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SPLICING OF AN ANTI-SENSE ALU SEQUENCE GENERATES A CODING SEQUENCE VARIANT FOR THE α-3 SUBUNIT OF A NEURONAL ACETYLCHOLINE RECEPTOR

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In this report we demonstrate that an α-3 acetylcholine receptor subunit transcriptional variant originates through alternative splicing of a complementary sequence of the right arm of an Alu element. This element is located within the 5.1Kb intron found between exons 5 and 6 of the α-3 acetylcholine receptor subunit gene. The transcriptional variant originates from the normal splicing process and carries an in-frame stop codon. If translated, it should encode for a peptide lacking the 4th transmembrane domain of the normal subunit.

Alternative splicing appears to be common amongst members of the acetylcholine receptor (AcChR) family. Splicing variants for the α-1 (1), α-3 (2), α-4 (3) and β-1 subunits (4) have been reported. PCR and sequence analysis of 14 cDNA clones isolated from a normal human thymic cDNA library indicated that 2 independent clones carried a 122bp insert corresponding to the complementary sequence of the right arm of an Alu element (Alu-c) after position 1296 of the mature transcript (2). Comparison of the variant human cDNA with the sequence of the chicken α-3 AcChR subunit gene indicated that Alu-c is found spliced between exons 5 and 6 of the normal α-3 AcChR subunit transcript (2). To corroborate these findings, the genomic make-up of the fragment comprising the intervening sequence found between exons 5 and 6 of the α-3 AcChR subunit gene was analyzed employing human genomic DNA and a genomic clone λ6, known to carry most of the α-3 AcChR subunit gene (5). To investigate the transcriptional expression of the variant form in different tissues we used amplification of reverse transcribed RNA (RT-PCR). Our findings show that the alternative splicing of a complementary right arm of an Alu element occurs as part of the normal splicing process and may originate peptide diversity. The translation of the α-3 transcriptional variant may have important biological and immunological consequences.

Abbreviations: AcChR: acetylcholine receptor; MG: Myasthenia gravis; RPA: ribonuclease protection assay; RT-PCR: amplification of reverse transcribed RNA.

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MATERIALS AND METHODS

Clones. cDNA clones were obtained from a λZAPII normal thymic library (6). Genomic clone λ6 was isolated from a Charon 4A library (5).

Sequencing analysis. Sequencing of PCR amplified products and λ6 subclones was performed employing the f-mol sequencing kit from Promega (Madison, WI) as previously reported (7) or spectrophotofluorimetrically utilizing the Applied Biosystems DNA sequencer 373A (Foster City, CA) and methodology recommended by the manufacturer. Variant cDNA clone 42NTBSZ carrying the 122bp antisense Alu element was used as control for both PCR and sequencing analysis. Primers employed in the procedure were[1]: Exon 5 sense primers α-3 1012F: CCGAGGCCCCTCTACGGT and α-3 1252F: AAGTATATTGCTGAAAAT; exon 6 anti-sense primers α-3 1412R: CTGGCCATCAGGGGTTGC and α-3 1440R: CACACAGCTTAGTGCTTA; Alu-c sense primer Alu-c 23F:AGAATGGAGTGCAGTAGTACA and Alu-c anti-sense primer Alu-c 120R: GTAGTCCCAGACACTTGG.

Amplification of reverse transcribed RNA (RT-PCR) analysis. RT-PCR was performed as indicated in reference 7, employing rTh DNA polymerase (Perkin-Elmer Cetus, Norwalk, CR). Thirty cycles of amplification were used to detect the normal α-3 product and 35 cycles to detect the variant α-3 product. Primers utilized in the analysis of the normal α-3 transcript correspond to the exon 5 sense primer α-3 1012F and the anti-sense exon 6 primer α-3 1440R. For the α-3 variant transcript analysis the primers correspond to the sense α-3 variant primer Alu-c 23F and the anti-sense exon 6 primer α-3 1440R (for primer sequences, see above).

PCR analysis. Analyses of the α-3 genomic clone λ6 were carried out employing Hot tub DNA polymerase (Amersham, Arlington Heights, IL) and primers that correspond to: 1) the exon 5 sense sequence α-3 658F: CCCTGCCTGCTCATCTCC, 2) the Alu-c sense primer Alu-c 23F, 3) the antisense Alu-c primer Alu-c 120R, and 4) the exon 6 anti-sense primer α-3 1412R (for primer sequences, see above). The reaction was carried out as recommended by the supplier, employing the following cycling conditions: 7 min denaturation at 93°C, followed by 30 cycles consisting of 30 sec of denaturation at 93°C, 1 min of annealing at 58°C and 8 min of extension at 65°C followed by a final extension at 65°C for 15 min.

RESULTS AND DISCUSSION

Variant α-3 subunit transcript expression.

Variant transcripts are expressed in all thymic tissues tested including thymomas (Fig. 1), thymocytes and in a pheochromocytoma (Fig.2B). Only tissues or cells known to express the normal transcript have been shown to express its variant counterpart (2, Fig. 2). Earlier we demonstrated through RPAs that the transcriptional levels for the alternatively spliced α-3 form is between 3 and 6% of the normally spliced transcript (2). RT-PCR assays confirmed these findings; thus RT-PCR assays extending from exon 5 through 6 of the α-3 AcChR transcripts produce mainly a product corresponding to the normally spliced poly(A)+ RNA (Fig. 2A). Under these conditions, the variant RT-PCR product can only be detected through blotting of the electrophoretically resolved RT-PCR products and probing with nested radiolabeled α-3 oligonucleotides (not shown). Moreover, to visualize the α-3 RT-PCR generated variant product we had to use 5 additional cycles of amplification over those required for the visualization of its normal RT-PCR product and higher concentrations of poly(A)+ RNA samples (Fig. 2). These observations suggest that the variant form is a minor transcriptional product of the gene encoding for the α-3 subunit of a neuronal AcChR.

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[1] AcChR α-3 subunit transcript position #1 is that of the first nucleotide of the sequence that encodes the mature subunit. It serves as reference for the identification numbers assigned to the exon 5 and exon 6 primers used in the study. For the Alu-c primers it is considered that the Alu-c position #1 corresponds to the first nucleotide of the 122bp insert found in the variant cDNA clone 42NTBSZ.

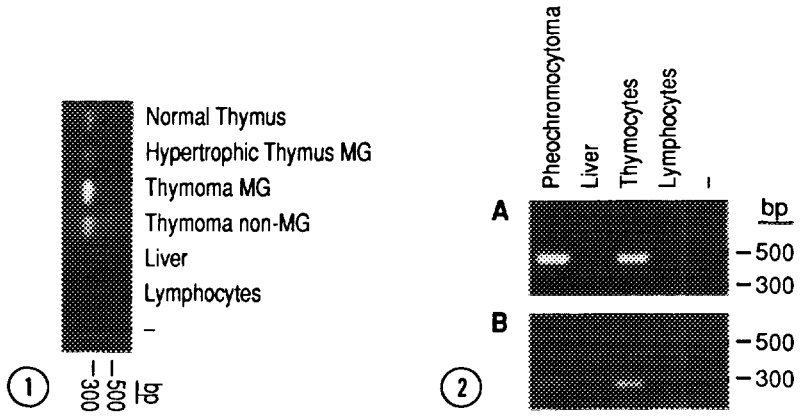

FIGURE 1. RT-PCR analysis for the variant α-3 AcChR subunit transcripts. RT-PCR analysis was performed as indicated in Methods employing 400 ng of poly(A)+ RNA.

FIGURE 2. RT-PCR analysis for the normal and variant α-3 AcChR subunit transcripts. RT-PCR analysis was performed as indicated in Methods employing A). 200 ng of poly(A)+ RNA for the analysis of the normal α-3 sequence and B). 400 ng of poly(A)+RNA for the variant sequence.

Organization of the genomic segment extending from exon 5 to exon 6 of the α-3 AcChR subunit gene.

To investigate the origin of the α-3 variant transcript, we partially sequenced the clone λ6 through the splice junctions of exon 5, exon 6 and the putative Alu-c exon (see Methods). Comparison of the genomic and variant cDNA sequence corresponding to the cDNA clone 42NTBSZ is shown in Fig. 3. The genomic sequence carries consensus donor and acceptor sites flanking the 122bp insert (Table I) indicating that the 122bp Alu element could be alternatively spliced (see below). To investigate the genomic location of the Alu-c element with respect to exon 5 and 6 of the α-3 AcChR subunit gene we performed a PCR analysis on the genomic λ6 clone spanning this region. Based on the genomic structure reported for the α-3 and other 6-exon type AcChR α subunit genes in the chick (8) and the rat (9), we expected an intron of 1.0 to 2.0 Kb in lenght. As shown in fig. 4, however,a large intervening sequence of approximately 5.0 Kb, contains the inverted Alu element 1.1 Kb downstream of exon 5 and 3.9 Kb upstream of exon 6. This PCR-generated data was confirmed through restriction map analysis of the region employing human genomic DNA and clone λ6. Analysis of digests utilizing combinations of 2 restriction enzymes resulted in the construction of a map for the region shown in Fig. 5. This map was further confirmed through subcloning of three independent HindIII and one BglII restriction fragments that extend through exon 5 and 6 of the α-3 AcChR subunit gene and human genomic DNA restriction analysis (not shown). Sequencing of the intron/exon boundaries in these subclones confirmed previous sequencing data obtained with the λ6 clone (Fig. 3, Table I). Analysis of the consensus splice junction (10) and branch point sequences (11) for splicing of the Alu-c element between exons 5 and 6 of the α-3 normal transcript have shown that the splicing of the variant form is likely to be less efficient than that of the corresponding normal transcript for at least two reasons. First, the presence of a G instead of the consensus C in the second position downstream from the poly-pyrimidine track of the consensus acceptor site NCAG/G (as it occurs in the Alu-c element (Table I)) results in a weaker acceptor

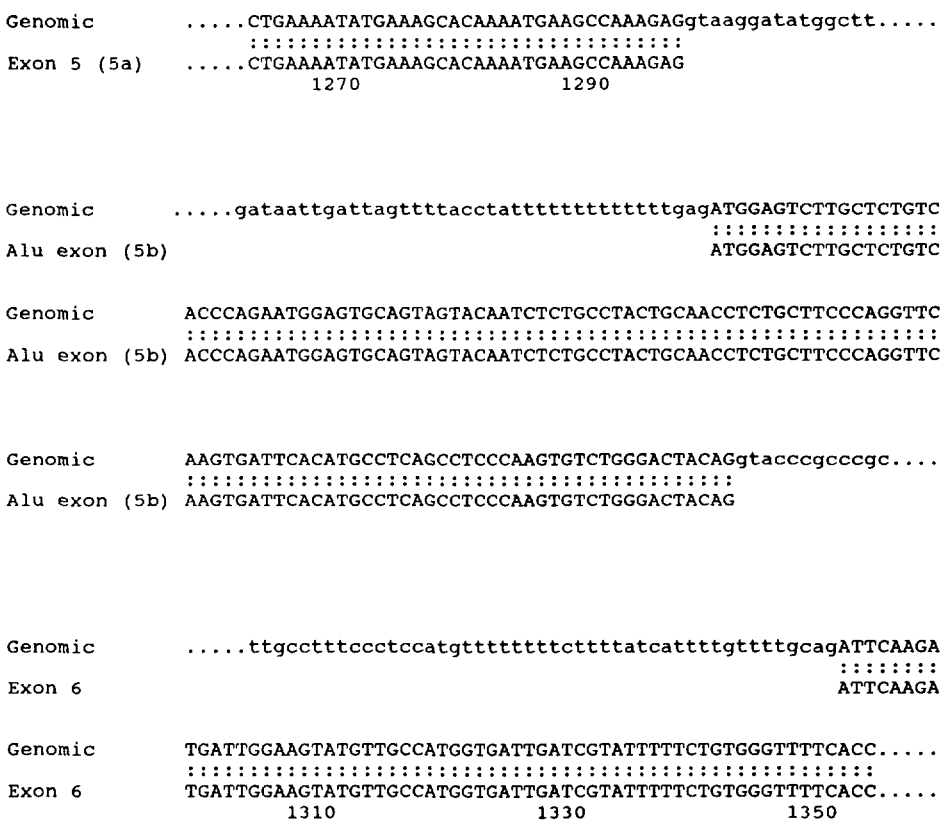

FIGURE 3. Comparison of the nucleotide sequence extending between exons 5 and 6 of the α-3 AcChR subunit gene and of the variant cDNA clone 42NTBSZ. In the variant clone the complementary right arm of an Alu sequence is spliced between exons 5 (5a) and 6. Consensus sequences for the splice junctions of exon 5 (5a) and 6 of the human gene are conserved (30).

site (12,13). The presence of a pyrimidine track close, but not adjacent, to this site, as is seen downstream from exon 6 (Table I), may still, however, provide an efficient consensus element because the usage of an acceptor site also depends

Table I

Splice junctions and branch point consensus sequences for exon 5, exon 6 and antisense Alu exon

	Donor site	Branch point (downstream from exon)	Acceptor site
Exon5	AG/GTAAGG*		
Alu exon	AG/GTA*CCC*	*A*ATTGAT^{-30}	T_{13}GAG/A
Exon6		TATTCAT^{-48}	$Y_{15}AY_2AY_4GY_4$GCAG/A
Consensus	AG/GTRAGT	YNYTRAY$^{-18\text{ to }-50}$	Y_nNCAG/G

* Italics show divergence from the accepted consensus sequences (10,11).

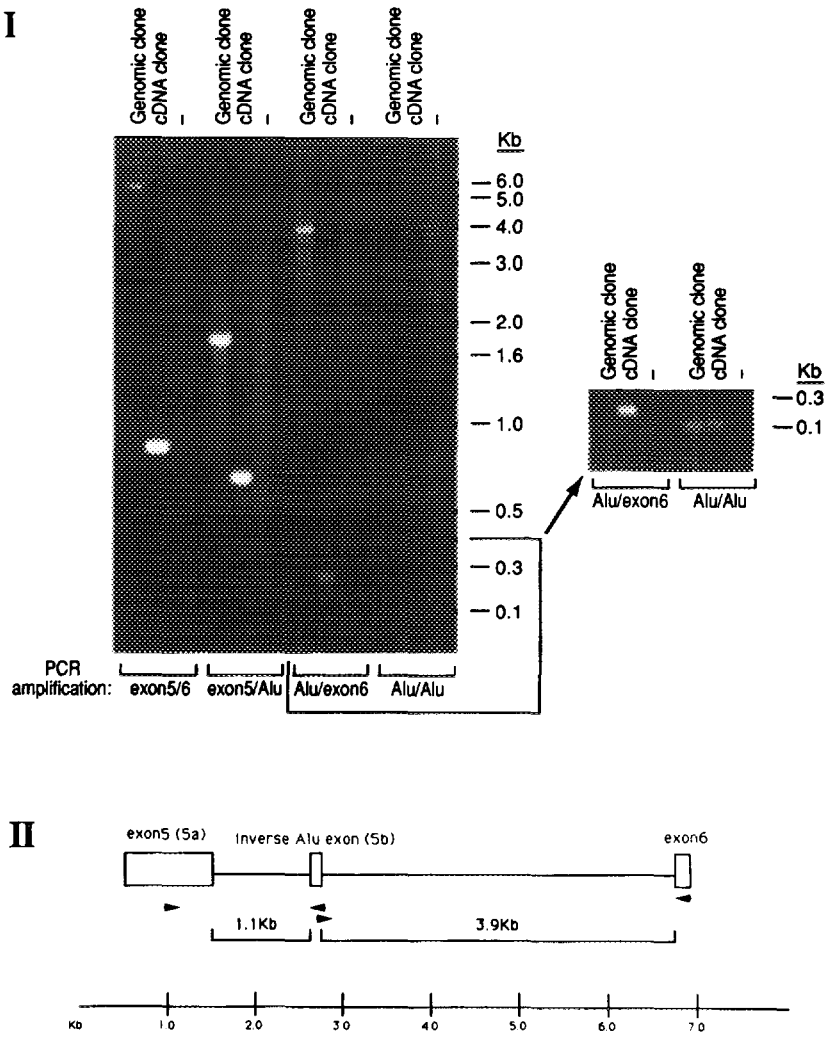

FIGURE 4. PCR analysis of genomic clone λ6 employing Hot tub DNA polymerase. PCR was performed as indicated in Methods employing 0.25 ng of the cDNA clone 42 NTBSZ or 1.0 ng of the genomic clone λ6. Primers utilized in the assay are shown as arrowheads (see Methods for exact primer sequences) priming within exon 5 (5a), exon 6 and the Alu-c exon 5b[2]. Insert: partial area of the same gel electrophoresed for a shorter length of time shows resolution of low molecular weight PCR products.

on the distance between the pyrimidine track and the lariat formation site (12). Secondly, based on statistical considerations, the Alu-c donor site AG/GTACCC is expected to be a less efficient donor than the consensus AG/GTAAGG which comprises the exon 5 3'end (10). These characteristics may explain the low level of variant transcript production in tissues expressing the α-3 transcripts.

Significance of the splicing of complementary Alu elements.

The analysis of the consensus inverse Alu sequence reveals that it carries multiple potential acceptor and donor sites (14,15). Upstream from the 5'end of

[2] Genbank accession # for Alu exon 5b: L18973.

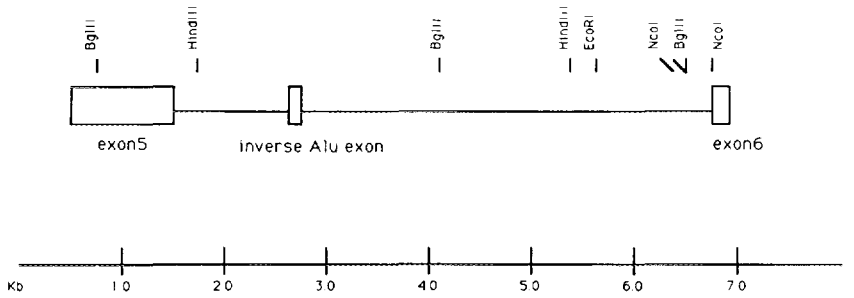

FIGURE 5. Human genomic map extending from exon 5 (5a) through exon 6 of the gene encoding for the α-3 subunit of an AcChR. The map was built through approaches that involved restriction mapping, PCR analysis and subcloning of DNA restriction fragments (see text). The Alu-c exon is shown as exon 5b.

most complementary Alu sequences there is a poly-pyrimidine track that, in conjunction with the potential 5'end acceptor site sequence found at the 5' end of inverted Alu elements, constitutes a functional splice acceptor site. These features of the inverse Alu element are utilized in the generation of alternative spliced transcripts from genes encoding the complement protein decay accelerating factor (16,17), the ornithine δ-amino-transferase (15) and possibly the rel proto-oncogene (14). To these reports, we add our own findings. Analysis of the normal gene encoding for the rel proto-oncogene (14), the decay accelerating factor (17) and our own data show that the complementary Alu elements -carrying adequate acceptor and donor splicing consensus sequences- are normally spliced and represent true exons. Alu-c splicing, however, also originates from mutations involving an Alu element as reported in the case or ornitine δ-amino transferase. In this case, the mutation generates a donor splice site resulting in the splicing of an inverted Alu sequence that leads to enzymatic deficiency and ultimately to chorioretinal degeneration (15).

Both transcriptional and translational data suggest that the splicing of a complementary Alu sequence is a mechanism through which peptide diversity is generated. The translation of the alternative spliced transcripts of the complement protein decay accelerating factor appears to generate 2 products encoding for a membrane bound and a soluble form of the factor (16). The translation of alternatively spliced transcripts encoding the rel proto-oncogene product and the α-3 subunit of an AcChR has not been demonstrated, but the transcriptional-deduced characteristics of the corresponding peptide indicates that the Alu exon incorporated in the rel proto-oncogene transcripts preserves the open reading frame of the peptide, while a similar Alu-c element incorporates a premature termination codon in the α-3 AcChR subunit peptide. Given the abundance of the Alu elements, representing 6% of the human genome, it is possible that this splicing mechanism plays a role in the evolution of proteins.

It has been postulated that in MG a thymic "cholinergic-neuromuscular type" immunogen could be the factor mediating the autoimmune response in this disorder (18,19). Because in MG there is occasional compromise of the central and peripheral nervous systems, it is possible that neuronal "cholinergic" antigens could also be involved in this disorder (20-25). Sequence analysis has shown that the 122bp exon found between exons 5 and 6 carries a stop codon which results in the coding of a truncated α-3 peptide lacking the 4th membrane domain

for this subunit, but carrying instead a dodecapeptide whose sequence does not resemble any 'cholinergic' sequence. The biological characteristics of such a peptide are expected to differ from those of its normal counterpart. If expressed in the thymus, either in thymocytes (see Fig.2B, 7) or other cells in the thymic tissue, it may influence the process of thymocyte maturation and/or the outcome of the immune responses to cholinergic antigens. Alternative splicing of exons carrying in frame stop codons have important developmental and metabolic implications as seen in the case of glutamic acid decarboxylase (26) and the sex-lethal sex determination switch genes (27). In the mature thymus, however, variant α-3 subunit AcChR transcripts appear not to be differentially regulated as judged by our inability to detect major differences in the expression of the variant α-3 AcChR transcripts in thymic tissues known to differ in their microenvironmental characteristics (2,28,29, Fig. 1). It has to be determined, however, whether or not there is developmental regulation of the splicing variant.

In conclusion, The structure of the human α-3 gene differs from that of chicken and rat neuronal α AcChR subunit genes, because it carries a large 5.1 Kb segment between exons 5 and 6 of the gene. This segment contains an additional exon corresponding to a complementary Alu sequence 1.1 Kb downstream from exon 5 (exon 5a). Splicing of the Alu-c exon (exon 5b) generates an α-3 AcChR subunit transcriptional variant. The thymic expression of the corresponding variant peptide could have important immunological implications. The description of the Alu-c exon strengthen the notion that the splicing of Alu sequences generates peptide diversity. Work is underway to assess the immunogenic potential of the variant α-3 peptide in normal individuals and MG patients.

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