceptible to MG, were characterised between AChR α-subunit exons P3 and P4. Although regions homologous to the P3A exon were identified for each of these species, analysis by RT-PCR showed that they are not expressed. At variance with a previous report, constitutive expression of mRNA encoding the human P3A+ α-subunit isoform was not detected in heart, kidney, liver, lung or brain. Differential expression of the two α-subunit isoforms was not seen during fetal muscle development or in muscle from MG patients. In all cases where mRNAs encoding the two α-subunit isoforms have been detected, they are present at an approximate 1:1 ratio.

INTRODUCTION

The AChR mediates synaptic transmission at the vertebrate neuromuscular junction. It is a transmembrane glycoprotein which when purified may be separated into four subunits with a stoichiometry $2\alpha:\beta:\gamma/\epsilon:\delta$ (1). The γ-subunit is present in mammalian embryonic muscle, but is replaced by an isoform of the γ-subunit, termed the ε-subunit, in adult muscle (2, 3).

The autoimmune response in the disease MG, which has the characteristic features of muscle weakness and fatiguability, is directed against the AChR at the neuromuscular junction (4). With the exception of penicillamine-induced MG (5, 6, 7), the provoking factors for MG are unknown. However, experimental autoimmune myasthenia gravis can be induced in animals by immunising with AChR (8), even if autologous AChR is used without adjuvant (9). Thus, an abnormality of the AChR, in either its subunit structure or expression, may underlie the pathogenesis of some forms of MG.

Each AChR α-subunit contains a site for acetylcholine binding and a separate 'main immunogenic region' (MIR) against which the majority of myasthenic antibodies are targeted (10, 11). In contrast to AChR α-subunits characterised from other species, the human muscle α-subunit exists as two isoforms (12, 13). The isoforms are generated through alternate splicing of the primary RNA transcript, giving rise to mRNAs that either do (P3A+ isoform) or do not (P3A-isoform) contain an additional sequence corresponding to the exon P3A. The 25 amino acids encoded by the P3A exon are incorporated into the extracellular domain of the α-subunit in close proximity to the MIR. However, both the functional and possible pathogenic significance of the additional 25 amino acid sequence are unknown.

It has recently been reported that the P3A sequence is present in the genome of primates and that the P3A+ isoform is expressed constitutively in human non-muscle tissues such as heart, kidney, liver, lung and brain (14). Here we analyse the genomic sequences between exons P3 and P4 of the AChR α-subunit gene from species known to be susceptible to MG (15) and look for the expression of analogous α-subunit isoforms. Thus far, man is the only species in which mRNA encoding the P3A+ isoform has been detected. Translation of the P3A+ isoform mRNA has also been demonstrated (13). We have also looked for differential expression of the human α-subunit isoforms in developing muscle, muscle from MG patients and in certain non-muscle tissues. In all human muscle examined we found an approximate 1:1 ratio of the α-subunit isoforms. However, we were unable to detect mRNA encoding either α-subunit isoform in heart, kidney, liver, lung or brain.

MATERIALS AND METHODS

Dog and cat genomic DNA were obtained from Promega and rhesus monkey genomic DNA from Clontech Laboratories. Post-mortem tissues were obtained from the John Radcliffe Hospital, Oxford, and were frozen immediately following dissection. Fetal muscle was obtained from the Nuffield Department of Obstetrics and Gynaecology. MG muscle was obtained from intercostal muscle biopsies taken at thoracotomy. cDNA derived from adult muscle, fetal muscle, brain, liver and lung were a gift of Dr Derek

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Blake, John Radcliffe Hospital, Oxford. cDNA libraries derived from temporal cortex and liver were from Clontech Laboratories.

Oligonucleotide primers were obtained from British Biotechnology and Oswell DNA Services. Restriction endonucleases and DNA modifying enzymes were from Bioline, Pharmacia, Promega USB and Life Technologies. Reactions were performed according to the conditions recommended by the manufacturer.

mRNA preparation and cDNA synthesis

Total RNA was prepared by the guanidium isothiocyanate method (16) using RNAzol B (Cinna/Biotecx Laboratories). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (17). First strand cDNA was synthesised from 1−5 μg poly(A)$^+$RNA in a final volume of 50 μl using MMLV reverse transriptase (Life Technologies).

Polymerase chain reaction

Polymerase chain reaction (PCR) amplifications were carried out according to the Perkin-Elmer/Cetus instructions. In general, 1 μg genomic DNA and 5−50 ng cDNA were used for amplifications. For the amplification of AChR α-subunit genomic DNA and cDNA, a forward primer containing the first 27 nucleotides of exon P3 (18), 5′ GATGAAGTAAATCAGATCGTGACAACC 3′ was used. Two reverse primers (1 and 2), complementary to the sequence of exon P4 (18), were used. Reverse primer 1 containing 30 nucleotides, 5′ TTCACACCGCCATAGTCATCTGGATTCCAT 3′, complementary to positions 24−53 of exon P4, was used in the PCR amplification of rhesus monkey, dog and cat muscle. Reverse primer 2 containing 21 nucleotides, 5′ GTTATAGAGAACAAGGTCTGG 3′, complementary to positions 88−108 of exon P4, was used in the PCR amplification of human tissues.

Oligonucleotides for amplification of cytoplasmic β-actin were a gift from Dr Angela Flannery, Institute of Molecular Medicine, Oxford. The forward primer contained 20 nucleotides, 5′ ATGGATGATGATATCGCCGC 3′ (19), complementary to positions 22−61, and the reverse primer contained 23 nucleotides, 5′ ACTCGTCATACTCCTGCTTGCTG 3′ (19) complementary to positions 1112−1134.

Conditions for amplification of human AChR α-subunit cDNA were 94°C 1 min, 55°C 1 min, 72°C 1.5 min. For amplification of the animal genomic and cDNA, the primer annealing conditions were 50°C 1 min. For amplification of β-actin cDNA, the primer annealing conditions were 60°C 1 min. PCR products were run on agarose gels and visualised under UV light after staining with ethidium bromide. Size markers were Hind III cut PM2 DNA.

DNA sequencing

Appropriate subclones of genomic DNA or amplified cDNA fragments were ligated into vector pGEM-3Z. Sequencing was performed using dideoxy chain termination using the Sequenase Version 2.0 kit (United States Biochemical).

RESULTS

Sequencing of the P3A homologues

The region of the AChR α-subunit gene between exons P3 and P4 was amplified by PCR using genomic DNA isolated from rhesus monkey, dog and cat, and primers corresponding to coding sequences within the P3 and P4 exons. The AChR α-subunit exon P3 and the 5′ end of exon P4 have sequences that are highly conserved between species and were chosen as regions from which to derive oligonucleotide primers for cross-species PCR amplification. Amplified products of around 1700 bp for rhesus monkey and dog, and around 1100 bp for cat were visualised on a 1% standard agarose gel stained by ethidium bromide (data not shown). The amplified products were ligated into pGEM-3Z and sequenced from the P4 end until a region homologous with the P3A exon was obtained for each species. Figure 1A compares the genomic organisation of the human, monkey, dog and cat α-subunit genes within this region, and figure 1B the homologous DNA sequences within the vicinity of the P3A exon. The region analogous to the intron between human exon P3A and P4 shows a high level of sequence similarity, although for dog an additional 196 bp is present.

Rhesus monkey was found to have a P3A homologue that differs at only five positions from the 75 bp that make up the P3A exon. Moreover, the sequences immediately either side of the intron/exon boundaries are identical. The deduced amino acid sequence of the rhesus monkey P3A homologue differs from the human sequence at 2 out of the 25 positions. However, at another site (amino acid 23 of P3A), a codon for glutamine is replaced by a stop codon (CAG→TAG). The P3A homologues of dog and particularly cat, differ to a much greater extent. Both show a high level of sequence identity with the 3′ end of P3A, but like the rhesus monkey homologue, possess a stop codon at a site equivalent to position 23 of P3A.

A P3A homologue is not expressed in Rhesus monkey, cat and dog

First strand cDNA was synthesised from poly(A)$^+$RNA isolated from skeletal muscle of human, rhesus monkey, dog and cat. The cDNAs were subsequently used as templates for PCR amplification. Oligonucleotide primers used for the PCR reaction were derived from the human AChR α-subunit sequence. In order to ensure cross-species hybridisation of the primers, the 30mer, reverse primer 1, was used and the annealing temperature was reduced to 50°C. The alternative splicing of the primary RNA transcript for the human α-subunit results in the generation of two differently sized PCR products. Figure 2 shows the products generated by PCR amplification of cDNA derived from human, rhesus monkey, dog and cat muscle. Whereas the PCR on human cDNA generates amplified products of 173 bp and 98 bp, PCR on the rhesus monkey, dog and cat cDNAs gave a single band of 98 bp indicating that the P3A homologue is not expressed in these species. The single amplified band generated from the rhesus monkey, dog and cat cDNA was subcloned and sequenced and each was shown to contain the respective homologues for P3 and P4.

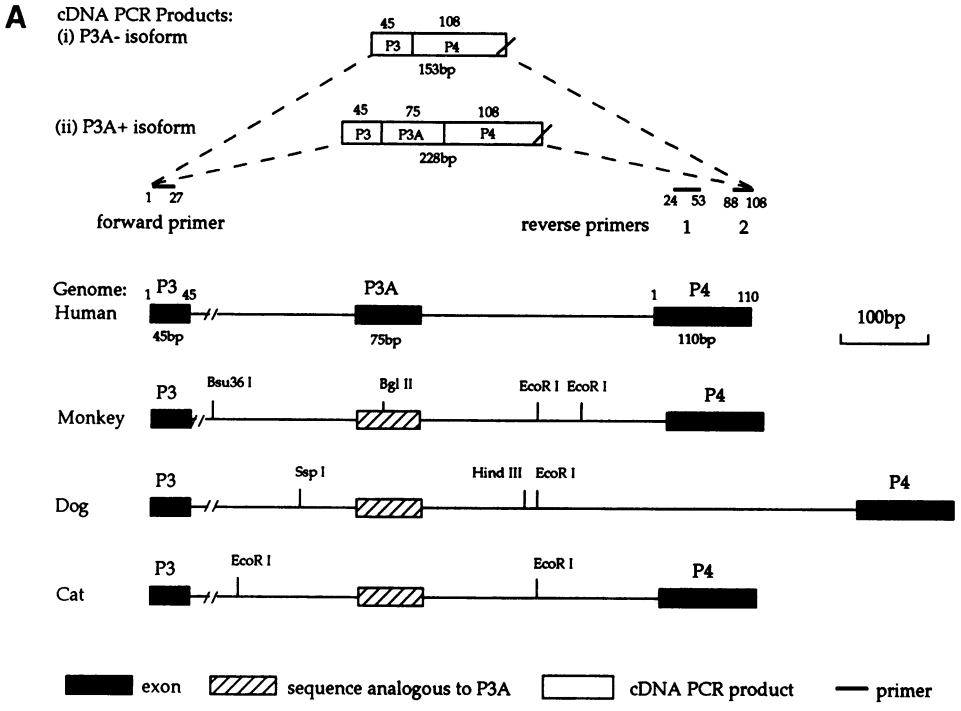
PCR on cDNA derived from human heart, kidney, liver, lung and brain does not show α-subunit expression

Following a report stating that mRNA encoding the human AChR α-subunit P3A+ isoform is constitutively expressed in heart, kidney, liver, lung and brain (14), we have attempted to confirm these findings. In order to help distinguish genuine PCR products from possible primer dimers we used reverse primer 2 for the human AChR α-subunit PCR amplification. The sequence of this oligonucleotide primer was complementary to the 3′ end of exon P4 and in PCR amplifications of muscle cDNA generates products of 153 bp and 228 bp (12). cDNA was synthesised from poly(A)$^+$RNA from post-mortem heart, kidney, liver, lung and

brain. In order to check the integrity of the mRNA/cDNA derived from post-mortem tissues, PCR was performed using primers which amplify mRNA encoding cytoplasmic β-actin, a protein that is ubiquitously expressed in man. Figure 3A shows PCR of cDNA from post-mortem tissues with primers which amplify the AChR α-subunit, and figure 3B shows PCR of the same cDNA samples with primers that amplify cytoplasmic β-actin. The strong amplification by the cytoplasmic β-actin primers demonstrates the integrity of the poly(A)$^+$RNA. However, we were unable to detect transcription of mRNA encoding either AChR α-subunit isoform. In order to confirm this result, we additionally used commercially available cDNA libraries of human adult temporal cortex and liver (Clontech Laboratories), and first strand cDNA derived from fetal brain, liver and lung (from Dr Derek Blake) as templates for PCR amplification of the AChR α-subunit. Again, we were unable to demonstrate transcription of mRNA encoding either of the AChR α-subunit isoforms from these non-muscle cDNA templates (data not shown).

Expression of the P3A+ α-subunit isoform in fetal and myasthenic muscle

We looked for differential expression of the two α-subunit isoforms both during fetal development and in muscle from MG patients. cDNA was synthesised from poly(A)$^+$RNA prepared from fetal muscle at 8, 14 and 19 weeks, and from muscle biopsies from two MG patients. PCR amplification of these cDNAs is shown in figure 4. In each case, bands corresponding



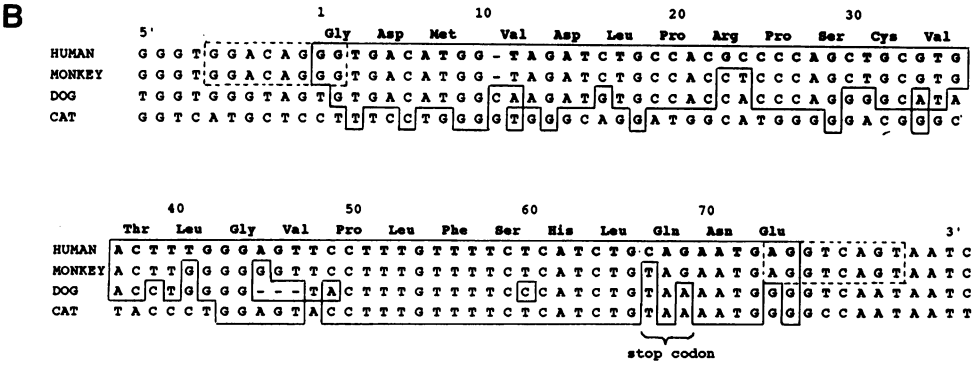


Figure 1. A. Structure of the genomic region spanned by AChR α-subunit exons P3 and P4 in man, rhesus monkey, dog and cat, and products generated by PCR amplification of cDNA synthesised from human mRNA transcribed from this region using the P3 exon forward primer and P4 exon reverse primer. Human exon P3A, sequences analogous to P3A, PCR primer positions, and restriction sites used for subcloning and sequencing are shown. **B.** Sequence of human P3A exon (12) (bold typed sequence) with corresponding amino acids above. Sequences analogous to P3A in the rhesus monkey, dog and cat, have been aligned underneath using the Pileup program (28). Solid boxing shows homology of these animal sequences with the human P3A exon. Dashed boxing indicates the identical intron/exon splice sites shared by man and rhesus monkey. A stop codon is found in monkey, dog and cat, aligned below human P3A amino acid 23 (a glutamine residue).

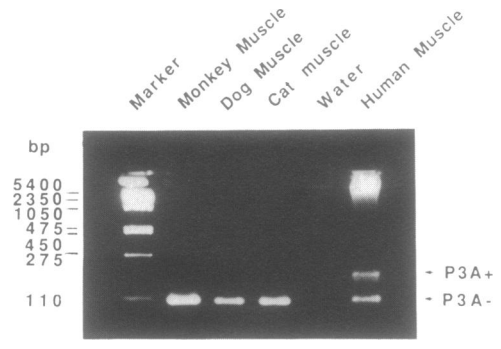


Figure 2. PCR amplification of cDNA using primers derived from P3 and P4 (reverse primer 1) exons of AChR α-subunit. cDNA was synthesised using poly(A)$^+$RNA isolated from skeletal muscle of rhesus monkey, cat and dog. PCR products run on a 2% agarose gel. 173 bp product corresponds to P3A+ isoform and 98 bp product to P3A-isoform.

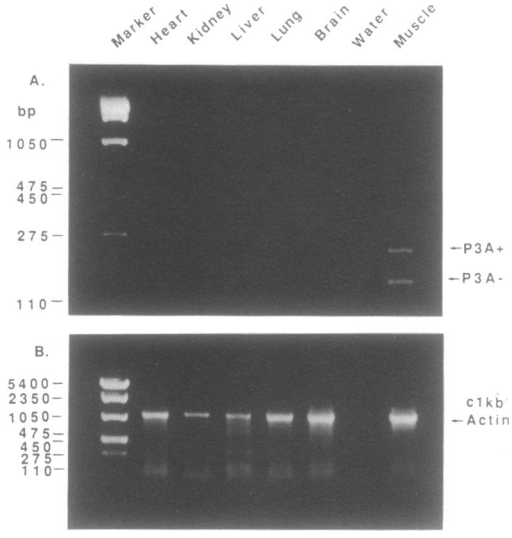


Figure 3. PCR amplification of cDNA made using poly(A)$^+$RNA from human post-mortem tissues. **A.** Using primers derived from AChR α-subunit P3 and P4 (reverse primer 2) exons. Products run on a 2% agarose gel. **B.** Using cytoplasmic β-actin primers. Products run on a 1% agarose gel.

to both α-subunit isoforms are present. Furthermore, in each case the two bands are of similar intensity suggesting equal levels of expression of the two isoforms.

The lower band present in the MG muscle samples is likely to be primer dimers caused by excess oligonucleotide primers used in these two amplifications. This lower band was not seen when amplifications were repeated with reduced primer concentrations.

DISCUSSION

We have examined the genomic sequences between α-subunit exons P3 and P4 from species known to be susceptible to MG, and have looked for expression of sequences analogous to the human P3A+ isoform. Each of these species has a region homologous to the P3A sequence in the intron between P3 and

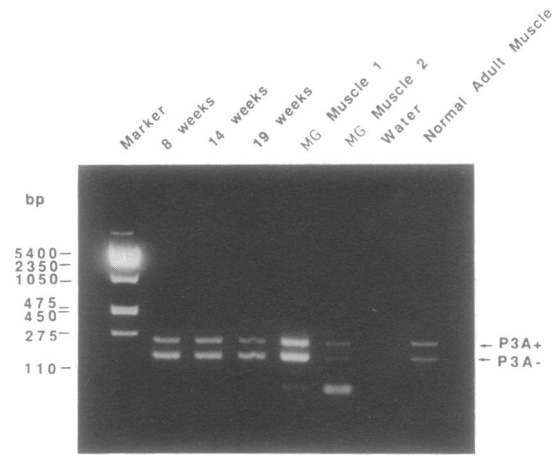


Figure 4. PCR amplification of cDNA using poly(A)$^+$RNA from muscle of 8, 14 and 19 week old fetuses and skeletal muscle from two MG patients using primers derived from AChR α-subunit P3 and P4 (reverse primer 2) exons. Products run on a 2% agarose gel.

P4. As might be expected, the highest level of sequence identity is seen in rhesus monkey, followed by dog and then cat. However, the level of sequence identity is no higher in the P3A region than in the intron between P3A and P4. This 270 bp intron in the human α-subunit gene shows 93% sequence identity with the analogous rhesus monkey sequence, if an additional 16 bp sequence found in the monkey intron is excluded from the calculation (data not shown).

Having established that sequences homologous to P3A are present in the genome of rhesus monkey, dog and cat, we looked to see if they were expressed in skeletal muscle of these animals. Using PCR amplification of muscle-derived cDNA, we have previously shown that a P3A homologue is not expressed in *Torpedo* electric organ, or chick or calf muscle (12). An S1 nuclease assay used by Goldman *et al.* (20) suggested that a P3A-like sequence is not expressed in mouse muscle, and this has since been confirmed (14, 21). It has recently been suggested from PCR analysis of primate genomic sequences that the P3A sequence is conserved through primate evolution and that the P3A+ isoform is likely to be expressed (14). However, our analysis, in which we examined mRNA expression as well as the DNA sequence, shows that although sequences homologous to P3A are present in the genome of rhesus monkey, dog and cat, in none of these is a P3A-like sequence expressed.

At present, man is the only species in which we have found expression of the P3A exon, which we believe arose as an allelic variant from mutations creating RNA splice sites in the intron between exons P3 and P4. It will be of interest to find out if the P3A exon is expressed by chimpanzees (hominoids), and at what stage of primate evolution the exon arose. The P3A exon splice sites and the sequences immediately 5′ and 3′ to the P3A exon are identical between rhesus monkey and man. It might be possible, using chimeric constructs and mutagenesis to determine which sequence change(s) are responsible for the splicing of the primary RNA transcripts which generate the two human α-subunit isoforms.

A comparison between the DNA sequences of the human P3A exon and the rhesus monkey homologue shows the sequence

change nearest an intron/exon boundary to be C→T at position 67 of P3A. It is not known whether this base change affects intron/exon splicing. However, were a mutation to occur elsewhere in the rhesus monkey α-subunit gene which resulted in RNA splicing of the P3A homologue, then this C→T change, which generates a stop codon in the rhesus monkey sequence, would lead to the abortion of α-subunit translation.

In response to a recent report (14) that the P3A+ isoform is constitutively expressed in a variety of human non-muscle tissues, we have looked for its expression in adult heart, kidney, liver, lung and brain. We were able to detect intact actin mRNA, but were unable to detect expression of mRNA encoding either α-subunit isoform. It is possible that the constitutive expression of the P3A+ isoform previously reported was the result of 'illegitimate' trace expression of the AChR α-subunit detected by PCR. However, in our PCR experiments we have always detected mRNA encoding both α-subunit isoforms rather than a single mRNA species. We conclude that if there is expression of the P3A+ isoform in these tissues, then it is at a level orders of magnitude lower than in muscle.

Two different cDNAs encoding α-subunits for the *Xenopus laevis* AChR have been isolated (22, 23). While both of these alternative α-subunits are expressed during development, the comparative ratio between them varies (23). Although we have previously shown by RT-PCR and RNAse A assays that mRNA encoding both human α-subunit isoforms are expressed at a ratio of approximately 1:1 in both denervated and innervated muscle (12), it is possible that there is a developmental role for the P3A+ isoform. We therefore carried out RT-PCR on 8, 14 and 19 week fetal muscle samples, as well as adult muscle. In each case, the bands corresponding to the α-subunit isoforms are of approximately equal intensity, suggesting that there is no specific developmental role for the P3A+ isoform and that it is not involved in the switch from γ-subtype to ε-subtype of the AChR (2, 3). It is not yet clear when the ε-subunit is first present in human muscle, but by 33 weeks the γ-subunit is no longer detected in muscle when staining with a monoclonal antibody specific for fetal AChR (24).

We also considered the possibility that abnormal expression of the P3A+ isoform may have a role in initiating or sustaining the autoimmune response in MG. RT-PCR on muscle biopsies from myasthenic patients again showed amplified bands of approximate equal intensity. This experiment does not exclude abnormal AChR expression in other muscles or tissues that express muscle AChR subunits such as the thymus (25–27, 21), from being involved in the myasthenic autoimmune response. However, it does suggest that in MG patients there is no generalised differential expression of the P3A+ isoform.

In contrast to a previous report (14), we have not found expression of the P3A+ isoform in rhesus monkey and do not see constitutive expression of this isoform in non-muscle tissues. In all cases where we have found expression of the human muscle AChR α-subunit, the two isoforms are equally represented. Thus far, the presence of the P3A exon has not been found to be polymorphic. This suggests a functional role for the additional 25 amino acids that it encodes.

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