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Preservation of the C-terminus of dystrophin molecule in the skeletal muscle from Becker muscular dystrophy

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Summary

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disorder of muscle in children. The DMD gene product, "dystrophin", is absent from DMD, while those allelic disease, Becker muscular dystrophy (BMD), exhibits dystrophin of abnormal size and/or quantity. But we are still uncertain about the scenario that internally deleted (or duplicated) dystrophin in BMD possess its carboxy (C)-terminal region, and severely truncated dystrophin in DMD does not. Here we use a new monoclonal antibody directed against a peptide in the C-terminal end of the dystrophin molecule to show that the C-terminus is preserved in 30 BMD and 24 control skeletal muscles but not in 21 DMD specimens. This result, taken together with data on deletions of the dystrophin gene, emphasizes both the diagnostic and biological importance of the C-terminal domain which will require for proper function and stability of dystrophin, and substantiates the validity of the reading frame hypothesis for DMD versus BMD deletions on a biochemical level.

Introduction

The past few years have seen tremendous advances in understanding the primary features of the pathogenesis of Duchenne muscular dystrophy (DMD), a common fatal X-linked muscle disease of children (Engel 1986; Emery 1987). Becker muscular dystrophy (BMD) is a milder allelic variant of DMD with a similar clinical distribution of proximal limb muscle weakness. The onset and progression of BMD is later and more variable than in DMD as patients often remain ambulatory into their third or fourth decade compared to 9–11 years of age for DMD (Engel 1986). Cloning of the coding sequences of the DMD gene (Monaco et al. 1986; Burghes et al. 1987; Koenig et al. 1987), and description of the gene product, dystrophin (Hoffman et al. 1987a,b; Koenig et al. 1988), have shed light on the molecular basis for this disease. Dystrophin is a previously unknown protein that is now thought to be a membrane

associated cytoskeletal component of muscle fibers (Sugita et al. 1988; Arahata et al. 1988, 1989a; Bonilla et al. 1988; Watkins et al. 1988; Zubrzycka-Gaarn et al. 1988). Sequence analysis predicts a 427 kDa dystrophin protein composed of 3685 amino acids arranged in four distinct domains (Koenig et al. 1988): (a) the amino-terminal domain that is highly homologous to the actin-binding domain of cytoskeletal α -actinin, (b) the largest second rod-like domain that resembles the repeat domains of spectrin and α -actinin, (c) the third cysteine-rich domain which is related to the C-terminus of α -actinin, and (d) the C-terminal domain, that has no resemblance to any other previously characterized proteins except the recently reported "B3" gene product which is found in muscle, and is autosomal (Love et al. 1989). Although there is as yet no direct evidence, it is thought that the amino-terminal and the C-terminal domains may mediate binding or other interactions between dystrophin and other components of the membrane cytoskeletal complex (Hoffman et al. 1989a). Recent work by Campbell and coworkers (Campbell et al. 1989; Ervasti et al. 1990) suggests that one of these proteins may be an integral membrane glycoprotein, and reduction

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of the 156 kDa glycoprotein was demonstrated in both *mdx* and DMD muscles.

Dystrophin is generally absent or undetectable in muscle from patients with DMD (Hoffman et al. 1988; Bonilla et al. 1988; Arahata et al. 1989b; Nicholson et al. 1989), and in 50–65% of the patients, this deficiency can be accounted for by gene deletions or duplications that disrupt the translational reading frame (Koenig et al. 1987, 1989; Den Dunnen et al. 1989; Gillard et al. 1989; Lindlof et al. 1989). As with DMD, the underlying molecular defects responsible for BMD are also mutations of the dystrophin gene. The phenotypic difference between these diseases is due to the fact that, in BMD, altered dystrophin is produced while it is usually undetectable in DMD muscle. On immunohistochemical analysis, BMD muscle generally has a faint, patchy and/or discontinuous pattern of immunostaining around the periphery of each muscle fiber (Arahata et al. 1989b; Nicholson et al. 1989). These findings suggest that the abnormal arrangement of dystrophin at the inner surface of the plasma membrane may be due to the altered size and/or instability of the mutant dystrophin in BMD muscle.

In contrast to DMD, most dystrophin abnormalities in BMD do not disrupt the translational reading frame, thus allowing the production of internally deleted or duplicated dystrophin molecules (Monaco et al. 1988; Koenig et al. 1989). There is, however, one major class of exceptions to the so called "reading frame rule". Patients with the predicted out-of-frame deletions of exons 3–7 can have quite variable phenotypes ranging from BMD to DMD. It has been hypothesized that some of these patients are capable of making low levels of dystrophin, either by some alternative splicing mechanism or by use of an alternative translational start site (Malhotra et al. 1988; Koenig et al. 1989).

Now, the considerable interest has centered on the structure and function of the C-terminal domain for several reasons: First, some frame-shifting deletions in DMD occur at the 3' - end of the central rod domain. The resulting loss of only the C-terminus seems apparently sufficient to destabilize the entire protein and may prevent proper subcellular localization and function (Koenig et al. 1989; Gillard et al. 1989). In contrast, internal deletions in patients with BMD can remove most of the amino terminal and central rod domains and yet still allow production of partially functional and perhaps rather stable dystrophin molecules (Koenig et al. 1989; England et al. 1990). Second, several different tissue-specific isoforms are apparently generated by alternative splicing of exons at the C-terminus (Feener et al. 1989). Presumably this might allow differential binding to various components of the cytoskeleton in different tissues. Finally, the strong evolutionary conservation of this region (Lemaire et al. 1988), and the

discovery of a second protein, B3 (Love et al. 1989), with homology to this region emphasizes the functional importance of the C-terminus of dystrophin.

To further our understanding of the function of the C-terminal domain of dystrophin, we have generated a new monoclonal antibody, "4-4C5" (abbreviated "4C5" in this article), which is directed specifically at this domain. Here we describe our results using this and other region-specific anti-dystrophin antibodies to analyze dystrophin in patients with DMD and BMD. By correlating these findings with DNA mutation data, we demonstrate that abnormal dystrophin in patients with BMD contains the C-terminus, thus validating the reading frame model at the protein level and emphasizing the importance of this region.

Materials and methods

Skeletal muscle materials

All 75 limb muscle specimens from various neuromuscular diseases were obtained for diagnostic purposes, with informed consent, and were frozen at -85°C . Of these, 21 DMD, 30 BMD and 24 OND (other neurological diseases as controls) were examined (Table 1).

Generation of a monoclonal antibody specific to the C-terminus of dystrophin

A mouse monoclonal antibody against the C-terminal portion of dystrophin was raised against a synthetic peptide deduced from the DMD complementary DNA (cDNA) clone. The peptide corresponds to the amino acid sequence of the C-terminal region of human fetal skeletal muscle dystrophin (peptide-IV, 3495–3544: LISLESEERGELER ILADLEEENRNLQAEYDRLKQQHEHKGGLSP-LPSPPE) (Koenig et al. 1988). The protected peptide-resin was synthesized by the solid phase method on Applied Biosystems model 430A peptide synthesizer, and deprotected with HF. The crude peptide was purified by preparative reversed phase HPLC and lyophilized. 100 μg of the peptide, 50 aa long, was injected intraperitoneally into BALB/C mice with Freund complete adjuvant. After 3 weeks, the antiserum titer was checked against the peptide antigen by the ELISA method. The mouse with the best response was immunized intravenously with 100 μg of the peptide as the final immunization. After 3 days, the spleen cells were taken out and fused with polyethyleneglycol (PEG, MW 4000) as a fusing agent. The reactivity of the supernatant against the peptide IV was checked by ELISA. The antibody is designated as "4-4C5" (4C5). Specificity of the 4C5 was characterized in several ways including immunoblot, blocking, and absorption tests. Immunohistochemically, positive reaction of the muscle fiber surface membrane was specifically absorbed by the synthetic peptide IV.

TABLE 1

IMMUNOBLOT AND IMMUNOFLUORESCENT ANALYSES OF DYSTROPHIN IN SKELETAL MUSCLES FROM OND^a, BMD AND DMD PATIENTS

Clinical diagnosis	No. of cases	Immublot ^b			Immunofluoresce for		
		Status	kDa (<i>n</i> = 400)	% (<i>n</i> = 100)	I ^c A-B ^d	II ^c A-B ^d	4C5 ^c A-B ^d
OND	24	normal	400	100	N-N	N-N	N-N
BMD ^e	30	abnormal		reduced			
	(27)		< 400	10 ~ 60	F-P	F-P	F-P
	(3)		> 400	10 ~ 30	F-P	F-P	F-P
DMD ^f	21	undetectable	-	0	O-O	O-O	O-O

^a OND includes: idiopathic scoliosis, arthrogryposis multiplex congenita, myotonic dystrophy, fascioscapulohumeral dystrophy, limb-girdle dystrophy, distal muscular dystrophy, polymyositis, malignant hyperthermia, and spinal muscular atrophy.

^b The molecular weight and amounts of dystrophin were evaluated qualitatively by inspection with reference to adjacent control lanes.

^c Region specific antibodies to dystrophin (I: anti-DMDP I, II: anti-DMDP II, and 4C5).

^d "A" indicates staining intensity (N = normal, F = faint, O = negative), and "B" indicates staining pattern of a section (N = normal, P = patchy, O = negative) (see Materials and Methods for details).

^e Muscles from all BMD patients expressed detectable dystrophin at the plasmamembrane by all antibodies tested including C-terminus (4C5).

^f Immunoblot test did not detect dystrophin, and majority of the muscle fibers showed completely negative immunofluorescent staining for dystrophin with only rare isolated positive fibers (<0.3%).

Immunoblotting

Immunoblot detection of dystrophin was done as previously described (Hoffman et al. 1989b). A final loading volume for the SDS-polyacrylamide gel electrophoresis was adjusted after densitometric measurements (by TIAS Image Processor/NEC PC 9801 RX) of myosin heavy chain (MHC) of the Coomassie blue-stained gel. The molecular weight and amounts of dystrophin were evaluated qualitatively by inspection with reference to the normal muscle. In some selected cases, quantitation of dystrophin were performed by the densitometric analysis of the immunoblots.

Immunofluorescence

Indirect immunofluorescence method was employed as described previously (Arahata et al. 1988). For the mouse monoclonal antibody 4C5, the second layer antibody was substituted to the affinity-purified FITC-labeled goat F(ab')₂ anti-mouse IgG (Tago Inc., 10 µg/ml in PBS/BSA). Each coverslip contained 6-10 frozen sections including a normal muscle biopsy, a biopsy from a known DMD patient, and a biopsy from a known BMD patient as controls for each of the experimental biopsies on the same coverslip. All fluorescent photographs were taken by the same condition under a Zeiss Axiophot microscope with epifluorescence.

Classifications of the immunocytochemical staining patterns of dystrophin

Relative fluorescence staining intensities (A) was classified in three ways, with normal being clearly detectable

fluorescence staining (N), faint (F), and negative (O). At the same time, fluorescence staining patterns of a section (B) was judged in each biopsy and classified also in 3 ways (N,P,O). When all muscle fibers in a given section were equally reacted as a continuous ring of fluorescence at the plasma membrane, it was classified as normal (N). But when a discontinuous 'patchy' immunostaining pattern appeared between and within fibers, it was classified as 'patchy' (P). When majority of muscle fibers in a given section were not immunostained, the section was classified as negative (O) in our study, if very few isolated positive fibers were present (less than 0.3%). Then, each muscle sample was scored according as these 2 (A-B) combinations.

Molecular genetic analysis of dystrophin

DNA was extracted from peripheral blood lymphocytes and Southern blotted as described (Aldridge et al. 1984). Dystrophin cDNA probes were used to assess structural alterations throughout the dystrophin gene (Koenig et al. 1987, 1989). In some cases, PCR analysis using primers specific for dystrophin exons was used to supplement Southern blot data (Chamberlain et al. 1988).

Results

Generation of a region-specific monoclonal antibody for the C-terminus of dystrophin protein

Figure 1 illustrates the regions of dystrophin to which various antibodies have been raised. To generate region-

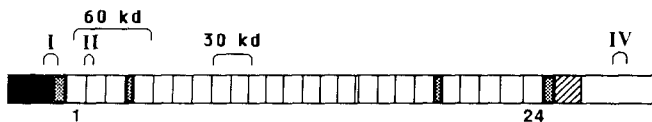


Fig. 1. Schematic diagram of dystrophin showing locations of regions used to raise antisera. Adapted from Koenig and Kunkel (1990). The four main domains are indicated as follows: amino-terminus (black box), central repeating rod (repeats 1 and 24 are indicated below), cysteine-rich region (diagonal lines) and carboxy-terminal domain (open box). Four putative hinge regions are indicated by gray boxes (Koenig et al. 1990). Origin of fusion proteins and synthetic peptides used as immunogens are indicated above. The monoclonal 4C5 antibody was raised against peptide DMDP IV.

specific antibody to the C-terminus of dystrophin, a synthetic peptide (peptide IV) corresponding to amino acids 3495–3544 (Koenig et al. 1988) was constructed. A monoclonal 4C5 antibody was generated as described in the methods, and specificity was determined by Western blots, blocking and absorption tests.

Additional antisera, anti-DMDP I and anti-DMDP II have been described previously (Sugita et al. 1988; Arahata et al. 1989). They are polyclonal antisera raised against peptides corresponding to amino acids 215–264 (DMDP I) and 440–489 (DMDP II) and each has been shown to be specific for dystrophin. Anti-30K and anti-60K polyclonal antisera were raised against fusion proteins of dystrophin and have been described extensively (Hoffman et al. 1987a,b, 1988, 1989b; Arahata et al. 1989b). Thus, this battery of antibodies allowed us to determine the presence or absence of several different regions of dystrophin including the C-terminus (Fig. 1).

Immunoblot analysis of dystrophin

Abundance and molecular weight of dystrophin in each of 75 biopsied muscle specimens was examined by immunoblotting using the 4C5 monoclonal and 30K or 60K polyclonal antibodies, and the similar results were obtained with each of these antibodies. Skeletal muscles from 24 patients with OND contained dystrophin of normal molecular weight and abundance (Table 1). On the other hand, dystrophin was undetectable in 21 muscle biopsies from male patients with DMD, and patients with BMD had dystrophin of altered size and/or abundance (Table 1) (Fig. 3).

Immunofluorescence analysis of dystrophin using region specific antisera

To determine which portions of dystrophin were preserved in patients with DMD and BMD, tissue sections from muscle biopsies were stained with antisera raised against several different portions of dystrophin (anti-DMDP I, II, and 4C5). All 24 OND patients with normal size dystrophin also had normal immunostaining patterns with each of the antisera tested (Table 1, Fig. 2). In con-

trast, none of the DMD patients had detectable staining with anti-DMDP I, II or 4C5 (Table 1, Fig. 2). Thus, the level of dystrophin expression in immunoblot-negative DMD patients was considered too low to be detected even by immunofluorescence, except those very rare isolated positive muscle fibers (less than 0.3%, as suggested by Nicholson et al. 1989) (Fig. 5).

Twenty eight of the thirty BMD muscle sections were stained at the surface membrane of the fibers by all of the antibodies tested including 4C5 (Table 1, Fig. 2). In each case, the immunostaining was fainter than normal, and the pattern was “patchy” between and within fibers as has been previously reported for BMD muscle (Arahata et al. 1989b). The two exceptions (No. 11 and 220) reacted as above with the anti-DMDP II and 4C5 antibodies but both were negative when anti-DMDP I antisera was used (Fig. 4). These findings suggest that these patients may have deletions that remove coding sequences at the 5'-end of the gene.

Deletion analysis of the dystrophin gene

The molecular defects responsible for dystrophin alterations were studied in patients for whom DNA was available. Polymerase chain reaction and Southern blot based testing were done on the dystrophin genes of 3 patients with DMD and 18 with BMD (Table 2). The DMD patients had deletions of exons 52 or 45, a mutation predicted to cause a frame shift (Koenig et al. 1989b).

Fifteen of the BMD patients had in-frame deletions in the “hot spot” region (exons 45–53) in the distal portion of the rod domain (Table 2). Interestingly, the two BMD patients (No. 11 and 220) were the apparent exception to the reading frame rule (Malhotra et al. 1988; Koenig et al. 1989).

Discussion

Our results describing the molecular differences between the dystrophin molecules produced by BMD and DMD patients. We confirmed that all BMD patients have dystrophin gene products which include the C-terminal domain using a new monoclonal antibody directed specifically against the C-terminal portion of the dystrophin molecule. In contrast, we have shown that the C-terminus is indeed absent in all patients with DMD. These observations strongly support the hypothesis that the C-terminal domain is critical for producing the proper function of dystrophin molecule. This will be also important for the proper clinical diagnosis of DMD and BMD.

To date, much attention has been paid to the relationship between clinical severity and patterns of deletion mutations. The initial observations that deletion size did not

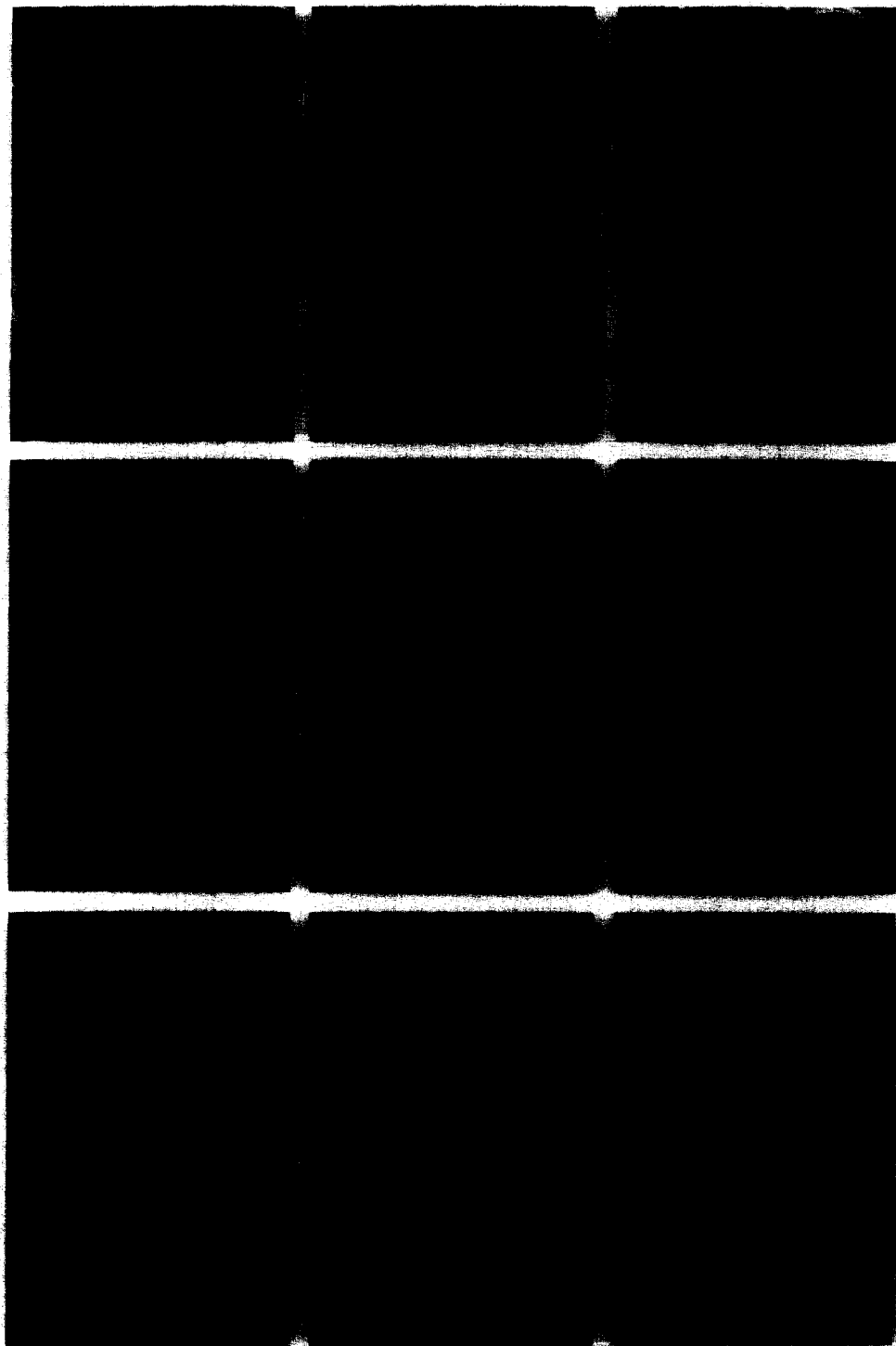


Fig. 2. Immunofluorescent analysis of dystrophin expression in skeletal muscles from OND (other neurological diseases as controls; a,b,c), BMD (Becker muscular dystrophy; d,e,f) and DMD (Duchenne muscular dystrophy; g,h,i) are shown. Four micron Frozen sections were immunostained with three different region specific antibodies to dystrophin, i.e., polyclonal anti-DMDP I (N-terminal domain) antiserum (a,d,g), anti-DMDP II (2nd rod-like domain) antiserum (b,e,h), and a monoclonal 4C5 (C-terminal domain) antibody (c,f,i). In OND, dystrophin appeared normally as a continuous clear fluorescence at the plasma membrane of each fiber. All BMD sections had fainter and discontinuous 'patchy' immunostaining at the plasma membrane. On the other hand, DMD sections lacked immunoreactivity. Thus, the C-terminal portion of dystrophin is preserved in all BMD and OND, but is absent in DMD muscle (250 × for OND, BMD; 125 × for DMD).

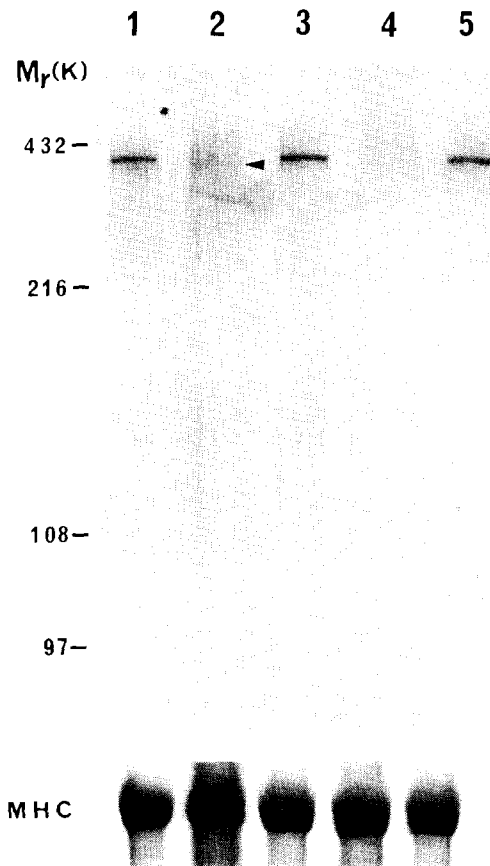


Fig. 3. Western blot analysis of dystrophin using the C-terminal region specific monoclonal anti-dystrophin antibody 4C5. Lanes 1, 3, 5, OND; lane 2, BMD; lane 4, DMD skeletal muscles. Cryostat thin sections from each biopsy were boiled in the loading buffer, and the amounts of myosin heavy chain (MHC) were adjusted by densitometric analysis of the Coomassie blue-stained gel. Then, muscles were loaded in lanes 1 through 5. Mr standards (tetramer, dimer and monomer of β -galactosidase, and phosphorylase *b*) are shown on the left. Dystrophin is evident in OND as the expected protein of approximately 400 kDa, but

TABLE 2

EXTENT OF DELETED EXONS IN BMD AND DMD PATIENTS FOR WHOM DNA WAS AVAILABLE

Patients ^a No.	Clinical diagnosis	Age (yrs)	Exons deleted ^b
1	BMD	16	45-47
2	BMD	11	45-47
3	BMD	9	45-53
4	BMD	15	45-47
5	BMD	29	45-48
6	BMD	16	45-48
9	BMD	41	45-47
11	BMD	15	3-7
15	BMD	9	45-47
19	BMD	32	45-49
24	BMD	37	45-47
32	BMD	15	no deletion
89	BMD	26	45-48
93	BMD	33	45-48
99	BMD	31	45-48
105	BMD	42	45-47
106	BMD	23	45-49
220	BMD	16	3-7
38b	DMD	18	52
91	DMD	3	52
223	DMD	25	45

^a All BMD patients are still ambulatory but DMD patient Nos. 38b and 223 entered the wheelchair at age 10 and 12 years, respectively, and No. 91 already has a positive Gowers' sign at age 3.

^b Exon numbering according to Koenig (1989).

is missing in DMD muscle. By densitometric analysis, the BMD patient had dystrophin of low molecular weight (390 kDa) with reduced quantity (27% of normal; arrowhead).

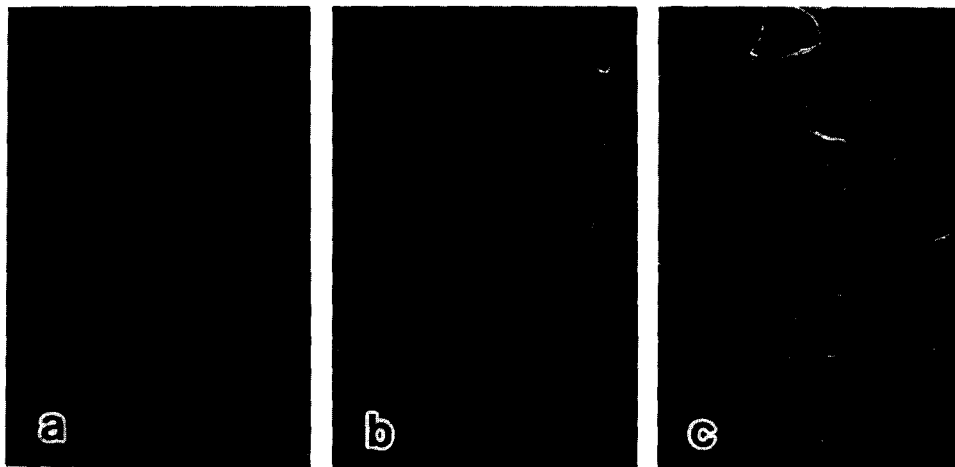


Fig. 4. Immunofluorescence characterization of dystrophin in a BMD patient with No. 11 a deletion between exons 3-7 who exhibits low molecular weight (370 kDa) dystrophin of reduced quantity (10% of normal). Immunoreaction was not observed for the anti-DMDP I (a), but faint, patchy staining was seen with the other antibodies (anti-DMDP II, b; 4C5, c). (125 \times).

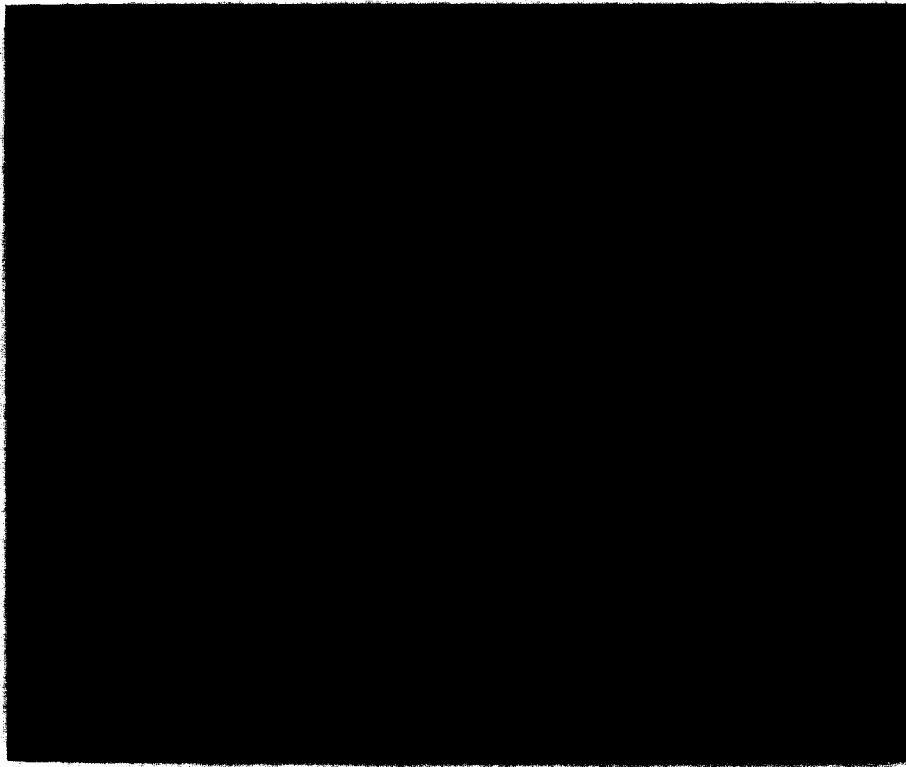


Fig. 5. Frozen section of skeletal muscle from a DMD patient who had deletion of exon 52, a mutation predicted to cause a frame shift. Most muscle fibres had no detectable dystrophin at all, but a very few percentage (less than 0.3%) of isolated myofibers (arrow) exhibited positive immunostaining. This type of immunostaining pattern was classified as negative (0) in this study (see Materials and Methods for details) (250 ×).

correlate with severity (Monaco et al. 1988; Forrest et al. 1988; Lindlof et al. 1988) were explained by the reading frame hypothesis of Monaco and co-workers (Monaco et al. 1988). Patients with BMD have been shown to have intragenic deletions that maintain the translational reading frame, thus allowing production of an altered but semi-functional protein. In contrast, patients with DMD have deletions that remove an uneven number of triplet codons causing a frame-shift and leading to production of a truncated protein missing sequences distal to the deletion. With the exception of one notable class of deletions at the 5' end of the gene (e.g. deletions of exons 3–7) (Malhotra et al. 1988), the reading frame hypothesis has been shown to be true for over 90% of deletions (Koenig et al. 1989; Baumbach et al. 1989; Gillard et al. 1989). If this is the case, then dystrophin in BMD patients should react with the 4C5 monoclonal antibody since virtually all deletions that have been mapped in BMD patients are in the amino terminal or central rod domains of dystrophin (Table 2) (Koenig et al. 1987, 1989; Forrest et al. 1988; Gillard et al. 1989). In fact, we have shown that the C-terminus is indeed absent in patients with DMD and it is invariably present in patients with BMD.

Deletion analysis of three DMD and eighteen BMD patients in our series also supports the reading frame hypothesis. Two DMD patients were missing a single exon

(No. 52) in the distal portion of the rod domain. This exon contains 39 1/3 triplet codons so we expect a frame shift to occur at the deletion junction (Koenig et al. 1989). Thus, although missing the C-terminal domains, the proteins produced by these DMD patients might be expected to contain the entire amino-terminus and the first 20 of 24 repeating units in the rod domain. Nevertheless, we did not detect immunoreactivity with any of our antisera in these patients. Therefore, we conclude that the truncated dystrophin molecules produced by these patients must be extremely unstable.

Fifteen of the eighteen BMD patients also fit the reading frame rule. Their deletions all occurred in the "hot spot" region of exons 45–53 where the majority of deletions causing BMD are found (Koenig et al. 1989). Since anti-DMDP I and II and the 30K and 60K antisera all recognize proximal portions of the molecule, it is not surprising that these antisera detected the mutant dystrophins. On the other hand, detection of these dystrophins by the 4C5 antibody could only occur if the mutations resulted in internally deleted proteins with intact C-termini.

In contrast, patient No. 11 and 220 had a deletion of exons 3–7 which code for amino acids 32–217. Since anti-DMDP I is raised against a synthetic peptide consisting of amino acids 216–264 (included in exon 8), we would expect this antisera to recognize the altered protein in these

patients. Since it does not, we conclude that exon 8 is either not present in the dystrophin produced by these patients, or that the configuration of the protein is disrupted by the deletion and the epitope(s) recognized by the antisera are destroyed. We favor the first hypothesis because it suggests an explanation for the low levels of dystrophin production in these patient's muscles. Splicing from exon 2 to exon 8 (caused by deletion of exons 3–7) is predicted to cause a frame shift and no protein production, however, if an alternative splice could occur between exon 2 and either 9, 10 or 11, then the reading frame would be restored and an internally deleted protein could be produced. This protein should be detected by anti-DMDP II, 30K and 60 K, and 4C5, but it would not be recognized by anti-DMDP I – exactly the pattern of immunoreactivity we observed (Fig. 4). Furthermore, this presumed alternative splicing might be expected to occur with low frequency, an assumption compatible with our observation of low dystrophin levels (about 10% of normal) in this patient. Another possible explanation for these findings is that translation is initiated from an alternative start site downstream of exon 8, as suggested by Malhotra et al. (1988). Future studies using antisera specific for exons 1 and 2, and/or examination of the mRNA produced by these patients may help distinguish between these possibilities.

The fact that some BMD patients may make dystrophin that does not react with certain antisera has several implications for diagnostic testing. Clearly, if only one antiserum is used in diagnostic tests (either immunoblots or immunofluorescence) there is a risk of mis-diagnosing a BMD patient as DMD. Since the C-terminus is probably one of the most important region for determining stability of mutant dystrophins, and hence clinical phenotype, we suggest that diagnostic tests be carried out using at least one antibody raised against this region (e.g., 4C5).

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