

demonstrate that there is a racial difference in the spectra of these polymorphisms.

It is remarkable that four of the 102 patients were heterozygous for p164E>K, which corresponds an allele frequency of 2.0% in the Japanese patients (Table 1). The p164E>K allele was identified in only 2 out of 189 individuals in the USA, and was not observed in 120 Italians and 57 Belgians [15-17]. In one of the Japanese DMD patients carrying p.164E>K, a second p.156L>I mutation was found in the same allele (Fig. 3). Considering that p.164E>K was identified in both the Americans and Japanese populations, p.164E>K may be an old polymorphism that originated in a common ancestor of the two populations or the polymorphism is located at hot spot for nucleotide changes.

### Conclusion

The present study, although limited to DMD cases, showed the rare occurrence of mutations in the myostatin gene in Japanese subjects. Our results indicate that heterozygous missense polymorphisms including two novel mutations did not produce an apparent increase in muscle volume or strength in Japanese DMD cases, even in a patient carrying two amino acid substitutions.

### List of abbreviations

DMD: Duchenne muscular dystrophy

### Competing interests

The author(s) declare that they have no competing interests.

### Authors' contributions

AN carried out the molecular genetic studies and drafted the manuscript. YT carried out the clinical and molecular genetic studies. KS and AN participated molecular genetic study. YO and MY participated in clinical examinations. MM conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

### Acknowledgements

We would like to acknowledge Ms. A. Hosoda for her secretarial help. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Health and Labor Research Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Research on Psychiatric and Neurological Diseases and Mental Health); a Research Grant for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare; and the Mitsubishi Foundation.

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### Pre-publication history

The pre-publication history for this paper can be accessed here:

<http://www.biomedcentral.com/1471-2350/8/19/prepub>

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## Identification of seven novel cryptic exons embedded in the dystrophin gene and characterization of 14 cryptic dystrophin exons

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Received: 2 April 2007 / Accepted: 10 May 2007 / Published online: 20 June 2007  
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**Abstract** The dystrophin gene, which is mutated in Duchenne and Becker muscular dystrophy, is characterized by its extremely large introns. Seven cryptic exons from the intronic sequences of the dystrophin gene have been shown to be inserted into the processed mRNA. In this study, we have cloned seven novel cryptic exons embedded in dystrophin introns that were amplified from dystrophin mRNA isolated from lymphocytes. All of these sequences, which ranged in size from 27 to 151 bp, were found to be cryptic exons because they were completely homologous to intronic sequences (introns 1, 18, 29, 63, 67, and 77), and possessed consensus sequences for branch points, splice acceptor sites, and splice donor sites. Compared with the 77 authentic dystrophin exons, the 14 cryptic exons were characterized by (1) lower Shapiro's splicing probability scores for the splice donor and acceptor sites; (2) smaller and larger densities of splicing enhancer and silencer motifs, respectively; (3) a longer distance between the putative branch site and the splice acceptor site; and (4) with one exception, the introduction of premature stop codons into their respective transcripts. These characteristics indicated that the cryptic exons were weaker than the authentic exons. Our results suggested that a mutation deep within an intron that changed these parameters could cause dystrophinopathy. The cryptic exons identified provide areas that should be examined for the detection of mutations in the dystrophin gene, and they may help us to understand the roles of large dystrophin introns.

**Keywords** Dystrophin · *Cryptic exon* · mRNA · Splicing

### Introduction

The human dystrophin gene, which is defective in patients with Duchenne or Becker muscular dystrophy (DMD/BMD), is the largest human gene; this gene spans more than 3,000 kb on the X-chromosome and encodes a 14-kb transcript that consists of 79 exons (Ahn and Kunkel 1993; Nishio et al. 1994). Therefore, some of the introns in the dystrophin gene are exceptionally large, covering more than 100 kb (Den Dunnen et al. 1989). However, the physiological roles of these introns are largely unknown, although fragments have been reported to contain alternative promoters and promoter-specific exons (Klamut et al. 1990; Boyce et al. 1991; Gorecki et al. 1992; Byers et al. 1993; Lederfein et al. 1993; Lidov et al. 1995).

Splicing, a process that removes intron sequences from pre-mRNA, is highly regulated to ensure the introns are removed and an ordered array of exons is assembled in mature transcripts. Splicing relies on the correct identification of exons, which must be recognized within pre-mRNA despite being relatively short compared to intronic regions. It is known that the presence of well-defined *cis*-elements, namely the splice donor and acceptor sites and the branch point, are necessary but not sufficient to define intron–exon boundaries (Senapathy et al. 1990). Other splicing regulatory elements include splicing enhancers and silencers in either exon or intron sequences (Ladd and Cooper 2002; Pozzoli and Sironi 2005). In the normal splicing reaction, introns are spliced out after the formation of a lariat that joins the 5' end of an intron to the branch point. Some controversy, however, remains regarding the

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mechanism for splicing out large introns. The resplicing theory states that a large intron is spliced out by repeatedly splicing out shorter fragments. Evidence in support of this theory comes from the splicing of the Ultrabithorax gene of *Drosophila melanogaster* (Hatton et al. 1998). In human genes, however, no evidence supporting this theory has been reported.

Intronic DNA frequently encodes potential exonic sequences called pseudoexons that are not recognized by the splicing machinery (Sun and Chasin 2000). A subpopulation of pseudoexons can become splicing competent after subtle nucleotide changes. Indeed, it has been reported that single base-pair mutations or microdeletions deep within intronic regions can define novel exons by altering pseudoexon sequences without creating novel splice sites (Metherell et al. 2001; Ishii et al. 2002; Pagani et al. 2002).

Part of the 190-kb intron 1 of the dystrophin gene was found to be inserted into dystrophin transcripts in lymphocytes and was named exon 1a (Fig. 1a) (Roberts et al. 1993). The physiological role of cryptic exon 1a, which is inserted between exons 1 and 2 and encodes an in-frame stop codon, remains uncharacterized. Subsequently, six additional cryptic exons (exons 2a, 2b, 2c-1, 2c-s, 3a-1, and 3a-s) were disclosed in introns in the 5' region of the dystrophin gene (Fig. 1a) (Dwi Pramono et al. 2000; Suminaga et al. 2002; Tran et al. 2005; Ishibashi et al. 2006). Because all of these exons encode a stop codon, their protein-coding abilities were not clear. Recently, a number of noncoding RNAs that are transcribed from the human genome have been cloned, which has shed new light on noncoding RNA (Carninci et al. 2005; Claverie 2005). In this study, we have identified seven novel cryptic dys-

trophin exons, and the characteristics of all 14 cryptic exons were compared with those of the 77 authentic dystrophin exons.

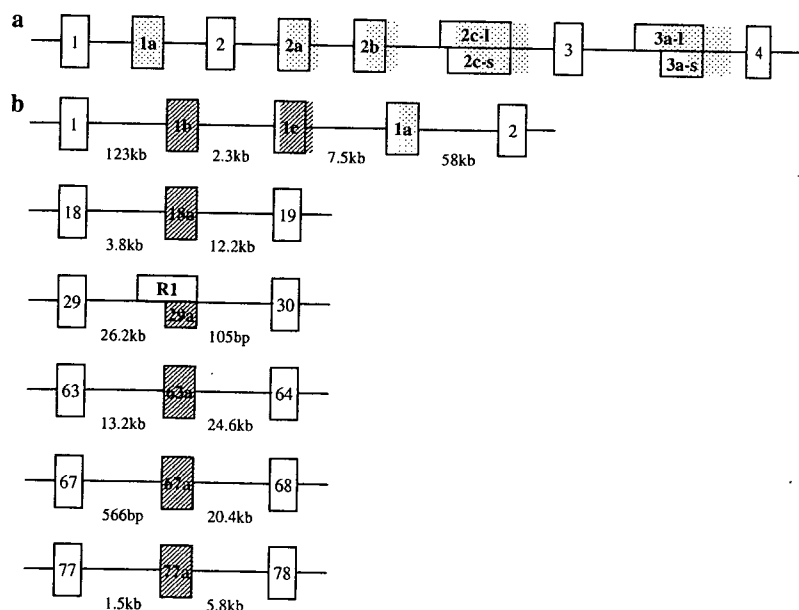
## Cases and methods

### Cases

More than 400 DMD patients from all over Japan were referred to the clinic for DMD patients at the Kobe University Hospital (Kobe, Japan). In clinically diagnosed cases of DMD, we attempted to identify the mutation in the dystrophin gene that was responsible. Genomic DNA was first analyzed by either conventional PCR amplification of the 19 deletion-prone exons of the dystrophin gene (Chamberlain et al. 1988; Beggs et al. 1990) or Southern blot analysis using *HindIII*-digested genomic DNA and amplified dystrophin cDNA as a probe (Mitsubishi Kagaku BCL Co., Tokyo, Japan) (Koenig et al. 1987). Then the 79 exons of the dystrophin gene were PCR amplified and directly sequenced.

In the cases that did not have any recognizable mutations in the analyzed genomic DNA, dystrophin mRNA expressed in lymphocytes was analyzed as described below. In some cases, dystrophin mRNA in lymphocytes was analyzed to clarify the molecular pathogenesis of the dystrophinopathy (Barbieri et al. 1996; Shiga et al. 1997; Adachi et al. 2003; Thi Tran et al. 2005). Consent for this study was obtained from the patient's parents, and the ethics committee of the Kobe University Graduate School of Medicine approved the study.

**Fig. 1a–b** Cryptic exons in the dystrophin gene. Seven previously described cryptic exons and seven novel cryptic exons are schematically described in panels a and b, respectively. Boxes and lines represent exons and introns, respectively. Dotted and shaded boxes represent known and novel exons, respectively. Numbers in the boxes indicate the exon numbers. Numbers between the boxes in b indicate distances from the known exons



### Analysis of dystrophin mRNA

Total RNA was isolated from the peripheral lymphocytes of DMD patients and a control subject. Total RNA from human heart, lung, prostate, and skeletal muscle were purchased from BD Biosciences (San Jose, CA, USA). cDNA was synthesized as described previously (Matsuo et al. 1991). The entire dystrophin cDNA was analyzed by amplifying ten separate fragments using reverse-transcription (RT)-nested PCRs (Roberts et al. 1991). To identify splicing errors, a second PCR amplification of exons 1 to 11, 18 to 21, 25 to 30, 61 to 64, 66 to 72, or 70 to 79 was performed using primers (Hokkaido System Science Co., Sapporo, Japan) designed from the respective exons (Table 1a). PCR reactions (20  $\mu$ l) contained 1  $\mu$ l of cDNA, 2  $\mu$ l of 10 $\times$  Ex Taq buffer, 1 U of Ex Taq polymerase (Takara Bio Inc., Kyoto, Japan), 200 nM for each primer, and 250 mM dNTPs. The first amplification was performed using an outer set of primers and 30 cycles of 94  $^{\circ}$ C for 1 min, 58  $^{\circ}$ C for 1 min, and 72  $^{\circ}$ C for 3 min. A 1- $\mu$ l sample of this reaction was then subjected to a second amplification using an inner set of primers and 30 cycles of 94  $^{\circ}$ C for 30 s, 60  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 1 min. A 5- $\mu$ l sample of each PCR reaction was analyzed on a 2% NuSieve agarose gel containing 0.2 mg/ml ethidium bromide prior to photography.

For DNA sequencing, amplified products were purified, subcloned into the pT7 vector (Novagen, Madison, WI, USA), and the inserted DNA was sequenced with a Big Dye terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ, USA) using an automatic DNA

sequencer (ABI PRISM model 310, Applied Biosystems, Foster City, CA, USA), as described previously (Suroño et al. 1997).

### Analysis of genomic DNA

In order to identify genomic nucleotide changes that enhanced the incorporation of an intron fragment into the spliced mRNA, the cryptic exon and its flanking regions were examined by PCR amplification and direct sequencing. Cryptic exon-encompassing regions were PCR-amplified using primers designed from the flanking intron sequences (Table 1b); the conditions used have been described previously (Matsuo et al. 1990). The amplified product was directly sequenced by the method described above.

### Characterization of cryptic exons

Shapiro's splicing probability scores for the splice acceptor and donor sites were calculated for the 5' and 3' ends of the inserted sequences, respectively (Shapiro and Senapathy 1987). The splicing enhancer and silencer sequences were analyzed using the ACESCAN2 web server (<http://genes.mit.edu/acescan2/index.html>), which is an online tool for identifying candidate *cis*-elements in the splicing of mammalian exons. The densities of the enhancer or silencer motifs were obtained by counting the number of elements in each 100 bp of genomic sequence. Putative splicing enhancer and silencer motifs were predicted and counted.

**Table 1** Primer sequences

Target region	Forward primer	Reverse primer
<b>(a) cDNA analysis</b>		
Exon 1-11	1C: 5' ATGCTTTGGTGGAAGAAGTAG 3'	1D: 5' TCCTGTTCCAATCAGCTTACTTC 3'
Exon 1b-1a	1b-F: 5' GCGGATGGATCATTTGAAGT 3'	1a-R: 5' GGACAAGGTATGAGGCCAGA 3'
Exon 18-21	c18f: 5' GCAGAGTCCTGAATTTGCAATC 3'	c21r: 5' TTGTCTGTAGCTCTTTCTCTC 3'
Exon 25-30	c25f: 5' ACTCAGTGGGATCACATGTG 3'	c30r: 5' CTCCTGGGCAGACTGGATGC 3'
Exon 61-64	c61f: 5' GCCGTCGAGGACCGAGTCAGGCAGCT 3'	c64r: 5' CTTCGGAGTTTCATGGCAGT 3'
Exon 66-72	5E: 5' ATTGAGCCAAGTGTCCGG 3'	c72r: 5' TATCATCGTGTGAAAGCTGAG 3'
Exon 70-79	c70f: 5' CAGGAGAAGATGTTTCGAGAC 3'	5F: 5' ATCATCTGCCATGTGGAAAAG 3'
<b>(b) Genomic DNA analysis</b>		
Exon 1b	5' CAATTGCAAGGTGCTTCCAT 3'	5' TCTCCTGTCTCAGCCTCCAG 3'
Exon 1c	5' GCCAGCTCTACTCAATTCTGG 3'	5' GGCACCTCAATTCGCATAGAAA 3'
Exon 18a	5' TTTCAAGCTTTTGTGGGGTA 3'	5' GGAACAAATGGCCAAGAATG 3'
Exon 29a	5' TCTCACTGGTGGAAAGCAGATT 3'	5' GCCAACTGCTTGTAATGAA 3'
Exon 63a	5' TGCCACTCAGTTGACTCTTCC 3'	5' TAGTGGCACACGCCTGTAGT 3'
Exon 67a	5' GCCTTTGTAGGCGAGTCTCTT 3'	5' CGATAAGGATGTTTCATTGTAGCA 3'
Exon 77a	5' CAGGCTTGACAGAATCAGGTG 3'	5' GTGGCCTGAATTGAGTTTCC 3'

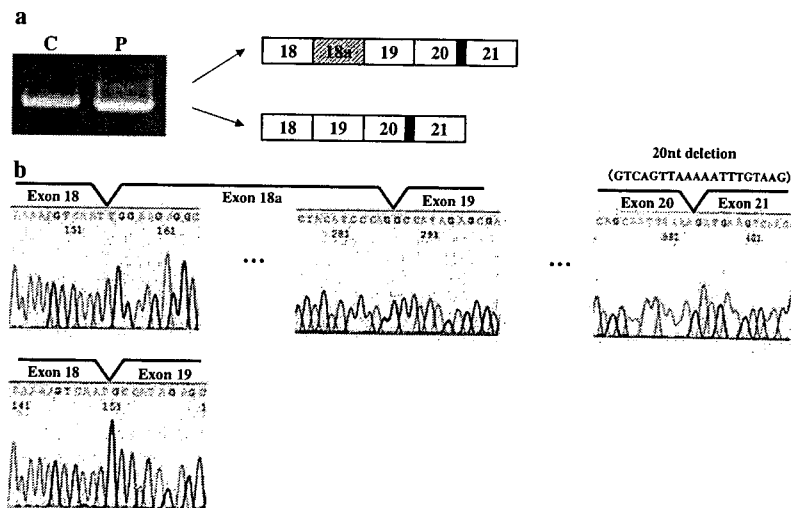
To assess the significance of the values, the nonparametric Mann–Whitney rank test was employed as described previously (Roca et al. 2003).

## Results

Dystrophin mRNA expressed in the lymphocytes of more than hundred DMD/BMD patients was analyzed by RT-nested PCR amplification to identify mutations in the dystrophin gene or to enhance molecular understanding of the dystrophinopathy. In some cases, subcloning sequencing of the amplified products disclosed some ambiguous clones, leading to the identification of cryptic exons. For example, the dystrophin mRNA in the lymphocytes of one DMD patient carrying a single G to A conversion at the first nucleotide of intron 20 (c.2622 + 1G>A), which destroyed a splice donor site, was examined for secondary splicing errors. When a fragment extending from exon 18 to 21 was amplified, two amplified products (one major and one minor) were obtained (Fig. 2). Subcloning sequencing of the major, normal-sized product disclosed a normal exon content. The size of exon 20, however, was shortened to 222 bp, because a cryptic splice donor site in exon 20 was activated due to inactivation of the authentic donor site, leading to a 20-bp deletion at the 3' end of exon 20. On the

other hand, the larger, minor product was composed of exons 18 to 21 and an additional 132-bp sequence between exons 18 and 19 (Fig. 2). A BLAST search with the 132-bp sequence revealed the sequence was derived from intron 18 of the dystrophin gene (nt 30314022–30313891 of GenBank NT\_011757). The inserted 132-bp sequence was located 3.8 kb downstream from exon 18 and 12.2 kb upstream of exon 19 (Fig. 1). AG and GT dinucleotides, which are absolutely conserved at splice acceptor and donor sites of all introns, were identified in the genomic sequence immediately adjacent to the 5' and 3' ends of the 132-bp sequence, respectively. The Shapiro's splicing probability scores for the splice acceptor and donor sites were 0.73 and 0.64, respectively (Shapiro and Senapathy 1987). Furthermore, the branch-point consensus sequence TGCTCAT was identified 38 bp upstream of the inserted sequence (Table 2). Seven splicing enhancer sequences and three splicing silencer sequences within the 132-bp sequence were identified (Table 2). Because the inserted sequence exhibited all of the characteristics typical of a genomic exon and was inserted between authentic dystrophin exons, we refer to this sequence as dystrophin exon 18a.

We then looked for a genomic mutation that could have caused the activation of exon 18a by sequencing the intronic region near the inserted 132-bp sequence. Because



**Fig. 2a–b** Identification of cryptic exon 18a. **a** RT-nested PCR amplification of a fragment containing exons 18 to 21 from the lymphocyte of a DMD patient carrying a single nucleotide conversion of the first nucleotide of intron 20 (c.2622 + 1G>A). Two products were visualized on the gel; one was barely visible, whereas the other one was clearly visible (P). One amplified product was obtained from the control sample (C). A schematic representation of the exonic organization in the amplified fragments is shown to the right of the products. The black boxes correspond to the 20-bp deletion in exon 20. C and P refer to the control sample and the index case,

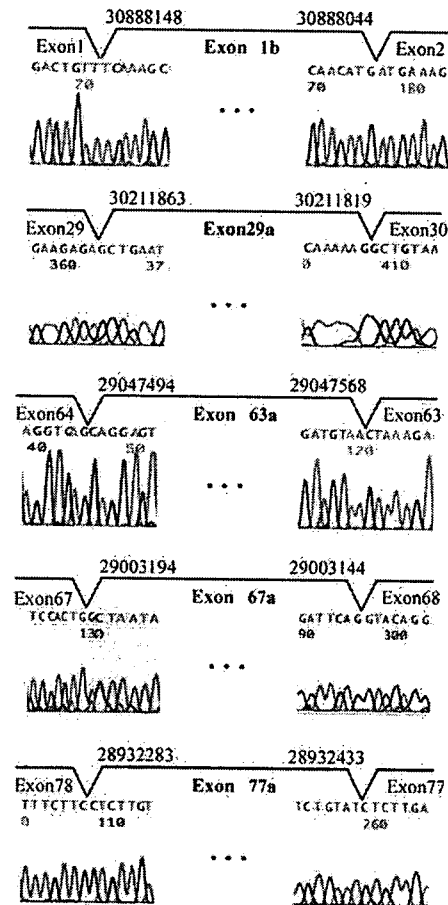
respectively. **b** The sequences at the junctions between the 132-bp inserted sequence and the flanking authentic exons and the junction between exon 20 and 21 are shown. The terminal sequence of exon 18 (CAAT) is joined to the 5' end of the 132-bp inserted sequence (TGGA), whereas the 3' end of the insert (CCAG) is joined to the 5' end of the sequence of exon 19 (GCCA) (upper panel). In the junction between exon 20 and 21, 20 bp of the 3' end of exon 20 are deleted (upper panel). The normal junction between exons 18 and 19 is shown (lower panel)

**Table 2** Novel cryptic exons in the dystrophin gene

Exon	Length (bp)	Shapiro's probability score		Branch site		Enhancer (number)	Silencer (number)	Dystrophin mutation
		Acceptor site	Donor site	Sequence	Distance (bp)			
1b	105	0.59	0.83	TACTAAC	21	15	5	Deletion of exons 3–6
1c	27	0.62	0.8	TACTAAT	40	0	2	Normal
18a	132	0.73	0.64	TGCTCAT	38	7	3	c.2622 + 1G>A
29a	45	0.93	0.82	CCCTGAT	57	3	0	c.3613 del G
63a	75	0.79	0.73	TTTCAAT	29	9	3	Unknown
67a	51	0.82	0.61	CACTGAC	61	3	0	Unknown
77a	151	0.76	0.9	CACCTAC	81	12	2	

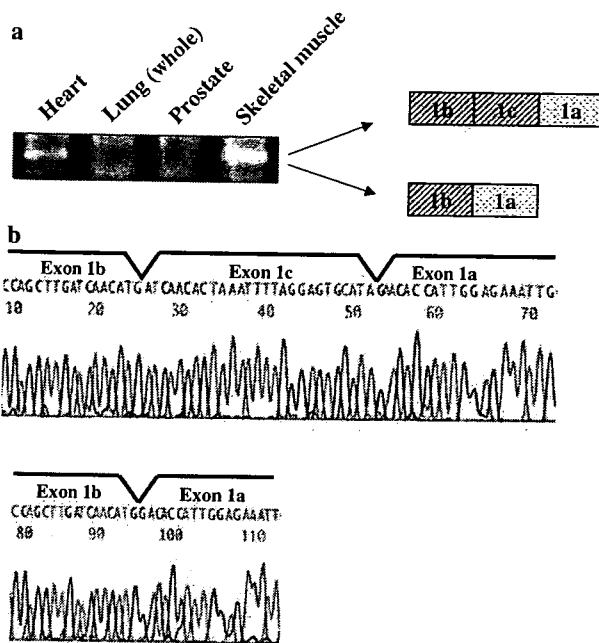
the genomic sequence was completely normal, however, the activation of exon 18a was attributed to a remote effect induced by a nucleotide change at some distant location (Suminaga et al. 2002). When the protein-coding ability of exon 18a was examined by analyzing the translational reading frame of the resulting dystrophin mRNA, a premature stop codon was identified in the 26th codon of exon 18a. Therefore, it is unlikely that transcripts containing exon 18a would code for the translation of a novel dystrophin protein isoform.

Subcloning sequencing of products amplified with RT-nested PCRs from dystrophin mRNA isolated from lymphocytes subsequently disclosed five unknown sequences that were inserted into at least one sequencing clone (Fig. 3). When a fragment containing exons 1 to 11 was amplified, one clone contained a 105-bp insertion between exons 1 and 2 together with a deletion of exons 3 to 6 (Table 2). When a fragment spanning from exon 25 to 30 was amplified, a 45-bp insertion between exons 29 and 30 was identified in one clone that also carried a single G nucleotide deletion in exon 27 (c.3613del G) (Table 2). In one clone containing an amplified fragment spanning from exons 61 to 64, a 75-bp insertion between exons 63 and 64 was identified, although no mutation was found yet in the dystrophin gene (Table 2). Remarkably, in one DMD patient in whom no mutation in the dystrophin gene had been identified thus far, two insertions were disclosed: a 51-bp insertion between exons 67 and 68 and a 151-bp insertion between exons 77 and 78 were cloned when fragments encompassing exons 66 to 72 and exons 70 to 79 were examined, respectively. All of the inserted sequences were completely homologous to the respective intron sequences (Fig. 2), and AG and GT dinucleotides immediately flanked the 5' and 3' ends of each insertion, respectively (Table 2). Furthermore, a consensus sequence for a branch point was identified upstream of each insertion (Table 2). Therefore, these insertions were named exons 1b, 29a, 63a, 67a, and 77a. Of these insertions, only exon 67a did not disrupt the open reading frame of the dystrophin mRNA.



**Fig. 3** Sequencing results for five of the novel cryptic exons. Sequences at the junctions of the inserted sequences and the flanking authentic exons are shown. Numbers over the sequencing charts indicate the nucleotide positions of the cryptic exons in the sequence of Genebank NT\_011757

The disclosure of exon 1b in addition to exon 1a in intron 1 suggested that these cryptic exons have physiological roles. A fragment encompassing exons 1b and 1a was examined using cDNA prepared from normal heart, lung, prostate, and skeletal muscle (Fig. 4). The amount of



**Fig. 4a–b** Cloning of cryptic exon 1c. **a** RT-PCR products are shown. Total RNA from four sources was analyzed by RT-PCR amplification of a fragment encompassing two cryptic exons (exon 1b and 1a). One product was amplified from heart and skeletal muscle, but not from lung or prostate. A schematic representation of the exonic organization in the amplified fragments is shown to the right of the products. **b** Sequences at the junctions of the exons are shown. Subcloning sequencing of the amplified products disclosed the presence of two different clones. In one clone, the 3' end of exon 1b was directly joined to the 5' end of exon 1a (*lower panel*), whereas the 27-bp cryptic exon was inserted between exons 1b and 1a in the other clone

the PCR-amplified fragment obtained from heart and skeletal muscle was sufficient for further analysis, whereas this was not the case for the samples from lung and prostate. Subcloning sequencing of the product disclosed two clones: one consisted of exons 1b and 1a and the other contained an unknown 27-bp insertion between exon 1b and 1a. The inserted sequence was completely homologous to a region in intron 1 (nt 30885727 to 30885701 of Genbank NT\_011757). In the genomic sequence, we identified AG and GT dinucleotides immediately adjacent to the 5' and 3' end of the insertion, respectively, in addition to a branch point consensus sequence upstream of the insertion; therefore, we named the insertion exon 1c (Table 2). Both of the cloned sequences, however, encoded stop codons in every frame and were not expected to code for proteins. BLAST homology searches in Genbank with the full-length clones disclosed no homologous sequences.

To date, 14 cryptic exons have been identified in introns 1, 2, 3, 18, 29, 63, 67, and 77. In order to identify elements that differentiate cryptic exons from authentic exons, the

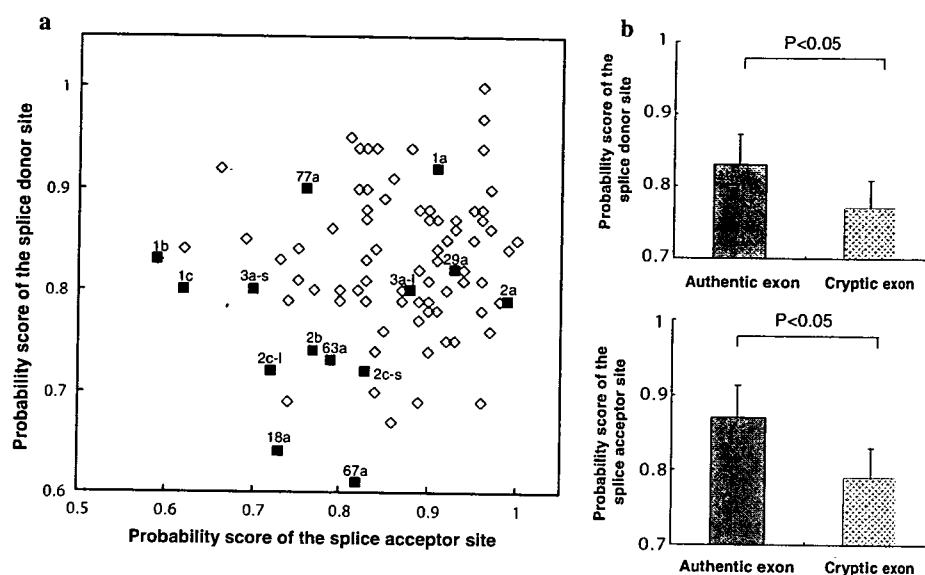
characteristics of the 14 cryptic and 77 authentic exons were examined. The length of the cryptic exons ranged from 27 to 357 bp (mean  $123 \pm 82$  bp), whereas that of the authentic exons ranged from 32 to 269 bp (mean  $143 \pm 49$  bp); the mean length of the cryptic exons was smaller than that of the authentic exons. The strengths of the splicing acceptor and donor sites were calculated using the Shapiro's splicing probability score (Fig. 5). In a plot of the scores for the acceptor versus the donor sites, many of the cryptic exons were plotted in the area where the two splicing scores were low, whereas many of the authentic exons were plotted in the area where the two scores were high. The Shapiro's scores for the splice donor sites of the cryptic exons ranged from 0.61 to 0.92 (mean  $0.78 \pm 0.07$ ), whereas those of the authentic exons ranged from 0.67 to 1 (mean  $0.83 \pm 0.07$ ) (Sironi et al. 2001); the scores for cryptic exons were significantly lower than those for the authentic exons (Fig. 5). In addition, the mean probability score for the splice acceptor sites of the cryptic exons was significantly lower than that for the splice acceptor sites of the authentic exons (Fig. 5; mean  $0.79 \pm 0.12$  vs.  $0.87 \pm 0.09$ , respectively).

Because the incorporation of exons into mRNA is controlled by splicing enhancer and/or silencer sequences in exons, the densities of the splicing enhancer and silencer sequences in each exon were analyzed (Fig. 6a,b). In a plot of the density of splicing enhancers versus the density of splicing silencers, most of the cryptic exons were plotted in the area corresponding to a larger density of splicing silencers compared to the density of enhancers. In contrast, authentic exons had a smaller density of splicing silencers and a larger density of enhancers. The mean density of splicing enhancers in the cryptic exons was smaller than that in authentic exons (9.8 vs. 16.2 per 100 bp of genomic sequence), and the mean density of splicing silencers was significantly larger in cryptic exons than in authentic exons (3.9 vs. 2.1 per 100 bp of genomic sequence) (Fig. 6b). This suggested that the spliceosome recognized the authentic exons better than the cryptic exons.

The distance between the splice acceptor site and the putative branch point was examined and compared in the two types of exons. The mean distance between the acceptor site and the branch point for the cryptic exons was 64 bp (range 20–116 bp), whereas that for the authentic exons was 44 bp (range 16–123 bp); the mean distance was significantly longer for the cryptic exons (Fig. 6c), which indicated that the cryptic exons were weaker than the authentic exons. Additionally, the pyrimidine nucleotide content, which is an indication of the strength of a splice site, was examined for the nucleotide sequences between the branch points and the splicing acceptor sites. For the cryptic exons, the mean pyrimidine content was 67% (range: 50–82%), whereas it was 63% (range 37–89%) for



**Fig. 5a–b** Strength of the splice sites. **a** A graphical representation of the Shapiro's splicing probability scores for the splice donor and acceptor sites. *Black and open boxes* represent the cryptic and authentic exons, respectively. **b** Comparison of the Shapiro's splicing probability scores. *Bars* indicate the mean values of the splice donor (*upper*) and acceptor (*lower*) sites. *Shaded and dotted bars* represent authentic and cryptic exons, respectively



the authentic exons; this difference was not significant (Fig. 6c).

The introns in the human dystrophin gene have increased in size through the acquisition of new elements into the introns (McNaughton et al. 1997). To examine whether the cryptic exons were products of exonization from retrotransposable elements, homology searches were done with each cryptic exon (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>). As a result, exon 1a and 2b were found within ER-V class I and Alu sequences, respectively. The other cryptic exons, however, did not contain repetitive elements, and the mechanism for exonization was consequently more difficult to establish.

Resplicing has been reported to explain the mechanism by which large introns are spliced out of pre-mRNA (Hatton et al. 1998; Burnette et al. 2005). Because the dystrophin gene has extremely large introns, it is possible that splicing proceeds via a resplicing mechanism that uses AGGT tetranucleotides as splice acceptor and donor sites. The sequences of the cryptic exons were examined for GT and AG dinucleotides at the 5' and 3' ends of the inserts, respectively. Among the 14 cryptic exons, only exon 2a contained a GT dinucleotide at its 5' end, which made the junction between exon 2 and 2a an AGGT tetranucleotide. At this tetranucleotide, the Shapiro's splicing probability scores for the splice acceptor and donor sites were 0.75 and 0.78, respectively, making this a good candidate for a resplicing site. At the 3' end of exon 2a, however, there was a GT but not an AG dinucleotide; therefore, this position was not suitable as a resplicing substrate. Therefore, it is unlikely that the cryptic exons were intermediate products of resplicing.

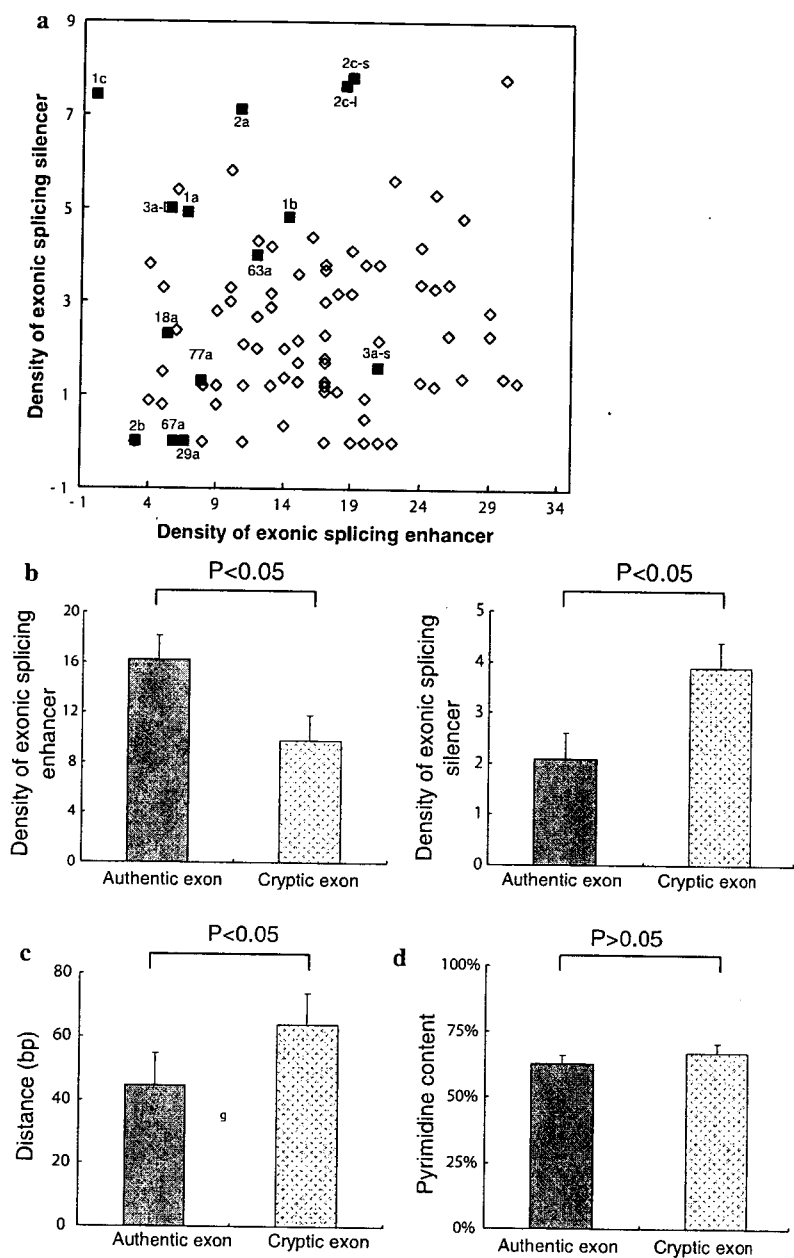
In conclusion, compared to the authentic exons, the cryptic exons were shorter, and had lower splicing acceptor and donor scores, smaller densities of splicing enhancers, larger densities of splicing silencers, and branch points that were farther away from the splicing acceptor sites. All of these characteristics suggest that the cryptic exons were weaker than the authentic exons.

## Discussion

The extremely large introns in the dystrophin gene suggest that a specific mechanism is required for the processing of dystrophin mRNA (Surono et al. 1997, 1999). Seven cryptic exons have been identified in dystrophin introns due to the incorporation of intron fragments into dystrophin mRNA (Fig. 1) (Roberts et al. 1993; Dwi Pramono et al. 2000; Suminaga et al. 2002; Tran et al. 2005; Ishibashi et al. 2006). In this study, seven additional fragments of introns of the dystrophin gene were shown to be inserted into dystrophin mRNA (Table 2). These cryptic exons are smaller and have weak splice site signals, which is commonly observed for alternatively spliced exons (Fig. 5) (Baek and Green 2005; Yeo et al. 2005).

It has been estimated that there is one pseudoexon for every kilobase of intron (Sironi et al. 2004). Therefore, more than 100 pseudoexons should be present in the 190-kb intron 1 of the dystrophin gene. In this report, we identified two additional cryptic exons in intron 1: exon 1b and exon 1c (Fig. 1). It is likely that other fragments of intron 1 will be identified as cryptic exons. In intron 1, one candidate sequence (121 nt from 56074 to 55954 of Gen-

**Fig. 6a–d** Examination of genomic sequences. **a** A graphical representation of the densities of the splicing enhancer and silencer motifs. *Black and open boxes* represent cryptic and authentic exons, respectively. **b** Comparison of splicing enhancer and silencer densities. *Bars* indicate mean values of the densities of splicing enhancer (*left*) and silencer (*right*) motifs. *Shaded and dotted bars* represent authentic and cryptic exons, respectively. **c, d** Comparison of length and the pyrimidine content of the sequences between the putative branch point and the splice acceptor site. The length (**c**) and pyrimidine content (**d**) of the sequence between the putative branch point and the splice acceptor site are shown. *Shaded and dotted bars* represent authentic and cryptic exons, respectively



bank AL049643) was identified 40 kb upstream from exon 1b with a computer-based search for exonic sequences. No incorporation of this sequence into dystrophin mRNA, however, was observed (data not shown). This suggests that not all sequences that have exonic characteristics are active, and that there are specific mechanisms to allow the spliceosome to recognize exon sequences.

Intron 29 is known to encode the promoter and the first exon (exon R1) of R-dystrophin, one of the dystrophin isoforms expressed in retina (Pillers et al. 1993). Exon 29a is different from the other cryptic exons in the dystrophin gene, because it shares a common splicing donor site with

exon R1 of the R-dystrophin transcript (Fig. 1). Though exon R1 is 95 bp long, exon 29 uses a cryptic splice acceptor site located 45 bp upstream from the 3' end of exon R1 (Fig. 1). Considering that the splice donor site of exon R1 is activated in the retina (Pillers et al. 1993), exon 29a may be specifically expressed in the retina, which should be addressed in future studies.

It is known that mRNA encoding a premature stop codon can be subjected to degradation by the nonsense-mediated mRNA decay mechanism (Maquat 2004). Because our results indicated that the cryptic exons eluded this surveillance (Hillman et al. 2004), the transcripts

containing the cryptic exons are suggested to have physiological roles. Five potential explanations could account for the presence of these cryptic exons in dystrophin mRNA. First, they could be aberrant splicing products. All of the cryptic exons except exon 1c were detected in dystrophin mRNA isolated from lymphocytes where the dystrophin gene is illegitimately expressed. Therefore, we believe that the splicing of dystrophin pre-mRNA is not as strictly controlled in lymphocytes as it is in skeletal muscle, which allows the splicing machinery to recognize cryptic exons. Second, these exons may constitute untranslated regions of dystrophin mRNA variants. This possibility is based on the fact that nine of the 14 cryptic exons were identified in introns located upstream of exon 8, which has been reported to include an alternative translation start site (Malhotra et al. 1988; Gangopadhyay et al. 1992). Therefore, the nine cryptic exons upstream of exon 8 could make up part of the 5'-untranslated region of a dystrophin mRNA variant. In fact, an alternative exon has been identified in the 5'-untranslated region the P-dystrophin transcript (Holder et al. 1996). Complex patterns of alternative splicing of the 5' region of the dystrophin gene have been reported and are considered to be a factor that can explain some cases in which there is a discrepancy between the phenotype and molecular genotype (Chelly et al. 1991; Reiss and Rininsland 1994; Torelli and Muntoni 1996; Surono et al. 1997).

A third possibility is that the cryptic exons are intermediate products of a resplicing process. Whether one-step splicing is used to remove the large intron sequences has long been debated (Hatton et al. 1998). The resplicing theory states that repetitive splicing of small fragments allows the removal of large introns (Hatton et al. 1998). Because the dystrophin gene has large introns, the identified cryptic exons may be intermediate products of resplicing that uses AGGT tetranucleotides as splice donor and acceptor sites. Only exon 2a, however, had a GT dinucleotide at its 5' end, and exon 2a did not have an AG dinucleotide at its 3' end, preventing the formation of an AGGT tetranucleotide with the downstream exon sequence. In addition, the sizes of the introns containing the cryptic exons are 189, 170, 4.8, 16, 26, 38, 21, and 7.4 kb for introns 1, 2, 3, 18, 29, 63, 67, and 77, respectively. Therefore, it is unlikely that all of these cryptic exons are resplicing intermediate products.

The fourth possibility is that these cryptic exons are part of noncoding RNAs. It has been suggested that noncoding transcripts play important roles in the downregulation of gene expression (Hillman et al. 2004; Lareau et al. 2004). Recently, a large number of noncoding RNAs were identified in humans (Carninci et al. 2005), and it is likely that there are many noncoding RNAs that are yet to be

uncovered. Therefore, the dystrophin cryptic exons may be parts of noncoding RNAs that regulate gene expression.

Finally, the exons may be intermediates of exonization. Recent data suggest that newly created exons result in an expansion of proteomic diversity (Lev-Maor et al. 2003; Sorek et al. 2004). This is probably accomplished through a multistep process in which the novel exon is initially included in a minority of the transcripts (due to weak splicing signals) and is thereby allowed to evolve without compromising the original gene function. Repeated genomic elements have been reported to be exonized (Lev-Maor et al. 2003; Sorek et al. 2004). Sequence analysis of the 14 cryptic exons disclosed some homology in exons 1a, 1b, and 2b to the repeat sequences. Future studies are required to address this possibility.

In the dystrophin gene a single nucleotide change located at deep intron has been reported to cause dystrophinopathy; creation or reinforcement of a splicing donor site consensus sequence has been reported in introns 9, 25, 60, and 62 (Ikezawa et al. 1998; Tuffery-Giraud et al. 2003) or 62 (Beroud et al. 2004), respectively. In addition, creation or reinforcement of a splice acceptor site consensus sequence has been reported in introns 1 and 2 (Yagi et al. 2003; Beroud et al. 2004) or 25 (Tuffery-Giraud et al. 2003), respectively. Until now any single nucleotide change affecting exonic splicing silencer or enhancer of cryptic exons of the dystrophin gene has not been reported. Pseudoxon insertions, however, have been reported in the ATM gene (Eng et al. 2004). Further studies may disclose mutations deep in introns that cause dystrophinopathy. In addition, our identification of these cryptic exons may shed light on the diverse functions of dystrophin.

**Acknowledgments** We would like to thank Ms. A. Hosoda for her secretarial help. This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science; Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan for research on psychiatric and neurological diseases and mental health; and a grant for nervous and mental disorders from the Ministry of Health, Labour and Welfare.

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