another method before concluding that there is a single-exon deletion. However, there has never been any example showing the ambiguous result of an intermediate-grade product amplification by MLPA.

In this study, MLPA analysis of the dystrophin gene was conducted in Japanese DMD/BMD patients. MLPA worked well to identify deletion or duplication. One patient showing a single-exon deletion by MLPA turned out to have a four-nucleotide deletion. Remarkably, ambiguous amplification products representing intermediates between a loss and a single-genome copy were found in two patients, and subsequently two novel small mutations were disclosed.

Patients and Methods

Seventy-seven Japanese male DMD/BMD patients were enrolled in this study. They were referred to Kobe University Hospital between January 2007 and May 2008 for their genetic diagnosis. Their clinical diagnosis was based on family history, clinical findings such as weak muscle strength, muscle biopsy findings, and markedly elevated serum creatine kinase levels. DNA samples were extracted from peripheral blood using the method described before (Tran *et al.*, 2006). This study was approved by the Institutional Review Board (Approval number 28).

MLPA analysis

MLPA analysis was performed using the MLPA DMD kit (SALSA MLPA KIT P034/P035 DMD/Becker; MRC-Holland), and was carried out following the manufacturer's protocol (MRC-Holland) by the Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Seventy-nine dystrophin exons were amplified in two separate reactions. After electrophoresis of amplified products on DNA sequencer, the relative peak ratio (RPR) of each exon to the internal control was obtained by the recommended method (http://www.mrc-holland.com). The interpretation of the calculated RPR was as follows: loss,<0.1; single, 0.65–1.3; double,>1.3.

PCR amplification and DNA sequencing

In patients who showed a single-exon deletion or ambiguous result by MLPA, an exon-encompassing region of the respective exon was amplified by conventional PCR using primers designed on flanking introns. Exons 25, 31, and 33 of the dystrophin gene were amplified in conditions essentially the same as described before (Surono *et al.*, 2004) using a set of exon-specific primers (exon 25: g25F, tgtggcagtaatttttttcag and g25R; aggaaatcttagttaagtacg; exon 31: g31F, atggtagaggtggttg agga and g31R, tataatgcccaacgaaaaca; exon 33: g33F, tggaatag caattaaggg and g33R, gctaagactctaatcatac).

Sequencing of the amplified DNA fragment from genomic DNA was performed using a DNA sequencer as described previously (Tran *et al.*, 2006).

Results

A total of 77 samples were analyzed for mutation by MLPA. Deletions detected by a complete loss of one or more corresponding peaks were identified in 35 patients. Duplication of exons with an approximately 2.0 RPR were identified in 15 patients. In our series, the detection rate of deletion and duplication was 45.5% and 19.4%, respectively. As a result, the mutation detection rate was 64.8% in Japanese DMD/BMD patients.

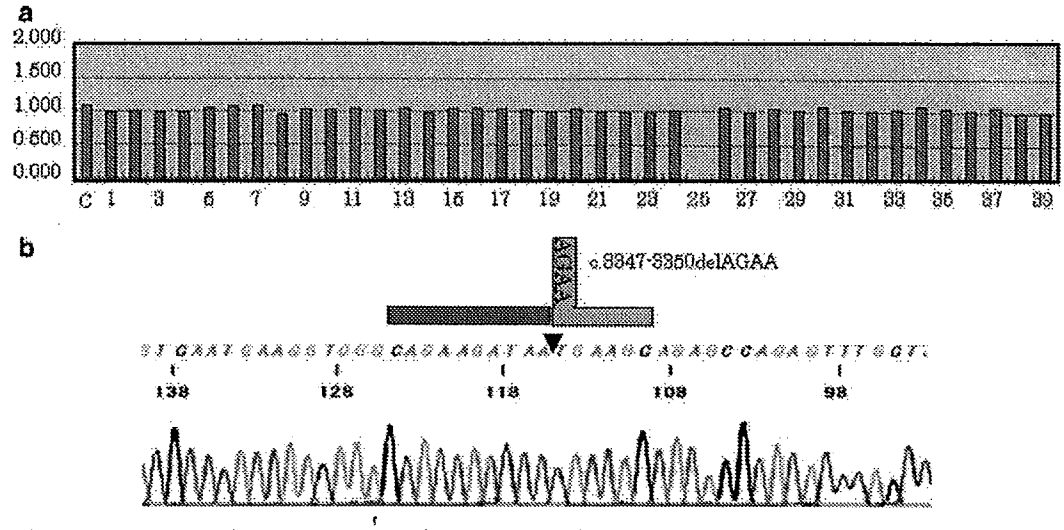

FIG. 1. Apparent loss of exon 25. (a) RPRs of exons 1 to 39 of the dystrophin gene are shown. Bars indicate RPRs of respective exons. The numbers below bars indicate exon number, while C indicates control. The RPR of exon 25 is 0, while that of others is nearly 1. (b) A part of the exon 25 sequence is shown. Four nucleotides, AGAA (c.3347–3350delAGAA), are not present between A and T in the sequence (arrow). The two bars over the sequence show regions where the upstream and downstream probes hybridize, respectively. The four-nucleotide deletion is located at the 5′ end of the downstream probe-hybridizing region.

A single-exon deletion was identified in 10 male patients, and their respective exon was further examined by conventional PCR amplification using primers on flanking introns. In nine patients the respective exon-encompassing region could not be obtained by conventional PCR amplification, confirming an exon deletion mutation. Remarkably, one DMD patient (KUCG 434) showed a PCR amplified product of exon 25, even though the peak of exon 25 was completely absent by MLPA (Fig. 1). These incompatible results from two different amplification methods were not surprising, because the genotype of the exon 25 deletion did not match with his severe phenotype of DMD. According to the reading frame rule (Monaco *et al.*, 1988), the exon 25 deletion that removed 156 bp from the dystrophin mRNA was supposed to produce an in-frame, semifunctional dystrophin mRNA, resulting in a mild BMD phenotype. Remarkably, sequencing of the amplified exon 25–encompassing region revealed the deletion of four nucleotides, AGAA (c.3347–3350delAGAA) (Fig. 1). We concluded that this deletion, creating a premature stop codon in exon 26, was the cause of DMD. The four-nucleotide deletion apparently inhibited amplification completely in MLPA by preventing the ligation of two probes.

In addition to cases simply diagnosed as deletion or duplication by MLPA, two male DMD patients unexpectedly showed ambiguous values in their RPRs: 0.43 and 0.16. These values were not consistent with either a loss or a single-genome copy and were thought to be artifacts. However, the patient showing 0.43 RPR in exon 31 (KUCG797) was further examined. The exon 31–encompassing region could be PCR amplified and obtained at an expected size and amount. Direct sequencing of the amplified product disclosed a novel single-nucleotide change from G to T at the 4303th nucleotide (c.4303G>T). This nucleotide change shifted the GAG codon for Glu to a TAG stop codon, so we concluded that this mutation was the cause of DMD. The nucleotide change partially hampered amplification of exon 31 in MLPA, leading to an ambiguous amplification result.

In the other patient (KUCG795), exon 33 in MLPA had a 0.16 RPR. However, conventional PCR amplification of exon 33 confirmed the presence of the exon at an expected size and amount. Direct sequencing of the amplified product disclosed a novel five-nucleotide deletion of GAGTG (c.4536–4540delGAGTG) within exon 33. We concluded that the identified five-nucleotide deletion was the cause of DMD. When the genomic sequence of exon 33 was carefully examined, we found that the deletion resulted in a novel eight-nucleotide sequence around the deletion site consisting of TCT/AAGTG ("/" indicates the site of the deletion) and that the new sequence was very similar to the probe-hybridizing site sequence (TCTGAAGTG; underline indicates a match with the mutated sequence). Therefore, we concluded that the deletion disturbed amplification in MLPA by preventing proper probe hybridization to the probe-hybridizing site.

Discussion

In this study, ambiguous amplification products by MLPA were found due to inefficient amplification caused by novel small mutations (Figs. 2 and 3). Further, a single-exon deletion identified by MLPA turned out to be a four-nucleotide

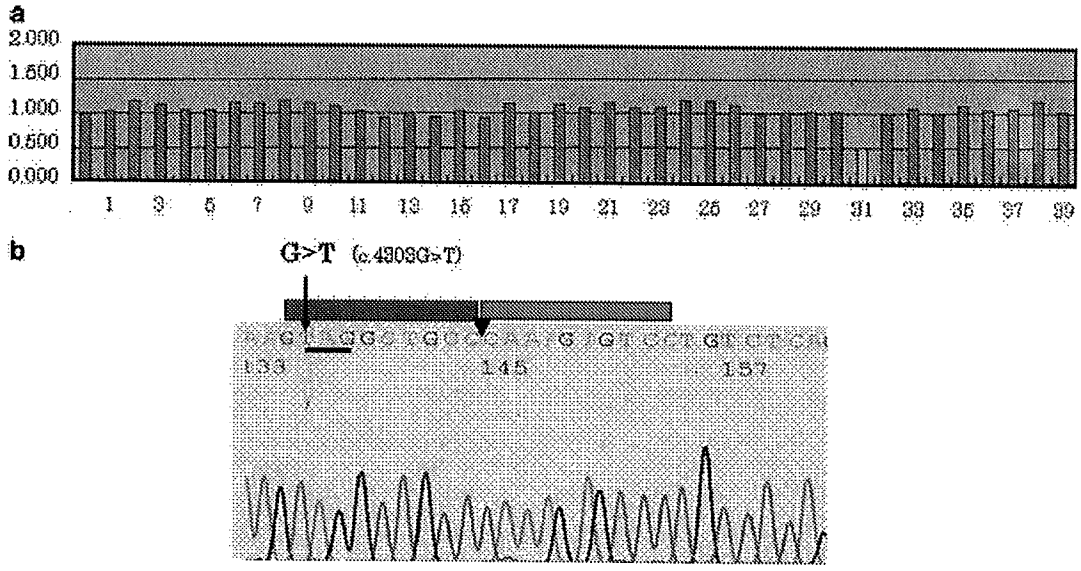

FIG. 2. A nonsense mutation in exon 31. RPRs of exons 1 to 39 of the dystrophin gene are shown. Bars indicate RPRs of respective exons. The numbers below bars indicate exon number, while C indicates control. The RPR of exon 31 is 0.43. A part of the exon 31 sequence is shown. A single-nucleotide change from G to T at the 4303th nucleotide of the dystrophin cDNA (c.4303G>T) is marked (arrow). This nucleotide change shifted the CAG codon for Glu to TAG for a stop codon (underlined). The two bars over the sequence show regions where the upstream and downstream probes hybridize, respectively. The nucleotide change is located at the 9th nucleotide from the ligation site (inverted triangle).

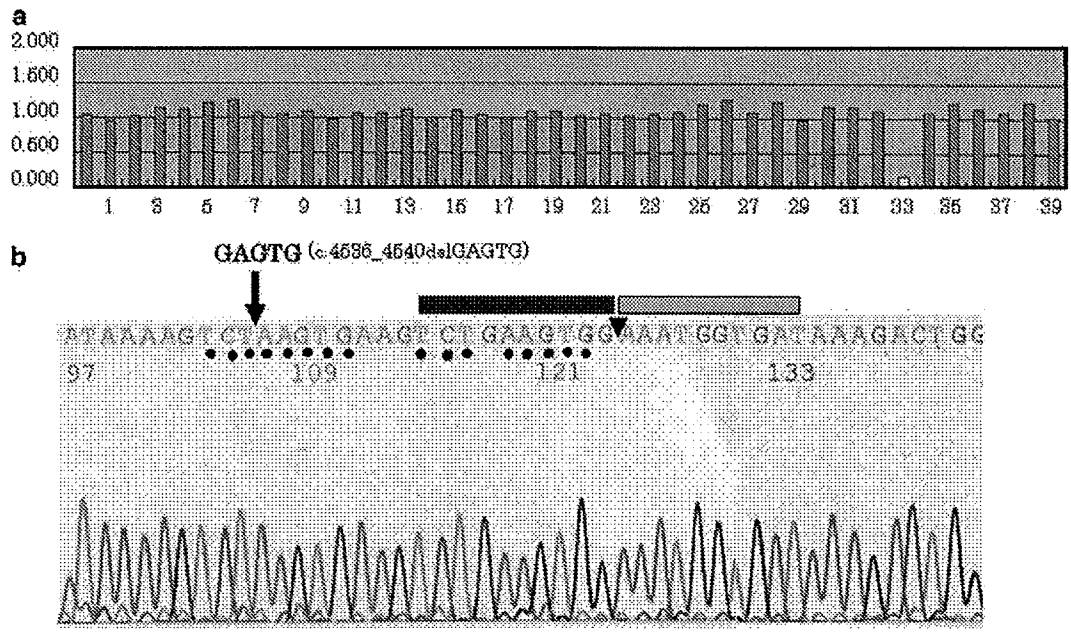

FIG. 3. Five-nucleotide deletion in exon 33. (a) RPRs of exons 1 to 39 of the dystrophin gene are shown. Bars indicate RPRs of respective exons. The numbers below bars indicate exon number, while C indicates control. The RPR of exon 33 is 0.16. (b) A part of the exon 33 sequence is shown. Five nucleotides, GAGTG (c.4536_4540delGAGTG), are not present in sequence (arrow). The two bars over the sequence show regions where the upstream and downstream probes hybridize, respectively. The five-nucleotide deletion is located at the 9th nucleotide from the probe-hybridizing region. Remarkably, the deletion resulted in a novel eight-nucleotide sequence consisting of TCTAAGTG (dots) that is similar to the nine nucleotides of the probe-hybridizing region (dots).

deletion in the probe-hybridizing region (Fig. 1). Our results indicate that MLPA is useful to screen even small mutations, in addition to its utility as a very powerful tool to screen deletions or duplications of all 79 exons in the dystrophin gene (Lalic *et al.*, 2005).

In the literature, more than 30 patients have been described to have a single-exon deletion by MLPA, and 8 of them have turned out to have small mutations in MLPA probe-binding regions (Janssen *et al.*, 2005; Lai *et al.*, 2006; Schwartz *et al.*, 2007; Todorova *et al.*, 2008). In this situation, small mutations have the possibility to be misdiagnosed as exon deletions in MLPA. Therefore, one must be very cautious when diagnosing a single-exon deletion. In our series, 1 out of 10 such patients had a small mutation (Fig. 1). Notably, deletion of single exon 25 identified by MLPA did not explain the severe DMD phenotype of patient KUCG 434, but the four-nucleotide deletion did. When there is a mismatch between genotype disclosed by MLPA and phenotype, we suggest that the mutation be analyzed further.

A total of 11 small mutations were screened by MLPA. It is reasonable to expect inefficient PCR amplification when nucleotide mismatch is present between the genome sequence and MLPA probe sequences. This can result in either nonamplification or intermediate amplification. From thorough examination of nonamplification mutations (Janssen *et al.*, 2005; Lai *et al.*, 2006; Schwartz *et al.*, 2007; Todorova *et al.*, 2008), we hypothesized that the location or size of the mutations is the determining factor for nonamplification; mutations within three nucleotides from the probe ligation site (c.2991C>G, c.5404C>T, c.8608C>T, c.10368 del T, c.128 ins GA, and c.2225–2241del TGCAGAGTCCTGAATTT) or a mutation of more than two nucleotides within the 10 bp probe-hybridizing region (c.5401–5402 del AT, c.128 ins GA, c.2225–2241del TGCAGAGTCCTGAATTT, and c.5800–5806del GAGGGC) (underlined are common to both criteria) result in nonamplification. When this hypothesis is applied to our patients, the nonamplification observed in patient KUCG 434 can be explained, because the deletion removed four nucleotides at the probe ligation site (Fig. 1).

Ambiguous results showing intermediate amplification are reported for the first time in this study. In fact, two mutations had neither of the characteristics proposed by the above hypothesis. The single-nucleotide change of c.4303G>T is present at the 9th nucleotide from the ligation site, resulting in a 0.43 RPR. This indicates that a single-nucleotide change does not necessarily result in complete loss of amplification. We suggest that c.4303G>T did not inhibit the ligation of the two probes but affected the hybridization of the upstream probe, thereby resulting in partial amplification.

It was unexpected to find the mutation in patient KUCG795 with a 0.16 RPR. When sequencing results disclosed a five-nucleotide deletion within 33, it was unclear why this induced

an intermediate RPR. This could be explained by the fact that the deletion created a novel sequence similar to the probe-hybridizing sequence, thereby inducing competition between two sequences for probe hybridization. This appears to be a new mechanism that can disturb the MLPA reactions.

An intermediate grade of amplified product is described here for the first time in two Japanese patients (Figs. 2 and 3). We suspect that ambiguous amplification results shown in this study have been dismissed as artifacts in previous studies without further examination. Therefore, we recommend that detailed examination of ambiguous MLPA results should be conducted, especially in patients with genotype–phenotype mismatch.

In conclusion, MLPA is a powerful tool for screening not only exon deletions and duplications, but also small mutations in the dystrophin gene, and could be used in routine clinical diagnosis.

AU1 ▶ **Disclosure Statement**

No competing financial interests exist.

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In vitro splicing analysis showed that availability of a cryptic splice site is not a determinant for alternative splicing patterns caused by +1G→A mutations in introns of the dystrophin gene

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ABSTRACT

Background: Splicing patterns are critical for assessing clinical phenotype of mutations in the dystrophin gene. However, it is still unclear how to predict alternative splicing pathways in such cases of splice-site mutation in the dystrophin gene.

Objective: To identify elements determining alternative splicing pathways in intron +1G→A mutations of the dystrophin gene.

Results: We found that exon 25 is spliced out in the +1G→A mutation in intron 25, resulting in mild Becker muscular dystrophy, and that a cryptic splice site within exon 45 was activated in severe Duchenne muscular dystrophy with a mutation of +1G→A mutation in 45. Furthermore, in vitro splicing analysis using a pre-constructed expression vector showed that the mutant intron 25 produced one transcript that lacked exon 25. In contrast, the same splice-site mutation in intron 45 produced three splicing products. One product used the same cryptic donor splice site within exon 45 as the in vivo donor site and another product used a cryptic splice site within the vector sequence. Notably, the available cryptic splice site was not activated by the same G→A mutation of intron 25.

Conclusion: It was concluded that sequences inserted into the in vitro splicing assay minigene contain *cis*-elements that determine splicing pathways. By taking other +1G→A mutations in the introns of the dystrophin gene reported in the literature into consideration, it seems that cryptic splice-site activation is seen only in strong exons. This finding will help to elucidate the molecular pathogenesis of dystrophinopathy and to predict efficiency of induction of exon skipping with antisense oligonucleotides for treatment of Duchenne muscular dystrophy.

Duchenne muscular dystrophy (DMD) is the commonest inherited myopathy in humans, and is caused by a mutation in the dystrophin gene on the X chromosome, which causes progressive muscle wasting. Paitints with DMD usually succumb to cardiac or respiratory failure in their 20s. Becker muscular dystrophy (BMD) is caused by mutations in the dystrophin gene and is a milder muscle-wasting disease that usually affects adult men. A reading frame rule explains the difference between DMD and BMD; mutations that create premature termination codons in the reading frame of dystrophin mRNA usually result in a DMD phenotype, whereas mutations that maintain the original reading frame cause the milder BMD phenotype.[1] Therefore, it is important to consider how alternative splicing events will alter translation products of the dystrophin gene and thus whether they will lead to either the DMD or the BMD phenotype. For this reason, dystrophinopathy is one of the most extensively studied diseases related to splice-site mutations and their resultant products.

Pre-mRNA splicing is a process that removes introns during the joining of exons to form mature mRNAs. A critical step in pre-mRNA splicing is the recognition and pairing of donor splice and acceptor sites. GT and AG dinucleotides are strictly conserved sequences at donor splice and acceptor sites, respectively. Therefore, mutations at these consensus sequences result in splicing errors such as exon skipping and cryptic splice-site activation and are responsible for nearly 15% of all genetic diseases.[2] In spite of the identification of a large number of splice-site mutations, there is still no established way to predict how these mutations will affect how the pre-mRNA is spliced.[3 4] It has been proposed that exon skipping is the preferred phenotype when the immediate vicinity aroudn the affected exon–intron junction is devoid of alternative cryptic splice sites.[5]

More than 30 point mutations have been reported in the consensus sequences at donor splice sites of the dystrophin gene,[6] of which mutations changing G to A at the first nucleotide of the relevant intron (+1G→A) are the most common. Even within this limited subset, the resulting splicing pathways can differ from mutation to mutation, leasing to either exon skipping or cryptic splice-site activation.

In this study, we show that the availability of a cryptic splice site is not a determinant for splicing pathways but rather that the inserted sequences into the minigene determine splicing pathway. Elements determining the splicing pathways of intron +1G→A mutations of the dystrophin gene and the application of our results to the treatment of DMD are discussed.

METHODS

The protocols used in this study were approved by the ethics committee of the Kobe University School of Medicine. Blood and muscle samples were taken after written informed consent was obtained from the patients.

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Patients

Mutation analysis of the dystrophin gene was conducted in >400 patients with dystrophinopathy at Kobe University Hospital. In one patient with BMD (KUCG293), direct sequencing of the amplified product of the region encompassing exon 25 found a single nucleotide change from G to A at the first nucleotide of intron 25 (c. 3432 +1G→A, intron 25+1A) (fig 1A). In one patient with DMD (KUCG701), a G→A transition was identified at the first nucleotide of intron 45 (c.6614 +1G→A, intron 45 +1A) (fig 1B).

Dystrophin mRNA analysis

Dystrophin mRNA in peripheral lymphocytes was analysed as described previously.[7 8] A fragment consisting of exons 24 to 28 in the KUCG293 case was amplified by nested reverse transcription (RT)-PCR using two sets of primers (2A and 2B; 2E and e28r; able 1). Muscle dystrophin mRNA was analysed in thin-sliced muscle sections of frozen muscle samples from patient KUCG701 as described previously.[9] A region encompassing exons 44 to 52 was also amplified using forward and reverse primers (4C and 4D; table 1) . Amplified fragments were sequenced directly or by subcloning as described previously.[10]

In vitro splicing analysis

Minigenes were constructed by inserting a test sequence consisting of exons and their flanking introns into the multi-cloning site of the pre-constructed expression vector H492 that encodes two exons (exons A and B), and then for splicing analysis (fig2A).[11] The inserted fragments were obtained from both control DNA and the patient's genomic DNA by PCR with a primer set (YH366 and YH367; table 1) for exon 25 and with an another set of primers (YH341and YH342; table 1) for the region encompassing exon 45. Amplified products were digested with *Nhe*I and *Eco*RV restriction enzymes (New England Biolabs, Hitchen, UK) and inserted into the H492 vector that was pre-digested with the same enzymes. After checking their sequences, the resultant plasmids were transfected into HeLa cells for splicing assays as described previously.[11–14]

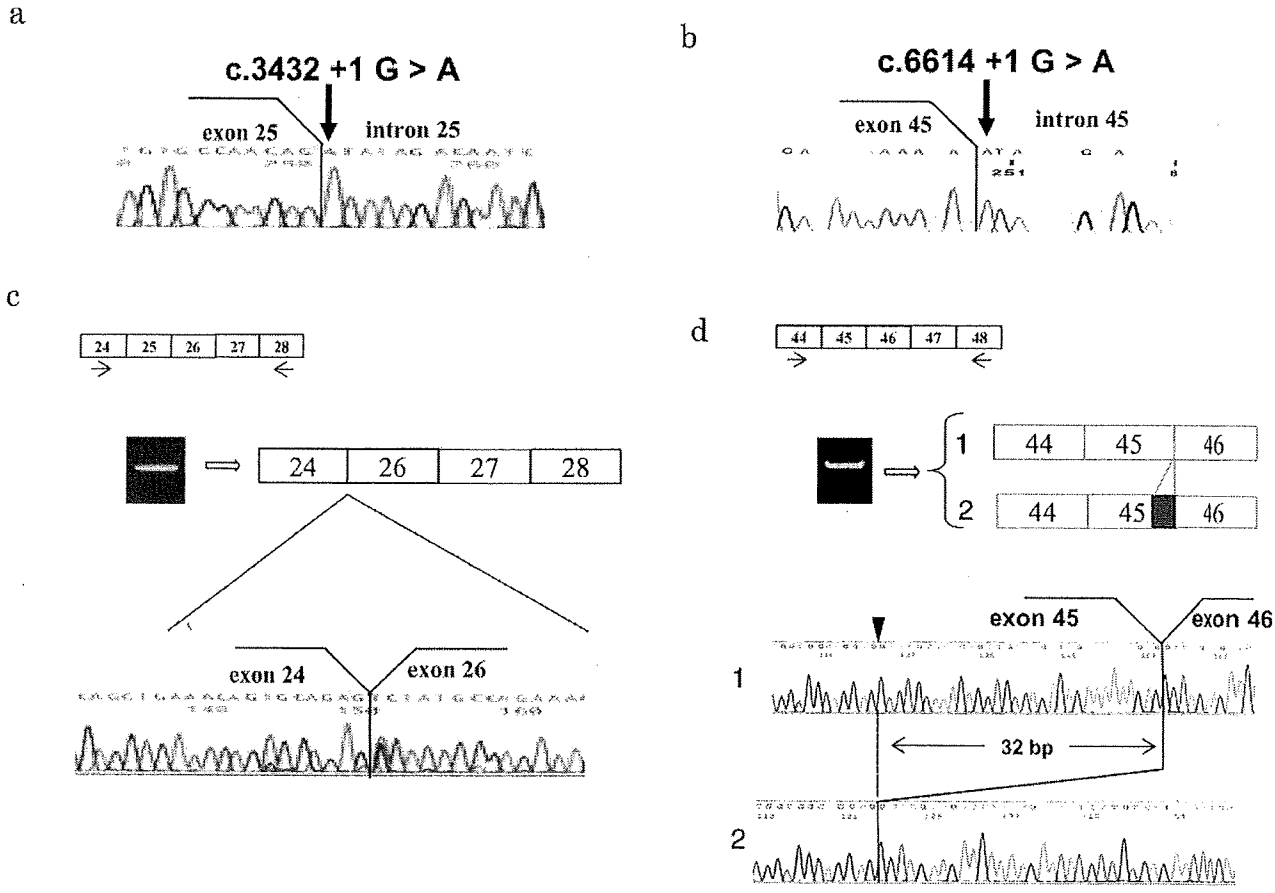

Figure 1 Results of genomic and mRNA analysis. (A) Partial sequencing of the region encompassing exon 25. A PCR product from one patient (KUCG293) was directly sequenced. The first nucleotide of intron 25 (c.3432 +1) was found to be A, indicating a G to A transition mutation (c.3432+1G→A). (B) The junction sequence of exon 45 and intron 45. The PCR product of the region encompassing exon 45m obtained from genomic DNA of one patient with DMD (KUCG701), was directly sequenced. The first G nucleotide of intron 45 (c.6614 +1) was replaced with A in the patient (c.6614+1G→A). (C) (Top) Dystrophin mRNA resulting from exon skipping. Reverse transcriptase (RT) nested PCR amplification extending from exon 24 to 28 was conducted for patient KUCG293. The amplification found one clear product. (Middle) On the right, the exon structure of the product is schematically described. (Bottom) Sequences at the junction of exons 24 and 26. (D) Dystrophin mRNA resulting from cryptic splice-site activation. RT-PCR amplification of the area encompassing exon 44 to 48 was conducted on a sample of muscle from a patient with DMD (KUCG701) (top). (Middle) The amplification produced one clear band that was found to consist of two transcripts (1 and 2). (Right) Schematic of the 5′ part of the exon, indicating the absence of 32 bp in one transcript (black box). (Bottom) Sequencing of the product revealed a mixture of two transcripts and the sequence at the junction of exons 45 and 46); one transcript has the normal exon content (1) and the other has a 32 bp deletion on the 3′ end of exon 45 (2). Inverted triangle represents a cryptic donor splice site.

Table 1 Primers used in experiments

Primer	Sequence (5'→3')
2A	ACAAGGGAACAGATCCTGGTAA
2B	CTGCTTTTTCTGTACAATCTGACG
2E	GAGCATTGTCAAAAGCTAGAGGA
e28r	GGGTCTTTTGCCAACTCATT
4C	GCTGAACAGTTTCTCAGAAAGACACAA
4D	CGATCCGTAATGATTGTTCTAGC
YH366	GCGCTAGCCATATGCAATGCCATCAGTC
YH367	GCGATATCATAGGAACAAAGCCTTAACC
YH341	GGGCTAGCGGGAAATTTTCACATGGAGC
YH342	GCGATATCTTAGTGCCTTTCACCCTGC

Dystrophinopathy cases with intron +1G→A mutation

To analyse the splicing pathways resulting from the intron +1 G→A mutations, cases of dystrophinopathy with the mutation were reviewed in the literature or a DMD website (http://www.dmd.nl/) and their splice pathways were summarised.

Cis-element analysis

The Shapiro splice-site probability score was calculated as described previously.[15] The ESE and ESS sites were predicted by the ACESCAN2 web server (http://genes.mit.edu/acescan2/index.html).[16 17] The binding sites for SR proteins (SF2/ASF, SC35, SRp40 and SRp55) were predicted using the ESEfinder web site (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) with default thresholds.[18]

RESULTS

Mutation analysis of the dystrophin gene revealed a G→A transition mutation at the first nucleotide of the intron in two patients with dystrophinopathy(fig 1A, 1B). One patient with BMD (KUCG293) had a +1G→A mutation in intron 25 (intron 25+1A) and one patient with DMD had a +1G→A mutation in intron 45 (intron 45+1A). As the G at the first nucleotide is absolutely conserved, these mutations were expected to produce the splicing error of exon skipping. Their splicing products were analysed by RT-PCR amplification as described above. In the patient with BMD (KUCG293) a region extending from exon 24 to exon 28 was amplified by nested RT-PCR from lymphocytes, producing one product (fig 1C). Sequencing of the product disclosed the complete absence of exon 25, indicating that the

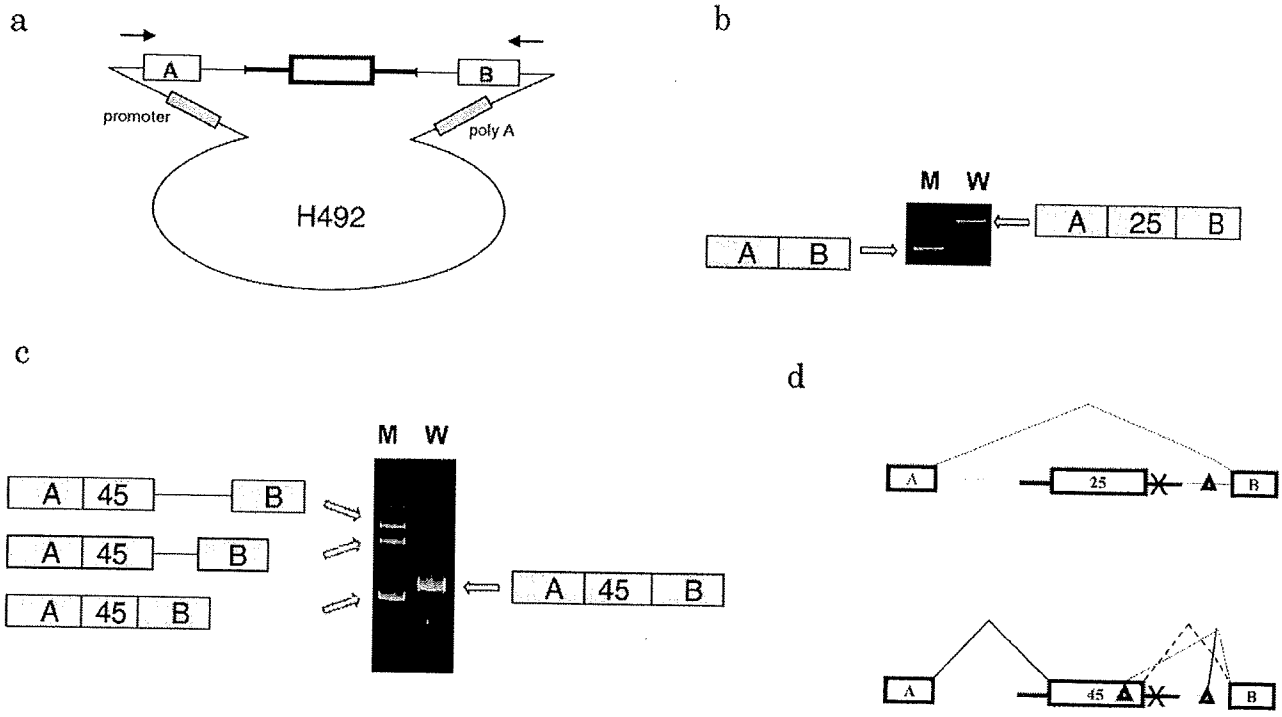

Figure 2 In vitro splicing analysis. (A) Schematic of the pre-constructed expression vector (H492). The vector contained a cytomegalovirus (CMV) enhancer-promoter and a bovine growth hormone gene (BGH) polyadenylation signal (coloured boxes). One intron encoding a multi-cloning site (thin line) was inserted between exons A and B. Exons 25 or 45 and their respective adjacent introns (bold line) was inserted into this multicloning site. (B,C) Reverse transcription (RT) PCR products of exon 25 and 45 minigene transcripts. A transcript was generated from a minigene carrying the wild-type exon 25 or 45 sequence (lane W). The minigene carrying the mutation generated transcripts (lane M); one shorter transcript for exon 25 and three transcripts for exon 45. Using sequencing, it was found that exon 25 was skipped entirely. For exon 45, sequencing of each product showed that the largest one retained the entire sequence between exon 45 and 'B'; the middle one retained a 144 bp sequence between exons 45 and 'B', and the smallest had a 32 bp deletion at the 3' end of exon 45. Their schematic structures are shown. (D) Splicing pathways of transcripts from two mutant minigene. (Top) In the case of intron 25+1A mutation (mutant intron 25), exon 25 was skipped even though a cryptic donor splice site was present (triangle). (Bottom) For the intron 45+1A mutation (mutant intron 45), two cryptic donor splice sites including the one vector sequence (triangle) were activated but no splicing of the second intron occurred. Open boxes represent exons; thin and bold lines represent introns from the vector or the dystrophin gene, respectively; cryptic splice sites are indicated by triangles; splicing patterns are designated by diagonal lines; dotted diagonal lines represent the non-spliced pattern.

mutation caused skipping of exon 25. The resulting dystrophin mRNA lacked the 156 bp exon 25 but was in frame, which is consistent with the patient's BMD phenotype. The splicing product in the patient's lymphocytes matched his phenotype, despite the fact that splicing patterns in lymphocytes may sometimes differ from that in skeletal muscle.[19]

In one patient with DMD (KUCG701), RT-PCR amplification of a region that includes exons 44–48 showed one clear product (fig 1D). However, sequencing of the product produced ambiguous results. Subcloning and sequencing unexpectedly revealed two clones, one of which had completely normal sequences consisting of exons 44 to 48. The other clone had a deletion of a 32-nucleotide segment at the 3′ end of exon 45, although all other sequences were completely normal (fig 1D). As the shortened fragment was considered a splicing product, the sequence of the donor splice site of exon 45 was examined more closely. Remarkably, GT dinucleotides conserved at the donor splice site were found at a position 32 nucleotides from the 3′ end of exon 45 (fig 1D) and this site had a high Shapiro probability score (0.73), whereas the intron 45+1G→A mutation decreased the score for the authentic site from 0.84 to 0.67. Therefore, it was determined that the deletion was due to the use of a cryptic donor splice site within exon 45. From their peak heights in the direct sequencing results (data not shown), the out-of-frame dystrophin mRNA with 32 nucleotides deleted was more abundant than the normal dystrophin product. This was consistent with the patient's clinical phenotype of DMD.

These two cases had identical G→A mutations at the first nucleotide of an intron but differential splicing products, and thus can be considered a good model for elucidating the factors that regulate splicing. It was hypothesised that the different splicing pathways seen in these cases were due to activation of cryptic donor splice sites. As shown above, exon 45 had a cryptic donor splice site within its sequence (fig 1D). Accordingly, the genomic sequence near the junction of exon 25 and intron 25 was also examined for a cryptic donor splice site. Such a cryptic site was not found, either within exon 25 or within the first 200 bp of intron 25, therefore availability of a cryptic site seemed to be one determinant of splicing in these two mutations.

To further validate the above findings, an in vitro splicing system using minigenes was used. Wild-type or mutant sequences encompassing exon 25 or 45 and their flanking introns were amplified by PCR and inserted into the multicloning site within the intron of the H492 vector (fig 2A). Four such minigenes were subjected to in vitro splicing in HeLa cells, and the resultant splicing products amplified by RT-PCR. From the minigenes containing inserts of wild-type sequences of exon 25 or 45, one mature transcript comprising three exons was obtained (fig 2b and fig 2C), which indicated that normal splicing occurred in this in vitro system. From the minigene containing intron 25+1A, one clear product was obtained but its size was smaller than expected (fig 2b). Sequencing of the product disclosed the complete absence of exon 25, exemplifying exon 25 skipping in vitro.

In contrast, the minigene encoding intron 45+1A produced three different RT-PCR products in similar amounts (fig 2C). Subcloning and sequencing of the three products disclosed identical sequences joining exon A to exon 45 in their 5′ ends, but their 3′ end sequences were different (fig 2C). The smallest product had precisely the same 32 bp deletion of exon 45 as that found in vivo. This indicated usage of the same cryptic splice site in this in vitro system as that used in vivo. In addition, two other splicing products were found. The middle-sized band contained an additional 139 bp insert between exons 45 and B (fig 2C). The sequence of the 139 bp insert precisely matched the 5′ end of the second intron of the minigene, which consisted of 105 bp of intron 45 and 34 bp of the vector intron. As this product was considered to be a splicing product resulting from activation of a cryptic donor splice site, the second intron sequence was examined further. GT dinucleotides that form a donor splice site (Shapiro probability score: 0.74) were found at 140 bp. Therefore it was determined that this product resulted from the activation of a cryptic donor splice site in the vector intron. The third product contained a 139 bp insertion between exons 45 and B. The inserted sequence precisely matched the entire second intron of the minigene, indicating that the second intron was not spliced out in this product (fig 2C). Even after in vitro splicing of mutant exon 45, exon skipping was not seen. However, the use of cryptic splice sites was seen in this in vitro system similar to the in vivo results.

Although both of the minigenes with intron 25+1A or intron 45+1A had in common an identical cryptic donor splice site in the vector part of the intron, the splice site was used only in the intron 45+1A minigene (fig 2C), but not in the intron 25+1A minigene (fig 2B). This suggested that a factor other than the availability of a splice site consensus sequence might determine the splicing pathway.

From the in vitro results, it was hypothesised that there are unknown factors present in the short inserted sequences. Comparing the two inserts, it was found that the Shapiro probability scores for the acceptor splice sites of the two exons were quite different: the score for exon 25 (0.83) was lower than that for exon 45 (0.92) and below the average score for dystrophin exons (0.87).[20] This suggested the possibility that weak exon recognition as represented by a lower score for the acceptor splice site leads to exon skipping whereas strong recognition facilitates exon retention, thereby promoting cryptic splice-site activation in intron +1G→A mutations.

To test this hypothesis, cases of BMD and DMD with intron +1G→A mutations were identified in the literature and a DMD database. In total,13 mutations in 13 introns were found, in addition to our 2 cases. Of these, 4 cases were due to cryptic splice-site activation, and the other 11 cases were due to exon skipping. Mutations in introns 3, 13, 25, 36, 44, 50, 51, 62, 66, 69 and 70 cause exon skipping,[21 22] and mutations in introns 32,[21 22] 43 (DMD database), 45 (this study) and 65[23] activate cryptic splice sites. In these two categories of mutations (exon skipping and cryptic site activation), the strengths of these acceptor splice sites were compared for all 15 cases. However,the differences in scores alone were not sufficiently large to discriminate the two groups of exons.

Next, exon length was analysed, as this has been reported to be important for exon recognition.[2] Notably, all 4 exons with cryptic splice-site activation were clearly separated from those with exon skipping, all of which had higher acceptor splice-site scores (>0.90) and longer lengths (>170 bp) than the other 11 exons (fig 3). Considering that exonic splicing enhancers or silencers influence exon recognition, the number of these elements associated with each intron as predicted by ACESCAN2 was used as another parameter but it was determined that these do not appreciably affect splicing pathways (data not shown). It was therefore concluded that the combination of high acceptor splice-site score and longer exon length is a major factor determining splicing pathways in intron +1G→A mutations in the dystrophin gene.

DISCUSSION

In this study, we found that availability of a cryptic splice site is not a determinant for splicing pathways of intron +1G→A mutations in the dystrophin gene and it was thus hypothesised that strong exon recognition resulting from the combination of a high acceptor splice-site score and a long exon length is a determining factor for splicing pathways (fig 3). It is possible that this conclusion may only apply in cases of intron +1G→A mutations but not for other mutations that disrupt the donor splice site of the dystrophin gene (data not shown).

The major consequences of mutation at splice sites are exon skipping and cryptic splice-site activation.[4] It has long been suspected that the determination of which of these two outcomes occurs depends upon a variety of factors, including exon and intron length, RNA secondary structure, open reading-frame conservation and the local DNA/RNA sequence context of the affected exon–intron junction.[24–26] In particular, it has been shown that the step that determines whether the mutation causes exon skipping or cryptic splice-site activation is influenced by the relative abundance of alternative splice-site motifs in the local DNA sequence environment of a mutated splice site,[27] and it has been proposed that exon skipping is the preferred phenotype when the immediate vicinity of the affected exon–intron junction is devoid of alternative splice sites.[5] In fact, exon skipping was induced in an intron 25 +1G→A mutation that had no candidate donor splice-site nearby (fig 2). Although the above theory could be applied to explain splicing pathways, our in vitro experiment yielded a result incompatible with this hypothesis; a cryptic donor splice site located in the second intron was not activated in the minigene with intron 25+1A (fig 2B), whereas the identical site used in the minigene with intron 45+1A (fig 2C) did exhibit cryptic splice-site activation. Therefore, it was concluded that mere availability of a cryptic splice site that can be activated by mutation is not sufficient to determine the splicing pathway (fig 2D).

The minigene containing intron 45+1A produced three transcripts: one non-spliced transcript of the second intron and two with activation of one of two cryptic donor splice sites (fig 2D). We hypothesise that the first step is recognition of the 5′ part of exon 45. Next, the splicing mechanism looks for a proper donor splice site. During this search, the mechanism may locate one of the two cryptic splice sites, which would lead to production of the two short transcripts. If the mechanism does not locate a splice site, this would lead to the retention of the full intron (fig 2D).

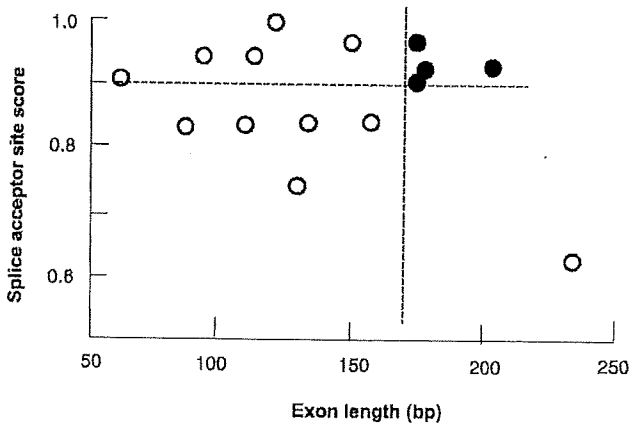

Figure 3 Factors determining exon skipping or cryptic splice-site activation. Each exon was plotted by the acceptor splice-site score on the *y* axis and exon length on the *x* axis. Black circles represent exons that lead to cryptic splice-site activation; open circles represent exons that are susceptible to exon skipping.

Table 2 Exon recognition markers of exons in the central deletion hot spot of the dystrophin gene

Number	Size	Score	I/O
40	153	0.75	I
41	183	0.85	I
42	195	0.80	O
43 *	173	0.96	O
44	148	0.96	O
45 *	176	0.92	O
46	148	0.97	O
47	150	0.91	I
48 *	186	1.00	I
49	102	0.96	I
50	109	0.83	O
51	233	0.83	O
52	118	0.89	O
53	212	0.82	O
54	155	0.59	O
55 *	190	0.96	O
56	173	0.84	O
57	157	0.97	O
58	121	0.93	O
59	269	0.75	O
60	147	0.84	I

I, in frame; O, out of frame.
*Strong exon classified by our criteria.
Exons from 40 to 60 in the central deletion hot spot are listed.

Fifteen intron +1G→A mutations of the dystrophin gene were categorised into two groups based on resulting splicing pathways: 11 mutations were associated with exon skipping and 4 were associated with activation of a cryptic splice site. Exon skipping was more common than cryptic donor splice-site activation in the dystrophin gene mutations. To identify any *cis*-acting elements that may be able to discriminate between these two groups, the two parameters of acceptor splice-site strength and exon length were taken into consideration. Remarkably, all 4 exons exhibiting cryptic splice-site activation had higher acceptor splice site scores (Shapiro score 0.90) and longer exon length (>170 bp) (fig 3) than those of the 11 other exons. It has been reported that skipped exons contain more candidate splicing enhancers and fewer silencers compared with exons with activated cryptic sites.[28] In our study other parameters such as densities of splicing enhancers and silencers in the affected exons and the first 180 nucleotides of the adjacent introns[29] were not influential in determining the splicing pathway (data not shown).

Three transcripts were produced equally by the minigene containing intron 45+1G→A (fig 2C). The lower number of transcripts identified in muscle of the patient with DMD suggested the possibility that transcripts retaining very long introns were not detected. It is possible that these multiple transcripts modify the clinical phenotype of dystrophinopathy such as in cases incompatible with the reading-frame rule.[1]

Induction of exon skipping in the dystrophin gene has been proposed as one of the most promising strategies of DMD treatment.[9 30 31] For this purpose an antisense oligonucleotide has been used to suppress exon recognition by blocking splicing enhancers in exons or by blocking splice-site consensus sequences. Our results suggest that artificial induction of exon

skipping will be difficult in exons with strong exon-recognition markers. Currently, induction of skipping of exons in the central deletion hot spot of the dystrophin gene is under intensive studies.[32–34] To predict efficiency in induction of skipping, exons 43–60 , located in the deletion hot-spot, were examined for their exon recognition markers based on our findings (table 2). Four exons were classified as strong exons. Three of these (exons 43, 45 and 55) are out of frame, and exon 48 is in frame. In treatment of DMD, it is out-of-frame exons that are a target for exon skipping.[50] In artificial induction of skipping of exons 43, 45 and 55 using antisense oligonucleotides against the donor splice site, therefore, cryptic splice-site activation should be expected and unexpected splicing products should be carefully analysed to evaluate the treatment.

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Competing interests: None.

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Letter to the Editor

Wide ranges of serum myostatin concentrations in Duchenne muscular dystrophy patients

Dear Editor,

Duchenne muscular dystrophy (DMD), a common inherited myopathy that affects approximately 1 in 3500 males, is characterized by progressive muscle wasting due to a deficiency in muscle dystrophin. DMD progresses with a rather uniform pattern of muscle weakness: i.e., DMD causes affected individuals to lose their ability to walk by the age of 12 y, and patients succumb during their twenties due to either respiratory or cardiac failure. The deficiency in dystrophin is caused by translational reading frame shift or nonsense mutations in the dystrophin gene [1]. However, the existence of a modifying gene has been suggested by the identification of unusually mild DMD phenotypes [2–4].

Although some phenotypic variability may arise due to environmental factors, such as diet or exercise, genetic components are likely to contribute to this variability. Myostatin, also known as growth and differentiation factor 8 (GDF8), is a muscle-specific secreted peptide that limits muscle growth [5,6]. However, genotyping of the myostatin gene failed to disclose any nucleotide changes that behaved as a phenotypic modifier of DMD [7]. Remarkably, blocking endogenous myostatin has been shown to result in anatomic, biochemical, and physiologic improvements in the dystrophic phenotype of *mdx* mice, a mouse model for DMD, including particularly prominent enlarged fiber diameters and greatly reduced fatty fibrosis [8–10]. These results suggest that blocking endogenous myostatin is a potential strategy to treat DMD [11]. We examined the hypothesis that serum myostatin is increased in DMD, thereby enabling treatment by myostatin blockage.

Forty-one DMD patients followed at Kobe University Hospital were enrolled in this study. All but 1 of the mutations in the dystrophin gene were found to introduce premature stop codons in the dystrophin mRNA: 24 cases with mutations that induced a translational reading frame-shift due to exon deletion or duplication; 5 cases with nonsense mutations; 7 cases with exon mutations involving one or a few nucleotides deleted or inserted; 3 cases with intron mutations that induced splicing errors; and one case with an abnormal chromosome (Table 1). The subjects' ages ranged from 1 to 22 y (average: 8.3 y). Regular clinical checkups, including determination of serum creatine kinase (CK) concentrations, were performed at the outpatient clinic. All protocols were approved by the ethics committee of the Kobe University School of Medicine. Blood samples were taken after written informed consent was obtained from all patients, and serum was separated using a clinical centrifuge.

Serum myostatin was measured using the Human Myostatin ELISA (Prodomain Specific) kit purchased from BioVendor Laboratory Medicine, Inc. (Brno, Czech Republic). The upper limit of determination was 50 ng/ml, and normal adults have serum concentrations of 0.19 to 9.02 ng/ml (BioVendor Laboratory Medicine, Inc.). The Pearson product-moment coefficient was calculated to quantify the relationship.

Serum myostatin concentrations in DMD patients ranged from 1.1 to >50 ng/dl (Table 1). Remarkably, 13 samples were >50 ng/ml, and the lowest concentration was 1.1 ng/ml (Table 1). Though age differences were examined in 2 conditions either including or deleting 13 samples with >50 ng/ml, no significant correlationship between age and serum myostatin concentration was found (Fig. 1). We next examined whether concentrations of myostatin were related to the type or location of mutations in the dystrophin gene. Though serum myostatin concentrations were compared based on their mutation types (exon deletion/duplication or others), no clear difference between two groups was revealed (Fig. 1). There found no significant difference in serum myostatin concentration between patients with mutation in the 5′ and 3′ regions of the dystrophin gene (Table 1).

Considering that myostatin is an inhibitor of muscle growth, cases with high serum myostatin concentrations were predicted to present rather severe phenotypes. Transgenic overexpression of myostatin in mice was shown to result in cachexia [12]. However, the ages when DMD patients with high myostatin concentrations became wheelchair-bound were not different from those of patients with low myostatin concentrations, and signs of muscle weakness appeared mostly between ages 4 and 5. Furthermore, serum CK concentrations were not significantly lower in DMD cases with high myostatin concentrations than in those with low concentrations (Table 1). This indicates that serum myostatin did not modify the DMD phenotype even though blocking endogenous myostatin has been shown to result in improvements in the dystrophic phenotype of *mdx* mice [8–10]. In this study, we measured myostatin that reacted with a monoclonal antibody recognizing the prodomain of myostatin. Considering that myostatin is secreted as an inactive propeptide and is cleaved to produce the active form, further studies would be required to measure active or latent myostatin individually.

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Expression of the myostatin gene has been examined previously in skeletal muscle by measuring mRNA and protein concentrations [13]. Serum myostatin concentration has been determined by the Western blot analysis in a patient with a mutation in the myostatin gene, disclosing an absence of myostatin [14]. Furthermore the Western blot analysis disclosed that serum myostatin concentration was lower than that of rat [14]. But no further study has been conducted on serum myostatin concentrations. Our results disclosed a wide range in serum myostatin concentrations in DMD patients. Though myostatin blockage is attracting attention as a novel target for increasing muscle growth in cases of DMD [11], our results suggest that myostatin blockage therapy would only be effective in DMD cases involving high serum myostatin concentrations. Therefore, myostatin blockage therapy should be applied carefully as a treatment for DMD.

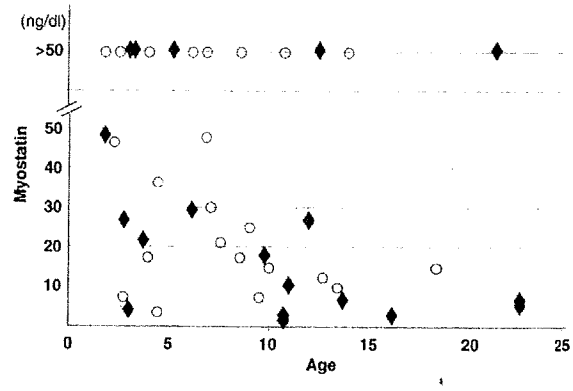

Fig. 1. Serum myostatin concentrations and patients' ages. Serum concentrations of myostatin (vertical axis) are plotted versus patient age (horizontal axis). Open circles and black diamonds represent exon deletion/duplication and other mutations, respectively.

Table 1
List of DMD patients

Case	Age	Mutation	CK (IU/L)	Myostatin (ng/ml)
749	21	ex45–50del	603	>50
643	14	ex57G8460A	3296	>50
765	13	ex51del	2219	>50
225	11	ex41C5899T	2848	>50
300	8	ex2dup	11.560	>50
761	7	ex63 c.9262delA	10.300	>50
344	6	ex46–49dup	20.008	>50
394	5	ex53–54del	22.680	>50
579	4	ex27 3613delG	27.160	>50
767	3	ex51del	10.492	>50
764	2	ex36 5071–2insA	25.040	>50
715	2	c.8669–1G>C	32.700	>50
581	2	ex46–51del	26.488	>50
755	1	ex46–52del	18.640	49.2
434	6	ex25 AGAA3347–50del	20.527	47.8
651	2	ex41C5899T	9860	46.7
736	4	ex2–7dup	8259	35.8
294	6	int17 2168G+1C	27.718	30.2
502	6	ex17del	15.500	30
763	2	ex44del	27.647	28.2
112	12	ex48–50del	2221	25.9
343	8	ex46–49dup	6119	24
570	3	ex46–53del	6389	22.5
481	6	46Y,inv(X)(p21.2 q28)	12,635	20.4
427	10	ex46–48del	8954	18.5
536	7	?	11,760	17.9
414	4	ex38 5434–7delTTCA	22,004	16.7
145	18	ex56dup	1209	15.4
401	10	ex70 C10171T	2528	14.1
13	12	ex2dup	3828	11.6
67	11	ex45–52del	2804	9.9
58	13	ex5 G354A	4669	9.8
453	22	ex10–44del	2899	7
453	22	ex10–44del	3345	6.6
436	9	ex39 T5561del	9160	6.6
641	2	int4 13insGT	16.165	6.3
788	13	ex46–52del	1726	6.2
759	4	ex45–48dup	13.402	3.4
689	2	ex8–24del	25.920	3.2
H.H	16	ex48–50del	1436	2.3
701	10	int45 6641+1G>A	7121	1.9
107	10	ex51–54del	4497	1.1

DMD patients are listed according to their serum myostatin concentrations. Detail mutation of the dystrophin gene is described in addition to their age and serum CK concentration. One question mark indicates an unrevealed mutation (case 536) and one has an abnormal chromosome (case 481).

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Dystrophin Nonsense Mutations Can Generate Alternative Rescue Transcripts in Lymphocytes

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Summary

Secondary alterations in splicing have been reported to produce semi-functional mRNA from several nonsense mutations in the dystrophin gene. Disruptions of exonic splicing enhancers by single nucleotide changes are thought to underlie such alterations. The precise frequencies of such nonsense mutation-dependent splicing alterations, however, remain unknown. Here we analyzed the splicing patterns of dystrophin mRNA in lymphocytes from 38 patients with dystrophinopathies due to nonsense mutations in the dystrophin gene. In seven of the cases (18%), we observed partial skipping of the nonsense-encoding exon. Two of the seven cases, however, exhibited complex activation of a nonsense mutation-created splice site, which resulted in the generation of novel transcripts. Examination of *cis*-regulatory splicing elements through calculation of splicing probability scores and identification of potential splicing enhancer or silencer sequences failed to disclose a single cause for exon skipping. Remarkably, individual differences in splicing patterns were observed for cells from patients with identical nonsense mutations (C.5899C>T). Although five cases produced semi-functional dystrophin mRNAs, only one of these exhibited a mild clinical course. These results provide important insights about targets for exon skipping induced by candidate antisense oligonucleotides and for ribosomal read-through of nonsense mutations.

Keywords: dystrophin, Duchenne muscular dystrophy, nonsense mutation, exon skipping, splicing

Introduction

The splicing machinery that removes introns from pre-mRNA is strictly regulated to avoid aberrant splicing. The locations of splice sites are determined by conserved *cis*-elements or splicing conserved sequences located at intron/exon boundaries and branch points. Splicing enhancer or silencer sequences within exons also regulate the recognition of splice sites (Zhang et al., 2005; Wang et al., 2004). In addition, the translatability of an exon influences its splicing (Zhang et al., 2003). Nonsense-associated alterations in splicing have been shown to occur in minigenes and T-cell receptor transcripts (Li et al., 2002; Wang et al., 2002).

Mutations in the dystrophin gene cause either the severe Duchenne muscular dystrophy (DMD; MIM 310200) or the more benign Becker muscular dystrophy (BMD; MIM 300376). The gene has a complex structure, including a large number of exons, lengthy introns, and several alternative promoters. Therefore, dystrophin pre-mRNA splicing requires strict regulation. Single nucleotide changes creating nonsense mutations in the dystrophin gene, however, can cause secondary splicing alterations, including exon skipping and nonsense mutation-associated splice site activation (Barbieri et al., 1996; Shiga et al., 1997; Melis et al., 1998; Ginjaar et al., 2000; Fajkusova et al., 2001; Disset et al., 2006; Deburgrave et al., 2007; Tran et al., 2007). Exon skipping results in in-frame open reading frames that encode semi-functional dystrophin proteins; these proteins ameliorate the severity of the DMD phenotype (Barbieri et al., 1996; Shiga et al., 1997; Melis et al., 1998; Ginjaar et al., 2000; Fajkusova et al., 2001; Disset et al., 2006; Deburgrave et al., 2007). Nonsense mutation-associated splice site activation, following nonsense mutation of exon 42 of the dystrophin gene, produces a novel mRNA transcript (Tran et al., 2007). Thus, single nucleotide changes resulting in nonsense mutations in the dystrophin gene can alter splicing in two independent ways.

Drug-induced suppression of nonsense mutations by inducing the ribosomal read-through effect is currently being examined as a treatment for DMD patients carrying nonsense mutations in dystrophin. The possibility that dystrophin protein can be produced following the creation of semi-functional mRNA transcripts by altered splicing had not been

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considered during read-through effect stimulation (Howard et al., 2004; Politano et al., 2003). Artificial induction of exon skipping using antisense oligonucleotides specific for the exon sequence has attracted attention as a promising treatment for DMD, as it transforms out-of-frame dystrophin mRNAs into in-frame transcripts, thereby producing semi-functional internally deleted dystrophin protein. Although disruption of the splicing enhancer sequence with antisense oligonucleotides induces exon skipping (Takeshima et al., 1995), methods facilitating the identification of optimal antisense oligonucleotides with the maximal ability to induce exon skipping have not been established. To assist with the identification of ideal target sites for designing antisense oligonucleotides, it is crucial to identify key nucleotides involved in splicing regulation. Nonsense mutations are a good model with which to validate whether single nucleotide changes can alter splicing regulation.

Here we analyzed 38 unique nonsense mutations in the dystrophin gene for their ability to cause alterations in the splicing of dystrophin pre-mRNA isolated from patient lymphocytes. Secondary alterations in splicing were observed in seven cases with the identification of alternative rescue transcripts in five cases.

Cases and Methods

Cases

More than 400 DMD patients from throughout Japan were referred to the specialized clinic for DMD patients at Kobe University Hospital (Kobe, Japan). For each clinically diagnosed case of DMD, extensive testing was performed to identify the causative mutation in the dystrophin gene. Fifty-six cases were determined to have nonsense mutations in the dystrophin gene. We then analyzed dystrophin mRNA expressed by lymphocytes for 38 of these patients as described below. Subjects' ages ranged from two to 32 years old (average: 10 years old). The protocols used in this study were approved by the ethics committee of Kobe University School of Medicine. Blood samples were acquired after obtaining written informed consent.

Analysis of Dystrophin mRNA

Total RNA was isolated from peripheral blood lymphocytes. Dystrophin mRNA transcripts expressed by the lymphocytes were analyzed by reverse transcription (RT)-nested PCR (Roberts et al., 1991; Matsuo et al., 1992). The first amplification utilized an outer set of primers (Table 1). An aliquot of this reaction was then subjected to a secondary amplification using an inner primer set. An aliquot of each of the PCR reactions was separated on an agarose gel (Tran et al., 2006).

After purification, amplified products were subcloned into the pT7 vector (Novagen, Madison, WI); the inserted DNA was sequenced using a Big Dye terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) on an automatic DNA sequencer (ABI PRISM model 310, Applied Biosystems) as described (Surono et al., 1997).

Analysis of Exonic Splicing Enhancers and Silencers

Two web-based algorithms identified exonic splicing enhancers. The ESE finder (http://exon.cshl.edu/ESE/) is a web-based resource that rapidly analyzes exon sequences to identify binding motifs for four serine/arginine-rich (SR) proteins, SF2/ASF, SC35, SRp40, and SRp55 (Cartegni et al., 2003). RESCUE-ESE software (http://genes.mit.edu/burgelab/rescue-ese/) employs a computational method identifying exonic splicing enhancers in human genomic sequences by searching for specific hexanucleotides (Fairbrother et al., 2004).

Exonic splicing silencers were analyzed using the ACESCAN2 web-based computational method (http://genes.mit.edu/acescan2/index.html). All putative splicing enhancer and silencer motifs were identified, allowing us to calculate the density of enhancer or silencer motifs per 100 bp.

Results

After extensive mutation analysis, nonsense mutations in the dystrophin gene were identified in 56 Japanese patients with dystrophinopathies. We analyzed the dystrophin mRNA isolated from 38 of these patients' lymphocytes by RT-PCR analysis to identify secondary alterations in splicing patterns (Table 2). We have previously reported two examples of exon skipping due to nonsense mutations. The c.3631G>T mutation in exon 27 (p.1211E>X) induced partial skipping of exon 27, which resulted in a mild dystrophinopathy phenotype with production of semi-functional dystrophin (Shiga et al., 1997). The c.5899C>T mutation in exon 41 (p.1967R>X) induced the partial skipping of exon 41 (Surono et al., 2004). Although the c.5899C>T mutation was identified in two additional patients with DMD (cases 501 and 651), in both cases, amplification of a fragment spanning dystrophin exons 40 to 45 resulted in a single PCR product (Fig. 1a). Sequencing of this transcript revealed an authentic splicing product containing exons 40 to 45. These results demonstrated that exon 41 was not skipped in two of the three cases carrying the c.5899C>T mutation (Surono et al., 2004). This difference in the splicing patterns suggests individual differences in the splicing regulatory environment.

We also identified a novel nonsense mutation in a patient with DMD (case 496); a c.1793C>G mutation altering a serine-encoding TCA codon to a TGA stop codon (p.598S>X) within exon 15. RT-nested-PCR amplification of a fragment spanning exons 13 to 18 from this template produced two PCR products (Fig. 1b). The larger product was an authentic splicing product containing exons 13 to 18.

Table 1 Primers used to amplify dystrophin cDNA fragments

Exon	Outer primers (exon number)		Inner primers (exon number)	
	Forward	Reverse	Forward	Reverse
5	1A : TTTTTAATCCCTGCCTTGATATACA (1)	1B : ACTCTGCAACACAGCTTCTGAG (18)	1C : ATGCTTTGGTGGGAAGAAGTAG (1)	c8r : GATGGACTTCTTATCTGGATA (8)
8	1A	1B	1C	1D
10	1A	1D : TCCTGTTCCAATCAGCTTACTTC (11)	c7f : GTGGTTTGCCAGCAGTCAGCCACA (7)	1D
14	1A	1B	c13f : GCTGCTTTGGAAGAACAACTT (13)	1F : CTTCTGAGCGAGTAATCCAGCT (18)
15	1A	1B	c13f	1F
17	1A	1B	c13f	1F
18	2A : ACAAGGGAACAGATCCTGGTAA (17)	2B : CTGCTTTTTCTGTACAATCTGACG (33)	2C : AGGCAGATTACTGTGGATTCTGA (17)	2D : CCCACCTTCATTGACACATGTT (25)
19	2A	2B	2C	2D
20	2A	c25r : CCCACCTTCATTGACACTGTT (25)	2C	c21r : TTGTCTGTAGCTCTTTCTCTC (21)
23	2A	2B	c21f : CAACCTCAAATTGAACGATT (21)	
26	2A	2B	2E : GAGCATTGTCAAAGCTAGAGGA (24)	2D
27	2A	2B	c25f : ACTCAGTGGGATCACATGTG (25)	c28r : CAATAACTCATGCCAACATGCCCA (28)
28	2A	2B	2E	c30r : CTCCTGGGCAGACTGGATGC (30)
33	3A : GCTTGAACAGAGCATCCAGTC (30)	3D : TTAAACTGCTCCAATTCCTTCAA (38)	3C : GCCCAAAGAGTCCTGTCTCA (31)	2F : TCCACACTCTTTGTTTCCAATG (32)
34	3A	3B : ACTGGCATCTGTTTTTGAGGAT (45)	3C	3D
38	3A	3B	3E	3D
39	3A	3B	3E	3F : CTTCCCCAGTTGCATTCAAT (45)
41	3A	3B	3E	c41r : TGCGGCCCCATCCTCAGACAA (41)
42	3E : TTTGACCAGAATCTGGACCA (36)	3B	c40f : AGCCTACCTGAGCCCAGAGATG (40)	3F
43	3A	3B	c40f	3F
44	3A	3B	3E	3F
47	4A : TGGCGGCGTTTTCATTAT (44)	4B : CGGAGTGCAGGTTCAATTTT (52)	4C : GCTGAACAGTTTCTCACAAAGACACAA (44)	4B
53	c48f : CAAGGAGAAATTGAAGCTCAA (48)	c56r : GTAACAGGACTGCATCATCG (56)	4E : TGGACAGAACTTACCGACTGG (51)	4B
59	5A : CTATTGCAGGCCCACTTTCCA (57)	5B: TTATTCTGCTCCTTCTTCATCTGTC (79)	5C	c56r
66	5C : CACAGAGCAGCCTTTGGAAG (58)	c72r : TATCATCGTGTGAAAGCTGAG (72)	c64f	5D : TTTCTGCAGCAGCCACTCT (68)
68	c64f: CTCCCAACACTGCAGAAGGC (64)	5F : ATCATCTGCCATGTGGAAAAG (79)	5E	5D
70	c64f	5F	5E	c72r
76	5E : ATTGAGCCAAGTGTCCGG (67)	5F	c70f : CAGGAGAAGATGTTCGAGC (70)	5F

Table 2 Nonsense mutations exhibiting splicing alterations

Case	Exon	Mutation	Aberrant splicing pattern	ESE	ESS	Phenotype
38	5	c.354G>A	No			DMD
571	5	c.355C>T	No	create		DMD
65	8	c.724C>T	No	disrupt	create	DMD
377	10	c.1062G>A	No	disrupt		DMD
313	10	c.1087C>T	No	disrupt		DMD
454	14	c.1684C>T	Yes*			DMD
496	15	c.1793C>G	Yes	create		DMD
582	17	c.2047G>T	Yes	disrupt	disrupt	DMD
264	17	c.2089A>T	No	disrupt	create	DMD
102	18	c.2236G>T	No	disrupt		DMD
78	19	c.2302C>T	No	disrupt		DMD
85	19	c.2365G>T	No	disrupt		DMD
203	20	c.2449C>T	No			DMD
326	23	c.3151C>T	No	disrupt		DMD
54	26	c.3562A>T	No			DMD
28	26	c.3580C>T	No	disrupt		DMD
N1	27	c.3631G>T	Yes	disrupt		BMD
522	28	c.4003G>A	No		disrupt	DMD
652	33	c.4527T>G	No	disrupt		DMD
517	33	c.4666G>T	No		disrupt	DMD
44	34	c.4729C>T	No	disrupt		DMD
52	34	c.4757G>A	No	disrupt		DMD
148	38	c.5350G>T	No	disrupt		DMD
557	39	c.5551C>T	No	disrupt		DMD
577	39	c.5551C>T	No	disrupt		BMD
225	41	c.5899C>T	Yes	disrupt	create	DMD
501	41	c.5899C>T	No	disrupt	create	DMD
651	41	c.5899C>T	No	disrupt	create	DMD
593	42	c.5985T>G	Yes*	disrupt	create	DMD
30	43	c.6283C>T	No			DMD
185	44	c.6432T>A	No	disrupt		DMD
341	47	c.6805C>T	No	disrupt		DMD
475	53	c.7855A>T	No	disrupt		DMD
110	59	c.8745G>T	No	disrupt		DMD
724	66	c.9568C>T	No	disrupt	create	DMD
445	68	c.9851G>A	No	disrupt		DMD
703	70	c.10108C>T	Yes			DMD
N2	76	c.10873C>T	No	disrupt	create	BMD

The mutations are shown in the table with the corresponding effect on the exonic splicing enhancers and silencers. Nonsense mutation-associated splice site activation was observed in two cases (asterisks; cases 454 and 593).

The smaller product, however, lacked exon 15 completely. Thus, the c.1793C>G mutation induced the skipping of exon 15, despite the fact that this nucleotide change was 89 bp downstream of the 5′ end of the exon and did not influence the splicing consensus sequences. The transcript lacking exon 15 was expected to produce an internally-deleted dystrophin protein lacking the in-frame exon 15 and the associated premature stop codon. The phenotype of this patient, however, was severe, classified as DMD (Table 2).

In one patient carrying a c.2047G>T mutation (p.683E>X) within exon 17 (case 582), we observed the partial skipping of exon 17. RT-nested PCR amplification of a fragment spanning exons 13 to 18 generated two PCR products (Fig. 1c). Sequencing of these products determined that the larger, more abundant product contained all six authentic exons, while the smaller product lacked exon 17. As a result of the absence of the 176-bp exon 17, the translational reading frame was disrupted, and no dystrophin protein was expected to be produced.

In a patient carrying a c.9851G>A mutation (p.3284W>X) within exon 68 (case 445), amplification of a fragment from exon 67 to exon 72 produced two products; the larger, more abundant product represented the full-length transcript, while the smaller lacked exon 68 (data not shown). As exon 68 has previously been shown to be alternatively spliced in normal controls (Feener et al., 1989), it was difficult to determine if exon 68 skipping was enhanced by this mutation.

We also observed the skipping of two consecutive exons in dystrophin mRNAs carrying the c.10108C>T mutation (p.3370R>X) within exon 70 (case 703). RT-nested PCR amplification of exons 67 to 72 generated two products (Fig. 1d). The major product represented the authentically spliced transcript, while the minor product lacked both exons 70 and 71. Subcloning and sequencing of the minor product failed to identify any transcripts lacking only exon 70 or exon 71, indicating that exon 70 skipping only occurred in conjunction with the previously reported alternative splicing of exon 71 (Feener et al., 1989).

We previously reported that a nonsense mutation in exon 42 (case 593) generated two altered splicing products: one demonstrating exon 42 skipping and the other incorporating a truncated exon 42 due to activation of a nonsense mutation-created splice acceptor site (Tran et al., 2007). In case 454, the c.1684C>T mutation identified in exon 14 altered a CAA codon to a TAA stop codon (p.562Q>X). Two bands were amplified from RT-nested-PCR of a fragment spanning exons 13 to 18 (Fig. 1e). Sequencing of the smaller product identified three consecutive missing exons (exons 14, 15, and 16). The larger band was a mixture of two products, one with the normal amplified product carrying a stop codon within exon 14 while the second PCR product lacked 22 bp from the 3′ end of exon 14. The truncation in exon 14 was likely due to aberrant splicing, as this unexpected product contained the normal sequences of exons 13 and 15. The c.1684C>T mutation, located 21 bp upstream from the 3′ end of exon 14, created a GT dinucleotide splice donor site. Therefore, the c.1684C>T mutation induced both skipping of three consecutive exons and aberrant splicing due to a nonsense mutation-generated splice donor site.

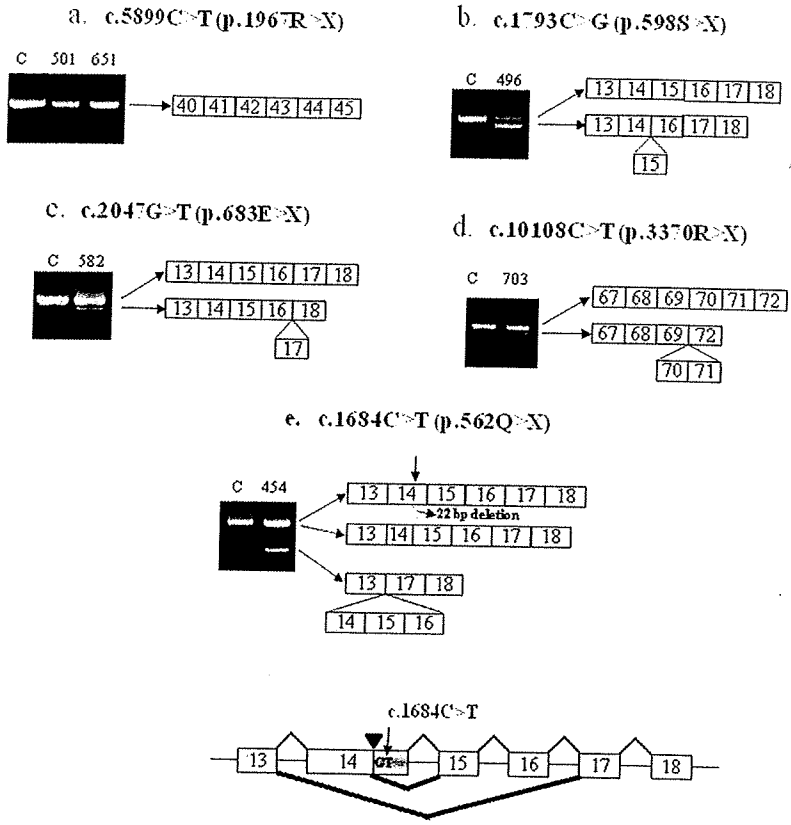

Figure 1 Analysis of dystrophin mRNAs isolated from lymphocytes. Products spanning exons indicated schematically at the right of each panel were amplified from cDNAs prepared from lymphocytes of each patient denoted by the case numbers over the amplified products. In panel e, the splicing patterns identified in the index case are represented schematically (bottom). Skipping of exons 14, 15, and 16 and activation of the splice site created by the mutation (inverted triangle) were observed. The diagonal lines above and below the boxes indicate the authentic and aberrant splicing events, respectively. The boxes and horizontal lines indicate the exons and introns, respectively. The figure is not drawn to scale.

In the remaining 30 cases, we did not detect any additional mRNA products, suggesting that the underlying nonsense mutations did not cause any aberrant splicing. Therefore, seven of the 38 nonsense mutations (18%) altered the splicing of dystrophin mRNA expressed in lymphocytes. The absence of an effect for the majority of nonsense mutations on the splicing pattern indicated that the translatability of the mRNA was not the primary cause determining splicing alterations.

Exons with low Shapiro's probability scores for the splice donor and acceptor sites are prone to exon skipping following single exonic nucleotide substitutions (Cartegni et al., 2002). We therefore calculated the splicing probability scores for the splice donor and acceptor sites of all exons that contained a nonsense mutation. No significant differences in these scores were observed between skipped exons and included exons (data not shown). When the exons were plotted based on their splicing probability scores, the skipped and non-skipped exons were distributed similarly in the plot (Fig. 2). In addition, we identified two exons (exons 17 and 41) that were either included or skipped, depending on the patient. Thus, the strength of the splice site was not a predictor of exon skipping.

Exonic splicing enhancer and silencer sequences also regulate splicing. The nonsense mutations identified in this study may have disrupted exonic splicing enhancers or created splicing silencers in skipped exons. Disruption of a splicing enhancer sequence was identified in four of the seven skipped exons (Table 2). This disruption, however, was also observed at a similar frequency (23/30) in the non-skipped exons (Table 2). Similarly, splicing silencer sequences were created in both skipped exons (two of seven cases) and non-skipped

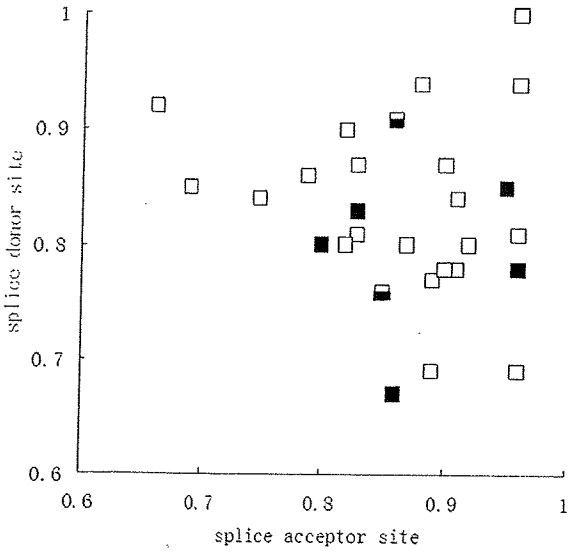

Figure 2 Quantitation of splice site strength. The Shapiro's splicing probability scores for the splice donor (vertical axis) and acceptor (horizontal axis) sites are represented graphically. Closed and open boxes represent the skipped and non-skipped exons, respectively. The half-filled boxes represent exons indicated in Table 2 that were skipped or included in a patient-dependent manner.

exons (six of 30 cases). Therefore, we concluded that splicing alterations are not primarily due to changes in the exon's splicing regulatory motifs.

As nonsense-mediated mRNA decay destabilizes mRNAs containing premature stop codons (Maquat, 2004), it was thought that detection of mRNAs containing nonsense mutations would be difficult (Ito et al., 2003). For the seven mutations producing skipped exons, five of the resulting transcripts contained an intact open reading frame, allowing them to avoid nonsense-mediated mRNA decay. In fact, the detection frequency of exon skipping was higher for in-frame exons (five of 15, 33%) than for out-of-frame exons (two of 13, 15%). This result suggested that detection of exon skipping was influenced in part by mRNA stability.

Although the observed alternative rescue transcripts would be predicted to lead to the production of semi-functional dystrophin in five cases, only one (case N1) exhibited a mild phenotype (Table 2). In the four remaining cases of DMD, dystrophin mRNA expressed in muscle was not analyzed. Rescue transcripts were demonstrated in lymphocytes. Since the splicing and processing patterns of dystrophin mRNA transcripts in myofibres may differ in lymphocytes, it is premature to conclude the effect of the presence of semi-functional dystrophin mRNAs on the DMD phenotype without specifically analyzing muscle dystrophin mRNAs. Semi-functional dystrophin mRNA transcripts may be inferred to modify the phenotype only when they are expressed in skeletal muscle.

Discussion

A few reports of nonsense-associated altered splicing have been reported in the literature (Zatkova et al., 2004; Vuoristo et al., 2004). In dystrophinopathy patients, nonsense mutations that induce exon skipping resulting in semi-functional dystrophin protein have been discovered for several exons of the dystrophin gene; such mutations appear to modify the clinical phenotype (Shiga et al., 1997; Disset et al., 2006; Deburgrave et al., 2007). The use of lymphocyte cells to identify mutations and to determine splicing patterns of transcripts have been established by both our group (Ito et al., 2003, Tran et al., 2007) and others (Tuffery et al., 1996; Barbieri et al., 1996; Roberts et al., 1991; Tay et al., 2006). Accordingly, we have previously shown that the splicing patterns of dystrophin mRNA active in lymphocytes are similar to those in skeletal muscles (Shiga et al., 1997). Similar splicing patterns of dystrophin mRNA have been observed in both lymphocyte and skeletal muscle (Shiga et al., 1997). In our current study, we observed secondary splicing alterations in nearly one-fifth (19%) of transcripts bearing nonsense mutations. The dystrophin splicing pattern in patients carrying nonsense mutations in the dystrophin gene should be analyzed in an attempt to understand those cases exhibiting a mild phenotype (Shiga et al., 1997).

An identical nonsense mutation has been reported to result in different dystrophinopathic phenotypes among siblings (Ginjaar et al., 2000). While this phenotypic difference was explained by differences in dystrophin mRNA splicing, no further examination was conducted. In this study of nonsense mutations in Japanese dystrophinopathy patients, we observed different splicing outcomes for an identical nonsense mutation (c.5899C>T) (Table 2). The c.5899C>T mutation has been identified in eight dystrophinopathy cases worldwide; these patients exhibit diverse phenotypes, ranging from severe DMD to mild BMD (http://www.dmd.nl/dmd_all.htmle. These differences in clinical phenotype may result from differences in rescue transcript production. These data suggest that there are individual differences in the regulation of splicing between patients.

We have described two types of splicing alterations that occur for dystrophin mRNA bearing nonsense mutations: exon skipping and the use of nonsense mutation-created splice sites. Exon skipping was more commonly observed than the generation of nonsense mutation-associated splice sites. Neither maintenance of the open reading frame nor the strength of the splice boundaries surrounding an exon was the primary factor determining exon skipping (Fig. 2). It was surprising that one such mutation induced the skipping of three consecutive