A frameshifting mutation in CHRNE unmasks skipping of the preceding exon

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INTRODUCTION

Congenital myasthenic syndromes (CMS) are caused by genetic defects of presynaptic, synaptic, or postsynaptic molecules at the motor endplate (EP) (1). Mutations in CHAT encoding the choline acetyltransferase impair acetylcholine (ACh) resynthesis at the motor nerve terminal and cause CMS with episodic apnea (2). Mutations in COLQ encoding the collagen tail subunit of acetylcholinesterase cause EP acetylcholinesterase deficiency (3,4). Mutations in acetylcholine receptor (AChR) subunit genes alter AChR channel kinetics and/or cause EP AChR deficiency (1,5). Mutations in CHRNE encoding the collagen tail subunit encoded by CHRNE show weak splicing signals for exon 6 also cause EP AChR deficiency (6,7). Mutations in other AChR subunits are probably fatal due to lack of a substituting subunit, low-expressor or null mutations of CHRNE account for most cases of CMS.

Nonsense-mediated mRNA decay (NMD) is a quality-control surveillance mechanism that eliminates mRNA harboring a premature termination codon (PTC) (12–14). In mammalian cells, an exon–exon junction protein complex (EJC) is deposited 20–24 nucleotides upstream of exon–exon junctions (15). EJC recruits some other factors and promotes mRNA export to cytoplasm (16). If translation terminates less than 50–55 nucleotides upstream of the most 3′ exon–exon junction, translating ribosomes remove the junction-bound EJC (17). If translation terminates more than 50–55 nucleotides upstream of the last exon–exon junction, the remaining EJC and the associated hUpf1-3 initiate removal of the 5′ cap and the exonucleolytic degradation of mRNA (18).

Splicing of pre-mRNA occurs in a macromolecular spliceosome composed of five small nuclear ribonucleoproteins (U1, U2, U4, U5 and U6 snRNPs) and non-snRNP proteins (19,20). Cis-acting elements for pre-mRNA splicing include: (i) the 5′ splice site with the consensus sequence of MAG/GURAGU (M = A or C, R = A or G) (21,22), where the delimiter represents an exon–intron boundary; (ii) the 3′ splice site with
the consensus sequence of YAG/G (Y = U or C) (21); (iii) the branch point sequence with the consensus sequence of CURAY, YNYURAy, or YVAY (V = A, G, or C), where an invariant A (underlined) is located 11–40 nucleotides upstream of the 3’ splice site (21,23); (iv) the polypyrimidine tract between the branch point and the 3’ splice site, in which U is preferred to C (24,25); and (v) exonic splicing enhancers (ESEs) and exonic splicing silencers (26,27). In splicesome assembly, U1 snRNP binds to the 5’ splice site, U2 snRNP to the branch point, U2AF65 to the polypyrimidine tract and to U2 snRNP, U2AF35 to the 3’ splice site, and SR proteins to ESEs (20,28).

Mutations in any of the cis-acting elements above may cause aberrant splicing (29,30), and splicing mutations constitute at least 10% of human disease-causing mutations (31). Common consequences of splicing mutations include exon skipping, activation of a cryptic splice site, creation of a new splice site and intron retention, with ratios of 51, 32, 11 and 6%, respectively (32). In addition to the commonly observed aberrant transcripts, splicing mutations also result in aberrant transcripts affecting multiple contiguous exons (33–45) or remote exons (46–49). Skipping of multiple contiguous exons is probably accounted for by ordered removal of introns and consequent clustering of neighboring exons (36,37). The mechanism that underlies skipping of remote exons has not been studied to date, and the present study focuses on elucidating this mechanism.

Here we report that a frameshifting mutation in CHRNE exon 7 (e553del7) observed in seven CMS patients remarkably increases the aberrantly spliced transcript that skips the preceding 101 bp exon 6 in skeletal muscle and in transfected COS cells. Inhibition of protein synthesis and NMD by anisomycin and optimization of splicing signals for exon 6 reveal that inherently weak splicing signals for exon 6 cause skipping of exon 6 even in wild-type CHRNE, but the exon 6-skipped transcript is degraded by NMD, whereas, in the presence of e553del7, NMD degrades the normally spliced transcript rather than the exon 6-skipped transcript. Presence of weak splicing signals for CHRNE exon 6 also prompted us to search in other CMS patients for ESE-disrupting mutations in exon 6, and we found that two CMS mutations, eEF157V and eEF154X, cause aberrant splicing of exon 6.

### RESULTS

#### Endplate studies in patients 1, 2, 3 and 8

The number of α-bgt binding sites per EP was reduced to 10% or less of normal in patients (Pts) 1, 2, 3 and 8. The amplitude of the miniature EP potentials and currents was decreased to 30% or less of normal, indicating a diminished synaptic response to ACh. Single channel patch clamp analysis of patient EPs revealed that the dominant component of the burst open durations was 1.6- to 3.3-fold longer than normal, and that the conductance of channel events was reduced to 43–48 pS, consistent with expression of the fetal γ-AChR instead of the adult-type ε-AChR.

Ultrastructural studies showed that many EP regions were smaller than normal but the structural integrity of the pre- and post-synaptic regions was preserved. The density and distribution of AChR on the postsynaptic membranes, visualized with peroxidase-labeled α-bgt, were greatly attenuated.

#### Mutation analysis

Direct sequencing of AChR subunit genes revealed that all patients carry two mutant CHRNE alleles. Pts 1–7 harbor a 7 bp deletion of TGGGCA at nucleotides 553–559 (e553del7), which occupies positions 12–18 of exon 7. Pts 1, 6 and 7 are heterozygous, and Pts 2–5 are homozygous for e553del7 (Table 1). e553del7 predicts a frameshift, but turned out to have an unexpected splicing effect on the preceding exon 6, as will be shown below. Pt 1 also carries a 3’ splice site mutation in intron 9 (eIVS9-1G→C). Pt 6 harbors a 20 bp duplication at nucleotides 1002–1021 (e1021ins20). Pt 7 carries duplication of a G nucleotide at 1098 (e1098insG).

Pt 8 is homozygous for a nonsense mutation at codon 154 (eEF154X). Pt 9 carries an inframe 3 bp deletion at nucleotides 470–472 (e470delAGT) that predicts to mutate glutamate and phenylalanine at codons 157 and 158 to valine (eEF157V). Pt 9 also harbors a 3’ splice site mutation in intron 4 (eIVS4-2A→C).

### Table 1. CHRNE mutations in nine patients

<table>
<thead>
<tr>
<th>Pt</th>
<th>Mutation</th>
<th>Position</th>
<th>Major transcript</th>
<th>Consequence</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>ε553del7</td>
<td>Exon 7</td>
<td>Skipping of exon 6</td>
<td>Δcodons 148–187β</td>
</tr>
<tr>
<td>1</td>
<td>εIVS9-1G→C</td>
<td>Intron 9</td>
<td>Retention of intron 9</td>
<td>PTC in exon 10</td>
</tr>
<tr>
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<td>ε553del7</td>
<td>Exon 7</td>
<td>Skipping of exon 6</td>
<td>Δcodons 148–187β</td>
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<tr>
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<td>Exon 7</td>
<td>Skipping of exon 6</td>
<td>Δcodons 148–187β</td>
</tr>
<tr>
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<td>ε553del7</td>
<td>Exon 7</td>
<td>Skipping of exon 6</td>
<td>Δcodons 148–187β</td>
</tr>
<tr>
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<td>ε553del7</td>
<td>Exon 7</td>
<td>Skipping of exon 6</td>
<td>Δcodons 148–187β</td>
</tr>
<tr>
<td>6</td>
<td>ε553del7</td>
<td>Exon 7</td>
<td>Skipping of exon 6</td>
<td>Δcodons 148–187β</td>
</tr>
<tr>
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<td>ε1021ins20</td>
<td>Exon 10</td>
<td>n.e.</td>
<td>PTC in exon 10</td>
</tr>
<tr>
<td>6</td>
<td>ε553del7</td>
<td>Exon 7</td>
<td>Skipping of exon 6</td>
<td>Δcodons 148–187β</td>
</tr>
<tr>
<td>7</td>
<td>ε1098insG</td>
<td>Exon 10</td>
<td>n.e.</td>
<td>PTC in exon 10</td>
</tr>
<tr>
<td>8</td>
<td>εE154X (ε460G→T)</td>
<td>Exon 6</td>
<td>Activation of a cryptic 3’ splice site in exon 6</td>
<td>PTC at the boundary of exons 6 and 7α</td>
</tr>
<tr>
<td>9</td>
<td>εEF157V (ε470del3)</td>
<td>Exon 6</td>
<td>Skipping of exon 6</td>
<td>PTC in exon 7e</td>
</tr>
<tr>
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<td>εIVS4-2A→C</td>
<td>Intron 4</td>
<td>Skipping of exon 5α</td>
<td>Δcodons 96–147e</td>
</tr>
</tbody>
</table>

aHomozgyous mutation.
bDeleted codons 148–187 are replaced with ERRV missense codons.
cAChR expression studies are not performed in HEK cells.
dDetailed splicing analysis will be presented in another report.

Nucleotide annotations are shown in parentheses, if applicable. n.e., not examined.

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The above text is a natural reading of the document, free of hallucinations and presented in a plain text format.
We previously reported e553del7 in another patient with EP AChR deficiency, but we did not analyze pre-mRNA splicing (case 3 in 9). The other mutations have not been previously reported.

**Family analysis**

Family analysis revealed that unaffected members carry no or a single mutant allele, whereas affected siblings harbor two mutant alleles, indicating that each mutation is recessive (Fig. 1A). As no DNA was available from the father of Pt 1, we examined the allelic distributions of e553del7 and eIVS9-1G→C using allele-specific RT–PCR. This revealed that the two mutations are heteroallelic and also that eIVS9-1G→C causes retention of intron 9 (Fig. 1B). RT–PCR spanning exons 9–11 also showed that eIVS9-1G→C does not cause skipping of exon 10 (data not shown). Heteroallelic distribution of eEF157V and eIVS4-2A→C in Pt 9 was established by cloning a PCR fragment spanning the two mutations.

**Aberrant transcripts in patients 1 and 2**

During optimization of the allele-specific RT–PCR in Pt 1, we noticed amplification of unexpected fragments. To examine the origins of the unexpected fragments, we amplified and cloned the entire coding region of *CHRNE* cDNA by RT–PCR using muscle mRNA in Pt 1. We characterized 56 clones and identified seven different transcripts (Fig. 2). Transcripts I–V originated from the e553del7 allele, whereas transcripts VI and VII originated from the eIVS9-1G→C allele. Transcript V was the dominant species and was the only transcript that harbored no PTC. Retention of intron 11 observed in transcript IV was also present in control cDNA samples (transcript VIII in Fig. 2).

We also cloned *CHRNE* cDNA in Pt 2, characterized 33 clones, and identified three transcripts that were identical with transcripts I, IV and V in Pt 1 (Fig. 2). Transcript V was again the dominant species in Pt 2 as in Pt 1.

**Expression studies of aberrant transcripts in HEK cells**

To examine the effects of the identified aberrant transcripts on AChR expression, we expressed each mutant *CHRNE* transcript with wild-type *CHRNA1, CHRNBI* and *CHRND* cDNAs in HEK cells. As a control, we coexpressed wild-type *CHRNA1, CHRNBI* and *CHRND* cDNAs with or without wild-type *CHRNE* cDNA.

The mutant *CHRNE* transcripts reduced the surface expression of [125I]α-bgt-binding sites on HEK cell to 9–29% of normal (data not shown). As α-bgt-binding sites of the e-omitted α2βδ3 pentamers were 26±4% of normal, the mutant *CHRNE* transcripts may not be incorporated into cell surface pentamers.

To confirm this, we measured ACh binding by competition against the initial rate of [125I]α-bgt binding (50). Wild-type α2βδ3 pentamers bind ACh in a monophasic manner, whereas e-omitted α2βδ3 pentamers bind ACh in a biphasic manner (51). Transcript I demonstrated a biphasic ACh binding as we observed with the e-omitted α2βδ3 pentamers, indicating that transcript I is not incorporated into cell surface pentamers (data not shown). A very low expression level of transcript V precluded using this transcript in the ACh competition assay, but lack of 40 essential residues (codons 148–187) that constitute the F-loop in the extracellular agonist binding domain (52,53) predicts that gene product of transcript V will not assemble into cell surface pentamers.

![Figure 1](image-url)
We also determined the ability of mutant ε subunits to dimerize with wild-type α subunit, an early step in AChR assembly. When the mutant CHRNE transcripts were coexpressed with wild-type CHRNA1 cDNA, the number of α-bgt binding sites was reduced to 16–36% of normal (data not shown). As the number of α-bgt binding sites of wild-type α subunit alone was 35 ± 3%, the mutant ε subunit is unlikely to dimerize with the α subunit.

To summarize, the expression studies show that each mutant CHRNE transcript is a null or a functionally null mutation, in which αε dimerization and incorporation into cell surface pentamers are probably compromised.

**Recapitulation of skipping of exon 6 in COS cells**

Presence of the major transcript V in Pts 1 and 2 was unexpected, because e553del17 was in exon 7, whereas the preceding exon 6 was skipped. We first confirmed by RT–PCR that e553del17 is associated with skipping of exon 6 in muscle mRNA in Pts 1, 2, 3, and in a previously reported patient (case 3 in 9) whose mRNA was not analyzed before. RT–PCR fails to amplify fragments retaining introns 5 and/or 6 (transcripts II, III and VI in Fig. 2) probably due to replication disadvantage of longer fragments. Ratios of the exon 6-skipped transcript are indicated by mean ± SD below the gel. Ratios of total CHRNE cDNAs to β-actin cDNA were 0.03 ± 0.009, 0.006 ± 0.0003, 0.006 ± 0.001, 0.021 ± 0.005 and 0.0006 ± 0.0002 for lanes 1–5, respectively, which probably represent variable numbers of EP regions included in muscle specimens. (B) Schematic diagrams of minigene and entire CHRNE. Exons (boxes) and introns (horizontal line) are drawn to the scale. Exon 6 is shaded. Arrowheads point to e553del17. (C) RT–PCR spanning CHRNE exons 5–7 of cytoplasmic RNA of transfected COS cells. Both wild-type and mutant minigenes predominantly result in an exon 6-skipped transcript. Wild-type entire CHRNE mostly yields a normally spliced transcript, whereas e553del17–CHRNE predominantly produces an exon 6-skipped transcript. Ratios of the exon 6-skipped transcript are indicated by mean ± SD below the gel. Ratios of total CHRNE cDNA to β-actin cDNA were 0.03 ± 0.002, 0.03 ± 0.006, 0.95 ± 0.31 and 0.94 ± 0.15 for lanes 1–4, respectively.
Real-time PCR revealed that homozygous patients (Pts 2 and 3) harbor more exon 6-skipped transcripts than heterozygous patients (Pt 1 and the previously reported case 3) and that even a normal control carries a small amount of an exon 6-skipped transcript (Fig. 3A).

To understand why e553del7 promotes accumulation of the exon 6-skipped transcript, we first constructed wild-type and mutant minigenes spanning **CHRNE** exons 4–8, and expressed each in COS cells. We chose COS cells, because expression of **CHRNE** was not detected in native COS cells. These constructs, however, failed to recapitulate the aberrant splicing: exon 6 was similarly skipped in both wild-type and e553del7-**CHRNE**s (lanes 1 and 2 in Fig. 3C). As different splicing patterns between a minigene and patient-derived cells have been previously reported (38,54), we next constructed wild-type and mutant entire **CHRNE**s spanning 12 exons and 11 introns, and expressed each in COS cells. These constructs successfully recapitulated the aberrant splicing (lanes 3 and 4 in Fig. 3C). In contrast to muscle mRNA of homozygous patients (Pts 2 and 3), cytoplasmic RNA of COS cells transfected with e553del7-**CHRNE** showed only a faint band arising from the normally spliced transcript.

Does e553del7 affect an exonic splicing enhancer?

We next asked whether e553del7 causes skipping of exon 6 by disrupting an ESE. Although ESEs are known to work on an ESE-bearing exon, disruption of an ESE possibly affects ordered removal of introns and subsequently results in skipping of exon 6. We thus scanned for the effect of a 7 bp deletion from four nucleotides upstream to four nucleotides downstream of e553del7 (Fig. 4A). This showed that none affected skipping of exon 6. We further deleted six- and four-nucleotides beginning at nucleotide 553 (e553del6 and e553del4), and found that an inframe 6 bp deletion resulted in normal splicing, whereas a frameshifting 4 bp deletion predominantly yielded an exon 6-skipped transcript (Fig. 4A). These results indicate that no specific nucleotides in exon 7 are required for inclusion of exon 6, but a shift in the ORF is causally associated with skipping of exon 6.

To confirm that a shift in the ORF accounts for skipping of exon 6, we deleted two nucleotides at 578 and 579 in exon 7 (e578delTG) in the e553del7-**CHRNE** construct so that a normally spliced transcript should have no PTC, whereas...
skipping of exon 6 should place a PTC in exon 7 (Fig. 4B). As expected, e578deTG efficiently prevented accumulation of exon 6-skipped transcript in the e553del7–CHRNE construct, which provides additional evidence that a shift in the ORF is associated with skipping of exon 6 (Fig. 4C).

**Does nonsense-mediated mRNA decay contribute to skipping of exon 6?**

We next examined if NMD contributes to exon 6 skipping. We expressed wild-type and e553del7–CHRNEs in COS cells and treated the transfected COS cells with 100 μg/ml of anisomycin for 0.5, 1.0, 1.5 and 2.0 h, which inhibits protein synthesis as well as NMD (55,56). Anisomycin treatment revealed that both normally spliced and exon 6-skipped transcripts were produced from both wild-type and e553del7–CHRNEs (Fig. 5). This indicates that exon 6 is spliced out even in wild-type CHRNE, but the exon 6-skipped transcript is probably degraded by NMD. e553del7 removes a PTC from an exon 6-skipped transcript and makes the transcript immune to NMD, whereas a normally spliced transcript harboring e553del7 is likely degraded by NMD.

Why is CHRNE exon 6 easily skipped?

We inspected splicing signals spanning CHRNE exon 6 to understand why exon 6 is easily skipped. The polypyrimidine tract of CHRNE intron 5 has 19 pyrimidines and is interrupted by multiple trains of Gs (Fig. 6A) to give a pyrimidine to total nucleotide ratio of 19/31 (61.3%), which is the second lowest among 11 introns of CHRNE (mean ± SD, 78.0 ± 10.1%; range 61.3–100%). The consensus value (CV) is an indicator of the similarity of any 8 bp sequence to the consensus splice-site consensus sequence between the putative branch point (underlined) and the 3′ splice site. We substituted T for each G (arrows) to make uninterrupted stretch of 30 pyrimidines (‘Int 5 Plus’ construct). The native 5′ splice site of intron 6 harbors four mismatches against the RNA binding site (5′-ACUUACCUG-3′) of U1 snRNP. We engineered optimized nucleotides (arrows) that are complementary to U1 snRNP (‘Int 6 Plus’ construct). RT–PCR spanning CHRNE exons 5–7 of cytoplasmic RNA of COS cells transfected with indicated constructs with or without anisomycin. Alleles-specific RT–PCR faintly detected the exon 6-skipped transcript only in the ‘Int 6 Plus, e553del7’ construct in the presence of anisomycin (not shown), and the estimated ratio of the exon 6-skipped transcript was 0.10 ± 0.07% by real-time PCR. Ratios of total CHRNE cDNA to β-actin cDNA were 0.75 ± 0.06, 0.29 ± 0.04, 1.63 ± 0.20, 2.36 ± 0.16, 5.20 ± 1.42, 5.46 ± 0.75, 8.11 ± 1.07 and 1.98 ± 0.48 for lanes 1–8, respectively.

**Figure 5.** Inhibition of protein synthesis and NMD by anisomycin. (A) RT–PCR spanning CHRNE exons 5–7 of cytoplasmic RNA of COS cells transfected with the indicated constructs. Treatment of transfected COS cells with 100 μg/ml of anisomycin for 2 h results in production of both normally spliced and exon 6-skipped transcripts from both wild-type and e553del7–CHRNEs. (B) Ratios of the exon 6-skipped transcript after anisomycin treatment are shown by mean ± SD. Ratios of total CHRNE cDNA to β-actin cDNA were 0.95 ± 0.31 (0 h), 1.08 ± 0.37 (0.5 h), 2.08 ± 0.13 (1.0 h), 2.03 ± 0.06 (1.5 h) and 1.28 ± 0.19 (2.0 h) for wild-type CHRNE, and 0.94 ± 0.15 (0 h), 2.27 ± 0.20 (0.5 h), 1.61 ± 0.38 (1.0 h), 3.34 ± 0.56 (1.5 h) and 1.31 ± 0.04 (2.0 h) for e553del7. Note that the ratios increase up to 1.5 h after anisomycin treatment.

**Figure 6.** Optimization of splicing signals flanking exon 6 prevents its skipping. (A) Native polypyrimidine tract of intron 5 has 19 pyrimidines (Y) interrupted by multiple trains of Gs at the YNCAG consensus sequence between the putative branch point (underlined) and the 3′ splice site. We substituted T for each G (arrows) to make uninterrupted stretch of 30 pyrimidines (‘Int 5 Plus’ construct). The native 5′ splice site of intron 6 harbors four mismatches against the RNA binding site (5′-ACUUACCUG-3′) of U1 snRNP. We engineered optimized nucleotides (arrows) that are complementary to U1 snRNP (‘Int 6 Plus’ construct). (B) RT–PCR spanning CHRNE exons 5–7 of cytoplasmic RNA of COS cells transfected with indicated constructs with or without anisomycin. Alleles-specific RT–PCR faintly detected the exon 6-skipped transcript only in the ‘Int 6 Plus, e553del7’ construct in the presence of anisomycin (not shown), and the estimated ratio of the exon 6-skipped transcript was 0.10 ± 0.07% by real-time PCR. Ratios of total CHRNE cDNA to β-actin cDNA were 0.75 ± 0.06, 0.29 ± 0.04, 1.63 ± 0.20, 2.36 ± 0.16, 5.20 ± 1.42, 5.46 ± 0.75, 8.11 ± 1.07 and 1.98 ± 0.48 for lanes 1–8, respectively.
occuring mutations in exon 6 affects its splicing. We analyzed one polymorphism and four mutations in exon 6 (Fig. 7A). The polymorphism was a C-to-T substitution at nucleotide 459 predicting no amino acid substitution (c459CT); this polymorphism was observed in seven out of 208 alleles in our series. The four mutations were eE154X in Pt 8, eEF157V in Pt 9, and the previously reported eT159P (61) and eD175N (62) (Fig. 7A). eT159P engineered into a normally spliced CHRNE transcript does not express on cell surface (61); eD175N engineered into a normally spliced CHRNE impairs AChR channel opening efficiency (62).

We engineered each nucleotide change into the cloned entire CHRNE and expressed each mutant in COS cells. RT–PCR of cytoplasmic RNA of transfected COS cells revealed that c459CT, eT159P and eD175N did not affect splicing of exon 6; eE154X activated a cryptic 3' splice site located 47 nucleotides downstream of the native 3' splice site; and eEF157V caused skipping of exon 6 (Fig. 7B). RT–PCR of muscle mRNA of Pt 8, who was homozygous for eE154X, was almost identical to that of transfected COS cells (Fig. 7C). Muscle mRNA of Pt 9, who carried eEF157V, was not available.

**DISCUSSION**

**Skipping of a preceding exon in CHRNE**

Cloning of the entire coding region of CHRNE cDNA in Pts 1 and 2 (Fig. 2) and RT–PCR analysis of muscle mRNA in Pts 1–3, and a previously reported patient (Fig. 3A) demonstrate that e553del7 in exon 7 is unexpectedly associated with skipping of the preceding exon 6. The exon 6-skipped transcript restores the ORF after e553del7 and is the most abundant species (transcript V in Fig. 2).

To understand the mechanism of skipping of exon 6 in CHRNE, we constructed a series of site-directed mutants using the cloned entire CHRNE and analyzed cytoplasmic RNA of transfected COS cells. We found the following: (i) scanning mutagenesis in and around e553del7 established that the remote splicing effect is not owing to disruption of an ESE in exon 7, but to a shift in the ORF (Fig. 4); (ii) treatment of transfected COS cells with anisomycin resulted in production of both normally spliced and exon 6-skipped transcripts from both wild-type and e553del7-CHRNEs (Fig. 5); however, the exon 6-skipped transcript arising from wild-type CHRNE and the normally spliced transcript arising from e553del7-CHRNE are probably eliminated by NMD; and (iii) boundaries of exon 6 carry weak splicing signals, and optimization of the splicing signals prevented skipping of exon 6 (Fig. 6). On the basis of these observations, we attribute skipping of exon 6 associated with e553del7 to inherently weak splicing signals at the boundaries of exon 6 and transcript selection driven by NMD. Therefore, e553del7 does not directly affect splicing of exon 6, but unmasks skipping of exon 6 owing to its weak splicing signals.

Swapping of the NMD target is also observed in ‘nonsense-associated altered splicing’ driven by disruption of an ESE, in which a nonsense mutation alters splice site selection of the mutant exon by disrupting an ESE. Here the alternatively spliced transcript is immune to NMD because of lack of a PTC, whereas a normally spliced transcript carries a PTC and is

**Do mutations in CHRNE exon 6 affect its splicing?**

Weak splicing signals and the consequent unstable splicing of CHRNE exon 6 imply that ESEs could be involved in recognition of exon 6 (60). We thus tested if any of naturally
degraded by NMD (56,63,64). In the present study, however, skipping of CHRNE exon 6 is not owing to disruption of an ESE, but to inherently weak splicing signals at the boundaries of the skipped exon 6.

A recent report shows that ~20% of human genes undergo alternative splicing, and one-third of alternative transcripts harbor a PTC and therefore are targets of NMD (65). In glutaminase (66) and fibroblast growth factor receptor 2 (67), production of PTC-bearing alternative transcripts regulates gene expression levels. Skipping of exon 6 in wild-type CHRNE may or may not have the similar functional significance at the motor endplate.

**Skipping of remote exons in other genes**

Skipping of remote exons has been reported in other genes. Four exonic and two intronic disease-causing mutations in four genes are known to affect splicing of remote exons. In SLC25A20 encoding the solute carrier family 25 (carnitine–acylcarnitine translocase) member 20, a single nucleotide deletion in exon 1 results in skipping of exon 3. Here, an exon 3-skipped transcript is the major transcript and restores the deletion in exon 1 results in skipping of exon 3. Here, an exon 3-skipped transcript is the major transcript and restores the ORF (47).

In DBT encoding the dihydrolipoamide branched chain transacylase (E2 component of branched chain keto acid dehydrogenase complex), a 2bp deletion in exon 2 results in skipping of exon 4 or 6. In the same patient, a heteroallelic nonsense mutation in exon 6 gives rise to skipping of exon 4 or 6. The patient also carries a transcript with skipped exons 2–8, which is the only transcript that restores the ORF (49).

In BTK encoding the Bruton agammaglobulinemia tyrosine kinase, a 3′ splice site mutation in intron 17 deletes the first nucleotide of exon 18 by shifting the 3′ splice site, and also shows skipping of remote exon 16, which fails to restore the ORF (48). In the same patient, a heteroallelic nonsense mutation in exon 18 gives rise to three aberrant transcripts: (i) skipping of exon 16; (ii) skipping of exons 15 and 16, and (iii) creation of a new 3′ splice acceptor site in exon 18 that also skips exons 16 and 17. Only the last transcript restores the ORF.

In MLH1, which is defective in human nonpolyposis colorectal cancer, IVS1-11T→A is associated with skipping of exon 2 along with six different combinations of skipping of exons 6, 9 and 10, and none restored the ORF (46).

All remotely affected exons in the above genes carry weak splicing signals in the polypyrimidine tract, the 3′ splice site, and/or the 5′ splice site. Low polypyrimidine ratios are observed in SLC25A20 introns 2 and 3, DBT intron 5, BTK introns 14 and 15, and MLH1 introns 5 and 8. Low CVs for the 3′ splice site are observed at SLC25A20 intron 3 and MLH1 introns 8 and 9. Low CVs for the 5′ splice site are recognized at DBT intron 4, BTK intron 15, and MLH1 intron 6. Indeed, in SLC25A20, DBT and MLH1, some affected exons with weak splicing signals are skipped even in normal subjects.

In all the above mutations, each exonic mutation introduces a PTC into a normally spliced transcript, and each intronic mutation primarily affects splicing of the neighboring exon and results in a PTC. Accelerated degradation of normally spliced transcripts or of transcripts with a primary splicing error is likely prerequisite for detecting aberrant transcripts affecting remote exons. Three out of four genes yield a transcript with restored ORF. The predominant species is determined only in SLC25A20, in which a transcript with restored ORF is predominant, as in our patients. Thus, in other genes as well as in CHRNE, skipping of remote exons likely owes to weak splicing signals that are unmasked by transcript selection driven by NMD.

**Mutations in CHRNE exon 6 that affect splicing of exon 6**

Weak intronic splicing signals predict presence of ESEs in the flanked exon (60). Analysis of one polymorphism and four CMS mutations in CHRNE exon 6 revealed that two mutations affect splicing of exon 6 and yield frameshifting transcripts: eEF157V in Pt 8 causes exon 6 skipping and eE154X in Pt 9 activates a cryptic 3′ splice site in exon 6 (see Fig. 7).

eEF157V (e470delAGT) mutates a putative ESE motif (named 5A/3G) of TGGA (60) from TGAGT to TGGTCG, which is possibly recognized by an ESE-binding SR protein, SRp40 (68). The ESE finder release 2.0 (http://exon.cshl.org/ESE/) (69) predicts that eE154X (e460G→T) mutates a putative SF2/ASF-binding site of CCGAAGA to CCTAAGA. SF2/ASF is another ESE-activating SR protein (68). The RESCUE-ESE server (http://genes.mit.edu/burgelab/rescue-ese/) (60) similarly predicts that eE154X affects two candidate ESE hexamers of CGGAAGA and GAAGAG, which also probably bind to SF2/ASF (60). Altered splicing caused by eE154X is unlikely due to nuclear scanning of pre-mRNA as exemplified in other genes (70–74), because the activated cryptic 3′ splice site also results in a framshefing transcript.

The cryptic 3′ splice site activated by eE154X has a CV of 0.742, whereas that of the native 3′ splice site at intron 5 is 0.864, which is against the notion that a CV of a cryptic splice site is better than, or similar to, that of a native site (32,58). This indicates that other factors including disruption of an ESE should drive activation of a cryptic 3′ splice site. The putative branch point for the cryptic 3′ splice site is CGAAG at nucleotides 459–463 in exon 6, which is mutated to CTAAG by eE154X (see Fig. 7A). The mutant CTAAG conforms to the branch point consensus sequence of CTRAY better than the wild-type CGAAG. On the other hand, eEF157V shortens the polypyrimidine tract for the cryptic site and mutates a polypyrimidine stretch of TTC at nucleotides 472–474 in exon 6 to TC (see Fig. 7A). Differential alteration of these cis-acting elements likely accounts for differential splicing consequences between eE154X and eEF157V.

**PATIENTS AND METHODS**

**Patients**

Pts 1–9 (years of age and gender: 6F, 18F, 10M, 39F, 16M, 30F, 8F, 10M and 33F) have had moderate to severe myasthenic symptoms since birth or infancy. All patients have decremental EMG responses and no AChR antibodies, and respond partially to pyridostigmine. Parents of Pts 5 and 8 are first cousins. Pts 2 and 4 have an affected sibling. Pt 5 also has phenylketonuria. The gene for phenylalanine hydroxylase (PAH), however, is on 12q24.1, whereas CHRNE is on 17p13.
Endplate studies
Specimens of intercostal muscle were obtained intact from origin to insertion from Pts 1–3 and 8 and from control subjects without muscle disease undergoing thoracic surgery. All human studies were in accord with the guidelines of the Institutional Review Board of the Mayo Clinic. EPs were localized for electron microscopy and analyzed as previously described (75,76). The number of AChRs per EP was measured with \(^{125}\text{I}\) labeled bungarotoxin (77). Recordings of the miniature EP potentials and currents, and estimates of the number of transmitter quanta released by nerve impulse were performed as described elsewhere (77,78). Single channel currents were performed in the cell-attached mode as described (79).

Sequencing procedures
PCR-amplified fragments were purified by the QIAquick PCR Purification Kit (Qiagen). Plasmids were purified by the QIAprep Spin Miniprep Kit (Qiagen). PCR products and plasmids were sequenced with an ABI 377 DNA sequencer (Applied Biosystems) using fluorescently labeled dideoxy terminators.

Mutation analysis
Genomic DNA and mRNA were isolated from muscle specimens as described (80). Genomic DNA was also isolated from blood using the QiAmp DNA Blood kit (Qiagen). Using genomic DNA, we directly sequenced \(\text{CHRNA1, CHRNB1, CHRN D, and CHRNAE}\) in Pts 1 and 2, and \(\text{CHRNAE}\) in Pts 3–9 as described (80).

To trace mutations in family members, we used \(\text{Hinfl, EcoNI, and MboI}\) restriction enzymes (New England Biolabs) for \(\varepsilon_{553}\)del7, \(\varepsilon_{1VSV9-1G}\)–C, and \(\varepsilon_{E154X}\), respectively. \(\varepsilon_{553}\)del7 gains a \(\text{Hinfl}\) site; \(\varepsilon_{1VSV9-1G}\)–C loses an \(\text{EcoNI}\) site, and \(\varepsilon_{E154X}\) loses an \(\text{MboI}\) site. For \(\varepsilon_{1E021s1s20}, \varepsilon_{1E098s1sG}, \varepsilon_{EF157V}\) and \(\varepsilon_{EF157V}\)–C and \(\varepsilon_{EF157V}\)–2A–C, we directly sequenced genomic DNA from family members.

To determine allelic distribution of \(\varepsilon_{553}\)del7 and \(\varepsilon_{1VSV9-1G}\)–C in Pt 1, we employed allele-specific RT–PCR. The allele-specific forward primers were 5'–GAACGGCGAG·TCGACTTC-3' for the mutant allele with \(\varepsilon_{553}\)del7 (a dot indicates a 7 bp deletion), and 5'–GAACGGCGAG·TGCGGGCAT-3' for the normal allele without \(\varepsilon_{553}\)del7. The reverse primer was 5'–GTGCCCTCTG CCCCTCAA·A-3' in exon 10. Allele-specific RT–PCR would yield 607 and 614 bp fragments from \(\varepsilon_{553}\)del7 and wild-type transcripts, respectively.

Cloning of aberrant transcripts in muscle of patients 1 and 2
To determine aberrant transcripts associated with the identified mutations, we cloned RT–PCR products from muscle specimens of Pts 1 and 2. A 1579 bp fragment of \(\text{CHRNAE}\) cDNA spanning positions –61 to 1518 was amplified by nested RT–PCR, where position +1 represents the initial nucleotide of the mature protein and the coding region is from –60 to 1419. We introduced an \(\text{EcoRI}\) site at the 5' end of nested primers. The RT–PCR product was ligated into pBlueScript II SK(–) (Stratagene) using an \(\text{EcoRI}\) site. We screened for aberrant transcripts by double digestion of isolated clones with \(\text{Ddel}\) and \(\text{Hinfl}\), and determined aberrant transcripts by sequencing the entire inserts. We characterized 56 and 33 clones for Pts 1 and 2, respectively.

Construction of human wild-type and mutant AChR subunit cDNAs and expression in HEK cells
Sources of human \(\text{CHRNa1, CHRNB1, CHRN D, and CHRNAE}\) cDNAs were as previously described (51,81,82). All four cDNAs were cloned into the CMV-based expression vector pRBG4 (83) for expression in 293 HEK cells.

Among seven different transcripts obtained from Pts 1 and 2 (transcripts I–VII in Fig. 2), transcripts II, III and VI predicted the same truncated protein. Thus, five transcripts (I, II, IV, V and VII) were cloned into the expression vector pRBG4 for expression in HEK cells. Absence of PCR artifacts was confirmed by sequencing the entire inserts.

HEK cells were transfected with a mutant or wild-type \(\text{CHRNAE}\) cDNA along with the complementary wild-type \(\text{CHRNa1, CHRNB1 and CHRN D cDNAs}\) using the calcium phosphate precipitation method as described previously (80). Total number of \(\text{[^{125}I]}\) labeled bgt sites of intact or saponin-permeabilized HEK cells and the ACh competition measurements were determined as described (51).

Cloning of minigene and the entire gene of \(\text{CHRNAE}\)
To recapitulate the aberrant splicing in transfected COS cells, we first PCR-amplified wild-type and mutant DNA segments spanning nucleotide 176 in exon 4 to nucleotide 853 in exon 8, where position +1 represents the initial nucleotide of the mature protein, and cloned them into the CMV-based expression vector pRBG4. These minigene constructs, however, failed to recapitulate the aberrant splicing (Fig. 3C).

We next PCR-amplified the entire \(\text{CHRNAE}\) spanning 12 exons and 11 introns and cloned it into pRBG4. The insert spanned 4392 bp from 29 nucleotides upstream of the translational start site in exon 1 down to 35 nucleotides downstream of a TAG stop codon in exon 12. Mutations were engineered into each construct using the QuikChange Site-Directed Mutagenesis kit. Absence of PCR artifacts was confirmed by sequencing the entire insert.

Isolation of cytoplasmic RNA of transfected COS cells
For transfection into COS cells, \(~1.5 \times 10^5\) cells were seeded into a six-well plate 24 h before transfection. Cells were transfected with 1 \(\mu\)g of each pRBG4 construct using 3 \(\mu\)l of FuGENE 6 transfection reagent (Roche) according to the manufacturer's recommendations. Fresh medium was added 16 h after transfection. Cytoplasmic RNA was extracted 48 h after transfection using the RNeasy Mini kit (Qiagen) including the DNase I treatment according to the manufacturer's instructions. To suppress translation and subsequently NMD, we added 100 \(\mu\)g/ml anisomycin (Sigma) 0.5, 1.0, 1.5 and 2.0 h before harvesting cells (55,56).
RT–PCR analysis of cytoplasmic RNA of transfected COS cells

For RT–PCR, one-third of the isolated cytoplasmic RNA was used for cDNA synthesis using an oligo-dT primer and the Superscript II reverse transcriptase (Invitrogen). One-twentieth of synthesized cDNA was used for each RT–PCR in 25 μl with primers, 5'-CTTCGGATCTCTATCCGAGAACTGTGT-3' in exon 5 and 5'-AGGAACTCTTGCTGCTGCGAGAAGCGAC-3' in exon 7. With these primers, a normally spliced transcript would yield a 324 bp fragment, whereas a transcript skipping exon 6 (101 bp) would give rise to a 223 bp fragment. The PCR condition comprised 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using the FastStart Taq DNA polymerase (Roche) and the DNA engine (MJ Research). We always included a negative control that is transfected only with FuGene 6 but without any plasmids to confirm that there were no cross-contaminations in experimental procedures and that RT–PCR products were not from the native COS cells.

Real-time PCR analysis

To estimate the ratio of normally spliced to exon 6-skipped transcripts in muscle specimens and in transfected COS cells, we compared amplification of each transcript with those of standards using the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Each transcript was amplified using allele-specific RT–PCR. The allele-specific forward primers were 5'-AGGCGCTATTTTCCG-3' for the normally spliced transcript (a dot indicates the boundary of exons 5 and 6), and 5'-CCTTGCCTATTTTCCG-3' for the exon 6-skipped transcript (a dot indicates the boundary of exons 5 and 7). The reverse primer was 5'-GGGACGATGATGTÁTTAGTA-3' in exon 7. The normally spliced and exon 6-skipped transcripts would yield 267 and 167 bp fragments, respectively. One-twentieth or less of the above synthesized cDNA was used for each allele-specific RT–PCR in 25 μl, so that an estimated initial copy number fell within the range of standards. The PCR condition comprised 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min using the FastStart Taq DNA polymerase in the presence of 1×1.75 SYBR Green I (Molecular Probes). Each allele-specific RT–PCR amplified an expected single fragment and no primer dimers. Each sample was analyzed in triplicate.

We used the cloned CHRNε cDNA (pRBG4-CHRNE) and the cloned transcript V (pBluescript-transcript V) as standards. Copy numbers of two standards were matched by amplifying the same fragment with 5'-ATTGGACAGATTACCGACTC-3' in exon 4 and 5'-AGGGGAAAAATAAGCCGACAC-3' in exon 5 using the real-time PCR.

To monitor transfection efficiency, we estimated the copy number of β-actin cDNA in COS cells. To this end, we amplified the entire 1128 bp coding region of β-actin cDNA (accession number, AB004047) of African green monkey, from which COS cells were derived. The RT–PCR product was cloned into pGEM-T (Promega) and was used as a standard. We amplified a 213 bp β-actin cDNA fragment using 5'-AACCTTCCCTCTGG-GCAT-3' and 5'-CAGGGAGGAGC AATGAT- CTTGAT-3', where dots indicate presumptive exon boundaries. The primers were complementary to both African green monkey and human β-actin cDNAs, and amplified an expected single fragment from both species.

We amplified 1×10³ to 1×10⁴ copies of each standard in triplicate. Each standard was fitted to an equation: cycle threshold (Ct) = A ln (initial copies) + B, where A=−1.57, B=37.4, correlation coefficient r=0.996 for the normally spliced transcript; A=−1.49, B=37.5, r=0.997 for the exon 6-skipped transcript; and A=−1.08, B=27.4, r=0.997 for β-actin. Initial copy numbers of each CHRNε transcript and of β-actin transcript were estimated by the respective equations.

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