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Coordinate regulation of RNAs encoding two isoforms of the rat muscle nicotinic acetylcholine receptor β-subunit

Daniel Goldman* and Katherine Tamai

Mental Health Research Institute and Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA

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ABSTRACT

The nicotinic acetylcholine receptor (nAchR) mediates communication between nerve and skeletal muscle. The properties, levels and distribution of these receptors change during development of the neuromuscular junction. These changes may be due, in part, to expression of different gene products. We are using nuclease protection experiments and cDNA cloning to identify the RNA transcripts that encode nAchRs in rat muscle. This analysis has identified two β-subunit mRNAs. Complementary DNAs corresponding to these two RNAs have been isolated from a rat skeletal muscle cDNA library. Based on nucleotide sequence analysis, these RNAs differ by 9 bases in their 5′ coding sequence. The levels of both mRNAs change similarly during muscle development and upon denervation of adult skeletal muscle. These two β-subunit RNAs probably result from the use of different exon/intron splice sites in the β-subunit gene.

INTRODUCTION

The nAchR is a pentameric integral membrane protein with a subunit stoichiometry of $\alpha_2\beta\gamma\delta$. A number of changes occur in the nAchR during development of the neuromuscular junction, including distribution along the surface of muscle fibers, stability, binding of antibodies and channel open times (reviewed in 1). These changes may be due to expression of different gene products or to posttranscriptional modification of a single gene product. As the receptor matures from the embryonic to the adult form the γ-subunit is replaced by an ξ-subunit (2,3). This change likely accounts for the different channel open times of these two receptor types (2). However, many of the other changes that take place in the nAchR during development still cannot be explained at the molecular level (1).

The expression of nAchRs on the cell surface is controlled by regulating the levels of their respective RNAs (4,5,6). Muscle activity is one process that regulates expression of these RNAs (7,8). The number of different RNAs that encode the muscle nAchR subunits is not known. In mouse muscle the α- and γ-subunit proteins are encoded by multiple RNAs differing in their 3′ untranslated sequences (5,9). The significance of these differences is not known, although it is possible that they influence the distribution, stability or translation of the RNA.

Probes for the various nAchR gene products are required to investigate the molecular mechanisms by which muscle activity and the nerve influence nAchRs. This is most easily done by isolating cDNA clones corresponding to the various nAchR transcripts expressed in muscle. Complementary DNAs encoding the nAchR expressed in the mouse muscle cell line, BC3H1, have been isolated (9,10,11,12). These clones are useful probes to study nAchR gene expression in muscle. However, this cell line expresses an embryonic nAchR, suggesting mRNAs for adult type receptors are not expressed. For example, the adult type

ξ-subunit transcript, originally identified in calf muscle has not been found in BC3H1 cells. In addition to a lack of expression of certain genes, those that are expressed in vitro may be regulated/processed differently from those expressed in vivo. We report that two β-subunit mRNAs are expressed in rat skeletal muscle. These RNAs are coordinately regulated during development and after denervation of adult muscle.

EXPERIMENTAL PROCEDURES

RNA Isolation

RNA was isolated using the guanidinium isothiocyanate procedure (5,13). Poly(A)+ RNA was selected for by chromatography over an oligo(dT)−cellulose column (14).

Construction and Screening of cDNA Libraries

Complementary DNA libraries were constructed using rat poly(A) + RNA isolated from adult innervated muscle. The method of Gubler and Hoffman (15) was used to prepare size-fractionated double-stranded cDNA. The cDNA was ligated to phosphorylated EcoR 1 linkers and was cloned into the EcoR 1 site of the bacteriophage vector, lambda gt11 (16). About 5×10^5 plaques were screened with radiolabeled cDNA encoding the BC3H1 mouse muscle β-subunit.

DNA Sequence Determination

Complementary DNAs and various restriction fragments were subcloned into the M13 bacteriophage vectors, mp18 and mp19. Nucleotide sequence was determined using the dideoxynucleotide chain-termination method (17).

RNA Blots

RNA was denatured in formaldehyde at 65° C and subjected to electrophoresis in 1.4% agarose gels containing 2.2 M formaldehyde. The RNA was then transferred to a Gene Screen Plus (Dupont/NEN) membrane. Prehybridization and hybridization conditions were 5×SSPE (0.75 M NaCl, 57 mM Na_2HPO_4, 5 mM EDTA [pH 7.4]), 1% SDS, 10% dextran sulfate, and 50% formamide at 42° C. After hybridization the blot was washed in 5×SSC, 0.1% SDS at 65° C, and exposed to X-ray film with an intensifying screen at −80° C.

RNase A Protection Experiments

Poly(A)+ RNA was isolated from rat soleus muscle and hybridized with radiolabeled antisense RNA probes prepared by run-off transcription of linearized PGEM-4 vectors containing portions of the rat β-subunit cDNA (18). The hybridization reaction (0.03 ml) contained 50% formamide, 40 mM PIPES (pH 6.7), 0.4 M NaCl, and 1 mM EDTA (pH8), and was incubated at 45° C for 16 h. Following hybridization, 0.3 ml of RNase digestion buffer was added; it contained 0.04 mg/ml RNase A in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA and 300 mM NaCl. The incubation was for 1 h at 30° C and terminated by the addition of 0.02 ml 10% SDS and 0.05 mg proteinase K and the incubation continued for an additional 15 min at 37° C. The samples were then extracted with phenol:chloroform and the RNA precipitated with ethanol. Those hybrids surviving digestion were fractionated by size on a denaturing 5% acrylamide, 8 M urea gel. The gel was dried and exposed to X-ray film with an intensifying screen at −80° C.

S1 Nuclease Analysis

Heteroduplexes between poly(A)+ RNA and M13 subclones harboring the cDNA for the rat β-subunit were digested with nuclease S1 (5). Hybrids surviving digestion were analyzed by electrophoresis through a 1.4% agarose gel, containing 2.2 M formaldehyde, transferred to Gene Screen Plus, and probed with a nick-translated radiolabeled rat β-subunit cDNA.

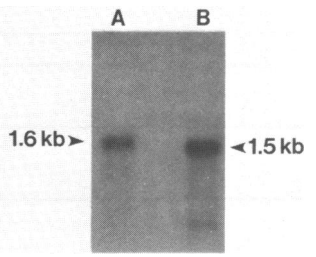

Figure 1: DNAs from β28 and β33 are different. Single-stranded DNA of the sense and antisense orientations of (A) clone β33 or (B) clone β33 and clone β28 were hybridized and subsequently digested with nuclease S1. Protected fragments were fractionated on an agarose gel, transferred to Gene Screen Plus and probed with nick-translated β28 cDNA.

RESULTS

Our long term goal is to determine the molecular mechanisms by which nerve regulates the level, distribution and properties of nAchRs expressed in rat skeletal muscle. Since all the muscle nAchR clones isolated to date were from species other than the rat we isolated them from rat muscle (unpublished data). This report focuses on the β-subunit of the receptor. In mouse muscle the β-subunit, like the other subunits, is encoded by a single gene (19). Southern blot analysis of restricted rat genomic DNA indicates a single gene also encodes the rat muscle nAchR β-subunit (data not shown). The β-subunit RNA is unusual in that the level of this RNA in innervated rat and mouse muscle is higher than that of the α, γ, or δ-subunit transcripts (4). In addition, denervation of rat skeletal muscle results in only a 3 to 10-fold increase in this RNA, in contrast to a 50-fold increase after denervation of mouse muscle (4); the involved molecular mechanisms are not known. Are the same RNAs expressed in innervated and denervated muscle?

Isolation and characterization of rat β-subunit cDNAs

A cDNA library was prepared with poly A(+)RNA isolated from adult innervated rat muscle. Approximately 500,000 lambda plaques were screened with a radiolabelled mouse β-subunit cDNA (4). Seven positive clones were isolated.

DNA:DNA S1 nuclease protection experiments were used to determine the relationship of these clones to each other. The EcoR 1 inserts from λgt11 were subcloned into the single-stranded phage, M13mp19, in both orientations. Single-stranded DNA from the clone containing the largest cDNA (β28, 1.7 Kb) was hybridized to single-stranded DNAs, of opposite orientation, from each of the seven M13 clones. Following digestion with the single-strand specific nuclease S1, duplexes were fractionated on a denaturing agarose gel, transferred to Gene Screen Plus and visualized by hybridization with a radiolabelled mouse β-subunit cDNA. DNAs from all of the clones except β33 were smaller versions of β28. The S1 nuclease-resistant duplex of β33:β33 hybridization is approximately 1.6 Kb, (Fig. 1, lane A) while the protected duplex from the β28:β33 hybridization is about 1.5 Kb (Fig. 1, lane B). This result indicated that either β33 extends about 100 bases 5′ or 3′ beyond the ends of clone β28, or that the β33 DNA sequence is different from β28 in its 5′ or 3′ region.

These possibilities were distinguished by determining the nucleotide sequence of these clones. The 5′ end of β28 starts at position −66 of the mouse cDNA (12), while that of β33 starts at position 44 (Fig. 2). Both clones contained identical 3′ ends (data not shown). An additional 9 base pairs found in the 5′ region of β28 were not present in β33 or the

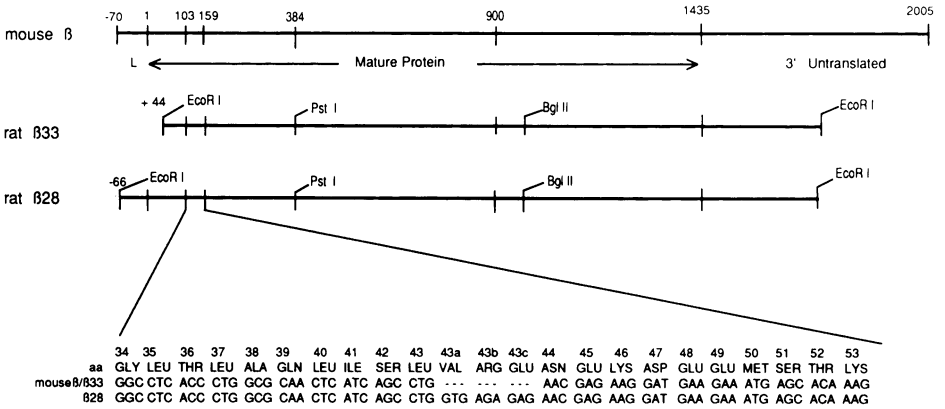

Figure 2: The relationship of the mouse β-subunit cDNA (12) and the two rat β-subunit cDNAs (β28 and β33). L refers to leader sequence. The regions of the nine nucleotide difference between β28 and either β33 or the mouse β-subunit cDNA is compared. The DNA sequence begins at nucleotide 103 and extends to base 159 of the published mouse β-subunit (12). In this region, the rat sequence of β33 is identical to that of the mouse β-subunit. The nucleotide sequence of β28 DNA is aligned below that of β33. A nine base gap was introduced in the β33 sequence to align it with the β28 sequence. This gap corresponds to the location of an additional nine bases in the β28 sequence. The amino acids encoded by these nine additional bases occur between residues 43 and 44 of the mouse β/β33 sequences and have been numbered 43a-43c.

mouse β-subunit cDNA (Fig. 2). These 9 bases code for 3 additional amino acids (val, arg, glu) situated between amino acid residues 43 and 44. We have determined the complete nucleotide sequence of one strand of β28 and found no other significant differences in the sequences of its coding region relative to that of the mouse β-subunit (12).

Expression of RNAs corresponding to clones β28 and β33 in innervated and denervated skeletal muscle

Do clones β28 and β33 represent different β-subunit encoding RNAs expressed in rat muscle? This question was addressed using RNase A and S1 nuclease protection assays.

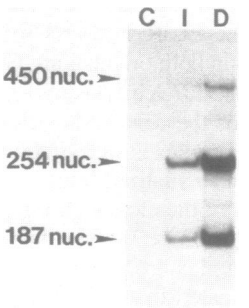

Figure 3: Clone β28 identifies two mRNAs that differ in their 5′ protein coding sequence. Hybrids were formed between mRNA isolated from innervated (I) or denervated (D) rat soleus muscle and a radiolabelled antisense RNA probe corresponding to the extreme 5′ 450 nucleotides of clone β28. Samples were digested with RNase A and fractionated on a denaturing acrylamide gel. The gel was dried and exposed to X-ray film, with an intensifying screen, overnight at −80° C. Lane C is a control in which no mRNA is added to the probe. The numbers to the left of the figure are the approximate lengths of the protected fragments in nucleotides.

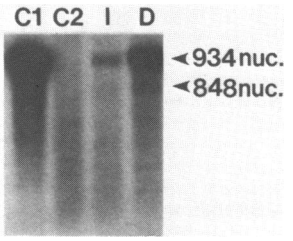

Figure 4: Clone β33 corresponds to the more abundant β-subunit isoform encoding mRNA. Heteroduplexes were formed between mRNA isolated from innervated (I) or denervated (D) rat soleus muscle and an M13 subclone harboring a 934 base pair 5′ fragment of clone β33. Samples were digested with S1 nuclease, fractionated on a denaturing agarose gel and transferred to a Gene Screen Plus membrane. S1 resistant heteroduplexes were identified by hybridization with a radiolabelled β33 probe. Lane C1 is a control in which two M13 subclones containing the β33 5′ insert in opposite orientations were hybridized to each other. Lane C2 is a control in which the M13 subclone containing the β33 5′ insert in the antisense orientation is allowed to hybridize without the addition of mRNA.

Single-stranded DNAs were generated from M13 clones containing β28 inserts in both orientations. This DNA was then hybridized to poly A(+) RNA isolated from either innervated or denervated rat muscle. Following digestion with S1 nuclease, heteroduplexes were fractionated on denaturing agarose gels, transferred to a Gene Screen Plus membrane and visualized by hybridization with nick-translated β28 cDNA. Two species of hybrids were protected during S1 nuclease treatment (data not shown): 1) a low abundance RNA corresponding to complete protection of clone β28 and 2) a more abundant molecule about 200 bases shorter than β28. These RNAs could represent heterogeneity at either the 5′ or the 3′ end of the molecule. If the S1 nuclease experiment was repeated with a subclone of β28 containing its 3′ 690 bases, a single species survived S1 nuclease digestion (data not shown). These results show that β28 hybridized to two skeletal muscle RNAs which differed at their 5′ ends.

Do the two β-subunit-encoding RNAs correspond to the two β-subunit cDNAs (β28 and β33) that differ by 9 base pairs in their 5′ coding sequence? For this analysis an EcoR 1/Pst 1 fragment corresponding to the extreme 5′ end of β28 (fig. 2) was subcloned into the plasmid pGEM 4. After linearization, this subclone was used to generate a strand-specific radiolabelled RNA probe (18). The probe was then used to hybridize to RNA isolated from muscle tissue and subsequently digested with RNase A. Those hybrids surviving RNase digestion were fractionated on denaturing polyacrylamide gels. When the 5′ 450 bases of β28 were hybridized to muscle RNA three fragments was protected (Fig. 3). The largest was about 450 bases and corresponded to complete protection of the RNA probe by muscle RNA. The other protected fragments were about 254 and 187 bases long and corresponded to incomplete protection by a second RNA expressed in rat muscle. The size of these fragments were determined by comparing their mobility with that generated from a DNA sequencing reaction. These fragments likely arose from hybridization of the RNA probe to a muscle RNA that lacks the 9 bases that are different between clones β28 and β33. Thus RNase A digested the probe in the region of these 9 bases generating the 254 and 187 nucleotide long fragments observed on the gel. In addition, the RNA that corresponded to complete protection by clone β28 was of lower abundance than the RNA that resulted in partial protection.

The second β-subunit encoding cDNA that we isolated (β33) is identical to β28 except that it is shorter by about 100 bases at its 5′ end and does not contain the 9 extra nucleotides

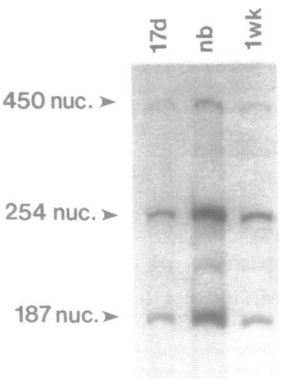

Figure 5: β-subunit RNAs identified by clones β28 and β33 are coordinately regulated during development. Duplexes were formed between mRNA isolated from 17 day old deviscerated rat embryos (17d), newborn rat leg (nb), or 1 week old neonatal rat leg muscle (1 wk) and a radiolabelled antisense RNA probe corresponding to the extreme 5′ 450 nucleotides of clone β28. Samples were digested with RNase A and fractionated in a denaturing acrylamide gel. The gel was dried and exposed to X-ray film with an intensifying screen for 2 days at −80° C. The amount of mRNA used in the hybridization was approximately 10 μg (17d), 10 μg (nb) and 5 μg (1 wk).

found in β28 (See Fig. 2). This molecule likely corresponds to the more abundant β-subunit RNA (Fig. 3) that results in protected fragments of 254 and 187 nucleotides when hybridized with the β28 5′ probe. This hypothesis was tested directly with β33 DNA as a probe in S1 nuclease protection experiments. A 934 base-pair EcoR 1/Bgl 11 fragment from the extreme 5′ end of β33 (Fig. 2) was subcloned into M13mp18. Single-stranded DNA containing the β33 fragment in the antisense orientation was hybridized with muscle RNA in a S1 nuclease protection experiment. This probe should completely protect the more abundant β-subunit RNA if it lacks the 9 bases found in clone β28, while a much less abundant RNA corresponding to clone β28 should protect two fragments; one of 86 bases and a second of 848 bases. Consistent with these predictions, two bands were protected from S1 nuclease digestion (Fig. 4). The more abundant band is about 934 bases long, corresponding to complete protection of the probe. The less abundant band is about 848 bases long and represents cleavage of the low abundant RNA in the region of the 9 extra nucleotides. We did not observe the 86 nucleotide long fragment probably because of its low abundance and small size.

Expression of β-subunit RNAs During Development

Are the β-subunit RNAs differentially regulated during development? RNA was isolated from rats at embryonic day 17, from newborn rats, and from 7 day-old neonates. Poly A(+) RNA was hybridized to a radiolabelled RNA probe corresponding to the 5′ 450 bases of clone β28. RNase A was then added and those molecules surviving digestion were fractionated by size on a denaturing acrylamide gel. Both β-subunit RNAs are expressed throughout development (Fig.5). The RNA lacking the additional 9 bases, corresponding to clone β33, is the more abundant molecule at all developmental stages examined. Quantitation of these blots by densitometry reveals no significant changes in the ratio of the two β-subunit RNAs at any stage of development. The apparent lower level of β-subunit RNA in lane 17d versus nb (Fig. 5) probably reflects the source of the RNA, i.e. deviscerated embryos versus leg, and not absolute levels of expression.

DISCUSSION

We report here the identification of two RNAs encoding different β-subunits of rat muscle nAchRs. The proteins encoded by these RNAs should differ by three amino acids (val, arg, glu) (Fig. 2). These three additional amino acids are located between residues 43 and 44 of the deduced amino acid sequence of the mouse β-subunit (12). The amino acids at positions 43 and 44 (leu, asn) are conserved in torpedo, mouse, and bovine β-subunits (12,20,21). This is the amino terminal region of the mature β-subunit protein and is probably exposed on the extracellular side of the membrane. These residues contribute to a very hydrophilic domain in the β-subunit which is also conserved in the γ- and δ-subunits, and to a lesser extent in the α-subunit. The three additional amino acids that β28 encodes should contribute to the hydrophilicity of this domain. The role these residues play in the structure or function of the nAchR is not known. However, we are now in a position to determine the influence on receptor function of this region of the β-subunit by comparing the properties of receptors containing these two β-subunits.

These two β-subunit encoding RNAs may result from alternate use of splice junctions in the rat β-subunit gene. In both the human α- and γ-subunit genes (22,23) and the chick γ-subunit and δ-subunit genes (24), the amino acids at positions 43 and 44 , the last and first residues of exons P2 and P3 respectively, are interrupted by an intron. We predict that this will also be true for the β-subunit gene. Thus a likely mechanism for the generation of the two rat β-subunit RNAs is the use of two different splice junctions in bringing exons P2 and P3 together. The more abundant RNA corresponding to clone β33 would result from use of a P2 exon/intron border consisting of residues TG/GT. We predict the GT dinucleotide is on the intron side of the exon/intron border based on the DNA sequence of clone β28 in this region. The less abundant β-subunit RNA corresponding to clone β28 is predicted to arise from a less efficient splice site found 9 bases into the intron separating exons P2 and P3. This splice site would consist of an AG/− − exon/intron junction. This putative splice site occurs at the junction of a hydrophobic/hydrophilic domain, and the three additional amino acids in β28 preserve this organization. The use of alternative splice junctions as a mechanism to generate receptor diversity was proposed for neural nAchRs (25). This type of mechanism may also generate diversity in muscle nAchRs.

The two β-subunit-encoding RNAs were coordinately expressed in innervated, denervated and developing rat muscle (Figures 3, 4 and 5). The RNA with nine additional nucleotides was always expressed at a lower level than the RNA lacking this sequence. Thus the preferred splice site is represented by the more abundant RNA. Although our paradigms have failed to reveal differential regulation of these two RNAs it remains possible that they contribute to different receptor populations. For example, a change in RNA/protein sequence may alter the function, stability or targeting of a mRNA or protein in the cell. Functional expression of nAchRs containing one or the other of these two β-subunits will help to determine their significance and possibly the functional role of residues in the region of amino acids 43 and 44.

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*To whom correspondence should be addressed

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