of each nonsense mutation while we used original full-length dystrophin from the genome. Considering that the mechanism of read-through by gentamicin is complicated, we speculate that the differences in our results may be attributed to the different methods. Therefore, our monitoring system is important to identify DMD patients for gentamicin therapy.

Wagner et al. [9] showed that four Duchenne/Becker muscular dystrophies with various stop codons including TGA were treated once daily with intravenous gentamicin at 7.5 mg/kg/day for 2 weeks. The peak serum concentration of gentamicin was 40 μg/ml. However, full-length dystrophin was not detected in pre- and post-treatment muscle biopsies. They concluded that the lack of detection was caused by the lower doses and short duration of gentamicin therapy.

The quantity of dystrophin was calculated by density of band of MHC and dystrophin. The results show that the quantity of dystrophin in differentiated myotubes derived from patient 7 was approximately 36% of non-DMD patients. The results suggested that muscle weakness may be improved by this therapy by the increase in dystrophin.

Fuji et al. (submitted for publication, I. Fuji et al., Adenoviral mediated MyoD gene transfer to human fibroblasts and application to genetic diagnosis of myogenic diseases) showed that dystrophin expression was detected in normal myotubes that differentiated from human fibroblasts 2 weeks after transfection with AdMyoD. The efficiency of differentiation of fibroblasts to myotubes using AdMyoD is approximately 80%, which is much higher than other transfection methods. Although we only obtained an efficiency of 30–40%, this method is still convenient and easy for inducing fibroblasts to differentiate into myotubes in vitro. We therefore, conclude that our system is useful for finding DMD patients eligible for aminoglycoside treatment.

Acknowledgements

The authors thank Ryan Pruchnic for his assistance. This study was supported by the research grant (13B-1, 14B-4) for Nervous and Mental Disorders from the Ministry of Health, Labour, and Welfare, the grant for research in Brain Science from the Ministry of Health, Labour and Welfare, and the grant from the Ministry of Education, Science, Culture and Sports of Japan.

References

[1] Hoffman EP, Brown RHJ, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919–28.

[2] Koenig M, Hoffman EP, Bertelson CJ, Monaca AP, Feener C, Kunkel LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 1987;50:509–17.

[3] Gillard EF, Chamberlain JS, Murphy EG, Duff CL, Smith B, Burghes AH, et al. Molecular and phenotypic analysis of patients with deletions within the deletion-rich region of Duchenne muscular dystrophy (DMD) gene. Am J Hum Genet 1989;45:368–72.

[4] Forrest SM, Cross GS, Flint T, Speer A, Robson KJ, Davies KE. Further studies of gene deletions that cause Duchenne and Becker muscular dystrophies. Genomics 1988;2:109–14.

[5] Lindlof M, Kiuru A, Kaariainen H, Kalimo H, Lang H, Pihko H, et al. Gene deletion in X-linked muscular dystrophy. Am J Hum Genet 1989;44:397–401.

[6] Prior T, Bartolo C, Pearl D. Spectrum of small mutations in the dystrophin coding region. Am J Hum Genet 1995;57:22–33.

[7] Mendell JR, Buzin CH, Feng J, Yan J, Serrano C, Sangani DS, et al. Diagnosis of Duchenne dystrophy by enhanced detection of small mutations. Neurology 2001;28:645–50.

[8] Barton-Davis ER, Cordier L, Shoturma DI, Leland SE, Sweeney HL. Aminoglycoside antibiotics restore dystrophin function to skeletal muscle of mdx mice. J Clin Invest 1999;104:375–81.

[9] Wagner RW, Hamed S, Hadley DW, et al. Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. Ann Neurol 2001;49:706–11.

[10] Howard MT, Shirts BH, Petros LM, Flanigan KM, Gesteland RF, Atkins JF. Sequence specificity of aminoglycoside-induced stop codon read-through: potential implications for treatment of Duchenne muscular dystrophy. Ann Neurol 2000;48:164–9.

[11] Pinney DF, SH P-W, Konieczny SF. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. Cell 1988;53(781):93.

[12] Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108:1933–2000.

[13] Kimura S, Fujishita S, Ikezawa M, Ogawa M, Abe Kand Miike T. Muscle type promoter and its first intron abnormalities in dystrophin gene in patients with Duchenne muscular dystrophy. J Child Neur 1998;13:202–90.

[14] Kimura S, Ikezawa M, Pruchnic R, Balkir L, Qu Z, Lowenstein J, et al. Persistent gene transfer to skeletal muscle mediated by stable transfected early myogenic progenitor cell. Basic Appl Myo 2000;10:237–48.

[15] Andrew HA, Kunkel LM. The structural and functional diversity of dystrophin. Nat Genet 1993;3:283–91.

[16] Lilienbaum A, Li Z, Butler-Browne G, Bolmont C, Grimaud JA, Paulin D. Human desmin gene: utilization as a marker of human muscle differentiation. Cell Mol Biol 1988;34:663–72.

[17] Kaufman SJ, Foster RF. Replicating myoblasts express a muscle-specific phenotype. Proc Natl Acad Sci USA 1988;85:9606–10.

[18] Li Z, Marchand P, Humbert J, Babinet C, Paulin D. Desmin sequence elements regulating skeletal muscle-specific expression in transgenic mice. Development 1993;117:947–59.

[19] Miller JB, Stockdale FE. Developmental origins of skeletal muscle fibers: clonal analysis of myogenic cell lineages based on expression of fast and slow myosin heavy chains. Proc Natl Acad Sci USA 1986;83:3860–4.

[20] Miller JB, Crow MT, Stockdale FE. Slow and fast myosin heavy chain content defines three types of myotubes in early muscle cell cultures. J Cell Biol 1985;101:1643–50.

[21] Holdwe E, Maeda M, Bies RD. Expression and regulation of the dystrophin Purkinje promoter in human skeletal muscle, heart, and brain. Hum Genet 1996;97:232–9.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Genetics and Metabolism 85 (2005) 213–219
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

A G-to-A transition at the fifth position of intron-32 of the dystrophin gene inactivates a splice-donor site both in vivo and in vitro

Hoai Thu Thi Tran [a], Yasuhiro Takeshima [a], Agus Surono [a], Mariko Yagi [a], Hiroko Wada [b], Masafumi Matsuo [a,*]

[a] *Department of Pediatrics, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo, Kobe 650-0017, Japan*
[b] *Department of Pediatrics, Sakura Ryoikuen Hospital, Sanda, Japan*

Received 8 January 2005; received in revised form 11 March 2005; accepted 12 March 2005
Available online 27 April 2005

Abstract

The splicing pattern of pre-mRNA is unpredictable in genes harboring a single-nucleotide change within the consensus sequence of a splice-donor site. In the dystrophin gene, a transition from G to A at the fifth position of intron-32 (4518 + 5G > A) has been reported as a polymorphism within the consensus sequence or a mutation identified in Duchenne muscular dystrophy (DMD). Here, we report both in vivo and in vitro evidence that shows inactivation of the splice-donor site caused by this mutation. In one Japanese DMD case, two novel dystrophin mRNAs were identified in the patient's lymphocytes, one with a 98 bp deletion of the 3′ end of exon-32 (dys32 − 98) and the other with a 28 bp intron retained between exons 32 and 33 (dys32 + 28). Genomic sequencing disclosed a single-nucleotide change from G to A at the fifth position of intron-32 (4518 + 5G > A). To demonstrate in vitro the inactivation of this splice-donor site by this nucleotide change, mini-dystrophin genes comprising three exons harboring either normal or mutant intron-32 sequences were expressed in HeLa cells, and the splicing products were analyzed by reverse-transcription PCR amplification. A normal transcript consisting of three exons was obtained from the normal construct. From the mutant, we obtained one product containing a 98 bp deletion at the 3′ end of exon-32, indicating complete inactivation of the native splice-donor site. Thus, both in vivo and in vitro experiments demonstrate that 4518 + 5G > A causes a splicing error leading to transcript termination; it did not behave like a silent polymorphism. Our results indicate that the in vitro splicing system is a powerful tool for determining the underlying mechanism of a disease-causing mutation in a splicing consensus sequence.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Dystrophin; DMD/BMD; Extra-exon; Splicing; Mutation

Introduction

Splicing is the process by which introns are removed from pre-mRNA. Consensus sequences located at intron/exon boundaries have been extensively characterized; they act as *cis*-elements that define the splice site. Mutations at consensus sequences have been reported to result in exon skipping, activation of cryptic splice sites, or both, producing aberrant mRNAs in all cases [1]. At the splice-donor site at the 5′ end of an intron, the sequence AG/GTAAG (/ denotes the exon/intron border) is the consensus sequence; the five bases in the intronic portion of the splice-donor site base-pair with U1 RNA as a prerequisite step for splicing [2,3]. The GT dinucleotide of the consensus sequence is strictly

* Corresponding author. Fax: +81 78 382 6098.
E-mail address: matsuo@kobe-u.ac.jp (M. Matsuo).

1096-7192/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2005.03.006

conserved, and changes in this sequence uniformly result in splicing errors. In contrast, only 20% of mutations at the +5 position cause aberrant splicing, even though the G at +5 position is very well conserved [1].

Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease, affecting approximately one in 3500 males, and is caused by mutations of the dystrophin gene, the largest human gene, which covers 3000 kb on the X chromosome. The dystrophin gene comprises 79 exons encoding 14 kb of cDNA, such that more than 99% of the sequence are intronic. More than 1000 single-nucleotide changes have been identified as disease-causing mutations (http://www.dmd.nl/, viewed on September 2004). Although most of them are nonsense mutations, nearly 15% are located in splicing consensus sequences, leading to either exon skipping or activation of cryptic splice sites [4–7].

Previous extensive sequence analysis of genomic DNA disclosed two instances of a single-nucleotide change from G to A at the fifth position of intron-32 of the dystrophin gene (4518 + 5G > A); one was a polymorphism (http://www.genomes.utah.edu/DMD/dystrophysnps, viewed on September 2004), whereas the second led to a case of DMD (http://www.dmd.nl/, viewed on September 2004) [8]. Although the latter suggests that 4518 + 5G > A is a disease-causing mutation, no evidence of the mutation affecting the coding sequence was shown. To conclude the 4518 + 5G > A is indeed a disease-causing mutation, evidence of the splicing error caused by 4518 + 5G > A must be provided.

Here, 4518 + 5G > A was first identified in a Japanese DMD patient. Subsequently, both in vivo and in vitro experiments disclosed inactivation of the native splice-donor site by 4518 + 5G > A.

Case and methods

Case

A 12-year-old boy was referred to Kobe University Hospital (KUHG329GA) for a genetic diagnosis of his muscle weakness. At age 1, his serum creatine kinase (CK) level was found to be markedly elevated, and Gower's sign was positive at age 3. At age 4, he was diagnosed with Duchenne muscular dystrophy (DMD) upon demonstration of a lack of dystrophin staining in a muscle biopsy. His muscle weakness progressed, and he was wheelchair-bound at the age of 10. There was no family history of neuromuscular disease. Laboratory examination disclosed an elevated level of serum CK (1646 IU/L, control: <169 IU/L). No abnormalities were detected by either X-ray or ECG examination. The Ethical Committee of Kobe University Graduate School of Medicine approved this study and consent for this study was obtained from his parents.

Methods

Mutation analysis

For mutation analysis of the dystrophin gene, DNA was isolated from peripheral blood of the index case and his family members by standard phenol–chloroform extraction methods. Southern-blot analysis was performed using *Hin*dIII restriction enzyme-digested DNA as a template as described previously [9]. This technique allows for the full extent of any deletions or duplications to be recognized.

To find a mutation not detectable by Southern-blot analysis, the dystrophin mRNA expressed in lymphocytes was analyzed using reverse-transcription nested PCR (RT-nested PCR) as described previously [10]. Full-length dystrophin cDNA was amplified as 10 separate, partially overlapping fragments [11]. A fragment showing an ambiguous sequencing result was further examined by narrowing the amplified region. A region encompassing exons 31–33 was amplified using a forward primer corresponding to a segment of exon-31 (3C = 5′-GCC CAA AGA GTC CTG TCT CA-3′) and a reverse primer complementary to a segment of exon-33 (2B = 5′-CTG CTT TTT CTG TAC AAT CTG ACG-3′).

To characterize the genomic nucleotide change, a region encompassing exon-32 (174 bp of exons 32 and 68, and 145 bp of neighboring introns 31 and 32, respectively) was PCR-amplified from the genomic DNA samples using the following pair of primers: a forward primer on intron-31: g32F = 5′-CAG AAA TAA AGG CAG ATC TAT CAA-3′ and a reverse primer complementary to intron-32: g32R = 5′-CAC AGA ATA GGC CAC AAT AC-3′.

All PCR amplifications were conducted essentially under conditions described previously [10]. PCR-amplified products were purified and subjected to sequencing either directly or after subcloning into T7 blue T vector [12]. Sequence analysis was done using a Dye terminator cycle sequencing kit (Amersham Bioscience, Piscataway, NJ) with an automatic DNA sequencer (model ABI Prism 310, Perkin Elmer Applied Biosystems, Foster City, CA).

In vitro splicing

To study splicing in vitro, three dystrophin exons (18, 19, and 20) and the intervening two introns [13] were inserted into a pcDNA3.0 mammalian expression vector (Invitrogen, Carlsbad, CA) that employs a cytomegalovirus (CMV) enhancer–promoter for high-level expression and contains a polyadenylation signal for complete synthesis of mRNA. To analyze any secondary effects of 4518 + 5G > A on splicing, the sequence of the central exon-19-encompassing region was replaced with that of

exon-32 and its flanking introns. An exon-32-encompassing fragment was amplified from DNA samples of a normal individual or the index case using primers containing restriction-enzyme recognition sites (g32F-*Xma*I = 5′-CgA CCC ggg TCT gTA TAT CTT CAg AAA TAA Agg-3′ and g32R-*Bam*HI = 5′-gCT ggA TCC AgT ATA ATT ATT ATg gTT ATC TgA-3′). After digestion of the amplified product with both *Xma*I and *Bam*HI restriction enzymes, the digested product was inserted into the dystrophin mini-gene that was predigested with the same two restriction enzymes. The resulting mini-dystrophin gene construct was then transfected into HeLa cells.

HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium) dissolved in distilled water (NANO pure DIAMOND VV/UF Water, Barnstead, Dubuque, IA) containing 10% fetal bovine serum (Trace Biosciences, Castle Hill, Australia) at 37 °C under 5% CO_2. The cells were plated on six-well tissue culture plates. One microgram of the plasmid was transfected into semi-confluent HeLa cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA) [13], and the cells were incubated for 24 h. Total RNA was extracted from HeLa cells and 3 μg RNA was subjected to reverse transcription using random hexamer primers as described previously [12]. To amplify the exogenous dystrophin mini-gene transcript, the following primers recognizing the vector/mini-gene junction were used: c18F*Kpn*I = 5′-CGA GGT ACC ACA GCT GGA TTA CTC GCT CA-3′ and c20R*Xho*I = 5′-GCT CTC GAG CAG CCA GTT AAG TCT CTC ACT-3′. PCR products were separated by 3% agarose gel electrophoresis and stained using ethidium bromide. To confirm the nucleotide sequences, each amplified product was excised from the gel and its sequence was determined as described above.

Results

Mutational analysis of the dystrophin gene was performed on genomic DNA of the index case using Southern blot. No gross gene rearrangement was disclosed. Dystrophin mRNA in lymphocytes was amplified as 10 separate fragments by RT-nested PCR amplification. Direct sequencing of these fragments revealed a completely normal sequence, except in the fragment encompassing exons 27–35; therefore, mutational analysis was focused on this region. Unexpectedly, amplification of a fragment from exons 31 to 33 disclosed two amplified products (Fig. 1A′). Subcloning and subsequent sequencing of both products disclosed two novel dystrophin mRNAs; the larger product showed a 28 bp insertion between exons 32 and 33 (dys32 + 28) (Fig. 1B′), whilst the smaller product had a 98 bp deletion of the 3′ end of exon-32 (dys32 − 98) (Fig. 1B′). Both novel dystrophin mRNAs were shown to result in a premature stop codon; this nonsense mutation accords with the lack of dystrophin staining in his muscle. However, the mechanism by which these two mRNAs were produced was not clear.

The 28 bp insertion in dys32 + 28 was found to be homologous to the 5′-end sequence of wild-type intron-32, except for the presence of a G-to-A transition at the fifth position of that intron. To confirm this transition in the patient's genomic DNA, we determined the genomic nucleotide sequence of intron-32 and found the same mutation to be present (4518 + 5G > A) (Fig. 1C′). The complete match of the inserted 28 bp sequence to the genomic sequence led us to conclude that the dys32 + 28 mutation constituted a retention of the 5′ end of intron-32.

These results led us to believe that the two novel dystrophin mRNAs were aberrant splicing products resulting from inactivation of the wild-type splice-donor site by 4518 + 5G > A. To validate this hypothesis, Shapiro's splicing probability score for the splice-donor site [14] was examined for the observed aberrant splice-donor sites (Fig. 2). These scores were found to be 0.68 and 0.82 at the sites 28 bp downstream and 98 bp upstream from the original splice-donor site, respectively; these scores are both higher than that of the mutated original splice-acceptor site (0.66; normal: 0.81), suggesting that the two aberrant dystrophin mRNAs (dys32 + 28 and dys32 − 98) are indeed splicing products that result from the use of cryptic splice-donor sites. Therefore, the in vivo results show that the 4518 + 5G > A mutation causes DMD by activating cryptic splice-donor sites.

Although our results indicated that 4518 + 5G > A is a disease-causing mutation, there has been a report describing 4518 + 5G > A as a polymorphism (http://www.genomes.utah.edu/DMD/dystrophysnps). To confirm the inactivation of the splice-donor site by 4518 + 5G > A, an in vitro splicing system was employed in which the dystrophin mini-gene was subjected to splicing in HeLa cells. Mini-gene constructs consisting of three dystrophin exons (18, 32, and 20) and the intervening intronic sequences containing either wild-type or mutant intron-32 sequences were prepared (Fig. 3) and transfected into HeLa cells. The mini-gene transcript was analyzed by RT-PCR using primers recognizing vector and exon junctions. When the construct containing the normal intron-32 sequence was transfected, one splicing product consisting of exons 18, 32, and 20 was obtained, indicating that splicing from the mini-gene transcript had occurred normally. On the other hand, a single smaller-sized amplified product was obtained from the mini-gene transcript containing the mutant intron-32 sequence (Fig. 3). Sequence analysis of the product disclosed that 98 bp of the 3′ end of exon-32 were deleted in this tran-

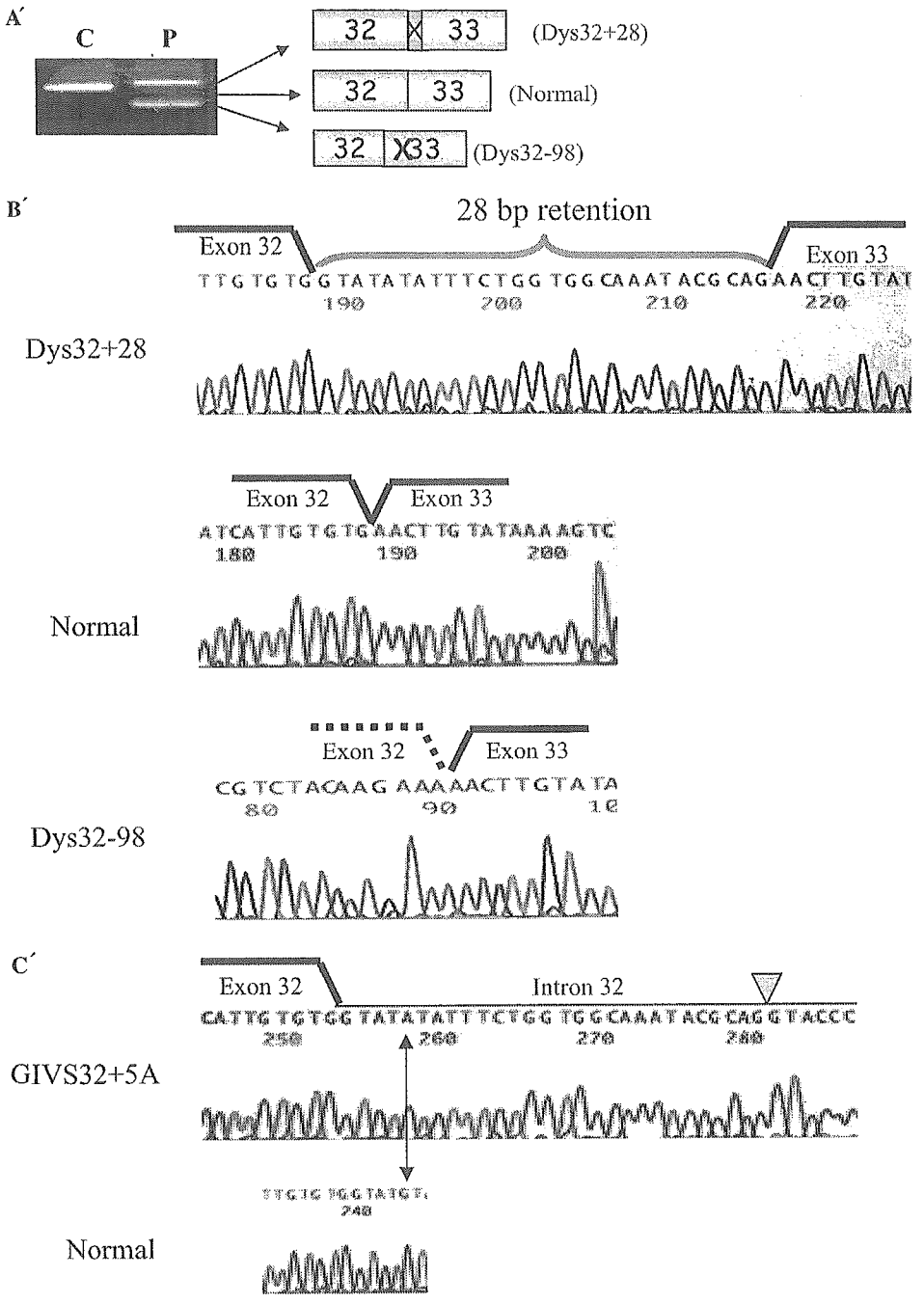

Fig. 1. Mutation analysis of the dystrophin gene. (A′) RT-PCR products. A fragment encompassing exons 31–33 was amplified from the patient's cDNA. One clear band was visualized from the normal (C), whereas two bands were visualized in the index case (P). The upper band contains an extra 28 bp between exons 32 and 33 (dys32 + 28), creating a stop codon in its sequence (X). In contrast, the lower band has a 98 bp deletion of the 3′ end of exon-32 (dys32 – 98), creating a stop codon in exon-33 (X). The structure of the product is shown schematically on the right side. Boxes and the numbers in boxes indicate the exon and exon number, respectively. The box inserted between exons 32 and 33 is the 28 bp of inserted sequence. (B′) cDNA sequences. Each sequence showed completely normal exons 31 and 33. The sequence of the 3′ end of exon-32 (5′-GTGTG-3′) is joined to two different sequences: GTATA (top) and AACTT (middle). At top, the 28 bp insertion was followed by a completely normal exon-33. In the bottom panel, 98 bp of the 3′ end of exon-32 were deleted, and the truncated exon-32 joined directly to exon-33. (C′) Intron-32 sequences. The PCR product amplified from genomic DNA was directly sequenced. The fifth nucleotide from the 5′ end of intron-32 was A in the index case (AIVS32 + 5) (upper) and G in the control (GIVS32 + 5) (lower). The inverted triangle indicates the activated cryptic splice-donor site.

script; all other sequences were normal. The sequence of the 98 bp deletion matched perfectly to that identified in the patient's lymphocytes (dys32 – 98). Neither the normal nor the dys32 + 28 transcript could be identified. These results showed that 4518 + 5G > A inactivates the original splice-donor site completely.

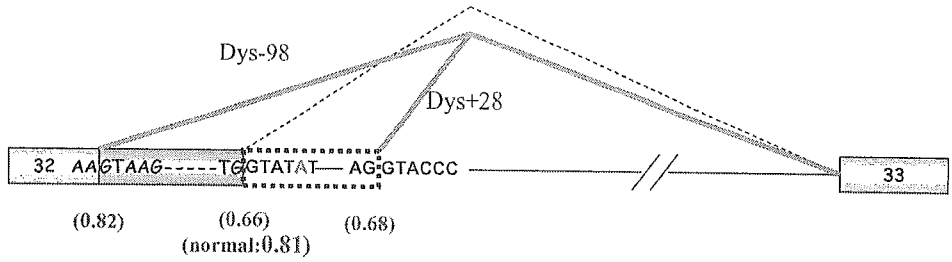

Fig. 2. Splicing patterns of intron-32 and cryptic splice-donor sites near the original splice-donor site. Two novel dystrophin mRNAs are produced by activating two cryptic splice-donor sites in 4518 + 5G > A. Splicing patterns identified in the index case are represented schematically (two solid diagonal lines; Dys + 28 and Dys – 98). One dotted line indicates a normal splicing pattern. Boxes and horizontal lines indicate exons and introns, respectively. The numbers in parentheses indicate the Shapiro's probability scores. The figure is not drawn to scale.

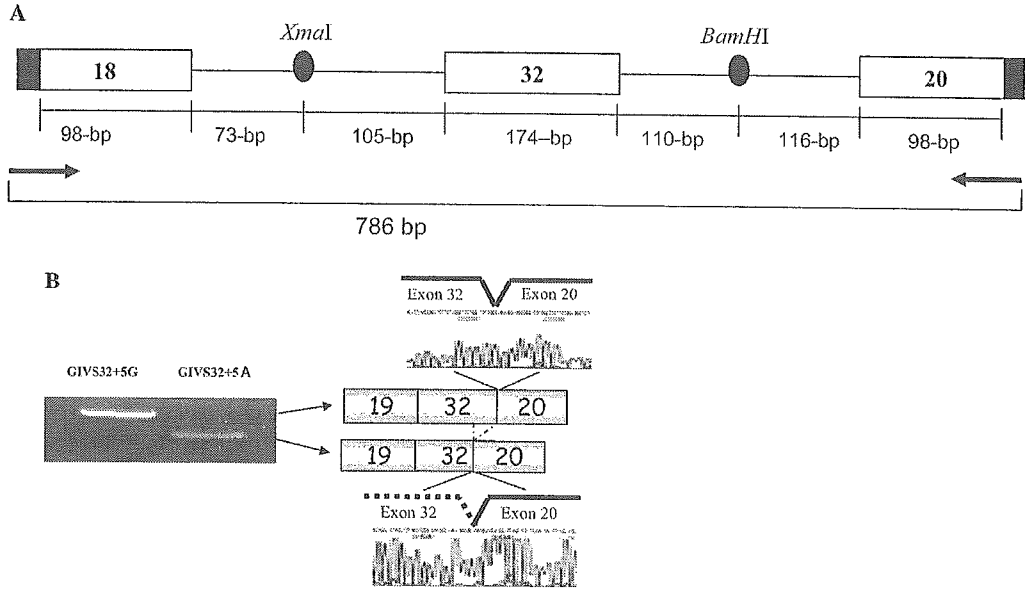

Fig. 3. In vitro splicing. (A) Structure of the mini-dystrophin gene. The mini-dystrophin gene contains sequences corresponding to exons 18 (98 bp), 32 (174 bp), and 20 (98 bp), and portions of introns 18 (73 bp) and 32 (105 bp) between exons 18 and 32, and introns 32 (110 bp) and 20 (116 bp) between exons 32 and 20, respectively. *Xma*I and *Bam*HI represent *Xma*I and *Bam*HI restriction-enzyme recognition site, respectively. Horizontal arrows indicate the positions and the directions of the primers. The bracket indicates the amplified product (786 bp). (B) RT-PCR products. The mini-gene transcript was analyzed by RT-PCR amplification of a fragment encompassing two vector–cDNA junctions. One amplified product was obtained each from the normal (GIVS32 + 5) and mutant (AIVS32 + 5), but the size differed clearly (left). Sequencing of the normal product disclosed the presence of exons 18, 32, and 19. From the mutant, however, 98 bp at the 3′ end of exon-32 were found to disappear, and the resulting truncated exon-32 was inserted between exons 19 and 20 (left).

Combining both the in vivo and in vitro results, we conclude that 4518 + 5G > A is a disease-causing mutation responsible for DMD.

Discussion

In this report, a G-to-A transition at the fifth nucleotide of intron-32 of the dystrophin gene (4518 + 5G > A) is shown to be a disease-causing mutation. Both in vivo and in vitro analyses disclosed complete inactivation of the original splicing donor site (Figs. 1 and 3). Though skipping of an upstream exon is a common outcome of a mutation of the consensus sequence of the splice-donor site [1], exon-32 skipping was not demonstrated in our patient. Instead, cryptic splice-donor activation was disclosed, and the resulting two dystrophin mRNAs were found to encode a premature stop codon (Fig. 1). Therefore, 4518 + 5G > A was concluded to be a disease-causing mutation.

Our conclusion was corroborated by in vitro splicing experiments showing inactivation of the original splice site (Fig. 3). Although two cryptic splice-donor sites were shown to be activated in vivo, only one was found in vitro. The limited activation of one cryptic splice site in vitro may be due to environmental differences in the splicing machinery, *including tissue difference and differences in pre-mRNA secondary structure or in the size of intron-32.* As demonstrated here, our current mini-gene is a powerful tool for confirming disease-causing mutations in a splicing consensus sequence.

Because some of the introns in the dystrophin gene are extraordinarily large (more than 100 kb), splicing of dystrophin pre-mRNA must be regulated by a very sophisticated mechanism. Nucleotide changes at the fifth position within introns of the dystrophin gene have been identified in 10 cases. Their resulting splicing patterns are described in several ways: five result in upstream exon skipping, one gives activation of nearby cryptic splice-donor sites, one results in both exon skipping and cryptic splice-site activation, one is a silent polymorphism, and the others were identified in DMD cases without examination of the mRNA transcripts. Our results add another example of cryptic splice-donor site activation. Although most previously described splice-site mutations induce exon skipping, 4518 + 5G > A induced cryptic splice-site activation only. It remains unclear what factors determine the splicing pattern when the fifth position within an intron is mutated.

At this moment, there is no effective way to treat DMD. Exon skipping of nonsense mutations present in exons 27, 29, or 72 is known to convert severe DMD to mild BMD [15–17]. Therefore, artificial induction of exon skipping is one candidate for alternative gene therapy for DMD, and antisense oligonucleotides targeting either splicing enhancer sequences or splicing consensus sequences have been reported to induce exon skipping successfully [18–23]. Our results, which disclose activation of nearby cryptic sites due to inactivation of the wild-type splice-donor site, suggest that antisense oligonucleotides against splicing consensus sequence induce not only exon skipping but also cryptic splice-site activation. Therefore, this possibility should be considered when a strategy for DMD treatment targeting splicing consensus sequences with antisense oligonucleotides is employed.

Acknowledgments

We thank Miss A. Hosoda for her help in preparing the manuscript. This work was supported by a grant from the Ministry of Education, Science, Sports, and Culture of Japan, and a Research Grant (14B-4) for Nervous and Mental Disorders from the Ministry of Health, Labor, and Welfare, Japan.

References

[1] K. Nakai, H. Sakamoto, Construction of a novel database containing aberrant splicing mutations of mammalian genes, Gene 141 (1994) 171–177.

[2] C.J. Alvarez, J.A. Wise, Activation of a cryptic 5′ splice site by U1 snRNA, RNA 7 (2001) 342–350.

[3] I. Carmel, S. Tal, I. Vig, G. Ast, Comparative analysis detects dependencies among the 5′ splice-site positions, RNA 10 (2004) 828–840.

[4] Y. Hagiwara, H. Nishio, Y. Kitoh, Y. Takeshima, N. Narita, H. Wada, M. Yokoyama, H. Nakamura, M. Matsuo, A novel point mutation (G^{-1} to T) in a 5′ splice donor site of intron 13 of the dystrophin gene results in exon skipping and is responsible for Becker muscular dystrophy, Am. J. Hum. Genet. 54 (1994) 53–61.

[5] M. Sironi, A. Bardoni, G. Felisari, R. Cagliani, M. Robotti, G.P. Comi, M. Moggio, N. Bresolin, Transcriptional activation of the non-muscle, full-length dystrophin isoforms in Duchenne muscular dystrophy skeletal muscle, J. Neurol. Sci. 186 (2001) 51–57.

[6] K. Adachi, Y. Takeshima, H. Wada, M. Yagi, H. Nakamura, M. Matsuo, Heterogous dystrophin mRNAs produced by a novel splice acceptor site mutation in intermediate dystrophinopathy, Pediatr. Res. 53 (2003) 125–131.

[7] M. Yagi, Y. Takeshima, H. Wada, H. Nakamura, M. Matsuo, Two alternative exons can result from activation of the cryptic splice acceptor site deep within intron 2 of the dystrophin gene in a patient with as yet asymptomatic dystrophinopathy, Hum. Genet. 112 (2003) 164–170.

[8] S. Tuffery-Giraud, C. Saquet, S. Chambert, B. Echenne, J. Marie Cuisset, F. Rivier, M. Cossee, C. Philippe, N. Monnier, E. Bieth, D. Recan, M. Antoinette Voelckel, S. Perelman, J.C. Lambert, S. Malcolm, M. Claustres, The role of muscle biopsy in analysis of the dystrophin gene in Duchenne muscular dystrophy: experience of a national referral centre, Neuromuscul. Disord. 14 (2004) 650–658.

[9] M. Koenig, E.P. Hoffman, C.J. Bertelson, A.P. Monaco, C. Feener, L.M. Kunkel, Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals, Cell 50 (1987) 509–517.

[10] M. Matsuo, T. Masumura, H. Nishio, T. Nakajima, Y. Kitoh, T. Takumi, J. Koga, H. Nakamura, Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy Kobe, J. Clin. Invest. 87 (1991) 2127–2131.

[11] R.G. Roberts, T.F. Barby, E. Manners, M. Bobrow, D.R. Bentley, Direct detection of dystrophin gene rearrangements by analysis of dystrophin mRNA in peripheral blood lymphocytes, Am. J. Hum. Genet. 49 (1991) 298–310.

[12] A. Surono, Y. Takeshima, T. Wibawa, M. Ikezawa, I. Nonaka, M. Matsuo, Circular dystrophin RNAs consisting of exons that were skipped by alternative splicing, Hum. Mol. Genet. 8 (1999) 493–500.

[13] Y. Takeshima, H. Nishio, H. Sakamoto, H. Nakamura, M. Matsuo, Modulation of in vitro splicing of the upstream intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe, J. Clin. Invest. 95 (1995) 515–520.

[14] M.B. Shapiro, P. Senapathy, RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression, Nucleic Acids Res. 15 (1987) 7155–7174.

[15] N. Shiga, Y. Takeshima, H. Sakamoto, K. Inoue, Y. Yokota, M. Yokoyama, M. Matsuo, 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, J. Clin. Invest. 100 (1997) 2204–2210.

[16] I.B. Ginjaar, A.L. Kneppers, J.D. vd Meulen, L.V. Anderson, M. Bremmer-Bout, J.C. van Deutekom, J. Weegenaar, J.T. den Dunnen, E. Bakker, Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family, Eur. J. Hum. Genet. 8 (2000) 793–796.

[17] M.A. Melis, F. Muntoni, M. Cau, D. Loi, A. Puddu, L. Boccone, A. Mateddu, C. Cianchetti, A. Cao, Novel nonsense mutation (C–>A nt 10512) in exon 72 of dystrophin gene leading to exon skipping in a patient with a mild dystrophinopathy, Hum. Mutat. (Suppl. 1) (1998) S137–S138.

[18] Z.A. Pramono, Y. Takeshima, H. Alimsardjono, A. Ishii, S. Takeda, M. Matsuo, Induction of exon skipping of the dystrophin

transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence, Biochem. Biophys. Res. Commun. 226 (1996) 445–449.

[19] Y. Takeshima, M. Yagi, Y. Ishikawa, Y. Ishikawa, R. Minami, H. Nakamura, M. Matsuo, Oligonucleotides against a splicing enhancer sequence led to dystrophin production in muscle cells from a Duchenne muscular dystrophy patient, Brain Dev. 23 (2001) 788–798.

[20] J.C.T. van Deutekom, M. Bremmer-Bout, A.A.M. Janson, I.B. Ginjaar, F. Baas, J.T. den Dunnen, G.J. van Ommen, Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells, Hum. Mol. Genet. 10 (2001) 1547–1554.

[21] J.C. van Deutekom, G.J. van Ommen, Advances in Duchenne muscular dystrophy gene therapy, Nat. Rev. Genet. 4 (2003) 774–783.

[22] Q.L. Lu, C.J. Mann, F. Lou, G. Bou-Gharios, G.E. Morris, S.A. Xue, S. Fletcher, T.A. Partridge, S.D. Wilton, Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse, Nat. Med. 9 (2003) 1009–1014.

[23] M. Yagi, Y. Takeshima, A. Suruno, M. Takagi, M. Koizumi, M. Matsuo, Chimeric RNA and 2′-*O*,4′-*C*-ethylene-bridged nucleic acids have stronger activity than phosphorothioate oligodeoxynucleotides in induction of exon-19 skipping in dystropin mRNA, Oligonucleotides 14 (2004) 33–40.