Trans-acting factors may cause dystrophin splicing misregulation in BMD skeletal muscles

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Abstract We analyzed dystrophin alternative splicing events in a large number of Becker muscular dystrophy (BMD) affected individuals presenting major hot-spot deletions. Evidence is shown that altered splicing patterns in these patients do not directly result from the gene defect but probably derive from modifications in *trans*- rather than *cis*-acting factors. Several potential CUG-binding protein 2 (CUG-BP2) binding sites were found to be located in the dystrophin gene region encompassing exons 43–60 and CUG-BP2 transcript analysis indicated that not only expression levels are increased in dystrophic muscles but also that different CUG-BP2 isoforms are expressed. The possibility that CUG-BP2 might have a role in dystrophin splicing regulation is discussed.
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1. Introduction

Duchenne and Becker muscular dystrophies (DMD and BMD) are allelic disorders caused by mutations in the dystrophin gene, located on Xp21.2. The gene is the largest known so far and is constituted by 79 relatively small exons [1]. The majority of DMD and BMD patients carry deletions in the gene (60–65% of cases) [2] and, in most cases there is a good correlation between the severity of the disease and the effect of the deletion on the reading frame: reading frame-disrupting mutations are usually associated with a severe DMD phenotype, whereas in-frame deletions determine a BMD clinical status [3]. However exceptions to the reading frame rule are found in about 8% of patients [4] and they are thought to be the result of alternative splicing events that modulate the clinical phenotype by editing the translational reading frame [5,6].

For both BMD and DMD, considerable variability in disease presentation is found across patients carrying the same mutation and, in both pathologies, muscle involvement can be associated with heart disease and/or mental retardation.

The dystrophin gene undergoes extensive alternative splicing. In particular, tissue-specific transcripts in brain neurons, cardiac Purkinje fibers, and smooth muscle cells [7,8] arise

from alternative splicing in the 3′-region, while at least 12 splicing patterns have been described in the 5′-portion of the gene in skeletal muscle [9]. More recently 16 different transcripts have been shown to originate from alternative splicing of the central gene region, some of them displaying tissue specificity [10].

Full-length dystrophin transcripts encode a rod-shaped 427 kDa protein consisting of four domains: an N-terminal actin binding domain, 24 spectrin-like repeats, a cysteine rich domain and an unique C-terminal domain [1]. Alternative splicing events that remove one or more in-frame exons from the spectrin-like region are thus expected to result in the production of dystrophin molecules displaying shortened rod-domains. The function of such gene products, if any, is presently unknown as well as their potential role in the modulation of the clinical status.

We have previously shown that at least seven alternative splicing events involve the portion of the gene encompassing exons 44–58 in healthy skeletal muscle [10], a region that also represent the major hot spot for intragenic deletions. Here we have analyzed a large number of BMD affected individuals presenting major hot-spot deletions. Our data indicate that splicing patterns involving this gene region are altered in BMD patients but the gene defect itself is not directly responsible for splicing misregulation.

2. Material and methods

2.1. Patients
Dystrophinopatic patients were selected according to standard international criteria and BMD subjects were classified as mild, moderate or severe as previously described [11].

Dystrophin gene deletion analysis was performed using routine procedures.

Mutation analysis in the *CAPN3* and *DYSF* genes was performed through direct sequencing of single exons amplified from genomic DNA. All patients reported here harbor pathological single base pair substitutions in either the *CAPN3* or the *DYSF* genes.

2.2. Transcript analysis
Tissue samples were derived, with informed consent of the family, from muscle biopsies of healthy individuals and patients. Dystrophin transcript analysis was performed as previously described [10]. One additional reverse primer was designed in exon 56 and used together with the exon 43 nested primer to verify the presence of transcripts lacking exons 45–55 in healthy muscles.

For semi-quantitative analysis of CUG-binding protein 2 (BP2) transcripts, an ABI-Tet modified forward primer (sequence: CAAATGGCAGGCATGAATGC) located in exon 8 and a reverse oligonucleotide (sequence: CGCTGCTCGTGGTAGAGAG) localized to exon 9 were used. These two exons do not undergo alternative splicing events in human tissues being common to all CUG-BP2 iso-

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Table 1

Subject	deleted exons	age at byopsy	clinical status	5' distance	3' distance	deletion size	resulting intron	skipped exons										CUG-BP2
								45-48[a]	45-49[a]	45-50[b]	44-50[b]	45-53[a]	45-55[a]	45-57[a]	44-56[a]	49-57[a]	44-57[b]	
CTR	-							+	+	+	-	+	-	-	+	+	-	7,06
1	45-47	32	moderate	201107	2289	138147	203395	-	-	-	+	-	+	-	-	-	-	20,36
2	45-47	27	typical	114316	16582	210645	130895	+	-	-	-	-	-	-	-	-	-	40,69
3	45-47	27	severe	42429	52283	246831	94713	-	+	+	+	+	-	+	-	-	+	41,13
4	45-47	31	typical	106396	52925	182222	159322	+	-	-	-	-	-	-	-	-	+	38,33
5	45-47	18	typical	182913	9118	149512	192033	-	-	-	-	-	-	-	-	-	-	30,72
6	45-47	35	mild	210238	50104	81201	260355	+	-	-	-	-	+	+	+	-	+	19,07
7	45-47	28	typical	79732	15338	246473	95069	+	-	-	-	-	-	+	-	-	-	66,06
8	45-47	8	severe	141897	10498	189148	152394	+	-	-	-	+	+	-	-	-	-	44,19
9	45-47	14	mild					-	+	+	-	-	-	+	+	+	+	5,40
10	45-47	2	presymptomatic					-	-	-	-	-	-	+	-	-	-	71,10
11	45-47	32	typical					-	+	+	-	-	-	-	+	+	-	10,51
12	45-47	15	mild					+	-	-	+	-	-	+	-	-	-	12,40
13	45-47	23	typical					+	-	-	-	-	-	+	+	-	+	7,29
14	45-48	48	typical	174710	17853	187534	192568	F.L.	+	-	-	-	+	-	-	n.a.	-	93,88
15	45-48	69	severe	220859	939	158299	221801	F.L.	+	+	-	-	-	+	-	n.a.	-	18,15
16	45-48	5	mild	183240	14636	182221	197877	F.L.	+	+	+	-	-	-	-	n.a.	-	20,16
17	45-48	31	mild	227435	43468	70640	270902	F.L.	-	-	-	+	-	-	+	n.a.	-	66,55
18	45-48	7	presymptomatic	247246	4945	127906	252192	F.L.	-	-	-	-	-	-	+	n.a.	-	36,87
19	45-48	11	mild					F.L.	-	-	+	-	-	+	-	n.a.	-	9,03
20	45-48	45	typical					F.L.	-	-	-	-	-	+	-	n.a.	-	27,99
21	45-48	20	typical					F.L.	-	-	-	-	-	-	-	n.a.	-	9,58
22	45-48	31	typical					F.L.	+	+	-	-	+	-	-	n.a.	-	8,14
23	45-48	23	typical					F.L.	-	-	-	-	-	-	-	n.a.	+	17,01
24	45-48	32	severe					F.L.	+	-	-	-	-	+	-	n.a.	-	6,19
25	48	1,6	presymptomatic					+	-	+	+	+	-	+	+	n.a.	+	57,98
26	48	3	mild	38907	8770	45100	47676	+	-	-	-	-	-	+	-	n.a.	-	8,67

Subject	gene defect																	
27	DMD (del 6-7)							-	-	-	+	-	+	+	-	-	+	20,43
28	DMD (del. 8-11)							-	-	-	+	-	+	-	-	-	+	138,30
29	DMD (del 34-43)							-	-	-	n.a.	-	-	-	n.a.	-	n.a.	n.d.
30	CAPN3							-	-	-	-	+	+	+	-	-	+	n.d.
31	CAPN3							-	-	-	+	+	+	-	-	-	+	5,14
32	DYSF							-	-	-	+	-	-	+	+	-	+	n.d.
33	DM1							-	+	+	+	-	-	+	-	-	-	5,37

Subjects 1–26: BMD patients; shading indicates novel splicing events. Legend: n.d.: not determined, n.a.: not applicable; F.L.: full-length transcript.
[a]In-frame transcript.
[b]Out-of-frame transcript.

Fig. 1. URE location along the dystrophin gene. Vertical lines represent exons, dots indicate UREs.

forms [12]. PCR reactions (22 cycles) were carried out with JumpStart REDAccuTaq DNA Polymerase (Sigma) using annealing and extension temperatures of 60 and 68°C, respectively. Amplified products were run on an ABI PRISM 310 Genetic Analyzer and quantified using the GeneScan program. CUG-BP mRNA abundance was normalized to GAPDH signal. To this aim GAPDH mRNA was amplified using an ABI-Hex modified forward primer (sequence: GTAT-GACAACAGCCTCAAG); sequence of the reverse oligonucleotide was GTGATGGCATGGACTGTGG. GAPDH amplification followed the same conditions reported for CUG-BP2 and reactions were stopped after 20 cycles.

Transcript analysis of CUG-BP2 was performed using exon 1-specific forward oligonucleotides (sequences: exon 1a, CTATGA-GAAATGAAGAGCTGC; exon 1b, GATTTCCTCCCGGACAT-GACG; exon 1c, CTCTGCTCGACAGCAGCACG) and a reverse primer located in exon 2 (sequence: GAGGACGTTGATCTGGTA-GAC). Reactions were performed with annealing and extension temperatures of 58 and 68°C, respectively.

3. Results and discussion

The dystrophin gene has been shown to undergo extensive alternative splicing events that determine the production of a number of transcripts, most of which still lack any functional definition. Given the potential relevance of translational frame editing on the clinical outcome of DMD and BMD patients, analysis of alternative splicing events in dystrophic muscles might be relevant to both diagnostic and therapeutic purposes.

We have previously shown [10] that at least seven alternative splicing events involve the portion of the dystrophin gene encompassing exons 44–58 in healthy skeletal muscle and that deletions involving the same exons can determine diverse splicing behaviors in different patients or even in different tissues of the same individual. Here we have analyzed 26 BMD patients that were selected according to deletion type so as to minimize genetic heterogeneity. In particular, 13 and 11 patients presented a 45–47 and 45–48 exon deletion, respectively, while two patients displayed loss of exon 48 alone. Data concerning analysis of dystrophin alternative splicing events in the region encompassing exons 43–58 are reported in Table 1.

Transcript analysis revealed that novel alternative splicing events are activated in BMD skeletal muscles. In particular, skipping of exons 44–50, 45–55, 45–57 and 44–57 had never been described in healthy muscles and we could not detect it in three control tissues from unrelated subjects. In order to rule out the possibility that small amounts of exon 45–55

skipped products might be present in control muscles and that their amplification might be selected against in favor of smaller products (i.e. transcripts lacking exons 44–56), a reverse primer was located in exon 56 and used instead of the nested exon 58 primer. No product corresponding to skipping of exons 45–55 was detectable in control muscle tissues. A similar procedure was not necessary in the case of transcripts lacking exons 44–50, 44–57 and 45–57 that yield very short fragments when primer pairs are located in exons 43 and 51 or 43 and 58, respectively.

The transcript lacking exons 45–57 was the most frequently found in patients' muscles, being present in 14 out of 26 samples. This product was either the only alternative transcript detectable in BMD tissues (patients 10 and 20) or was expressed together with other alternative gene products. Splicing events did not seem to correlate with deletion type since some patients displayed the same splicing pattern despite carrying different exon deletions. Moreover, as it is evident from Table 1, patients with the same deletion displayed different splicing behaviors with respect to the loss or preservation of alternative transcripts expressed in healthy skeletal muscle.

The clinical status of BMD patients was also evaluated and no splicing pattern or alternative transcript could be ascribed to any peculiar phenotypic class.

We have previously proposed that deletion extension in flanking introns might have some relevance with respect to splicing patterns. To verify this hypothesis, we analyzed deletion breakpoints in 14 BMD patients; sequencing of the 14 junctions has been previously reported [13]. Genomic distances of both 5′ and 3′ deletion ends from immediately upstream and downstream exons were calculated as well as deletion size and lengths of resulting introns deriving from the juxtaposition of deleted intervening sequences. None of these calculations gave any clear explanation of observed splicing patterns. As far as exclusion of exon 45 (i.e. the presence of transcripts skipping exons 44–50 or 44–57) is concerned, it did not correlate with deletion extension in intron 44 or with resulting intron lengths (Table 1). Similarly, the presence or absence of transcripts lacking exons 45–49 did not depend upon breakpoint location in intron 48. Overall, no evident explanation of splicing patterns could be derived from the analysis of breakpoint locations.

Taken together, these findings made us speculate that splicing patterns might be independent from the genetic defect and might instead be related to the patho-physiology of dystrophic
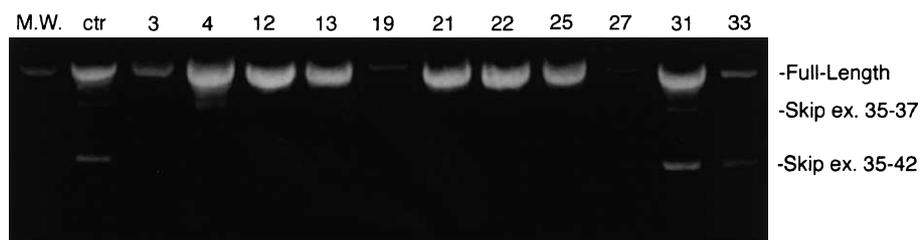

Fig. 2. RT-PCR amplification of dystrophin transcripts arising from alternative splicing of the gene region encompassing exons 33–43. Skipped exons are reported for each amplified product while lane headings indicate patient number. M.W.: Molecular weight marker.
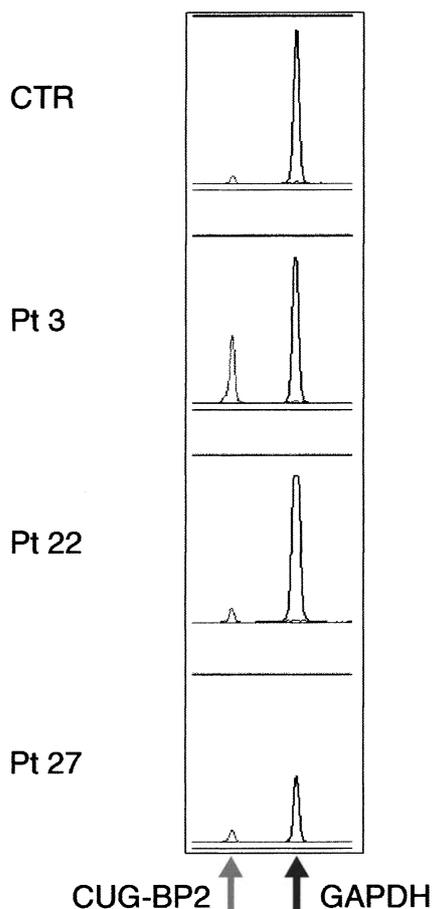
Fig. 3. CUG-BP2 transcript analysis. Patient numbers are indicated. CTR: Control muscle tissue.

muscles. To test this hypothesis, splicing events involving exons 44–58 were analyzed in DMD patients carrying proximal deletions and in skeletal muscles from dystrophic patients with different gene defects (two patients carried mutations in the calpain3 gene, one in the dysferlin gene). These patients displayed both loss of physiological dystrophin transcripts and the presence of one or more novel splicing products (Table 1). Interestingly, these same findings also applied to skeletal muscle transcripts from one patient affected by myotonic dystrophy (MD). These data indicate that dystrophin alternative splicing patterns do not directly result from the gene defect and implicitly suggest that modifications in trans- rather than cis-acting factors might be regarded as responsible for altered splice-site selection in dystrophic muscles.

It has recently been reported that CUG-BP2 mRNA is over-expressed in DMD skeletal muscles and its role in splicing regulation as well as disease progression has been proposed [14]. CUG-BP2 belongs to a family of highly conserved proteins that are involved in regulation of cell-specific and developmentally regulated alternative splicing [15]. CUG-BPs function is altered in MD, resulting in aberrant mRNA processing of several genes [16,17]. CUG-BP2 binds to repeated elements of purine and uridine (UREs) [18], which are located in intervening regions of CUG-BP2-regulated mRNAs. We searched for such elements throughout the dystrophin gene and their distribution is reported in Fig. 1. Several UREs were present in the gene region encompassing

exons 43–60, many of them being located close to intron–exon boundaries. Conversely, a lower URE concentration was evident in the portion of the gene spanning exons 7–43.

These observations, together with the identification of aberrant transcripts shared among dystrophinopatic patients and the MD1 subject, led us to speculate that CUG-BP2 might have a role in dystrophin splicing misregulation. We thus analyzed alternative splicing events involving exons 33–43, a region that displays a lower UREs concentration, and CUG-BP2 expression in BMD skeletal muscle.

Transcript analysis of the gene region encompassing exons 33–43 was performed for patients 3, 4, 12, 13, 19, 21, 22, 25 (BMD), 27 (DMD), 31 (carrying a CAPN3 mutation) and 33 (MD1). Data are reported in Fig. 2 and indicate that loss of physiological splicing patterns does occur in this region, but generation of aberrant novel products does not.

Semi-quantitative RT-PCR reactions were then performed to determine CUG-BP2 expression levels in dystrophic skeletal muscle (Fig. 3). Three healthy muscles were analyzed for CUG-BP2 mRNA expression, providing very similar results. As it is evident from Table 1, BMD patients displayed a variable up-regulation of CUG-BP2, with some of them expressing mRNA amounts similar to control tissues and other presenting a dramatic increase in transcript levels. Overall, 21 out of 26 BMD patients displayed increased CUG-BP2 mRNA expression and transcript levels did not correlate with age at biopsy; nonetheless, we were unable to identify any clear relation between CUG-BP2 expression levels and splicing patterns or clinical phenotype in BMD patients. Yet our data indicate that not only expression levels are increased in dystrophic muscles but also that different CUG-BP2 isoforms are expressed. Three CUG-BP2 gene products are known to date that differ predominantly in their first exons (exons 1a, 1b, 1c) [12]; analysis of CUG-BP2 transcripts in six BMD, one DMD and the MD1 samples (Fig. 4) revealed that while only the exon 1c isoform is expressed in control tissues, almost all patients express the exon 1b isoform and some of them also exon 1a. At present the function of CUG-BP2 first exon alternative usage is unknown and the possibility that it might regulate substrate specificity, including dystrophin transcripts, is intriguing.

Although the role of CUG-BP2 in dystrophin splicing regulation requires further studies, our data suggest a new role for trans-acting factors in dystrophin pre-mRNA processing in dystrophic muscles.

Since the 'reading frame rule' has been developed, all exceptions to it have been ascribed to the presence of alternatively spliced transcripts, indicating the importance of pre-mRNA editing on disease presentation in DMD/BMD patients. Coherently, novel gene therapy approaches based on
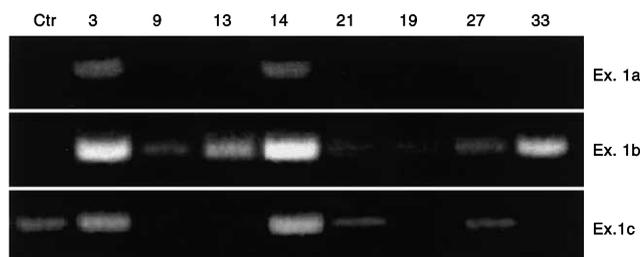
Fig. 4. CUG-BP2 isoform expression. Lane headings indicate patient number.

exon skipping induction have recently been proposed for the treatment of DMD. The observations reported here might offer a valuable insight into the patho-physiology of dystrophin splicing regulation and indicate that any such therapeutic approach should take into account possible splicing alterations taking place in pathological tissues. We consider that understanding the molecular mechanisms underlying splicing regulation will provide insight into novel targets for therapeutic intervention in DMD/BMD.

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