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A newly identified chromosomal microdeletion and an N-box mutation of the AChRε gene cause a congenital myasthenic syndrome

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Summary

Congenital myasthenic syndromes (CMSs) are frequently caused by mutations of the coding region of the acetylcholine receptor epsilon subunit (AChRε) gene leading to a reduced expression of the acetylcholine receptor (AChR) at the postsynaptic membrane. Two recent observations have linked two different N-box mutations of the human AChRε promoter to a clinical CMS phenotype. N-boxes are regulatory sequence elements of mammalian promoters that confer synapse-specific expression of several genes, including the AChR subunit genes. Here, we report on a novel point mutation (ε-154G→A) in the N-box of the AChRε promoter in a German CMS pedigree. Semiquantitative analysis of AChRε mRNA levels in the patient's muscle indicated significantly impaired AChRε expression. We provide additional evidence of a pathogenic role for this mutation using the mutated promoter (ε-154G→A) driving a heterologous gene (luciferase) in rat skeletal muscle. We show that agrin-induced gene expression is significantly reduced by the N-box mutant (mt) compared with the wild-type (wt) promoter. Refined haplotype analysis and direct sequencing revealed maternal inheritance of the mutant AChRε promoter (ε-154G→A) together with paternal inheritance of a chromosomal microdeletion (Δ1290 bp) encompassing the promoter and the first two exons of the AChRε gene in the index patient. In conclusion, we provide genetic and functional evidence that a mutation of the AChRε subunit promoter (ε-154G→A) causes CMS due to the reduction of gene expression in skeletal muscle. Moreover, this is the first report of a chromosomal microdeletion affecting an AChR gene. This type of mutation may be missed in standard screening techniques of CMS patients.

Keywords: acetylcholine receptor; congenital myasthenic syndrome; epsilon subunit; gene deletion; promoter mutation

Abbreviations: AChR = acetylcholine receptor; AChRε = epsilon subunit of the acetylcholine receptor; bp = base pair; CMS = congenital myasthenic syndrome; kb = kilobase pair; mt = mutant; RACE = rapid amplification of cDNA ends; RT–PCR = reverse transcription PCR; SNP = single nucleotide polymorphism; wt = wild type

Introduction

Congenital myasthenic syndromes (CMSs) are a heterogeneous group of disorders, where neuromuscular transmission is impaired by inherited defects (Engel, 1994; Beeson *et al.*, 1998; Vincent *et al.*, 2000; Engel, 2001). The underlying molecular defects identified to date include mutations in a gene encoding the collagenic tail subunit of acetylcholinesterase (ColQ) (Donger *et al.*, 1998; Ohno *et al.*, 1998) (synaptic defect), in a gene encoding choline acetyltransferase (Ohno *et al.*, 2001) (presynaptic defect) and mutations in acetylcholine receptor (AChR) subunit genes (Engel *et al.*, 1999) (postsynaptic defects).

The latter are most frequently affecting the gene encoding the epsilon subunit of AChR (AChRε). Some missense mutations of the AChRε gene lead to kinetic abnormalities of the channel. However, most AChRε mutations reported to date are missense, nonsense, splice site mutations or single base pair (bp) deletions that result in a loss of functional

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AChR at the postsynaptic membrane. Rearrangements and chromosomal deletions of AChR genes have not yet been reported. Severe deficiency of the adult AChR at the neuromuscular junction leads to the clinical phenotype of CMS, and is usually inherited in autosomal-recessive traits (Engel *et al.*, 1999; Vincent *et al.*, 2000; Croxen *et al.*, 2001).

Alternatively, mutation of regulatory elements may also cause reduced or inadequate gene expression. Therefore, the promoter region of the human AChRε-subunit gene was investigated thoroughly (Nichols *et al.*, 1999; Ohno *et al.*, 1999). This region contains several sequence elements such as E-boxes and one N-box that are crucial for the developmental and tissue-specific regulation of gene expression (Fig. 1A). N-boxes have been identified to direct the synapse-specific expression of mammalian genes encoding AChR subunits (Koike *et al.*, 1995; Duclert *et al.*, 1996) and other proteins such as utrophin (Gramolini *et al.*, 1999; Khurana *et al.*, 1999) and acetylcholinesterase (Chan *et al.*, 1999). The N-box of the AChRε gene is highly conserved among different species, including man (Ohno *et al.*, 1999). Indeed, two recent reports indicated that mutations of this promoter element may be responsible for CMS (Fig. 1A). In both reports (Nichols *et al.*, 1999; Ohno *et al.*, 1999), the mutant allele was shown to cosegregate with the congenital myasthenic phenotype. Analysis of an intercostal muscle biopsy of one patient revealed a reduction in the number of end-plate AChRs and a reduction in ε-subunit mRNA levels (Nichols *et al.*, 1999).

Here, we report on a new mutation (ε-154G→A) of the AChRε N-box motif in a German CMS family. We present direct evidence that this mutation results in severely impaired gene expression. We also, to our knowledge, provide the first report of a chromosomal microdeletion affecting an AChR gene.

Material and methods

Patients

A 36-year-old German CMS patient has been followed up in our department for the last 15 years. Starting in early childhood, he presented with myasthenic symptoms including ptosis, limited eye movements, and mild facial and limb weakness. On several occasions, tests for anti-AChR antibodies were negative. Currently, he presents with marked bilateral ptosis, severe bilateral reduction in eye abduction, and fatigable weakness more prominent in proximal than in distal limb muscles. Repetitive stimulation of a proximal motor nerve (N. accessorius) revealed a decremental response of the compound muscle action potential by 30%, indicative of disturbed neuromuscular transmission. The patient responded well to acetylcholinesterase inhibitors; currently he receives pyridostigmine bromide (10 mg/kg/day). An immunosuppressive treatment with azathioprine (100 mg/day) for 6 years was not successful and was therefore discontinued. Both parents and all six siblings (three male, three female) of the patient are clinically unaffected.

DNA samples, muscle biopsy and mRNA samples

Venous blood samples were obtained from the patient, his parents and four siblings. Genomic DNA was isolated from 10 ml of peripheral blood using a blood and tissue culture DNA extraction kit following the manufacturer's recommendations (Qiagen, Hilden, Germany). A muscle biopsy of the patient was obtained from the left biceps. Control muscle tissue was obtained from orthopaedic surgery for a non-muscle-related reason. RNA was isolated from muscle tissue using standard methods (Chomczynski and Sacchi, 1987).

Sequence analysis and restriction digest

PCR primers were designed to amplify the promoter region, all 12 exons and flanking intronic regions of the AChRε-subunit gene (Abicht *et al.*, 1999) (DDBJ/EMBL/GenBank accession no. AF105999). PCR-amplified fragments were purified by the QIAquick PCR purification kit (Qiagen) and sequenced with an Applied Biosystems model 377 DNA sequencer and fluorescein-labelled dideoxy terminators (Perkin-Elmer, Foster City, Calif., USA). Sequence numbering is given with respect to the start of the mature ε-subunit polypeptide sequence (nucleotide position +1) (Beeson *et al.*, 1993). Screening for the mutation ε-154G→A was performed by restriction digest of PCR products from the patient, his family members, 42 unrelated patients with CMS, and 50 normal controls. A 623 bp fragment containing the promoter region of the AChR ε-subunit was amplified by PCR using primers 5′-gaatctctgtaccgcagggcta-3′ and 5′-agcccctgtccg-taccgagaa-3′. The ε-154G→A mutation results in the loss of a *Msp*I site. The mutant allele remains undigested, whereas the wild-type allele yields two fragments (465 and 158 bp). Restriction enzyme digestion was carried out at 37°C for 2 h by adding 15 U of *Msp*I in 20 μl of reaction mixture. Restriction fragments were size fractionated on a 2% agarose gel containing ethidium bromide.

Genotype and polymorphism analysis

Five polymorphic microsatellite markers on chromosome 17p13 flanking the AChRε-subunit gene (D17S1175, D17S926, D17S849, D17S1828, D17S1810) were chosen based on information obtained from Genome Database (GDB) and Généthon online services. Markers were amplified by PCR using fluorescence-labelled primers, and the size of PCR products was determined on sequencing gels using the GeneScan and GenoTyper software according to the manufacturer's instructions (Perkin Elmer Biosystems, Norwalk, Conn., USA). To characterize intrafamiliar genotype variation further, the intronic polymorphism

IVS3+54C→A was analysed in all family members by direct sequencing of PCR products obtained with primers 5′-tgccctggacaagacctcacac-3′ (intron 3) and 5′-aacaataatcgtccgggcctcg-3′ (intron 5). Furthermore, in the 3′-untranslated region of the AChRε gene, UniSTS 83674 (GenBank UniSTS database RH76075) was analysed in all family members using the recommended primers.

Mapping of the transcriptional initiation site in humans

First-strand cDNA synthesis and quantification of specific RNA transcripts was performed using the SMART™ Rapid Amplification cDNA Ends (RACE) Amplification Kit (Clontech, Palo Alto, Calif., USA) according to the manufacturer's instructions. The reverse transcription reaction was performed under standard conditions in a reaction volume of 10 µl, using 1 µg of total RNA from muscle biopsies, 200 U Superscript™ RNase H⁻ (Gibco-BRL, Karlsruhe, Germany) and 1 µl each of the 5′-RACE cDNA synthesis primer CDS and the SMART II oligo (Clontech).

For mapping of the human transcriptional initiation site of the AChRε gene, RACE experiments using an AChRε exon 5-specific antisense primer 5′-cgtcacggagccgccctcgta-3′ and the Universal Primer Mix (Clontech) were conducted on control muscle cDNA. This was followed by nested PCR with the AChRε exon 2-specific antisense primer 5′-cagtgagatgagattcgtcag-3′ and Nested Universal Primer (Clontech). In total, eight RACE products of eight independently performed reactions were analysed.

Semi-quantitative reverse transcription–PCR (RT–PCR) analysis

First-strand cDNA synthesis was carried out as described above for both patient and control samples. For quantification of the AChRα-subunit gene transcripts (DDBJ/EMBL/GenBank accession no. XM 010902), PCR was performed using 1 µl of the reverse transcription reaction mixture and the gene-specific primers 5′-ccgaggtgaaaagtgccatcg-3′ (exon 8) and 5′-tgagtcgacctgcaaacacgg-3′ (exon 9). PCR cycling was carried out with 91°C for 30 s, 50°C for 30 s and 72°C for 1 min. The efficiency of quantification was monitored after cycles 30, 32, 36, 39, 42 and 46. All PCR reactions were performed in an Eppendorf Personal Cycler (Hamburg, Germany). For quantification of specific mRNA transcripts of the AChRε gene, 2 µl of the reverse transcribed products were amplified using Universal Primer Mix (Clontech) and the aforementioned AChRε exon 5-specific antisense primer according to the following protocol: 5 cycles at 94°C for 30 s, 55°C for 30 s and 68°C for 5 min, and 40 cycles at 94°C for 1 min, 58°C for 1 min and 68°C for 3 min. Subsequently, nested PCR (25 cycles at 94°C for 5 s, 50°C for 30 s, 72°C for 1 min) was performed in duplicate using Nested Universal Primer (Clontech) and the above-mentioned AChRε exon 2-specific antisense primer with 1.5 and 2.5 µl of the first PCR product, respectively.

Reporter constructs

PCR fragments of the 5′-untranslated region of the AChRε gene, including upstream regulatory elements, promoter and transcriptional initiation site (position –622 to position –90), were generated using the genomic DNA of the patient and of a normal control. To facilitate subcloning, restriction sites for *Mlu*I and *Hin*dIII were added using primers 5′-gcgacgcgtgaatctctgtaccgcagggcta-3′ and 5′-gcgaagcttagctctggcaggcttggag-3′. Promoter fragments were subcloned into pGL3-Basic Vector, a commercially available vector containing an SV40 enhancer located downstream of a luciferase gene (Promega, Madison, Wisc., USA). This system has been used previously to test promoter function (Si *et al.*, 1997). The nucleotide sequence of the subcloned fragments was confirmed by DNA sequencing. The wild-type (wt) and the mutant (mt) constructs differ only at position –154. Plasmid DNA was purified using an endotoxin-free kit (Qiagen) according to the manufacturer's instructions, and DNA was resuspended in sterile PBS (phosphate-buffered saline) to a final concentration of 2–4 µg/µl.

In vivo *reporter gene assays*

The mt or wt AChRε reporter constructs were mixed at a ratio of 18: 1 with the pRL-TK vector (Promega) encoding *Renilla* luciferase under the control of the herpes simplex virus thymidine kinase promoter. The AChRε reporter/pRL-TK mixture was diluted to 100 ng/µl and a cDNA encoding full-length chicken neural agrin $cAgrin_{7A4B8}$ (Denzer *et al.*, 1995) was added at a dilution of 10 ng/µl. For controls, empty pcDNA1 vector (Invitrogen, Carlsbad, Calif., USA) was used. The resulting plasmid mixtures were injected into ~ 40 single soleus muscle fibres of young adult rats (body weight 120 g) as described elsewhere (Jones *et al.*, 1997; Meier *et al.*, 1997; Briguet and Ruegg, 2000). Two weeks after injection, the muscle was removed and frozen in liquid nitrogen. The proximal part of the soleus muscle (injected region) was homogenized in 500 µl 'passive lysis buffer' (Promega). Firefly and *Renilla* luciferase activities of 20 µl lysate were measured using the dual-luciferase reporter assay (Promega). Firefly luciferase activity was normalized to the *Renilla* luciferase activity derived from the pRL-TK vector. For statistical analysis, an unpaired *t*-test was used with significance defined as $P < 0.1$.

Southern blot analysis

To test the hypothesis of a chromosomal rearrangement/deletion of the paternal AChRε allele, Southern blot analysis was carried out. Genomic DNA (10 µg each) from the patient, his father and a control person were digested with *Msp*I, separated by gel electrophoresis on agarose gels, transferred

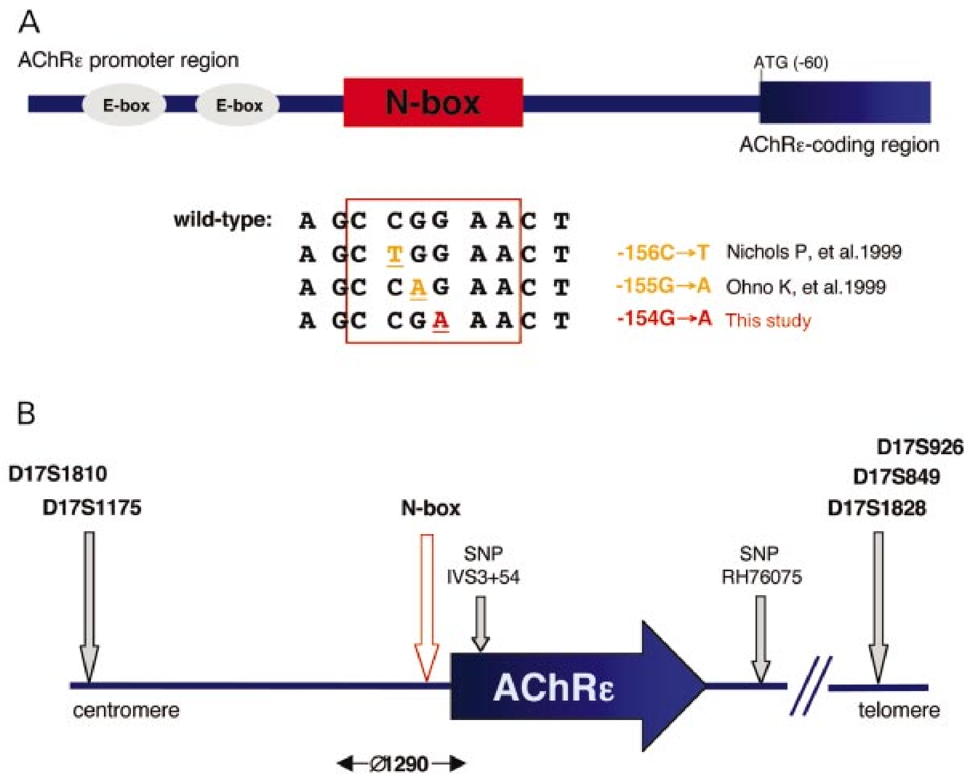

Fig. 1 (**A**) Schematic representation of the human AChRε gene promoter region, indicating the position of E-box and N-box elements and the mutations identified in the N-box. (**B**) Chromosomal position of the AChRε gene locus with flanking microsatellite markers and SNPs. In addition, the location of the chromosomal microdeletion (1290 bp) is indicated.

onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and hybridized with [α-^{32}P]dATP- and [α-^{32}P]dCTP-radiolabelled probes. Probes were generated by PCR and restriction digest as follows: a 740 bp fragment was amplified from control genomic DNA using primers 5′-gaatctctgtaccgcagggcta-3′ (AChRε promoter, 270 bp upstream of the N-box) and 5′-cagtgagatgagattcgtcag-3′ (AChRε exon 2, 470 bp downstream of the N-box), and digested by *Msp*I. The wt N-box contains a *Msp*I restriction site (see above). Two fragments (270 and 470 bp, respectively) were separated on a 2% agarose gel, purified and used as probes. Membranes were hybridized overnight at 65°C in an ExpressHyb™ Hybridization Solution (Clontech) and washed for 30 min each at 65°C in 2× SSC, 0.1× SSC and 0.01× SSC, respectively. Mt N-box alleles were detected as hybridization signals of 1260 bp with both probes, while wt alleles resulted in hybridization signals of 790 bp with the upstream probe and 470 bp with the downstream probe.

Determination of the deletion breakpoints

PCR amplification was carried out on the genomic DNA of the patient and of a control using the primer 5′-gtcggcacagtcagtaaagagg-3′ (AChRε intron 11). PCR conditions were as follows: 5 cycles at 94°C for 6 min, 52°C for 1 min, 68°C for 15 min, and 35 cycles at 94°C for 1 min, 55°C for 1 min, 68°C for 10 min. Subsequently, nested PCR was performed under the same conditions using the primers 5′-ccctggatcccgaccttagcat-3′ [~1.6 kilobases (kb) upstream of AChRε at a *Bam*HI restriction site] and 5′-tgccctggacaagacctcacac-3′ (AChRε intron 3). In addition to the expected band of ~2.2 kb in length (wt allele), a strong 950 bp was detected in the patient (deletion allele). Direct sequencing of the 950 bp band in the antisense direction revealed the proximal and distal deletion breakpoints. The same method has been used to detect the deletion allele in the patient's relatives.

Results

Transcription start site of the human AChRε gene in human skeletal muscle

Recently, we and others have analysed the human and rodent AChRε promoter (Ohno *et al.*, 1999; Stucka *et al.*, 2000). In the present study, we mapped the transcription start site of the human AChRε gene by direct sequencing of RACE products. Heterogeneity was observed in eight independent experiments using skeletal muscle from different healthy individ-

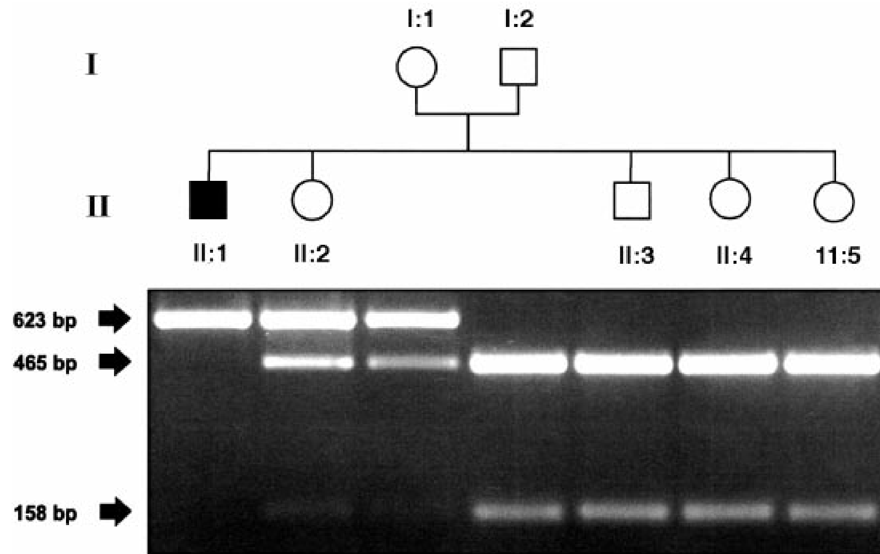

Fig. 2 Restriction enzyme analysis of the N-box mutation (ε-154GεA) in the CMS index patient (II:1), his asymptomatic parents (I:1, I:2) and four healthy siblings (II:2–5). A PCR fragment of 623 bp containing the promoter region of the AChRε was amplified. For the wt AChRε promoter N-box, the restriction digest with *Msp*I yields two fragments (465 and 158 bp). The ε-154G→A N-box mutation results in the loss of the *Msp*I site. In the patient (II:1), only mutated fragments (ε-154G→A) were observed. Both the wt and mt fragments were detected in the asymptomatic mother (I:1) and one asymptomatic sister (II:2), indicating heterozygosity for ε-154G→A. In the father and three asymptomatic siblings, only the wt fragments were observed.

uals and different amounts of RNA, indicating that multiple transcription start sites may exist. However, in five out of eight experiments the main transcription start site was assigned to position –88 (data not shown).

Mutation analysis in the German CMS pedigree

Sequence analysis of the promoter region of the AChRε gene revealed a G→A transition at nucleotide position –154 (ε-154G→A) in the index patient II:1 (Fig. 2) of our German CMS pedigree. The mutation affects the fourth position of the conserved 6 bp core of the N-box sequence (Fig. 1A). To exclude additional mutations we sequenced the entire coding region of the AChRε gene of the patient. No other mutation was found. The mutation ε-154G→A was detected in neither 42 unrelated CMS-patients nor 50 healthy controls. In index patient II:1, the wt AChRε promoter sequence was detectable neither by direct sequencing nor by restriction digest of PCR products, suggesting a homozygous mutation, i.e. two identically mutated N-box alleles (Fig. 2). Restriction analysis (Fig. 2) revealed that unaffected family members are either carrying the N-box mutation heterozygously (I:1 and II:2) or do not carry the mutation (I:2, II:3, II:4 and II:5). This is in accordance with an autosomal recessive trait. Surprisingly, the father (I:2) of the index patient did not carry a mutated N-box allele heterozygously. We hypothesized that one of the following three mechanisms may be responsible for this unexpected finding: (i) non-paternity; (ii) germline N-box mutation of the paternal AChRε allele; or (iii) chromosomal deletion/rearrangement of the paternal AChRε allele.

Refined haplotype analysis of the AChRε locus in the German CMS pedigree

By haplotype analysis using five polymorphic microsatellite markers on chromosome 17p13 (Figs 1B and 3) flanking the AChRε gene (D17S1175, D17S926, D17S849, D17S1828, D17S1810), non-paternity of the patient's father was found to be highly unlikely since the patient (II:1) and his father (I:2) share an identical allele for all markers tested.

Haplotype analysis confirmed that the index patient inherited the allele with the N-box mutation (ε-154G→A) from his mother (I:1), as did his unaffected sister II:2 (Fig. 3). To exclude a chromosomal rearrangement of the patient's AChRε locus, Southern blot analysis was performed. Only fragments with the mutated N-box allele were detected, suggestive of a chromosomal deletion but not suggestive of a chromosomal rearrangement (data not shown). Therefore, we hypothesized that the patient's paternal allele harbours a chromosomal deletion of the entire, or at least parts of the AChRε gene, resulting in a 'hemizygosity' for the N-box mutation in the patient (Figs 1 and 3).

The putative deletion was mapped between markers D17S1175 (5′ of the AChRε gene, distance 5 kb) and D17S1828 (3′ of the AChRε gene), which were found heterozygously in the index patient (II:1) and/or the patient's father (I:2) (Figs 1B and 3). A single nucleotide polymorphism (SNP) in the 3′-untranslated sequence of the AChRε gene

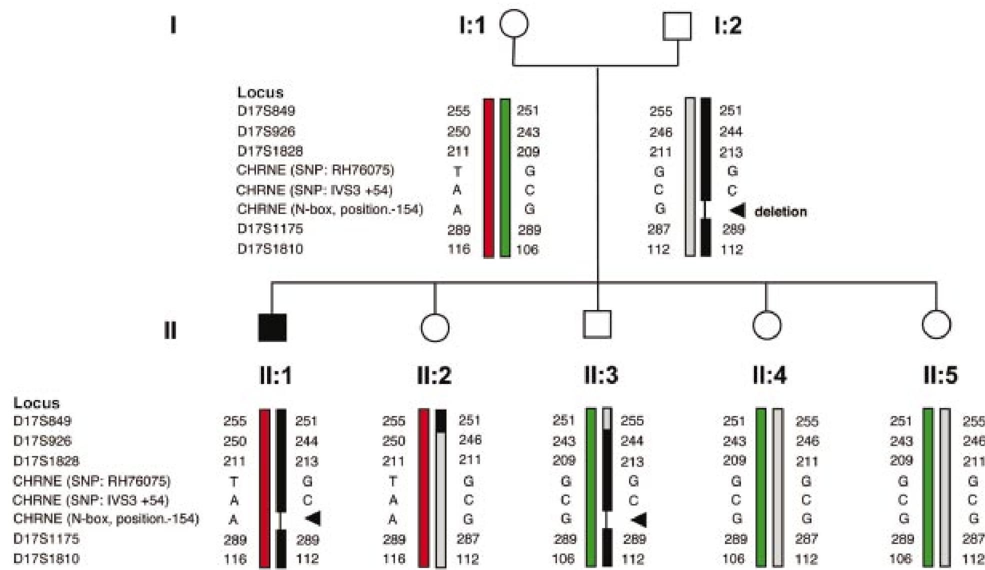

Fig. 3 Genotype analysis using five polymorphic microsatellite markers on chromosome 17p13 flanking the AChRε gene and two SNPs of the AChRε gene. Paternal haplotypes (black and grey) are on the right, and maternal haplotypes (red and green) on the left. The maternal allele harbouring the N-box mutation is shown in red. The paternal allele harbouring the chromosomal deletion is shown in black. Only the patient (II:1) carries both mutant alleles.

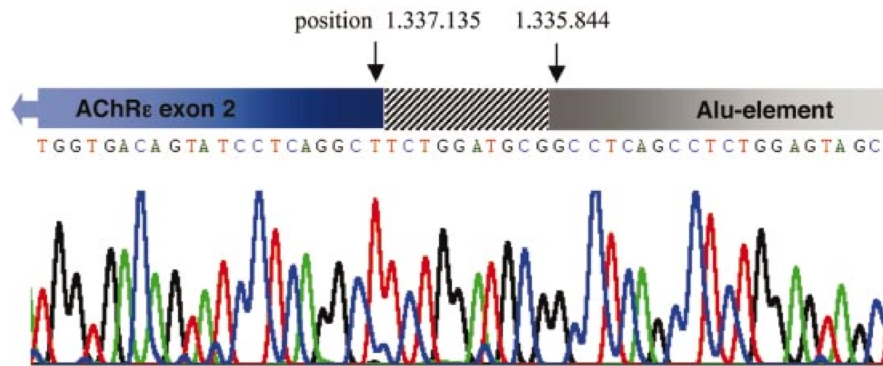

Fig. 4 Direct sequencing of the paternal deletion allele in antisense direction. The 5′-breakpoint is located within an Alu element [at position 1.335.844 on contig NT_010823 (GenBank/DDBJ/EMBL accession no.)]. The 3′-breakpoint is located within exon 2 of the AChRε gene (at position 1.337.135 on contig NT_010823). The deletion extends over 1290 bp (position 1.335.845 to 1.337.134 on contig NT_010823). Ten base pairs in between the two breakpoints do not show homology to any sequence at this locus.

(RH76075; NT_010823 variation 1341821: G→T) and a polymorphism in intron 3 of the AChRε gene (IVS3+54C→A) cosegregate with the N-box mutation on the maternal allele, but are not found in the father. The patient (II:1) was found to be heterozygous for both polymorphisms. Therefore, the paternal allele is deleted neither in the 3′-untranslated sequence of the AChRε gene, nor the coding part of the AChRε gene downstream of intron 3.

Identification of the deletion breakpoints in the AChRε gene

We wished to determine the deletion breakpoints of the paternal AChRε allele by direct sequencing. Therefore, the above-mentioned polymorphisms were used for allele-specific PCR in order to map the 3′ deletion breakpoint to exon 2 of the AChRε gene (data not shown). The deletion was confirmed by PCR and direct sequencing of the paternal allele. The deletion extends over 1290 bp [position 1.335.845 to 1.337.134 on contig NT_010823 (DDBJ/EMBL/GenBank accession no.)], thus removing the promoter region, exon 1 and the 5′-part of exon 2 of the AChRε gene (Fig. 4). The 3′-breakpoint is located within exon 2 of the AChRε gene, while the 5′-breakpoint is located within a sequence element highly homologous to Alu. Ten base pairs in between the two breakpoints do not show homology to any sequence at this locus. The deleted paternal allele is also present in the patient's healthy brother (II:3), but not in combination with the maternal allele carrying the mutated N-box (Fig. 3).

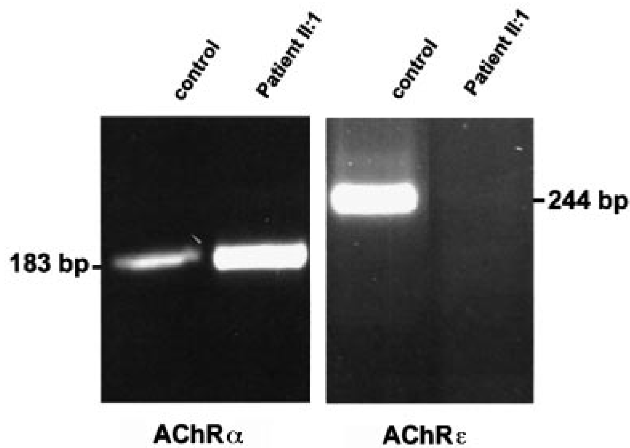

Fig. 5 Semi-quantitative RT–PCR of AChR subunits in skeletal muscle. Semi-quantitative RT–PCR analysis of α- and ε-subunit mRNA isolated from a muscle biopsy of the patient and an unaffected control revealed a significantly reduced AChRε expression compared with control. By contrast, robust expression of the AChRα-subunit cDNA was detected in the patient's muscle.

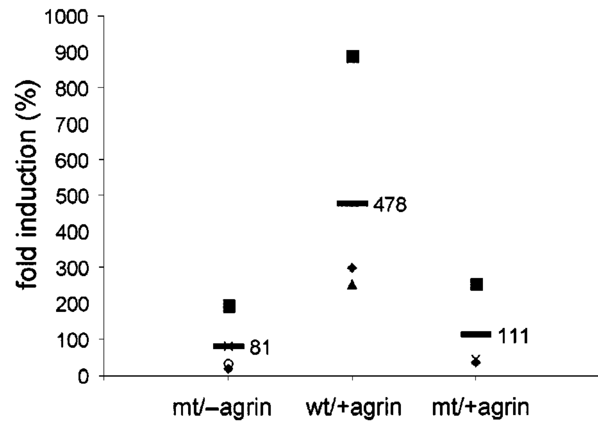

Fig. 6 *In vivo* studies using reporter gene constructs. Reporter gene constructs driven by a human wt or mt AChRε promoter fragment were injected into rat soleus muscle. In some experiments (+agrin) reporter constructs were injected in conjunction with expression plasmids encoding neural agrin. In each series, luciferase expression levels were normalized to the wt AChRε promoter construct. In the absence of agrin, expression levels of the mt AChRε promoter construct (mt/–agrin) did not significantly differ from wt promoter. Agrin co-injection led to a more than four-fold increase in luciferase expression of the wt AChRε promoter construct (wt/+agrin; $P < 0.1$). In contrast, agrin had no influence on the mutated AChRε promoter (mt/+agrin). Note: while absolute values between experiments varied substantially, agrin co-injection increased expression of wt promoter constructs within each series. Symbols represent individual measurements.

Semi-quantitative RT–PCR analysis

Semi-quantitative RT–PCR analysis of α- and ε-subunit mRNA isolated from a muscle biopsy of the patient and an unaffected control showed robust and approximately equal amounts of α-subunit cDNA in both muscles. By contrast, significantly reduced and barely detectable AChRε expression was seen in the patient's muscle compared with that of the control (Fig. 5). In the patient, direct sequencing of barely detectable fragments of a length consistent with wt transcripts was carried out after an additional reamplification step. Interestingly, besides correctly spliced, full-length AChRε transcripts, aberrantly spliced products were detected containing exon 2 of AChRε spliced to parts of the coding region of the GP1BA (glycoprotein Ib, platelet, alpha polypeptide) gene, located ~50 kb centromeric of the AChRε gene locus (data not shown).

In vivo *expression studies of AChRε promoter constructs*

In order to determine whether the mutation ε-154G→A of the N-box element would affect gene expression *in vivo*, reporter gene constructs driven by the human wt or mt AChRε promoter fragment were injected into rat soleus muscle. No difference in expression was observed between wt and mt promoter constructs in the absence of agrin (Fig. 6, mt/–agrin). While agrin increased expression of the wt promoter construct >4-fold (wt/+agrin), agrin had no effect on the expression of the mutant AChRε promoter (mt/+agrin).

Discussion

In this study, we report on a chromosomal deletion of the AChRε gene and a newly identified mutation (ε-154G→A) of the AChRε N-box motif in a German CMS family. So far, numerous AChR subunit gene mutations have been identified. Most of them are recessive loss-of-function mutations (Engel *et al.*, 1999; Croxen *et al.*, 2001). In our index patient, the hemizygous N-box mutation is uncovered by the heteroallelic AChRε deletion. We show that the hemizygous N-box mutation results in severe reduction of AChRε expression in the patient's skeletal muscle (Fig. 4), which is in agreement with a previously reported homozygous N-box mutation (Fig. 1) (Nichols *et al.*, 1999).

At present, the underlying genetic defects leading to CMS cannot be elucidated in all CMS families. As most of the studies have focused on the coding regions of the AChRε and other CMS-related genes, further analysis of regulatory sequences may reveal additional pathogenic mutations. In this study, we characterized the transcriptional start site of the human AChRε gene by RACE analysis and found a major transcription start site at position –88. Compared with transcription initiation sites of the rat or mouse AChRε gene (Sanes *et al.*, 1991; Durr *et al.*, 1994), the human transcripts initiate 40 and 60 bp downstream, respectively.

Mutations close to the transcription start site have not been detected in CMS patients so far, but may also impair AChRε gene expression.

In addition, we report for the first time a chromosomal deletion of the AChRε gene encompassing the entire promoter region and the first two exons. This deletion may have arisen through a non-homologous recombination of the paternal allele (Roth and Wilson, 1988). Whereas single bp mutations of AChR subunit genes are readily detected, chromosomal deletions or rearrangements may be missed by conventional PCR analysis employed to screen CMS patients. The frequency of chromosomal deletions of AChR subunit genes is currently unknown. In contrast, chromosomal deletions of the dystrophin gene account for up to 60% of all Duchenne muscular dystrophy cases (Oudet *et al.*, 1992; Mendell *et al.*, 2001). Since the AChRε gene is relatively small and does not contain repeated sequences prone to recombinational events, it may be speculated that large deletions occur at a lower frequency if compared with the dystrophin gene.

The promoter mutation ε-154G→A changes the fourth nucleotide of the 6 bp core of the N-box element. Recently, two other mutations in the AChRε promoter region have been identified in CMS families (ε-155G→A and ε-156C→T) (Nichols *et al.*, 1999; Ohno *et al.*, 1999), disrupting the conserved N-box motif at positions 2 and 3, respectively (Fig. 1). In both studies as well as in our report, the mt allele cosegregates with the congenital myasthenic phenotype in an autosomal recessive fashion. Homozygous carriers of the mutation show myasthenic symptoms, whereas heterozygous carriers remain unaffected. Similarly, only homozygous or compound heterozygous carriers of null mutations of the coding region of the AChRε gene cause a severe deficiency of the adult AChR at the neuromuscular junction that leads to the clinical phenotype of CMS. Therefore, it may be speculated that only a drastic reduction in AChRε promoter activity may lead to myasthenic symptoms. Indeed, analysis of an intercostal muscle biopsy of one patient (ε-156C→T) revealed a reduction in the number of end-plate AChRs and a severe reduction in ε-subunit mRNA levels (Nichols *et al.*, 1999). Similarly, in our patient functional AChRε mRNA is merely detectable (Fig. 4).

The AChRε promoter is highly conserved among different species and critical for synapse-specific gene expression. From mutagenesis studies in the mouse it is known that mutations of the N-box element and other promoter mutations directly affect gene expression at the endplate (Duclert *et al.*, 1996; Fromm and Burden, 1998; Gramolini *et al.*, 1998; Schaeffer *et al.*, 1998). The effect of the specific point mutation in the N-box motif identified in our patient altering CCGGAA to CCGAAA has been analysed in two studies using mouse promoter reporter gene constructs (Duclert *et al.*, 1996; Schaeffer *et al.*, 1998). These studies revealed reduced reporter gene expression at the endplate due to the N-box mutation. However, direct proof that impaired gene expression results from the N-box mutation of the human AChRε promoter has not yet been provided. Moreover, expression levels may not be extrapolated directly from the *in vitro* to the *in vivo* situation, since *in vivo* expression of AChR molecules is mediated via complex pathways (Schaeffer *et al.*, 1998) involving electrical activity and/or nerve-derived trophic factors that may be absent *in vitro*. Recent evidence strongly suggests that agrin mediates several mechanisms at the neuromuscular junction, including AChR clustering and AChR subunit gene transcription (Ruegg and Bixby, 1998; Briguet and Ruegg, 2000). In the current study, *in vivo* experimentation revealed that the wt AChRε promoter fragment is responsive to neural agrin-mediated activation of gene expression. In contrast, this was not seen using the mt promoter.

In conclusion, we report for the first time on a chromosomal deletion of the AChRε gene linked to a CMS phenotype. In addition, we provide genetic and functional evidence that a newly identified mutation of the AChRε gene promoter (ε-154G→A) causes a congenital myasthenic syndrome due to the reduction in gene expression in skeletal muscle.

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