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Myasthenic syndrome caused by plectinopathy

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ABSTRACT

Background: Plectin crosslinks intermediate filaments to their targets in different tissues. Defects in plectin cause epidermolysis bullosa simplex (EBS), muscular dystrophy (MD), and sometimes pyloric atresia. Association of EBS with a myasthenic syndrome (MyS) was documented in a single patient in 1999.

Objectives: To analyze the clinical, structural, and genetic aspects of a second and fatal case of EBS associated with a MyS and search for the genetic basis of the disease in a previously reported patient with EBS-MD-MyS.

Methods: Clinical observations; histochemical, immunocytochemical, and electron microscopy studies of skeletal muscle and neuromuscular junction; and mutation analysis.

Results: An African American man had EBS since early infancy, and progressive muscle weakness, hyperCKemia, and myasthenic symptoms refractory to therapy since age 3 years. Eventually he became motionless and died at age 42 years. At age 15 years, he had a marked EMG decrement, and a reduced miniature endplate potential amplitude. The myopathy was associated with dislocated muscle fiber organelles, structurally abnormal nuclei, focal plasmalemmal defects, and focal calcium ingress into muscle fibers. The neuromuscular junctions showed destruction of the junctional folds, and remodeling. Mutation analysis demonstrated a known p.Arg2319X and a novel c.12043dupG mutation in *PLEC1*. The EBS-MD-MyS patient reported in 1999 also carried c.12043dupG and a novel p.Gln2057X mutation. The novel mutations were absent in 200 Caucasian and 100 African American subjects.

Conclusions: The MyS in plectinopathy is attributed to destruction of the junctional folds and the myopathy to defective anchoring of muscle fiber organelles and defects in sarcolemmal integrity. *Neurology*® 2011;76:327-336

GLOSSARY

Ab = antibodies; **AChR** = acetylcholine receptor; **anti-C Ab** = antibody recognizing the C-terminal plectin domain; **anti-Rod Ab** = antibody recognizing the plectin rod domain; **EBS** = epidermolysis bullosa simplex; **EP** = endplate; **IF** = intermediate filament; **IgG** = immunoglobulin G; **MD** = muscular dystrophy; **MyS** = myasthenic syndrome; **P1** = patient 1; **P2** = patient 2.

Plectin is a ~500 kDa dumbbell-shaped molecule with a central coiled-coil rod domain flanked by globular N- and C-terminal domains. Owing to tissue and organelle-specific transcript isoforms, plectin is a versatile linker of cytoskeletal components to target organelles in cells of different tissues.¹⁻³ In skeletal muscle, multiple alternatively spliced transcripts of exon preceding common exon 2 link desmin intermediate filaments (IFs) to specific targets: the outer nuclear membrane (isoform 1), the outer mitochondrial membrane (isoform 1b), Z disks (isoform 1d), and costameres in the sarcolemma (isoform 1f).³ Plectin is also highly expressed at the neuromuscular junction where it provides crucial structural support for the junctional folds.⁴ Plectin deficiency in muscle results in progressive muscular dystrophy (MD).⁴⁻¹⁹ Plectin

Supplemental data at
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is also highly expressed in intercalated disks in the heart but only a single patient with EBS/MD and cardiomyopathy was identified to date.¹⁸ Plectin deficiency in skin causes epidermolysis bullosa simplex (EBS).²⁰ Some patients with EBS and MD (EBS-MD) also had symptoms suggesting a myasthenic disorder^{9,21–23} but this was not suspected or confirmed by specific studies. The association of EBS-MD with a myasthenic syndrome (MyS) was well-documented in a single patient (P1) in 1999.⁴ Although numerous autosomal recessive and one dominant mutation in *PLEC* have been detected,²⁰ the genetic basis of EBS-MD-MyS in P1 was not identified. We describe our findings in a second patient with EBS-MD-MyS (P2), report additional observations in P1, and identify the genetic basis of the disease in both patients.

METHODS All human studies described here were in accord with the guidelines of the Institutional Review Board of the Mayo Clinic.

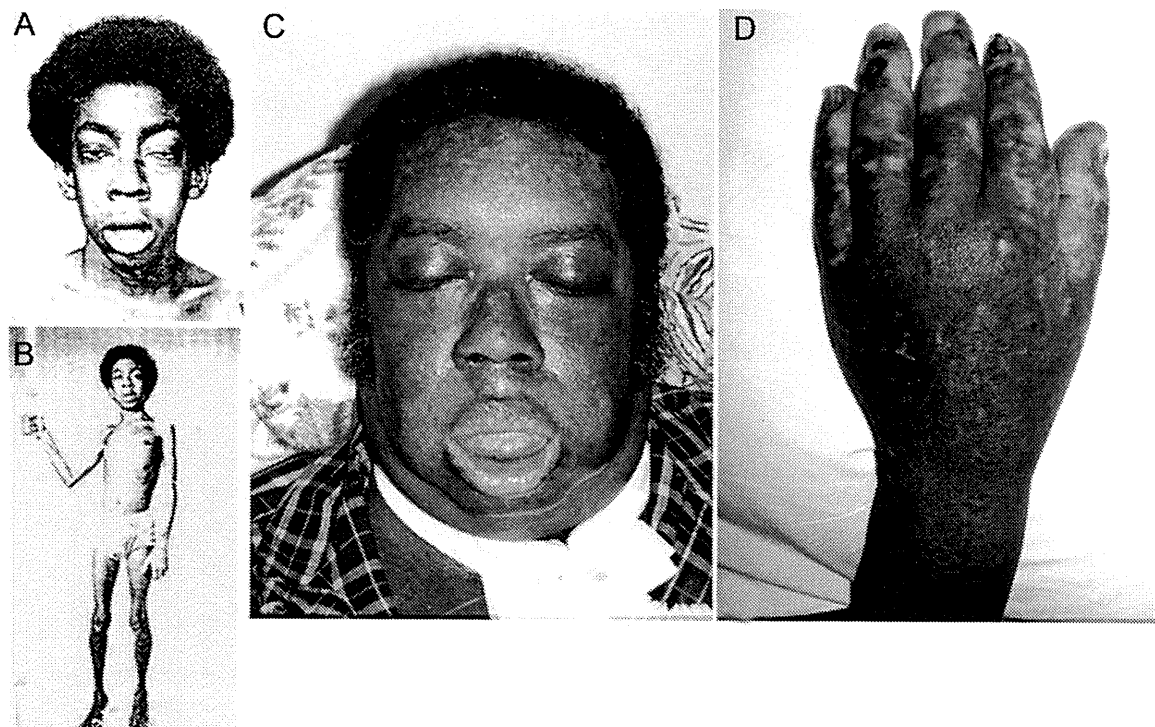
Structural observations. Routine histochemical studies on cryostat sections and electron microscopy studies were performed as previously described.²⁴ Immunoglobulin G and the C3 and C9 complement components were immunolocalized as previously reported.^{25,26} We immunolocalized the last 50 C-terminal residues of plectin with 4 $\mu\text{g}/\text{mL}$ goat polyclonal C-20 antibody (anti-C Ab), and the plectin rod domain with 4 $\mu\text{g}/\text{mL}$ 10F6 mouse monoclonal antibody (anti-Rod Ab) (both from Santa Cruz Biotechnology), followed by 3 $\mu\text{g}/\text{mL}$ biotinylated donkey antigoat or antimouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories) and the ABC peroxidase kit (Vector Laboratories). Intrafiber calcium excess was evaluated by the Alizarin red stain.²⁷ Synaptic contact regions were visualized on fixed, teased muscle fibers by a cytochemical reaction for acetylcholinesterase.²⁸ The acetylcholine receptor (AChR) and plectin were colocalized at endplates (EPs) with rhodamine-labeled α -bungarotoxin and the plectin anti-Rod Ab followed by fluorescent goat antimouse IgG. EPs were localized for electron microscopy²⁴ and quantitatively analyzed²⁹ by established methods. Peroxidase-labeled α -bungarotoxin was used for the ultrastructural localization of AChR.³⁰

Molecular genetic studies. Genomic DNA was isolated from blood of P1 and muscle of P2 and mRNA from intercostal muscles of both by standard methods. *PLEC* nucleotides were numbered according to the mRNA sequence (GenBank reference no: NM_000445). We used PCR primer pairs to amplify and directly sequence the 32 exons and flanking noncoding regions of *PLEC* isoform 1 and also first exons of isoforms 1b, 1d, and 1f. We screened for the identified novel mutations in 200 Caucasian and 100 African American control subjects using allele-specific PCR. To estimate expression of the rodless isoform of *PLEC* at the mRNA level, we used real-time PCR and SYBR green I (Roche) with 5' GTGTCATCCAGGAGTACGTG 3' as the forward primer in exon 30, 5' AGCGACAGCAGAGT-

GACCAT 3' as the forward primer in exon 31 that encodes the rod domain, 5' GCCTTCTCCTGCTCGATGAA 3' as the reverse primer in exon 32 for both forward primers, and *GAPDH* as the housekeeping gene. All experiments were done in triplicate.

RESULTS Clinical observations. P1 is an African American woman. Her case was reported in 1999 when she was 23 years of age.⁴ In brief, she was diagnosed with EBS as an infant and her myasthenic symptoms began around the age of 9 years. Since 1999, her weakness has worsened so she can now only take a few steps, has dysphagia, is dyspneic on slight exertion and at night, and is resistant to anticholinesterase drugs. However, her skin symptoms are mild, with new skin blisters appearing infrequently.

P2 is an African American man. He was a single child without similarly affected family members. He sucked poorly during infancy but this gradually improved. Since the age of 6 weeks, he had an intermittent vesicular eruption over his skin and oral mucosa and developed nail deformities. He attained his motor milestones on time, but had significant fatigue on exertion since age 3 years. At age 11 years he had difficulty running and rising from the floor and serum creatine kinase level was 827 U/L (normal <60 U/L). Prednisone therapy improved his strength but was discontinued because of abdominal pain. Nystatin therapy for thrush worsened the weakness. At age 12 years, a vastus medialis muscle specimen revealed a myopathy associated with necrotic and regenerating fibers, a sural nerve specimen was normal, and a skin biopsy showed EBS and secondary infection. In 1981, at age 15 years, the patient was evaluated at the Mayo Clinic. He now had reduced muscle bulk, bilateral eyelid ptosis, restricted eye movements, and mild facial and moderately severe diffuse cervical and limb muscle weakness, and was areflexic except at the ankles. Nerve conduction studies were normal. Repetitive stimulation at 2 Hz showed a decremental response (67% in hypothenar muscles) that was partially corrected by IV edrophonium chloride. Serratus anterior and intercostal muscles were biopsied. In vitro electrophysiology study of the intercostal specimen by Dr. Edward Lambert revealed reduction of the mean miniature endplate potential amplitude to 50% of normal; the quantal content of the endplate potential was in the low-normal range. Tests for anti-AChR antibodies were negative. A MyS was diagnosed but therapy with pyridostigmine bromide for a year was of no benefit. The weakness progressed more rapidly throughout adolescence and accelerated after routine illnesses. At age



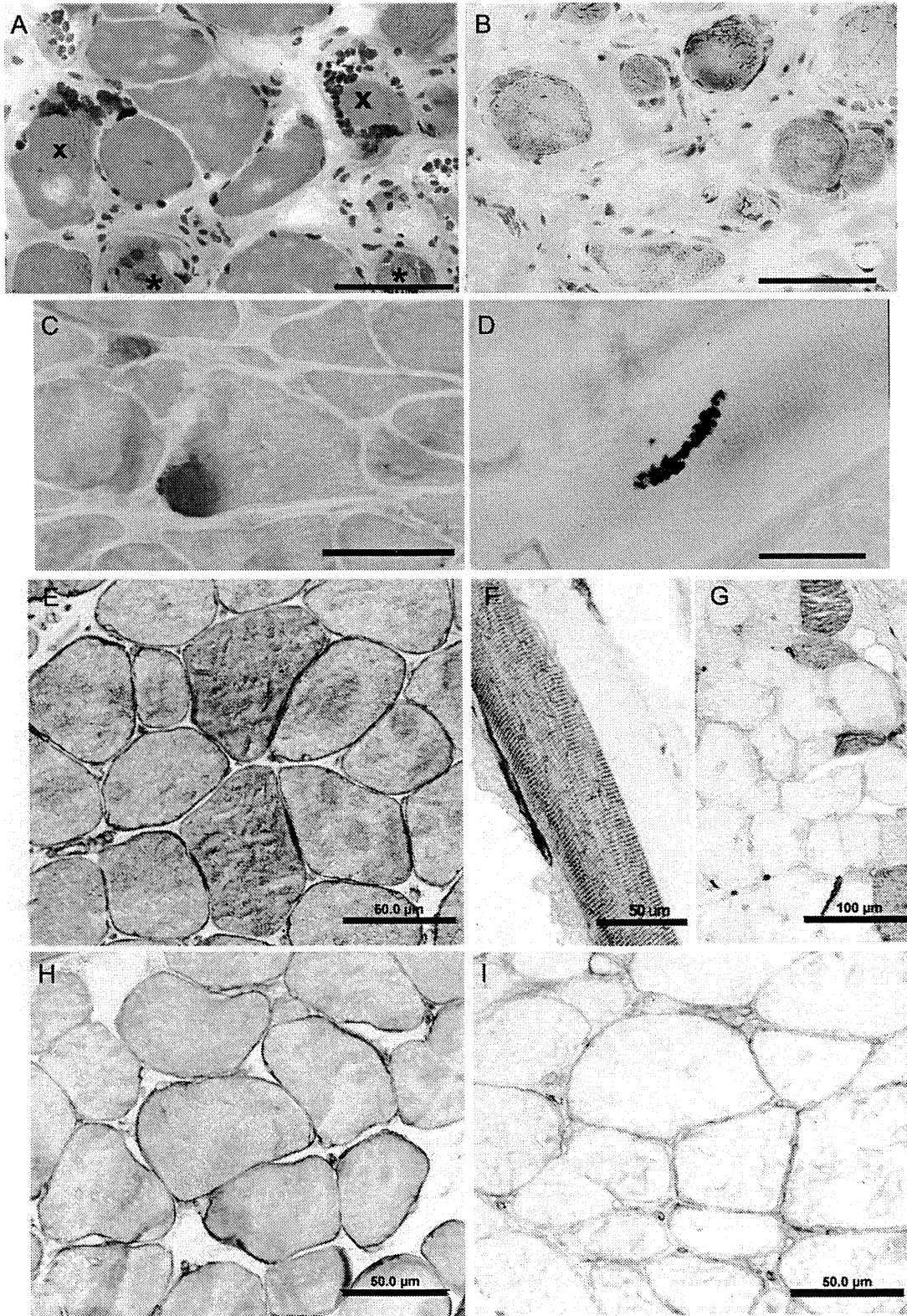
(A, B) Patient at age 17 years. Note severe asymmetric bilateral ptosis, hyperactive frontalis muscle, facial paresis, open mouth, cubitus valgus, Achilles tendon contractures, and diffuse muscle atrophy. (C, D) Patient at age 41 years. He has a tracheostomy, has facial diplegia, is unable to close his mouth or open his eyes, and shows the chronic skin changes of epidermolysis bullosa simplex. He also has blisters on his lip and tongue and oral monilliasis.

17 years, he could barely walk (figure 1, A and B). He was wheelchair-bound by age 18 years, and respirator-dependent by age 26 years. After age 35 years, he had dysarthria and dysphagia and needed a percutaneous gastrostomy. His cognitive functions and cardiac status remained normal. Subsequently, he became motionless (figure 1C), continued to have skin blisters (figure 1D), communicated with clicks and whispers, failed to respond to 3,4-diaminopyridine combined with pyridostigmine bromide, and died of pneumonia at age 42 years.

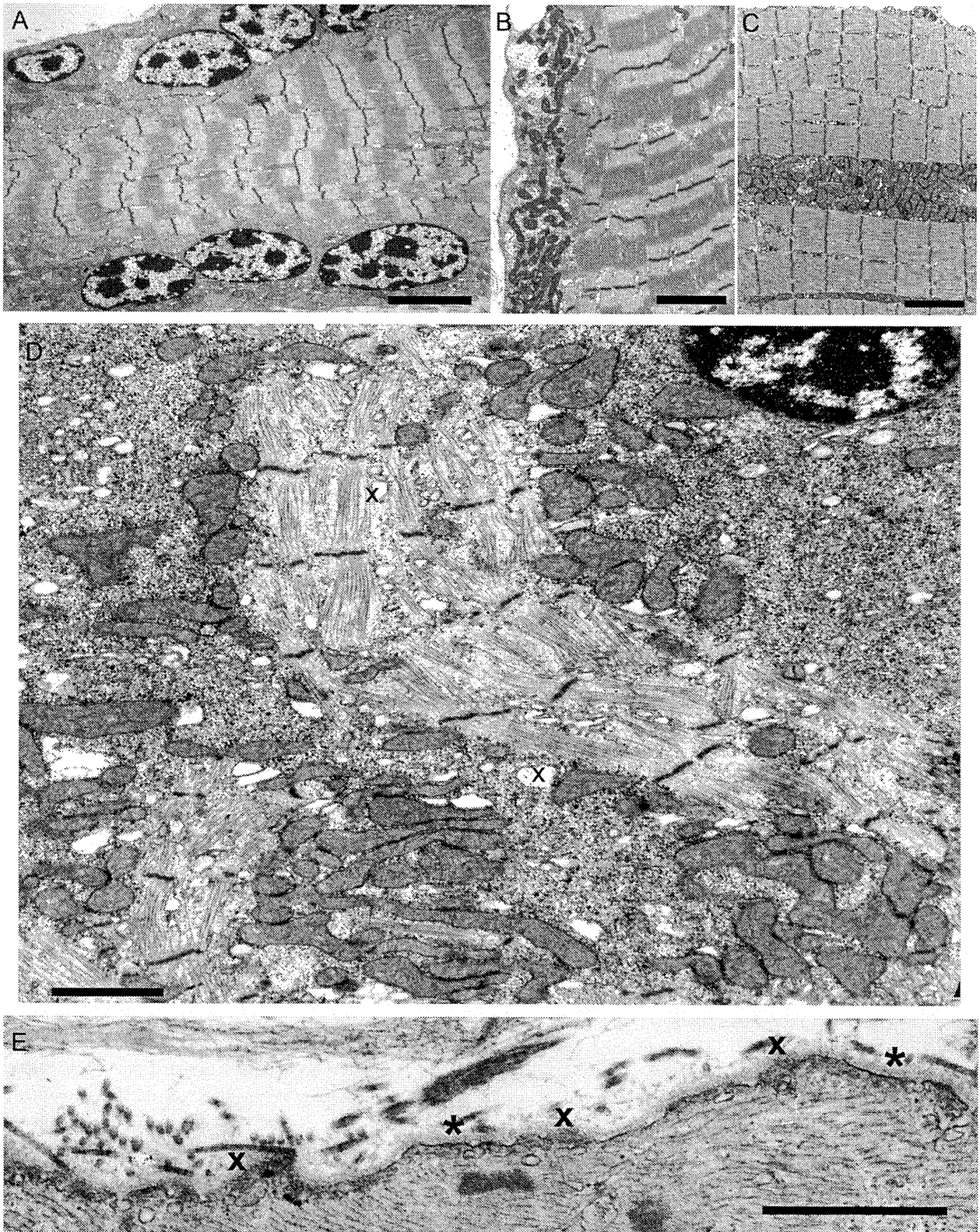
Histochemistry, P2. Serratus anterior and intercostal muscle specimens showed similar findings (figure 2, A and B). The muscle fiber diameters varied from 6 μm to $\sim 120 \mu\text{m}$. There was a mild to moderate increase of internal nuclei. Many nuclei were larger than normal and appeared in subsarcolemmal rows or clusters. Some fibers were necrotic or regenerating or subdividing by splitting, or displayed aberrant myofibrils. There was mild to marked (figure 2B) increase of perimysial and endomysial connective tissue. No immunoglobulin G, C3, or C9 deposits were present at patient endplates. In sections reacted for oxidative enzymes, some fibers showed attenuation or an irreg-

ular distribution of enzyme activity. Both muscle specimens showed type 1 fiber preponderance. Because plectin deficiency disconnects or weakens the link between the sarcolemma and the underlying cytoskeleton, it likely increases sarcolemmal vulnerability to mechanical stress. We therefore searched for signs of sarcolemmal injury evidenced by subsarcolemmal calcium deposits²⁷ and detected these in scattered fibers in both patients (figure 2C and figure e-1 [on the *Neurology*[®] Web site at www.neurology.org]).

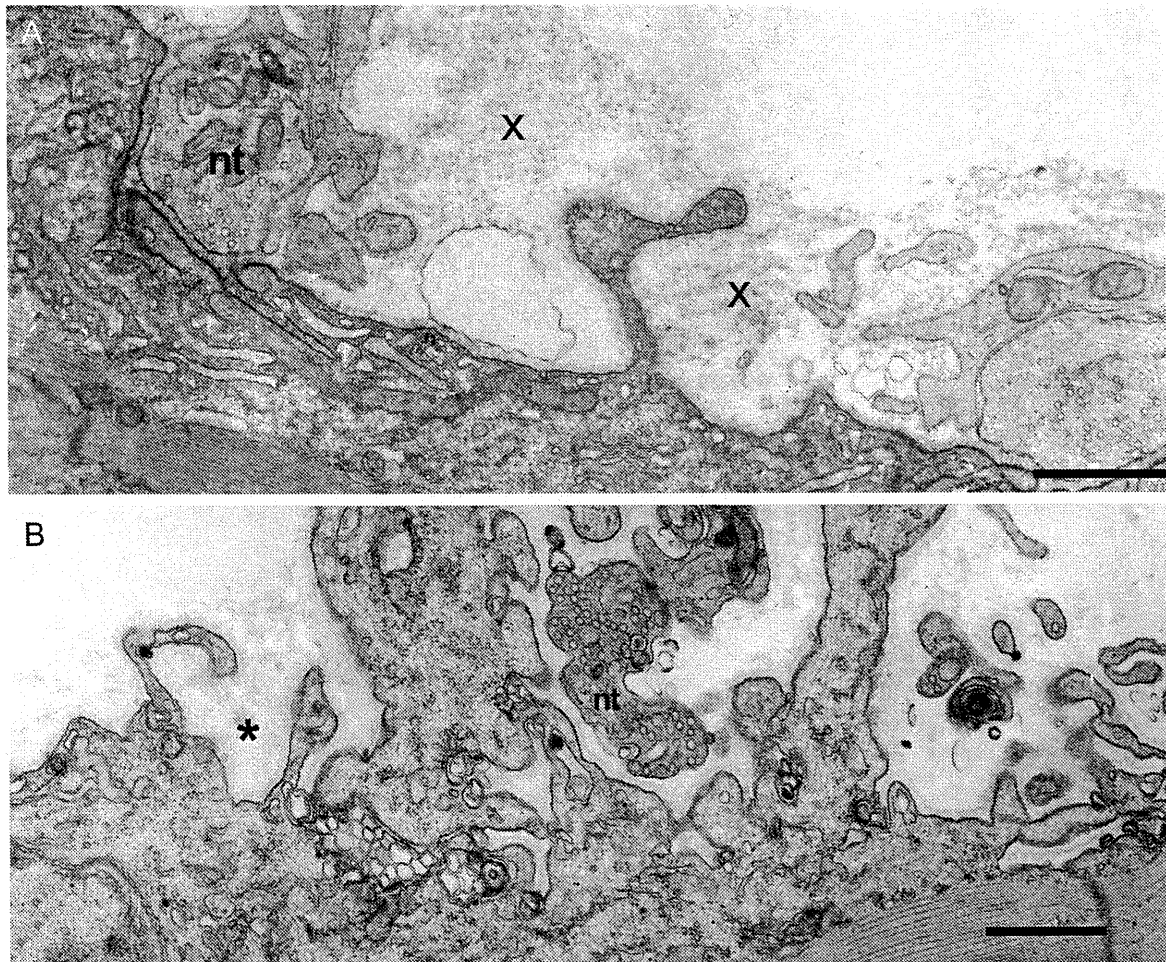
Plectin immunostains. These were performed on 6- to 10- μm -thick acetone fixed frozen sections. In 1999, an antibody recognizing the rod domain of plectin (gift from Dr. Owaribe) showed no immunoreactivity in P1 muscle fibers. As this antibody was no longer available, we used the 10F6 antibody directed against the plectin rod domain (anti-Rod Ab), and a C-20 antibody raised against the last 50 C-terminal residues of plectin (anti-C Ab), and immunolocalized plectin in P1, P2, and normal muscle (see Methods). In normal muscle, both antibodies immunostained the sarcolemma, the intermyofibrillar network, capillaries, and vascular smooth muscle (figure 2, E and H); the C-20 Ab also immunostained perineurium and myelin-



(A, B) Note marked variation in fiber size, regenerating fiber elements (asterisks), endomysial fibrosis (B), and clusters of large nuclei at periphery of several fibers. (C) Alizarin red stain reveals focal calcium deposits in 2 fibers. (D) Multiple small cholinesterase-reactive endplate regions arrayed over an extended length of the fiber. Plectin was localized in normal control muscle (E, H) and patient intercostal muscle (F, G, I) with antibody recognizing the plectin rod domain (anti-Rod Ab) (E-G) and antibody recognizing the C-terminal plectin domain (anti-C Ab) (H, I). (E, H) In normal muscle, plectin is localized to the sarcolemma and sarcoplasm with both Abs. The anti-Rod Ab shows plectin-depleted and plectin-positive muscle fibers (F, G), whereas the anti-C Ab shows sarcoplasmic loss and slight sarcolemmal expression of plectin in all muscle fibers (I). Bars indicate 50 μm in all panels except in (G), where they indicate 100 μm .



(A) Note subsarcolemmal rows of large nuclei harboring multiple prominent chromatin bodies. (B, C) Subsarcolemmal and intrafiber clusters of mitochondria surrounded by fiber regions devoid of mitochondria. (D) Aberrant and disrupted myofibrils surrounded by clusters of mitochondria intermingled with glycogen, ribosomes, and dilated vesicles (x). Note preapoptotic nucleus at upper right. (E) Focal sarcolemma defects due to gaps in the plasma membrane. Where the plasma membrane is absent, the overlying basal lamina is thickened (x). Small vesicles underlie the thickened basal lamina. Asterisks indicate segments of the preserved plasma membrane. Bars = 4 μm in (A), 3 μm (B, C), 1.4 μm in (D), 1 μm in (E).



(A) The imaged EP regions show partial occupancy of the postsynaptic region by the nerve terminal and remnants of degenerate folds (x). The nerve terminal (nt) occupies only part of the postsynaptic region. Degenerate remnants of the folds (x) appear over the simplified postsynaptic region from which folds were lost. Dark reaction product on postsynaptic membrane shows acetylcholine receptor localization with peroxidase labeled α -bungarotoxin. (B) Small nerve terminal occupies only part of a highly simplified postsynaptic region. Asterisk indicates remnants of basal lamina that surrounded preexisting folds. Bars = 1 μ m.

ated nerve fibers (not shown). In normal muscle, the anti-Rod Ab (figure 2E) was more reactive than the anti-C antibody (figure 2H) and stained type 1 fibers more intensely than type 2 fibers. In the patients, the anti-Rod Ab demonstrated loss of sarcoplasmic and trace sarcolemmal reactivity in type 1 fibers but, as noted by others,¹⁹ type 2 fibers retained plectin positivity (figure 2, F and G, and figure e-2). In contrast, the C-20 Ab revealed no sarcoplasmic and only slight sarcolemmal reactivity in all fibers (figure 2I).

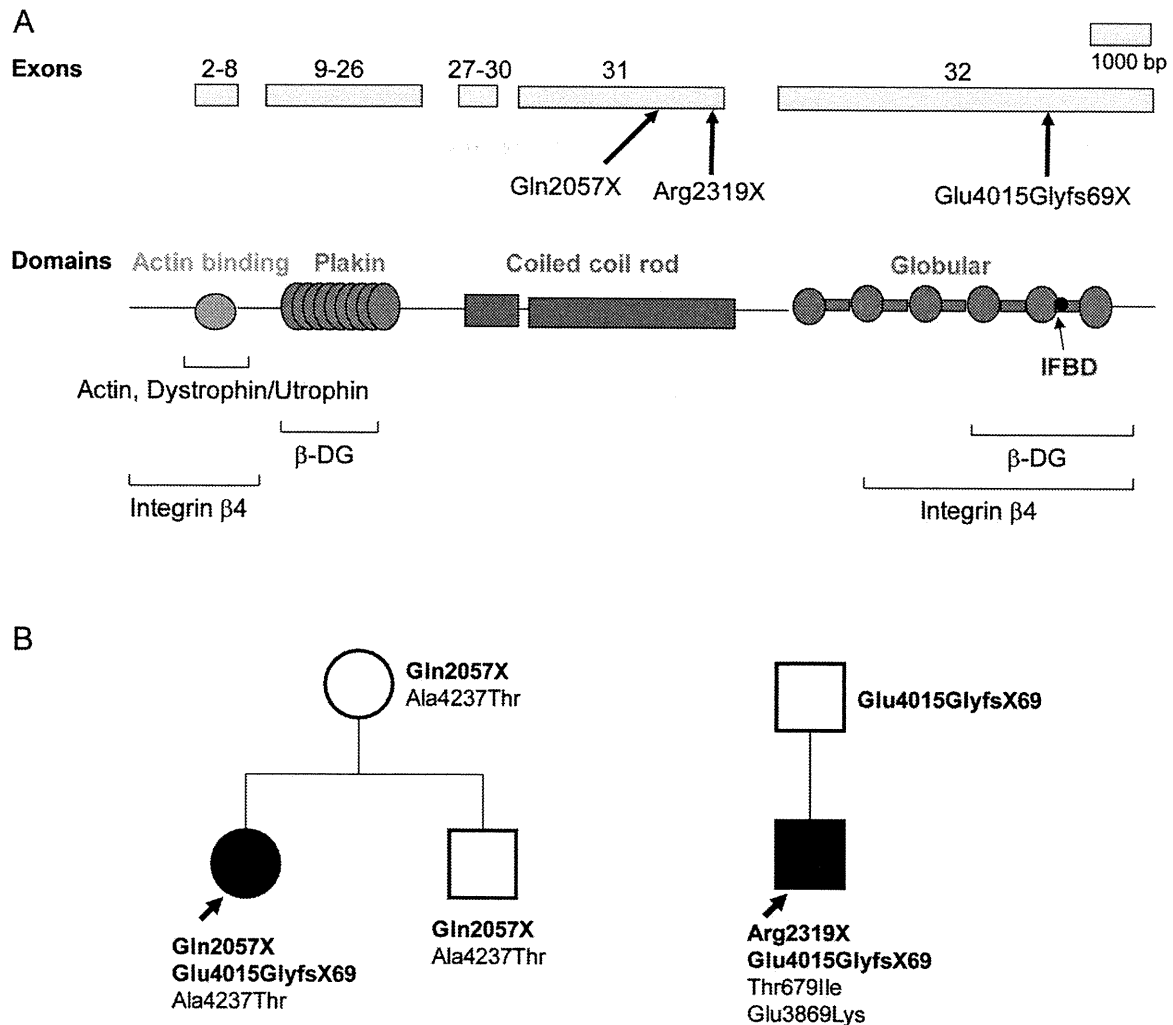
Muscle fiber ultrastructure in P2. Nuclear abnormalities. Consistent with light microscopy, numerous muscle fibers harbored subsarcolemmal rows or clusters of large ovoid nuclei containing 3 to 10 large, highly electron dense chromatin deposits (figure 3A). Nuclei in other fiber regions were of normal size and heterochromatic or euchromatic. Some nuclei harbored large clumps of

heterochromatin with small islands of euchromatin indicating preapoptotic changes (figure 3D).

Mitochondrial abnormalities. In some fibers, the mitochondria were normally aligned with the Z disk and evenly distributed in the muscle fiber. In other fibers, the mitochondria aggregated into clusters under the sarcolemma (figure 3B), near nuclei, or in other fiber regions (figure 3C), leaving adjacent fiber regions depleted of mitochondria. Some mitochondrial aggregates were interspersed with glycogen granules, ribosomes, and dilated vesicles (figure 3D).

Myofibrillar abnormalities. Aberrant myofibrils appeared among dislocated organelles in both atrophic and nonatrophic fibers (figure 3D). Disintegration and streaming of Z disks were detected in some fiber regions with or without myofibrillar disarray. A few abnormal fiber regions harbored small nemaline rods. End-stage muscle fibers contained few mitochondria, remnants of

Figure 5 Plectin domains and identified PLEC variants



(A) Schematic representation of PLEC exons 2-32 indicating identified patient mutations and binding domains associated with C- and N-terminal regions of plectin.³⁹ (B) Family analysis of P1 and P2 shows transmission of pathogenic mutations (bold face) and polymorphisms.

Z disks with attached short filaments (Z brushes), and debris. Small membrane-bound vacuoles of different sizes that likely represent dilated components of the sarcoplasmic reticulum appeared in fiber regions with or without other abnormalities (figure 3D).

Sarcolemmal defects. The focal subsarcolemmal calcium deposits in scattered muscle fibers prompted us to search for sarcolemmal defects at the ultrastructural level and we detected these in some fibers of both patients. Where the plasma membrane was discontinuous, it was often covered by focally thickened basal lamina (figure 3E).

Endplate studies in P2. Synaptic contacts on the muscle fibers, visualized by the cholinesterase reaction, consisted of multiple small EP regions arrayed over an extended length of the muscle fiber (figure 2D). This finding has been observed in other patients with ongoing destruction and remodeling of the postsynaptic region.³¹

Colocalization of plectin and AChR by fluorescence microscopy showed strong expressions of plectin and AChR at normal EPs. At patient EPs AChR expression was not appreciably attenuated but plectin expression was barely perceptible (figure e-3).

On electron microscopy, some EP regions appeared normal but many had an abnormal conformation, displaying one or more of the following: partial occupancy of the postsynaptic region by nerve terminals, small nerve terminals, atrophic and remnants of degenerated junctional folds resulting in highly simplified postsynaptic regions, and nerve sprouts near degenerating EPs (figure 4 and figure e-4). Table e-1 shows the frequency of the observed conformational changes. Table e-2 shows morphometry revealing reduced size of presynaptic and postsynaptic EP components.

Genetic analysis. Genetic analysis was challenging owing to the very large size of the PLEC transcript

(~14 Kb vs ~11 Kb for dystrophin), the very large exons 31 (3381 bp) and 32 (6219 bp), multiple splice variants of exon 1, and polymorphisms that may be race-dependent. Eventually we detected 2 truncating mutations in each patient (figure 5). P1 harbors a stop codon mutation at nucleotide 6169 in exon 31 (c.6169C>T/p.Gln2057X), and a duplication at nucleotide 12043 in exon 32 that predicts 68 missense codons followed by a stop codon (c.12043dupG/p.Glu4015GlyfsX69). P2 harbors a previously reported nonsense mutation at nucleotide 6955 in exon 31 which generates a stop codon (c.6955C>T/p.Arg2319X),¹⁶ and the same duplication mutation detected in P1 (figure 5B). Presence of the pathogenic mutation was confirmed at the cDNA level in both patients. Both stop codon mutations abrogate, and the c.12043dupG mutation disrupts, the IF binding site and one of the two β -dystroglycan and integrin β 4 binding sites (figure 5A).

Both patients also harbored polymorphisms not listed in the SNP database (see figure 5B). In P1, p.Ala4237Thr was deemed a polymorphism because it appeared together with Gln2057X in the unaffected mother. In P2, p.Thr679Ile was present in 1 of 60 African Americans and p.Glu3869Lys in 1 of 100 African Americans, although both variants were absent in 200 Caucasians.

It has been suggested that expression of the rodless plectin transcript may mitigate the plectinopathy phenotype.³² We therefore confirmed presence of the rodless domain by sequencing cDNA and used real-time PCR to compare the relative abundance of the rodless transcript in P1, P2, and 3 normal controls. The rodless transcript/full transcript ratio was 0.15 in P1, 0.32 in P2, and 0.22 ± 0.03 (mean \pm SD) in 3 controls.

DISCUSSION Although each patient carries a nonsense mutation in *PLEC* exon 31 and an identical frameshift mutation in exon 32, the tempo of the disease was faster in P2 than in P1. In P2, EBS presented at age 6 weeks, MyS at age 3 years, and he lost ambulation by age 18 years. In P1, MyS presented at age 9 years, EBS at age 18 years, and she can still take a few steps at age 31 years. It has been suggested that expression of the rodless transcript can mitigate the phenotype in patients who carry mutations in the plectin rod domain.³² However, real-time PCR indicates that expression of the rodless transcript was higher in the more severely affected P2. Thus in the patients studied by us the abundance of the rodless transcript was not a reliable indicator of the clinical phenotype.

Dislocation of the fiber organelles apparent at the ultrastructural level has multiple predictable consequences. Abnormal alignment and displacement of the myofibrils weakens or eliminates their contractile strength; separation of mitochondria from myofibrils renders energy delivery to contracting myofibrils inefficient. The eccentrically positioned large nuclei with multiple chromatin deposits may be dysfunctional or inefficient in their translational activities and in nuclear-cytoplasmic trafficking when not adjacent to organelles or fiber domains they subserve. Injury to the inadequately supported plasma membrane is evidenced by subsarcolemmal calcium deposits and sarcolemmal defects in a proportion of the fibers (see figure 2C, figure 3E, and figure e-1). Most of these were smaller than those in Duchenne dystrophy^{27,33} but they still likely contribute to fiber injury and, if large, they may contribute to fiber necrosis.

Electron microscopy of the EPs indicates that plectin deficiency targets the junctional folds for destruction. When sarcomeres contract and relax, the extrajunctional sarcolemma bulges and relaxes but the junctional folds maintain a constant architecture.³⁴ This mandates enhanced rigidity of the junctional folds and renders them especially vulnerable to mechanical stress. Thus loss of cytoskeletal support of the junctional folds due to the plectin deficiency, as depicted in figure e-3, readily explains the progressive destruction of the folds. Destruction of junctional folds decreases the density or eliminates the voltage-gated Na^+ channels which are concentrated in troughs between the folds^{35,36} and this increases the threshold for action potential generation.³⁷ Destruction of the folds also decreases the input resistance of the postsynaptic membrane and thereby the amplitude of synaptic potential.³⁸ Widening of the synaptic space reduces the concentration of acetylcholine before it reaches the junctional folds. The combination of these factors decreases quantal efficacy and compromises the safety margin of neuromuscular transmission. The relentless progression of the myasthenic symptoms in both patients implies that formation of new EP regions eventually fails to compensate for the ongoing destructive changes.

Why only a proportion of the muscle fibers is affected in a given muscle, and why some EPs are more severely affected than others, remain unanswered questions. It is uncertain that it can be attributed to preserved plectin expression in type 2 fibers, as shown by the 10F6 anti-Rod antibody. First, the anti-C terminal antibody showed nearly complete plectin deficiency in all muscle fibers in both patients. Second, the anti-rod domain antibody showed either no plectin reactivity in any fiber, or preserved immunoreactivity in type 1 instead of type 2 fibers.¹⁹

The fiber type specificities of the different anti-rod domain antibodies in plectinopathy are presently unexplained.

It is also unclear why some patients with EBS-MD have myasthenic symptoms and others do not. Possibly myasthenic weakness of the limb muscles is masked by the MD, or is overlooked in severely weak patients, but fatigable weakness of the oculobulbar muscles would be unlikely to go unrecognized. It also is not known whether some mutations are more prone to result in the MyS phenotype than others.

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AG-dependent 3'-splice sites are predisposed to aberrant splicing due to a mutation at the first nucleotide of an exon

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ABSTRACT

In pre-mRNA splicing, a conserved AG/G at the 3'-splice site is recognized by U2AF³⁵. A disease-causing mutation abrogating the G nucleotide at the first position of an exon (E⁺¹) causes exon skipping in *GH1*, *FECH* and *EYA1*, but not in *LPL* or *HEXA*. Knockdown of U2AF³⁵ enhanced exon skipping in *GH1* and *FECH*. RNA-EMSA revealed that wild-type *FECH* requires U2AF³⁵ but wild-type *LPL* does not. A series of artificial mutations in the polypyrimidine tracts of *GH1*, *FECH*, *EYA1*, *LPL* and *HEXA* disclosed that a stretch of at least 10–15 pyrimidines is required to ensure normal splicing in the presence of a mutation at E⁺¹. Analysis of nine other disease-causing mutations at E⁺¹ detected five splicing mutations. Our studies suggest that a mutation at the AG-dependent 3'-splice site that requires U2AF³⁵ for spliceosome assembly causes exon skipping, whereas one at the AG-independent 3'-splice site that does not require U2AF³⁵ gives rise to normal splicing. The AG-dependence of the 3'-splice site that we analyzed in disease-causing mutations at E⁺¹ potentially helps identify yet unrecognized splicing mutations at E⁺¹.

INTRODUCTION

In higher eukaryotes, generation of functional mRNA is dependent on the removal of introns from pre-mRNA by splicing (1). The splicing process occurs in the spliceosome, the major components of which include five small nuclear RNAs and their associated proteins (U1, U2, U4, U5 and U6 snRNPs) in addition to a large number of non-snRNP proteins (2). In the first step of assembly of the spliceosome, U1 snRNP, SF1, U2AF⁶⁵

and U2AF³⁵ bind to the splicing *cis*-elements at the 5' splice site (ss), the branch point sequence (BPS), the polypyrimidine tract (PPT) and the acceptor site, respectively, to form complex E (3).

Yeast has a well conserved BPS of UACUAAC (4), whereas we recently reported that human carries a highly degenerate BPS of yUnAy, where 'y' and 'n' represent pyrimidines and any nucleotides, respectively (5). Degeneracy of the human BPS supports a notion that the human BPS is likely to be recognized along with the downstream PPT where U2AF⁶⁵ binds and possibly with the invariant AG dinucleotide at the 3' ss where U2AF³⁵ binds (6,7). U2AF⁶⁵ and U2AF³⁵ also make a heterodimer (8). In PPT, uridines are preferred over cytidines (9,10). In addition, PPT with 11 continuous uridines is highly competent and the position of such PPT is not critical (10). On the other hand, PPTs with only five or six uridines are required to be located close the 3' AG for efficient splicing. In addition, phosphorylated DEK binds to and cooperates with U2AF³⁵ for proper recognition of the 3' ss (11).

In the next step of the spliceosome assembly, the bound U2AF⁶⁵ and U2AF³⁵ facilitate substitution of SF1 for U2snRNP at the branch point to form complex A. Introns carrying a long stretch of PPT do not require U2AF³⁵ for this substitution, which is called 'AG-independent 3' ss' (12–15). On the other hand, introns with a short or degenerate PPT require both U2AF⁶⁵ and U2AF³⁵ for this substitution, which is called 'AG-dependent 3' ss'. Thereafter, the U4/U6.U5 tri-snRNP is integrated into the spliceosome to form complex B and the initial assembly of the spliceosome is completed.

The invariant AG dinucleotides are frequently reported targets of mutations causing human diseases, and the most frequent consequence is skipping of one or more exons (16). In addition, even mutations in highly degenerate BPS (5) and PPT (17) give rise to aberrant splicing

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causing genetic diseases (18). Disease-causing mutations also affect the first nucleotide of an exon (E^{+1}), but their effects on pre-mRNA splicing have been rarely scrutinized. As far as we know, only three such mutations in *FECH* (19), *GHI* (20) and *EYAI* (21) have been reported to cause aberrant splicing. Similarly, two such mutations in *LPL* (22) and *HEXA* (23) have been reported to have no effect on splicing. In this communication, we dissected molecular bases that differentiate splicing-disrupting and splicing-competent mutations, and found that AG-dependent ss is vulnerable to a mutation at E^{+1} , whereas AG-independent ss is tolerant.

MATERIALS AND METHODS

Minigene constructs and mutagenesis

Human genes of our interest were PCR-amplified from HEK293 cells using the KOD plus DNA polymerase (Toyobo). We introduced restriction enzyme-recognition sites at the 5'-end of the forward and reverse primers. We inserted the amplicon into the pcDNA3.1(+) mammalian expression vector (Invitrogen). We introduced patients' or artificial mutations with the QuikChange site-directed mutagenesis kit (Stratagene). We confirmed the absence of unexpected artifacts with the CEQ8000 genetic analyzer (Beckman Coulter).

Cell culture and transfection procedures

HEK293 cells were maintained in the Dulbecco's minimum essential medium (DMEM, Sigma-Aldrich) with 10% fetal bovine serum (FBS, Sigma-Aldrich). At ~50% confluency ($\sim 5 \times 10^5$ cells) in a 12-well plate, 1 ml of fresh Opti-MEM I (Invitrogen) was substituted for DMEM, and 500 ng of a minigene with 1.5 μ l of the FuGENE6 transfection reagent (Roche Diagnostics) were then added. After 4 h, 2 ml of DMEM with 10% FBS was overlaid, and the cells were incubated overnight. The transfection medium was replaced with 2 ml of fresh DMEM with 10% FBS. RNA was extracted at 48 h after initiation of transfection.

RNA extraction and RT-PCR

Total RNA from HEK293 was extracted by Trizol reagent (Invitrogen) according to the manufacturer's protocols. The quantity and quality of RNA was determined by spectrophotometry (NanoDrop Technologies). Twenty percent of the isolated RNA was used as a template for cDNA synthesis with the Oligo(dT) 12–18 Primer (Invitrogen) and the ReverTra Ace (Toyobo). Ten percent of the synthesized cDNA was used as a template for RT-PCR amplification with T7 primer (5'-TAATACGACTACTATAGGG-3') and gene-specific primers for minigenes in pcDNA3.1(+). Image J software (National Institutes of Health) was used to quantify intensities of fragments. We employed JMP (SAS Institute) for statistical analysis.

RNA interference to knockdown U2AF³⁵

We synthesized siRNA of 5'-GGCUGUGAUUGACUUGAAUdTdT-3' (GenBank accession number NM_006758, nucleotides 459–479), which is against the shared sequence of U2AF^{35a} and U2AF^{35b} (15). We employed Lipofectamine 2000 (Invitrogen) to cotransfect plasmids and siRNAs according to the manufacturer's protocols. Briefly, the transfection reagent included 300 ng of the plasmid, 50 pmol of siRNA, and 2 μ l of lipofectamine 2000 in 100 μ l of Opti-MEM I. The cells were harvested by western blotting for 48 h after transfection. The primary antibodies were goat polyclonal antibody for U2AF³⁵ (Santa Cruz Biotechnology), and mouse monoclonal antibodies for U2AF⁶⁵ (Santa Cruz Biotechnology) and PTB (Zymed Laboratories). The secondary antibodies were HRP-conjugated mouse anti-goat (Santa Cruz Biotechnology) or sheep anti-mouse (GE healthcare) antibodies. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham Biosciences).

For the siRNA rescue assay, we cloned the human U2AF³⁵ cDNA (Open Biosystems) into the HindIII and EcoRI restriction sites of the p3XFLAG-CMV-14 vector (Sigma-Aldrich). We introduced four silent mutations into the siRNA target region using the QuikChange site-directed mutagenesis kit with a primer, 5'-GAAAAGGCTGTAATCGATTTAAATAACCGTTGGTT-3', where artificial mutations are underlined (24).

RNA probe synthesis

We synthesized [α -³²P]-CTP-labeled RNA using the Riboprobe *in vitro* transcription system (Promega) from a PCR-amplified fragment according to the manufacturer's instructions. We used the same forward primer for all the probes with the sequence of 5'-TAATACGACTCACTATAGGGAGACAGG-3', where the italicized is T7 promoter and the underlined is for annealing to the reverse primer. The four reverse primers were: wild-type *FECH*, 5'-TGGACCAACCTATGCGAAAGATAGACG AATGCGTAAGCCTGTCTC-3'; mutant *FECH*, 5'-TGGACCAACTATGCGAAAGATAGACG AATGCGTAAGCCTGTCTC-3'; wild-type *LPL*, 5'-TGGATCGAGG CCTTAAAGGGAAAAAAGCAGGAACACCCTGTCTC-3'; and mutant *LPL*, 5'-TGGATCGAGGACTTAA AAGGGAAAAAAGCAGGAACACCCTGTCTC-3', where the underlined is for annealing to the forward primer.

Expression and purification of recombinant proteins

The human U2AF³⁵ and U2AF⁶⁵ cDNAs were obtained from Open Biosystems. U2AF³⁵ and U2AF⁶⁵ cDNAs were subcloned into the *Bam*HI and *Eco*RI restriction sites of the pFastBac HTb vector. The recombinant baculoviruses were expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. Infected Sf9 cells were harvested after 48 h and resuspended in the lysis buffer containing 50 mM sodium phosphate, 10 mM imidazole, 300 mM NaCl, 1% Triton X-100, 2 mM

β -mercaptoethanol, the Complete Protease Inhibitor Cocktail (Roche Applied Science) and 5U endonuclease in pH 7.0. His-tagged U2AF³⁵ and U2AF⁶⁵ proteins were purified using the TALON metal affinity resins (Clontech) under the denatured and native conditions, respectively. Purified U2AF³⁵ was refolded by extended dialysis in dialysis buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7.0). We determined the protein concentrations using the Pierce 660 nm Protein Assay Reagent (Thermo Scientific).

RNA-electrophoretic mobility shift assay

The radioactively labeled RNA (1×10^5 cpm) was incubated at room temperature with varying concentrations of recombinant proteins, 16 μ g of yeast tRNA, and 1.6 U of RNasin (Toyobo) in a final volume of 20 μ l of the binding buffer (20 mM HEPES pH 7.8, 50 mM KCl, 3 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM EDTA and 5% glycerol). After 20 min, the RNA-protein complexes were separated on 5% non-denaturing polyacrylamide gels in $1 \times$ TBE buffer at 4°C. The gels were dried and complex formation was visualized using the Typhoon 8600 Imager (GE Healthcare).

In silico analysis of the human genome and ESE-motifs

We analyzed human genome annotations (NCBI Build 37.1, hg19) by writing Perl programs, and executing them either on the PrimePower HPC2500/Solaris 9 super-computer (Fujitsu) or on the cygwin UNIX emulator running on a Windows computer. To search for

ESE-motifs, we used the ESE Finder (<http://rulai.cshl.org/ESE/>) (25,26), the RESUCE-ESE server (<http://genes.mit.edu/burgelab/rescue-ese/>) (27), the FAS-ESS server (<http://genes.mit.edu/fas-ess/>) (28), the PESX server (<http://cubweb.biology.columbia.edu/pesx/>) (29,30), and the ESRsearch server (<http://ast.bioinfo.tau.ac.il/>) (31).

RESULTS

Recapitulation of normal and aberrant splicing in minigenes

We first constructed minigenes of *GHI*, *FECH*, *EYAI*, *LPL* and *HEXA*, and introduced a previously reported disease-causing mutation at E⁺¹ (Figure 1A). These minigenes successfully recapitulated normal and aberrant splicings: mutations in *GHI*, *FECH* and *EYAI* caused exon skipping, whereas those in *LPL* or *HEXA* did not (Figure 1B).

Down-regulation of U2AF³⁵ increased exon skipping in wild-type *GHI* and *FECH*, but not in wild-type *EYAI*, *LPL* and *HEXA*

We predicted that a mutation at E⁺¹ should disrupt binding of U2AF³⁵. We thus hypothesized that *GHI*, *FECH* and *EYAI* require binding of U2AF³⁵ for the assembly of spliceosome, whereas *LPL* and *HEXA* do not require it. To prove this hypothesis, we first knocked down U2AF³⁵ and analyzed its effect on the wild-type minigenes. We achieved an efficient down-regulation of U2AF³⁵ in HEK293 cells (Figure 2A). We also confirmed

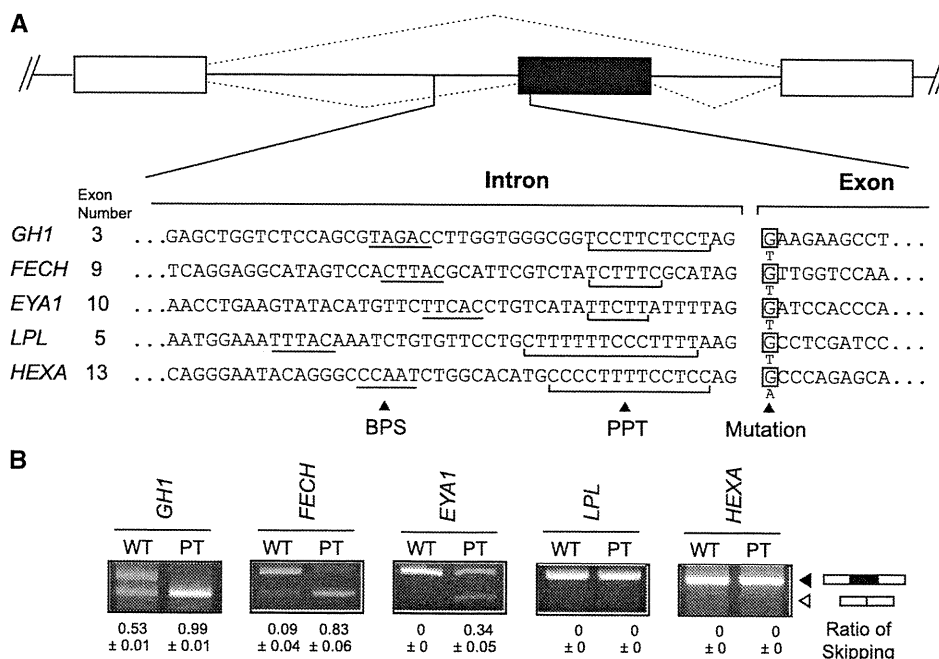


Figure 1. Recapitulation of normal and aberrant splicing of five genes. (A) Nucleotide sequences at the intron/exon junctions of five analyzed genes. Putative BPS is underlined. PPT is shown by a bracket. Mutant nucleotides are indicated at E⁺¹. (B) RT-PCR of minigenes expressed in HEK293 cells carrying the wild-type (WT) or patient's (PT) nucleotide. The mutations cause exon skipping in *GHI*, *FECH* and *EYAI*, but not in *LPL* and *HEXA*. Mean and SD of three independent experiments of the densitometric ratios of the exon-skipped product is shown at the bottom.

