Am. J. Hum. Genet. 53:409–416, 1993

Mild Deficiency of Dystrophin-associated Proteins in Becker Muscular Dystrophy Patients Having In-Frame Deletions in the Rod Domain of Dystrophin

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Summary

The dystrophin-glycoprotein complex spans the sarcolemma to provide a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix in skeletal muscle. In Duchenne muscular dystrophy (DMD), the absence of dystrophin leads to a drastic reduction in all of the dystrophin-associated proteins in the sarcolemma, thus causing the disruption of the dystrophin-glycoprotein complex and the loss of the linkage to the extracellular matrix. The resulting sarcolemmal instability is presumed to render muscle fibers susceptible to necrosis. In the present study, we investigated the status of the dystrophin-associated proteins in the skeletal muscle from patients with Becker muscular dystrophy (BMD), a milder allelic form of DMD. BMD patients having in-frame deletions in the rod domain of dystrophin showed a mild to moderate reduction in all of the dystrophin-associated proteins in the sarcolemma, but this reduction was not as severe as that in DMD patients. The reduction of the immunostaining for the dystrophin-associated proteins showed a good correlation with that for dystrophin in both intensity and distribution. Our results indicate that (1) the abnormality of the sarcolemmal glycoprotein complex, which is similar to but milder than that in DMD patients, also exists in these BMD patients and (2) the rod domain of dystrophin is not crucial for the interaction with the dystrophin-associated proteins.

Introduction

Duchenne muscular dystrophy (DMD) is caused by the absence of dystrophin, which is due to defects in the DMD gene on the X chromosome (Hoffman et al. 1987; Koenig et al. 1988). Dystrophin is a cytoskeletal protein tightly associated with a large oligomeric complex of sarcolemmal glycoproteins, including dystroglycan, which binds laminin, a major protein component of the extracellular matrix (Campbell and Kahl 1989; Ervasti et al. 1990, 1991; Yoshida and Ozawa 1990; Ervasti and Campbell 1991; Ohlendieck and Campbell 1991*a;* Ohlendieck et al. 1991*b;* Ibraghimov-Beskrovnaya et al. 1992). The amino-terminal domain of dystrophin interacts with F-actin (Hemmings et al. 1992; Way et al. 1992). These findings indicate that the dystrophin-glycoprotein complex is a trans-sarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix.

At present, a major goal for DMD research is a detailed understanding of the mechanism by which the absence of dystrophin leads to muscle fiber necrosis. Recently, we have demonstrated that the absence of dystrophin leads to a drastic reduction in all of the dystrophin-asociated proteins in the sarcolemma of DMD patients (Ervasti et al. 1990; Ibraghimov-Beskrovnaya et al. 1992; Ohlendieck et al. 1993). The disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix because of the loss, in all components, of the dystrophin-glycopro-

Received February 18, 1993; revision received April 20, 1993.
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0002-9297/93/5302-0013$02.00

tein complex is presumed to cause severe sarcolemmal instability, which, in turn, may render muscle fibers susceptible to necrosis (Ohlendieck et al. 1993; Matsumura and Campbell, in press).

Becker muscular dystrophy (BMD), a milder allelic form of DMD, is also caused by the defects in the dystrophin gene. While out-of-frame mutations in the dystrophin gene are frequently found in DMD patients, in-frame mutations are found in many cases of BMD (Malhotra et al. 1988; Monaco et al. 1988; Baumbach et al. 1989; Gillard et al. 1989; Koenig et al. 1989; Beggs et al. 1991). Dystrophin of abnormal size and/or reduced quantity has been detected by immunoblot analysis, and abnormal dystrophin staining has been demonstrated by immunohistochemical analysis in BMD (Arahata et al. 1988, 1989, 1991; Hoffman et al. 1988, 1989; England et al. 1990; Nicholson et al. 1990; Bulman et al. 1991; Slater and Nicholson 1991; Gangopadhyay et al. 1992; Ikeya et al. 1992). However, the precise mechanism by which the reported abnormalities of dystrophin lead to muscle fiber degeneration in this disease has not been clarified. The aforementioned structural organization of the dystrophin-glycoprotein complex, together with the findings in DMD patients, indicates that the clarification of the status of the dystrophin-associated proteins in BMD patients is important for the elucidation of this mechanism. In the present study, we performed immunohistochemical analysis of dystrophin-associated proteins in the biopsied skeletal muscle from BMD patients having in-frame rearrangements in the rod domain of dystrophin.

Material and Methods

Skeletal Muscle Biopsy Specimens

Skeletal muscle biopsy specimens were obtained from 11 BMD patients (table 1). Diagnosis of BMD was made on the basis of the history of illness, physical examination, family history, pathological examination of the biopsied skeletal muscle, immunohistochemical and immunoblot analysis of dystrophin, and analysis of the dystrophin gene. In-frame rearrangements in the rod domain of dystrophin were confirmed in all 11 patients. Deletion of the exons 45–47 and exons 45–48 were the most common, in accordance with the previous reports (Koenig et al. 1989; Beggs et al. 1991), and were each found in four patients. Deletion of the exons 35–44 and 13–44 and duplication of the exons 43–47 were found in one patient each. Skeletal muscle biopsy specimens with no obvious pathological abnormality and skeletal muscle biopsy specimens from patients with DMD, limb-girdle muscular dystrophy, myotonic dystrophy, facioscapulohumeral muscular dystrophy, oculopharyngeal muscular dystrophy, non-Fukuyama–type congenital muscular dystrophy, and spinal muscular atrophy were investigated as normal and disease controls, respectively.

Antibodies

Monoclonal antibodies IVD3$_1$ against the 50-kDa dystrophin-associated glycoprotein (50DAG) and VIA4$_2$ against dystrophin were characterized elsewhere (Ervasti et al. 1990; Ohlendieck et al. 1991*b*). The epitope of VIA4$_2$ is confined to the cysteine-rich and the carboxyl-terminal domains of dystrophin and thus is not involved in the BMD patients examined in the present study (table 1). Sheep antibodies against the 156-kDa dystroglycan (156DAG), 59-kDa dystrophin-associated protein (59DAP), 50DAG, 43-kDa dystroglycan (43DAG), and 35-kDa dystrophin-associated glycoprotein (35DAG) were affinity purified according to methods described elsewhere (Ohlendieck and Campbell 1991*b;* Ibraghimov-Beskrovnaya et al. 1992; Matsumuraet al. 1992*a,* 1992*b;* Ohlendieck et al. 1993). Affinity-purified rabbit antibodies against the first 15 amino acids of the N-terminus and the last 10 amino acids of the C-terminus of dystrophin were characterized elsewhere (Ervasti et al. 1991; Ohlendieck and Campbell 1991*b;* Ohlendieck et al. 1991*a,* 1991*b*).

Immunohistochemistry

Indirect immunofluorescence microscopy of 7-μm-thick cryosections from skeletal muscle biopsy specimens was performed according to methods described elsewhere (Ohlendieck and Campbell 1991*b;* Ohlendieck et al. 1991*a,* 1991*b,* 1993). Blocking was performed by a 30-min incubation with 5% BSA in PBS (50 mM sodium phosphate pH 7.4, 0.9% NaCl). Incubation with primary antibodies was performed for 1 h. In the case of rabbit or mouse primary antibodies, cryosections were incubated with 1:200 diluted fluorescein-labeled goat anti-rabbit IgG or anti-mouse IgG (Boehringer Mannheim) for 1 h. In the case of sheep primary antibodies, cryosections were incubated for 30 min with 1:500 diluted biotinylated rabbit anti-sheep IgG (Vector Laboratories) followed by incubation for 30 min with 1:1,000 diluted fluorescein-conjugated streptoavidin (Jackson ImmunoResearch Laboratories). Each incubation was followed by rigorous washing with PBS. Final specimens were examined under a Zeiss Axioplan fluorescence microscope. For reliable com-

Table 1

Summary of the BMD Patients Investigated in the Present Study

No.	Age (years)	Sex	Exons[a]	Effects on ORF[b]	Status of Dystrophin-associated Proteins[c]
1	6.5	M	13–44	In-Frame	near normal
2	11	M	35–44	In-Frame	moderate reduction
3	12	M	45–47	In-Frame	moderate reduction
4	14	M	45–48	In-Frame	mild reduction
5	27	M	45–48	In-Frame	mild reduction
6	32	M	45–48	In-Frame	mild reduction
7	32	M	45–47	In-Frame	mild reduction
8	43	M	45–47	In-Frame	mild reduction
9	46	M	45–48	In-Frame	near normal
10	50	M	43–47	In-Frame	mild reduction
11	55	M	45–47	In-Frame	mild reduction

[a] Deleted exons of the dystrophin gene, according to Koenig et al. (1989), except in patient 10, where exons were duplicated.

[b] Predicted effects on the open reading frame of the dystrophin gene, according to Koenig et al. (1989).

[c] The overall status of the dystrophin-associated proteins in the sarcolemma was scaled as the following, on the basis of the immunostaining intensity and distribution compared with normal and DMD muscles: near normal, mild reduction, moderate reduction, and severe reduction (near-DMD level).

parison, cryosections from BMD patients, normal controls, and disease controls were placed on the same microscopy slide and were processed identically. In addition, photographs were taken under identical conditions with the same exposure time.

Results

All components of the dystrophin-glycoprotein complex were colocalized to the sarcolemma in the skeletal muscle from normal humans and patients with various non-DMD neuromuscular diseases, as described elsewhere (Ervasti et al. 1990; Ibraghimov-Beskrovnaya et al. 1992; Matsumura et al. 1992*b;* Ohlendieck et al. 1993) (figs. 1 and 2). In the skeletal muscle from DMD patients, dystrophin was absent, and all of the dystrophin-associated proteins were greatly reduced in the sarcolemma (fig. 2) (Ervasti et al. 1990; Ibraghimov-Beskrovnaya et al. 1992; Matsumura et al. 1992*b;* Ohlendieck et al. 1993).

In the skeletal muscle from BMD patients, immunostaining for the components of the dystrophin-glycoprotein complex was reduced in the sarcolemma to varying degrees (table 1, figs. 1 and 2). For each patient, however, the reduction in the intensity of the staining for the dystrophin-associated proteins showed a good correlation with that for dystrophin (figs. 1 and 2). The distribution of staining for dystrophin and the dystrophin-associated proteins also showed a good correlation when examined on serial cryosections (figs. 1 and 2). When dystrophin staining was inhomogeneous and patchy along the sarcolemma, staining for the dystrophin-associated proteins was also inhomogeneous and patchy (figs. 1 and 2). In none of the patients investigated, however, was the reduction in staining for the dystrophin-associated proteins as severe as that in typical DMD patients (table 1) or in DMD patients lacking the cysteine-rich and carboxyl-terminal domains of dystrophin, as reported elsewhere (Matsumura et al., in press-*b*). In a young patient (patient 1) with a huge deletion of exons 13–44 of the dystrophin gene, dystrophin and all of the dystrophin-associated proteins were relatively well preserved in the vast majority of muscle fibers. However, all of these proteins were greatly reduced in the sarcolemma, in scattered clusters of regenerating muscle fibers (fig. 3).

Discussion

Here we reported a mild to moderate reduction of the dystrophin-associated proteins in the sarcolemma of BMD patients having in-frame rearrangements in the rod domain of dystrophin. The reduction of the immunostaining for the dystrophin-associated proteins showed a good correlation with that for dystrophin in both intensity and distribution, suggesting that the dys-

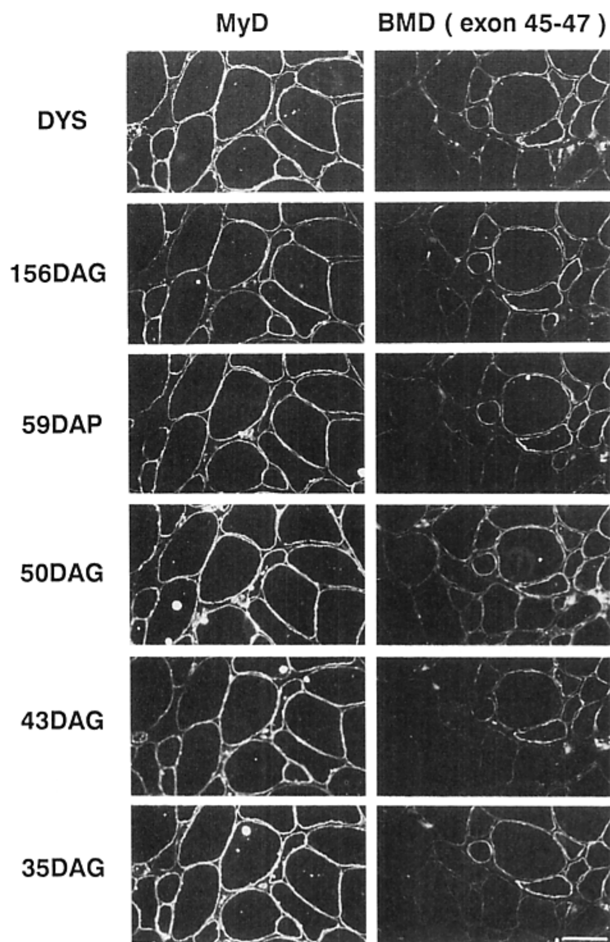

Figure 1 Immunohistochemical analysis of the dystrophin-associated proteins in the skeletal muscle from a BMD patient with deletion of exons 45–47 of the dystrophin gene. Transverse cryosections (7 μm) from biopsied skeletal muscle from a patient with myotonic dystrophy (MyD) and a BMD patient (patient 8) were immunostained with monoclonal antibody VIA4$_2$ against the cysteine-rich and carboxyl-terminal domains of dystrophin (DYS), and affinity-purified sheep antibodies against the dystrophin-associated proteins (156DAG, 59DAP, 50DAG, 43DAG, and 35DAG). Dystrophin and all of the dystrophin-associated proteins were reduced in the sarcolemma of some of the muscle fibers in the BMD patient. Also note the codistribution of dystrophin and the dystrophin-associated proteins in a patchy fashion along the sarcolemma. Bar in the lower-right corner = 50 μm.

trophin-associated proteins were reduced because of the reduction of dystrophin in these patients.

The structural organization of the dystrophin-glycoprotein complex suggests that the dysfunction/disruption of this complex may result in the loss of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix, which could lead to severe sarcolemmal instability and, eventually, to muscle fiber degeneration (Matsumura and Campbell, in press). The following observations are consistent with this hypothesis: (1) The absence of dystrophin causes a drastic reduction in all of the dystrophin-associated proteins in large-caliber skeletal muscles of mdx mice (Ohlendieck and Campbell 1991*b*). (2) In small-caliber skeletal muscles and cardiac muscles of mdx mice which are relatively free from degeneration, the dystrophin-associated proteins are well preserved, presumably because of the association with overexpressed utrophin, an autosomal homologue of dystrophin (Ohlendieck et al. 1991*b;* Matsumura et al. 1992*a;* Tinsley et al. 1992). (3) The absence of dystrophin causes a drastic reduction in all of the dystrophin-associated proteins in DMD skeletal muscle (Ervasti et al. 1990; Ibraghimov-Beskrovnaya et al. 1992; Ohlendieck et al. 1993). (4) All of the dystrophin-associated proteins are greatly reduced in the dystrophin-deficient muscle fibers, while they are well preserved in the dystrophin-positive fibers in a symptomatic DMD carrier (Matsumura et al., in press-*a*). (5) The deficiency of the 50DAG alone causes severe childhood autosomal recessive muscular dystrophy with a DMD-like phenotype (Matsumura et al. 1992*b*). (6) The dystrophin-associated proteins are expressed abnormally, despite the near-normal expression of dystrophin, in Fukuyama-type congenital muscular dystrophy (Matsumura et al. 1993).

Recently, the domain of dystrophin which interacts with the dystrophin-associated proteins was reported to exist in the cysteine-rich and the first half of the carboxyl-terminal domains, on the basis of the results of limited calpain digestion of the dystrophin-glycoprotein complex (Suzuki et al. 1992). This is also consistent with our recent findings on the status of the dystrophin-associated proteins in the unique DMD patients who had dystrophin lacking the cysteine-rich and carboxyl-terminal domains (Matsumura et al., in press-*b*). In these patients, the dystrophin-associated proteins were reduced in the sarcolemma as greatly as were those in typical DMD patients, despite the expression and localization of truncated dystrophin to the sarcolemmal region. The deficiency of the dystrophin-associated proteins in the sarcolemma was considered to be the cause of the severe phenotype, despite the proper expression and intracellular localization of truncated dystrophin in these patients. The present results, which demonstrated only a mild and comparable reduction of dystrophin and the dystrophin-associated proteins in the BMD patients having in-frame rearrangements in the rod domain of dystrophin, further indicate that the

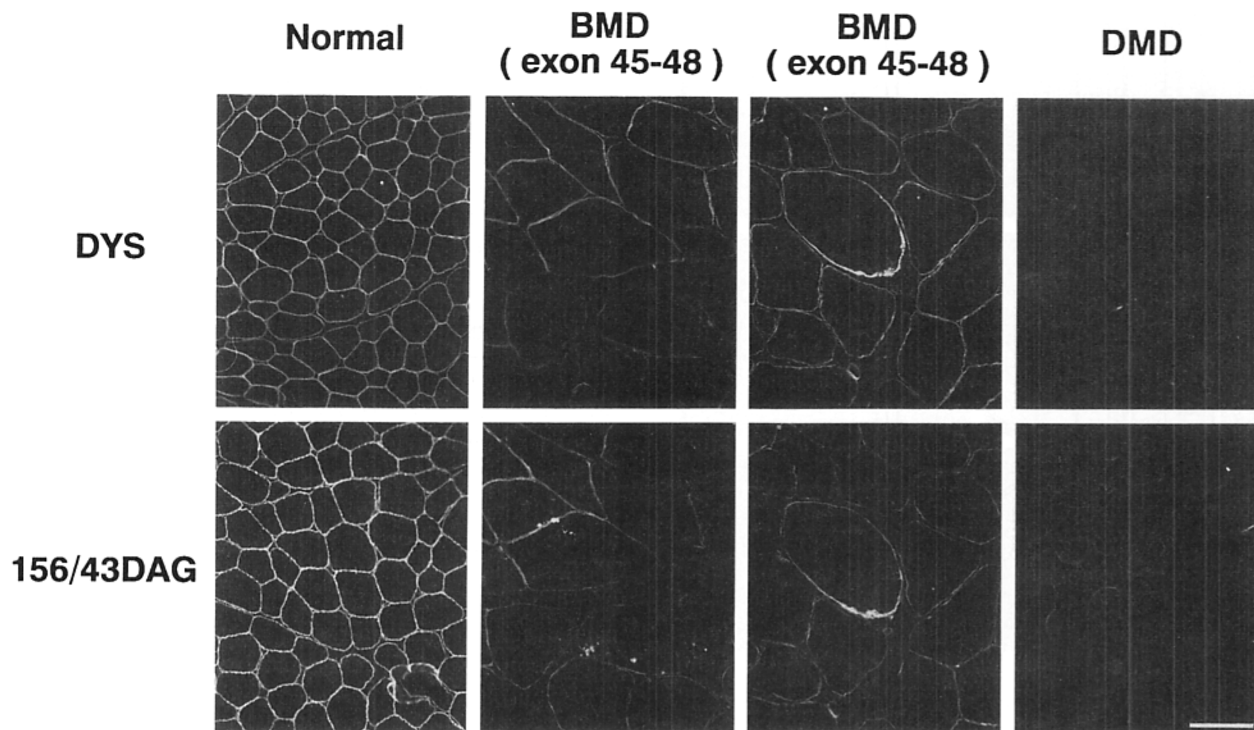

Figure 2 Immunohistochemical analysis of the dystrophin-associated proteins in the skeletal muscle from BMD patients with deletion of exons 45–48 of the dystrophin gene. Transverse cryosections (7 μm) from biopsied skeletal muscle from a human with no obvious pathological abnormality (Normal), two BMD patients with deletion of exons 45–48 of the dystrophin gene (patients 4 and 5), and a DMD patient were immunostained with monoclonal antibody VIA4$_2$ against the cysteine-rich and carboxyl-terminal domains of dystrophin (DYS) and affinity-purified sheep antibody against the dystroglycan fusion protein. Dystrophin and dystroglycan (156/43DAG) were slightly reduced in the BMD sarcolemma. Also note the codistribution of dystrophin and dystroglycan in a patchy fashion along the sarcolemma. Bar in the lower-right corner = 50 μm.

rod domain of dystrophin is not crucial for the proper interaction with and stabilization of the dystrophin-associated proteins.

The amino-terminal domain which interacts with the actin-cytoskeleton and the cysteine-rich and carboxyl-terminal domains which interact with the dystrophin-associated proteins are both presumed to be retained in the BMD patients having in-frame rearrangements in the rod domain of dystrophin (Hemmings et al. 1992; Levine et al. 1992; Suzuki et al. 1992; Way et al. 1992; Matsumura et al., in press-*b*). This suggests that the link between the subsarcolemmal actin cytoskeleton and the extracellular matrix via the dystrophin-glycoprotein complex is relatively intact in these patients. However, dystrophin which has defects in the rod domain may not have a normal function or may be unstable, and this may lead to a mild reduction in the density of the dystrophin-glycoprotein complex in the sarcolemma. This could explain the mild phenotype of these BMD patients (Gospe et al. 1989; Koenig et al. 1989; England et al. 1990; Beggs et al. 1991; Love et al. 1991). Thus, the specific site and size of the rearrangements in the rod domain of dystrophin could influence the functional status of the complex and, eventually, modify the phenotype of the patient.

On the other hand, deletions involving the amino-terminal domain of dystrophin are known to lead to a phenotype of severe BMD (Malhotra et al. 1988; Koenig et al. 1989; Beggs et al. 1991; Matsumura and Imoto 1991; Vainzof et al. 1992). In these patients, the link between the dystrophin-glycoprotein complex and the subsarcolemmal actin cytoskeleton could be disrupted because of the loss of or defects in the actin binding activity of truncated dystrophin (Hemmings et al. 1992; Levine et al. 1992; Way et al. 1992). This could explain the severe phenotype of these patients. Thus, it would be interesting to investigate the status of the components of the dystrophin-glycoprotein complex in

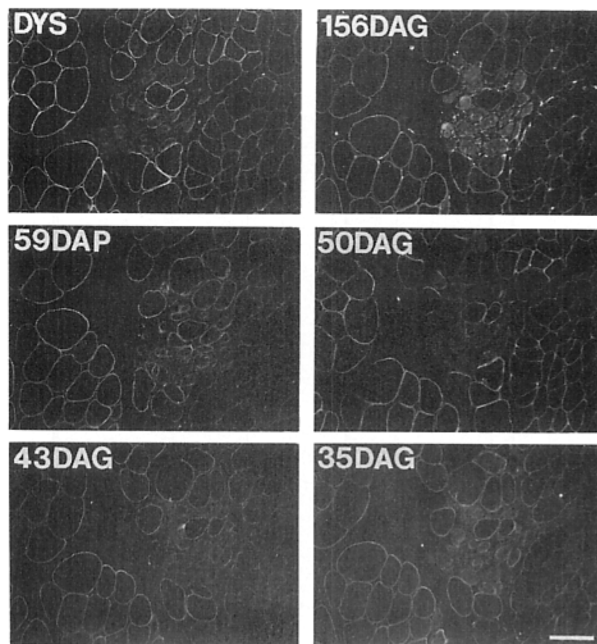

Figure 3 Immunohistochemical analysis of the dystrophin-associated proteins in the skeletal muscle from a BMD patient with deletion of exons 13–44 of the dystrophin gene. Transverse cryosections (7 μm) from biopsied skeletal muscle from a BMD patient (patient 1) were immunostained with affinity-purified rabbit antibody against the N-terminus of dystrophin (DYS), monoclonal antibody IVD3$_1$ against the 50DAG, and affinity-purified sheep antibodies against the 156DAG, 59DAP, 43DAG, and 35DAG. Dystrophin and all of the dystrophin-associated proteins were relatively well preserved in the sarcolemma of this patient. However, all of these proteins were drastically reduced in the sarcolemma in a cluster of regenerating muscle fibers. Bar in the lower-right corner = 100 μm.

these patients. Taken together with these observations, the present results would have significant implications on the development of potential DMD therapies using dystrophin minigenes, since dystrophin minigenes effective for the prevention of muscle fiber necrosis in DMD must have the binding activity for the dystrophin-associated proteins (Love et al. 1991).

Finally, a phenomenon called "delayed expression of dystrophin in the regenerating muscle fibers" was reported in young siblings with BMD (Tachi et al. 1992). We observed a similar phenomenon in a young patient with a large deletion in the rod domain of dystrophin (patient 1). Dystrophin staining was greatly reduced along the sarcolemma in clusters of regenerating muscle fibers in this patient, and, interestingly, all of the dystrophin-associated proteins were also greatly reduced in the sarcolemma of these muscle fibers. These findings suggest that the assembly of the dystrophin-glycoprotein complex or the incorporation of the complex into the sarcolemma is not completed or could be delayed in these muscle fibers. We have not encountered this phenomenon in a large number of patients with various neuromuscular diseases investigated in our laboratory. Whether this phenomenon is specific to certain cases of BMD, as originally suggested by Tachi et al. (1992), awaits further investigation.

Acknowledgments

K. P. Campbell is an Investigator of the Howard Hughes Medical Institute. This work was also supported by the Muscular Dystrophy Association, Uehara Memorial Foundation, and Association Française Contre les Myopathies.

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