Disruption of the splicing enhancer sequence within exon 27 of the dystrophin gene by a nonsense mutation induces partial skipping of the exon and is responsible for Becker muscular dystrophy.

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Disruption of the Splicing Enhancer Sequence within Exon 27 of the Dystrophin Gene by a Nonsense Mutation Induces Partial Skipping of the Exon and Is Responsible for Becker Muscular Dystrophy

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Abstract

The mechanism of exon skipping induced by nonsense mutations has not been well elucidated. We now report results of in vitro splicing studies which disclosed that a particular example of exon skipping is due to disruption of a splicing enhancer sequence located within the exon. A nonsense mutation (E1211X) due to a G to T transversion at the 28th nucleotide of exon 27 (G3839T) was identified in the dystrophin gene of a Japanese Becker muscular dystrophy case. Partial skipping of the exon resulted in the production of truncated dystrophin mRNA, although the consensus sequences for splicing at both ends of exon 27 were unaltered.

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Introduction

Pre-mRNA splicing demands remarkable accuracy. The cis-acting elements implicated in splicing are the 5′ and 3′ splice site sequences, branch point sequences, and their locations (1). Significantly, these elements are necessary but not sufficient for intron removal. Several lines of evidence indicate that a purine-rich region within the exon sequence, the splicing enhancer sequence (SES) or exon recognition sequence, operates as a cis-element to enhance splicing of the upstream intron (2–4). Recently, members of the SR family of essential splicing factors, characterized by alternating serine and arginine residues in their carboxyl domain, have been proposed to interact directly with purine-rich sequences (5, 6). Watakabe et al. (2) reported that a purine-rich sequence in exon M2 of the mouse IgM gene enhanced splicing of the upstream intron in an in vitro splicing system based on a heterologous intron of the Drosophila melanogaster doublesex (dsx) pre-mRNA that is spliced according to the nucleotide content of downstream exon sequence in the chimera. This model system could be used to analyze the splicing enhancer activity of the aforementioned purine-rich sequence, but has never been employed in studies of human diseases with splicing abnormalities.

As many as 15% of point mutations that result in human disease cause aberrant splicing (7). Recently, nonsense mutations located outside the splicing consensus sequence have been reported to cause skipping of the nonsense-containing exon in several human diseases (8–17). There are three possible mechanisms by which nonsense mutations could induce exon skipping: alteration of pre-mRNA secondary structures (10); a change in the mRNA coding potential (8); and disruption of the SES (9, 14). Although the second explanation, which was proposed to apply to Marfan syndrome, was confirmed in an in vitro study (18), there is no direct evidence that disruption of an SES can cause exon skipping in a genetic disease.

The severe disease Duchenne muscular dystrophy (DMD) and the more benign Becker muscular dystrophy (BMD) are allelic conditions characterized by progressive muscular degeneration and wasting caused by mutations in the dystrophin gene, which consists of 79 exons spread over >3,000 kb of the X chromosome (19, 20). The dystrophin gene is characterized by an extremely low exon to intron ratio (<0.01) and by the presence of multiple promoters and alternative splice sites (21). A purine-rich sequence within some exons of the complex dystrophin transcript is proposed to act as an additional cis-element to permit correct splice site selection. Several reports, all from our laboratory, of mutation-induced complete exon skipping in the dystrophin transcript that do not involve a
nucleotide change in the splicing consensus sequences support the above suggestion (22-24).

DMD cannot be treated therapeutically, but we have proposed it might be transformed into milder BMD by correcting the frame-shifted into the in-frame translational reading frame by artificially inducing an alternative splicing reaction (24, 25). However, there is no evidence that such alternative splicing events can occur naturally because dystrophin mRNA has not been subjected to detailed analysis in BMD patients. Here, we report a nonsense mutation that would be expected to cause DMD in the dystrophin gene of a patient with BMD. We show that the nonsense mutation is eliminated from a small part of transcript by exon skipping. The results of in vitro splicing experiments support the idea that SES disruption is one mechanism by which a nonsense mutation induces the exon skipping. This discovery provides direct evidence that DMD can be converted to BMD by modulating splicing.

Methods

Case. A 31-yr-old Japanese male was diagnosed as BMD in his early twenties on the basis of clinical features. At the age of 31, when cardiac symptoms of exercise fatigue and palpitation developed, cardiac examination disclosed dilated cardiomyopathy. At this time, the patient’s serum CK activity was 3,251 IU/liter (control 200 IU/liter). His elder brother also had a high serum CK value, and his mother, maternal grandmother, and aunts also had histories of cardiac disease, but no further information was available.

Mutation analysis. Genomic DNA and cDNA were analyzed basically as described previously (26-30). Unless otherwise specified, all other PCR amplifications of genomic DNA or cDNA were performed according to the conditions described previously (22). To locate the mutation, a smaller fragment encompassing exons from 25 to 30 was amplified from muscle cDNA using a forward primer corresponding to a region in exon 25 (5'-ACACGAGGATCA-CATGTG-3') and a reverse primer complementary to a sequence in exon 30 (5'-CTCTGCGGAGACTGGATGC-3'). To quantify each transcript, cDNA synthesized from 1 μg of total RNA was used for amplification and amplified products were quantified by measuring the density of each band (Densitograph; ATTO Co., Tokyo, Japan) after ethidium bromide staining.

Sequencing of the amplified DNA fragment from genomic DNA or cDNA was performed as described previously (30, 31).

Analysis of the genomic DNA sequence at the 5' end of intron 26. Since the sequence of the region upstream of exon 27 is not available, the 5' flanking region of exon 27 was cloned by nested PCR using the method described previously (32). A Sau3A1 cassette (Takara Shuzo Co., Kyoto, Japan) was ligated to Sau3A1-digested genomic DNA according to the protocol suggested by the supplier (Fig. 1 a). Then, two PCR reactions were carried out using first cassette primer C1 (Takara Shuzo Co.) and primer G27R corresponding to the 3' flanking region of exon 27 (5'-AATTCCGGATCCATTGG-3'), and then cassette primer C2 (Takara Shuzo Co.) and primer E27R corresponding to a region in exon 27 (5'-TCAGTAAAGCATTCCTTGCGGAT-3') (Fig. 1 b) was designed and used together with primer G27R to amplify the genomic region encompassing exon 27.

Examination of splicing enhancer activity. The splicing enhancer activity of natural and artificial purine-rich sequences was examined in vitro by analyzing splicing of the heterologous intron of the Drosophila melanogaster doublesex (dsx) gene pre-mRNA in HeLa cell nuclear extracts as described before (3). In this in vitro system, splicing of chimeric dsx pre-mRNA is dependent on the downstream sequence [43]. In the nucleotide sequence of the patient’s DNA, nucleotide G3839 (44), corresponding to nucleotide 28 of exon 27, was replaced by a T (asterisk). Lowercase and uppercase letters indicate intron 26 and exon 27 sequences, respectively. The underlined region (DW) within exon 27 indicates the purine-rich region, which consists of two purine-rich sequences indicated by dotted lines over each (DW1 and DW2). The horizontal arrow indicates the location and direction of the G27F primer.
Results

Once BMD had been diagnosed, the dystrophin gene was examined for the presence of a mutation. Deletion mutation analysis by multiplex PCR or Southern blot analysis failed to reveal any gross abnormality. Since RT nested PCR analysis of the dystrophin gene transcript in lymphocytes showed an aberrant splicing product (data not shown), the same region encompassing exons from 25 to 30 was amplified from muscle cDNA. Three products were obtained from the control (Fig. 3, lane C): the normal-sized product (717 bp) comprised the complete exon 25 to 30 sequence, a small (249 bp) product that lacked 468 nt corresponding to exons 27, 28, and 29 (exon 27, corresponding to nucleotide 3839 of dystrophin cDNA), and an intermediate-sized product that was a heteroduplex of the normal and exon 27, 28, 29 transcript, resulting in the elimination of the nonsense mutation and the restoration of the translation reading frame. In fact, Western blot analysis of skeletal muscle disclosed the presence of a dystrophin polypeptide that was ~27 kDa smaller and about five times less abundant than the 427-kD normal dystrophin (data not shown). Therefore, the nonsense mutation within exon 27 and the resulting exon 27 transcript were deemed to be the molecular explanation for the BMD phenotype of the index case.

The genomic DNA sequence around exon 27 was examined next, since exon skipping is usually induced by a mutation affecting the splicing consensus sequences. However, since the sequence of the region upstream of exon 27 was not available, this region was cloned from control DNA as described in

Figure 2. Analysis of splicing enhancer activity. (a) Schematic representation of Drosophila melanogaster doublesex (dsx) chimeric plasmid. The chimeric plasmid consists of the entire third exon (168 nt), the third intron (114 nt), and 30 nt of the 5’ portion of the fourth exon of the dsx gene and the linker sequence derived from the pSP72 vector (8 nt). The exons (boxes) and intron (line) are shown. The linker sequence (closed boxes) and the insertion site for the test sequence (open box) are indicated. B and X indicate BamHI and XhoI restriction enzyme recognition sites, respectively. The annealed test oligonucleotides were cloned into the insertion site. (b) Sequences examined for splicing enhancer activity.

Figure 3. Amplification of dystrophin cDNA encompassing exons 25 to 30. Amplified products encompassing exons 25 to 30 were obtained from muscle cDNA by RT-PCR. Two major products (717 and 249 bp) and other minor products were obtained from control (lane C). Three bands were visualized in the index case (lane P). Two of these (717 and 249 bp) corresponded to those from control cDNA, while the middle one (534 bp) was unique to the patient’s muscle. The amplified product from muscle cDNA of the patient’s elder brother showed the same pattern as lane P (lane B). Lanes P, B, C, and M represent the patient, his brother, control, and a size marker, respectively. The size marker was HaeIII-digested φX174 phage DNA (Takara Shuzo Co.).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>DW</td>
<td>AAAGAAGGCCCACAAGAACAAAGAGCAAAGGAAG</td>
</tr>
<tr>
<td>DM</td>
<td>AAAGAAGGCCCACAAGAACAAAGAGCAAAGGAAG</td>
</tr>
<tr>
<td>(DW1)x3</td>
<td>AAAGAAGGCCCACAAGAACAAAGAGCAAAGGAAG</td>
</tr>
<tr>
<td>(DW2)x3</td>
<td>AAAGAAGGCCCACAAGAACAAAGAGCAAAGGAAG</td>
</tr>
<tr>
<td>(DW2G28T)x3</td>
<td>AAATAGGCAAGAACAAAGAGCAAAGGAAGGAAG</td>
</tr>
<tr>
<td>(DW2A30T)x3</td>
<td>AAAGAAGGCCCACAAGAACAAAGAGCAAAGGAAG</td>
</tr>
<tr>
<td>ASLV</td>
<td>CTCCCAAGAAAGAGGCCCACAAGAACAAAGGAAG</td>
</tr>
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sequence (3, 33). Briefly, template plasmids coding for the chimeric dsx pre-mRNA were constructed as shown in Fig. 2 a. After confirming its sequence, radiolabeled pre-mRNA was prepared by in vitro transcription and subjected to in vitro splicing. The reaction products were electrophoresed and then quantified (BAS2000 Bio Image Analyzer; Fuji Photo Film Co., Tokyo, Japan) as described previously (24).

To evaluate splicing enhancer activity, various sequences were inserted between the BamHI and XhoI restriction enzyme recognition sites (Fig. 2 a). Several test sequences were prepared (Fig. 2 b) by annealing two strands of synthesized single-strand DNA. To minimize the effects of length variations, all prepared sequences were adjusted to ~30 nt. Pre-mRNA containing a segment of avian sarcoma-leukosis virus (ASLV) sequence that has been reported to be a good splicing enhancer (3) was used as positive control, and the BamHI-digested enhancer (3) was used as negative control. Several test sequences were prepared (Fig. 2 b) by annealing two strands of synthesized single-strand DNA. To minimize the effects of length variations, all prepared sequences were adjusted to ~30 nt. Pre-mRNA containing a segment of avian sarcoma-leukosis virus (ASLV) sequence that has been reported to be a good splicing enhancer (3) was used as positive control, and the BamHI-digested enhancer (3) was used as negative control.
Exon Skipping Induced by Disruption of Splicing Enhancer Sequence

Methods (Fig. 1a) and a segment of 151 nt from the 3' end of intron 26 was determined (Fig. 1b). Analysis of the patient's DNA failed to disclose any nucleotide change at the branch point and 3' splice acceptor and 5' splice donor sites. However, the amplified DNA contained the same G to T nucleotide change at nucleotide 28 of exon 27 (G3839T). Since the same sequence was obtained from 10 independent amplified products, the possible existence of a mosaic sequence pattern could be excluded. In addition, analysis of genomic DNA and muscle cDNA from the patient's elder brother, who was also a BMD case, disclosed exactly the same splicing pattern (Fig. 3, lane B) and the G3839T nucleotide change (data not shown).

Although it has been proposed that nonsense codons decrease mRNA levels relative to wild-type mRNA because of increased instability or elimination by a nonsense codon recognition system (34), the majority of dystrophin mRNA in the index case contained the premature stop codon (Fig. 3, lane P). This indicated that the appearance of nonsense codon in exon 27 did not have a major impact on the levels of dystrophin transcript, and suggested that the nonsense codon did not affect the fate of this transcript.

The case reported here is the first example of dystrophinopathy in which exon skipping was actually induced by the nonsense mutation. To identify the factor responsible for inducing the observed exon skipping, we examined the region around exon 27 for cis-acting elements known to control splicing (Fig. 1b). Remarkably, a long purine-rich stretch, including two copies of the tandem AAG that is presumed to be a strong SES (3), was identified from nucleotide 7 to 31 of exon 27. This suggests that correct splicing of exon 27 needs this purine-rich region as a splicing enhancer (2, 5). Since the G3839T substitution is in the middle of this purine-rich region and affects the second tandem AAG (Fig. 1b), we reasoned that exon 27 skipping identified in the index case could be induced by disruption of SES.

The splice-enhancing activity of the purine-rich region of
exon 27 was analyzed in vitro by its ability to activate splicing of chimeric dsx pre-mRNA, which is dependent on the downstream sequence (3). In this system, splicing of dsx pre-mRNA did not occur if any enhancer sequence was not present (negative control) (Fig. 5, lane NC), whereas splicing proceeded well when a part of the sequence of ASLV was inserted at the linker site (positive control) (Fig. 5, lane ASLV). When 31 nt of the purine-rich sequence from nt 7 to 37 of exon 27 (DW) was inserted into the linker site, the expected splicing product was observed (Fig. 5, lane DW). In contrast, the splicing product was not observed when the mutant sequence containing G3839T (DM) was inserted into the linker site (Fig. 5, lane DM). These in vitro studies clearly showed that the purine-rich sequence within exon 27 enhanced splicing of the upstream intron and that G3839T completely abolished this splicing enhancer activity.

The purine rich sequence from nt 7 to 37 of exon 27 comprises two purine-rich regions, nt 7 to 16 [AAAGAA-GAGG (DW1)] and nt 25 to 37 [AAAGAAGCGAAAG (DW2)] separated by three cytosines (Fig. 1b). To see which of the two purine-rich regions had stronger splicing enhancer activity, each part was examined individually. To avoid length effects and to facilitate manipulations, 30 nt long sequences were prepared by synthesizing three tandem repeats of each part of the sequence (Fig. 2b). Polypurines consisting of three repeats of the first part [(DW1)x3] and the second part [(DW2)x3] showed splicing enhancer activity, with the former exerting stronger splicing enhancer activity than the latter [Fig. 5, lanes (DW1)x3 and (DW2)x3]. These data showed that the purine-rich sequence itself had the splicing enhancer activity but indicated that the activity depended on its nucleotide content or sequence. To determine the effect of the G3839T nucleotide change, the third G of DW2 was changed to T in each of the three tandem repeat sequences in (DW2)x3 to give (DW2G28T)x3. In this case, the splicing product was not observed [Fig. 5, lane (DW2G28T)x3]. Thus, the G to T substitution corresponding to G3839T suppressed splicing enhancer activity both in the original 31 nt sequence of exon 27 and in the artificial purine-rich region.

There were two ways that G3839T might have suppressed splicing: the T insertion disrupted the purine-rich region (3) or a nonsense codon was created (8). Since the G to T change did indeed introduce a nonsense codon in the in vitro experiments, the effect of a simple T nucleotide change that did not create a stop codon in any frame was examined by preparing another polypurine sequence that consisted of three AAAGATGCGCGAAAG tandem repeats in which A was replaced by T at a position corresponding to nt 30 of exon 27 [(DW2A30T)x3] (Fig. 2b). However, this sequence showed splicing enhancer activity [Fig. 5, lane (DW2A30T)x3] which was lower than that of the polypurine [(DW2)x3] but higher than that of the polypurine with a nonsense codon [(DM2G28T)x3]. These results showed that the T insertion itself inhibited SES function but that the inhibition was strengthened by the creation of nonsense codon.

Our data showed that a case of BMD resulted from partial elimination of nonsense mutation from dystrophin mRNA by exon skipping, and that the exon skipping observed in this
BMD patient was due to disruption of an SES. This represents a novel case of BMD molecular pathogenesis that provides a natural model for how the severe DMD phenotype can be reduced to the less-severe BMD phenotype by altering the pattern of splicing.

Discussion

In this report, we describe in vitro evidence that disruption of the SES by a nonsense mutation is the cause of skipping of the mutation-containing exon. The mechanism of exon skipping induced by nonsense mutations has recently attracted considerable attention (8, 10–16). Three explanations have been proposed for the induction of exon skipping: a change in pre-mRNA structure, a change in the mRNA coding potential, and disruption of SES. In support of the first of these explanations, alteration of pre-mRNA secondary structure by a nonsense mutation has been reported to lead to exon skipping in the Fancomi anemia group C gene (10), as did a missense mutation (35). This is supported by the recent finding that a silent mutation of the platelet fibrinogen receptor gene induced exon skipping (36). The nonsense mutation scanning model (the second explanation) is based on the observation that skipping of a constitutive fibrillin gene exon containing a nonsense mutation occurred even though the consensus splice sequence was maintained (8). In this model, the exon might be removed from mature mRNA if an open reading frame is not detected due to the presence of a premature termination codon. This model was confirmed by an in vitro model splicing system (18). Furthermore, exon skipping has been reported to be due to the creation of a stop codon in several diseases (8, 11, 12, 16). However, this model is not universally accepted because nonsense mutations do not necessarily induce splicing alterations (37).

The third explanation for the induction of exon-skipping reactions by nonsense mutations, which invokes disruption of splicing enhancers, is based on the finding that a purine-rich sequence activates the splicing of upstream introns (2, 3, 38). This theory was supported by Santisteban et al. who reported that exon skipping was induced by a C to T change in the purine-rich region of exon 5 of the adenosine deaminase gene (14). Furthermore, disruption of an SES was thought to cause the skipping of the nonsense-containing exon resulting from mutations in the purine-rich sequence of the Factor VIII gene (9) and the cystic fibrosis transmembrane conductance regulator gene (13, 15). However, these examples were not confirmed by in vitro experiments. In addition to the above, the nonsense-containing exon has been reported to be eliminated spontaneously by alternative splicing, a mechanism proposed to explain the phenotypic rescue of a common inherited defect (39).

In our case, the secondary structure alteration theory is not plausible because the mutation was not predicted to cause a marked change in the secondary structure of the pre-mRNA region encompassing exon 27 (data not shown). The nonsense mutation scanning model also seemed not to apply because the transcript containing the nonsense codon remained a major component of the total transcript (Fig. 3, lane P). Finally, we focused on the idea that disruption of the SES could cause mis-splicing. In vitro splicing studies showed that the wild-type purine-rich sequence of exon 27 had splicing enhancer activity and that this activity was completely abolished by the G3839T substitution (Fig. 5, lane DM). However, induction of exon 27 skipping was observed only in a small proportion of the dystrophin transcripts in skeletal muscle (Fig. 3, lane P), even though the results of the in vitro splicing analysis showed that the mutant sequence completely abolished splicing enhancer activity (Fig. 5, lane DM). Since exon 27 has two separate purine-rich regions, each of which showed splicing enhancer activity [Fig. 5, lanes (DW1)x3 and (DW2)x3], it seemed likely that disruption of the second part of the purine-rich region by the G to T substitution might not be enough to abolish SES function in vivo. In fact, synergistic interactions of two splicing enhancers within an exon have been reported (33). Therefore, normal splicing presumably proceeded in this case even when one of two purine-rich regions was disrupted. Recently, members of the SR family of essential splicing factors have been proposed to interact directly with purine-rich sequences (5, 6), raising the possibility that SESs might function through their ability to recruit one or more SR proteins. Our observation that exon 27 skipping was incomplete in skeletal muscle suggested that nuclear proteins present in HeLa cell nuclear extracts were different from those present in skeletal muscle cells. Further investigation is required to clarify the exact interaction of each purine-rich region with nuclear proteins.

The in vitro splicing study showed that a sequence containing a nonsense codon inhibited splicing of an upstream intron more efficiently than a similar mutation that did not contain a stop codon [Fig. 5, lanes (DW2G287)x3 and (DW2 A30T)x3]. This evidence supported the idea that creation of a nonsense codon contributed to aberrant splicing (8) but it is not clear whether this mechanism contributed to the situation observed in the patient’s muscle because of the large amounts of nonsense codon-containing transcripts that were present. Thus, the in vitro splicing study did not faithfully reproduce the in vivo phenomenon and it remains possible that the nonsense codon recognition model could explain a small part of the induced exon skipping in this case. A survey of similar nonsense mutations in purine-rich regions that induce exon skipping (9, 13–15) revealed that they all introduced a T nucleotide, which conforms to the finding that thymine has a strong propensity for SES disruption (3). Therefore, exon skipping should be evaluated whenever a stop codon created by T replacement is identified in purine-rich region of a disease-causing gene.

This is the fourth report of a nonsense mutation of the dystrophin gene that caused BMD rather than DMD (40). These examples of an apparent lack of correlation between the presence of a nonsense mutation and the severity of the disease were explained as follows: one nonsense mutation was found to be a somatic mosaic (41), while the two others disappeared spontaneously from mRNA because they were located in exons that normally undergo alternative splicing (42). In the index case, partial skipping of exon 27 removed a nonsense mutation from some of the dystrophin mRNA and resulted in the production of truncated dystrophin. Among 79 exons of the dystrophin gene, only a limited number have a purine-rich region that might be an SES. Therefore, rescuing of nonsense mutation by exon skipping is likely to be a very rare occurrence in dystrophinopathy. Nevertheless, our finding has very important clinical implications for the proposed therapeutic conversion of DMD into BMD by artificially inducing a reading frame change through altered splicing using the antisense nucleotide strategy (25).
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