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EXPRESSION OF DYSTROPHIN-ASSOCIATED PROTEINS IN DYSTROPHIN-POSITIVE MUSCLE FIBERS (REVERTANTS) IN DUCHENNE MUSCULAR DYSTROPHY

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Abstract—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. This is presumed to lead to sarcolemmal instability which could render muscle fibers susceptible to necrosis. In DMD, a very small percentage of muscle fibers show dystrophin staining along the sarcolemma, presumably due to a second in-frame deletion in the dystrophin gene. However, the functional significance of these rare dystrophin-positive muscle fibers (revertants) in DMD has been unclear. Here we report the co-expression of the dystrophin-associated proteins with dystrophin in revertants of DMD skeletal muscle. Our results suggest that the entire dystrophin–glycoprotein complex is restored in revertants and, thus, the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix is restored in these muscle fibers.

Key words: Duchenne muscular dystrophy, revertant, dystrophin–glycoprotein complex, dystrophin-associated proteins, dystroglycan.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is caused by the absence of dystrophin due to defects in the DMD locus on the X chromosome [1, 2]. Dystrophin is a cytoskeletal protein tightly associated with a large oligomeric complex of sarcolemmal glycoproteins, including α-dystroglycan which binds the extracellular matrix component, laminin [3–10]. Dystrophin is a major component of the subsarcolemmal cytoskeleton, constituting 2% of total sarcolemmal proteins and 5% of sarcolemmal cytoskeletal proteins [6, 8]. Dystrophin also interacts with F-actin [11, 12]. These findings indicate that the dystrophin–glycoprotein complex (DGC) is a major trans-sarcolemmal structure which links the subsarcolemmal cytoskeleton to the extracellular matrix [10, 13].

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The structural organization of the DGC suggests that the disruption of the complex and/or the deficiency in the components of this complex may result in severe sarcolemmal instability and may, eventually, lead to muscle fiber degeneration [13]. This hypothesis is supported by the following findings: (1) the absence of dystrophin causes a great reduction in all of the dystrophin-associated proteins (DAPs) in large-caliber skeletal muscles of mdx mice [14]; (2) in small-caliber skeletal muscles and cardiac muscle of mdx mice which are relatively free from degeneration, the DAPs are well preserved, presumably due to the association with overexpressed dystrophin-related protein, an autosomal homologue of dystrophin [15–17]; (3) the absence of dystrophin causes a great reduction in all of the DAPs in DMD skeletal muscle [4, 10, 18]; (4) all of the DAPs are greatly reduced in the unique DMD patients who have dystrophin lacking the cysteine-rich and carboxyl-terminal domains, despite the proper expression and localization of truncated dystrophin to the sarcolemmal region

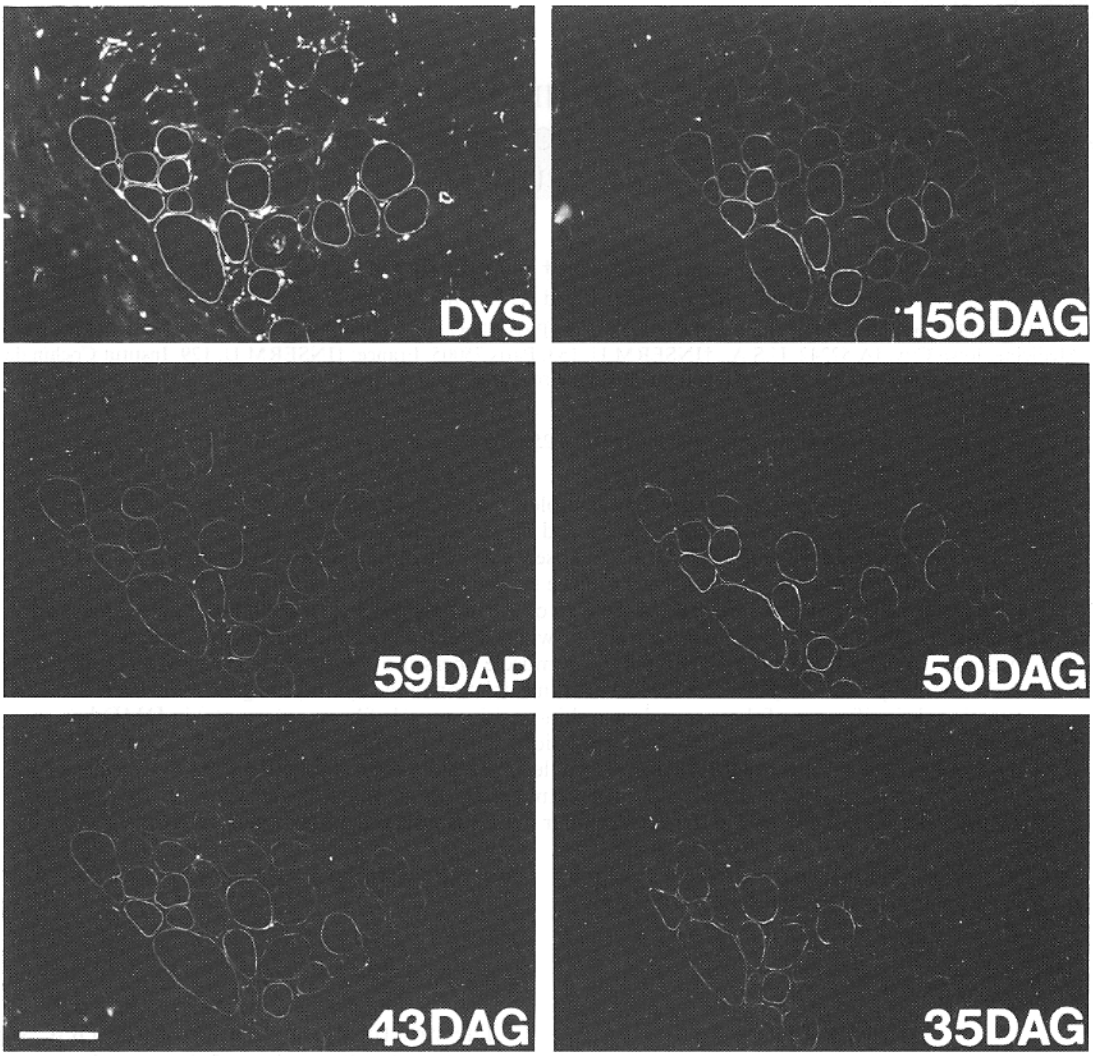

Fig. 1. Immunohistochemical analysis of dystrophin and the DAPs in the skeletal muscle from a 9-yr-old DMD patient who had a deletion of exons 12–43 of the dystrophin gene. Serial transverse cryosections (7 μm) were immunostained with a monoclonal antibody against the cysteine-rich and carboxyl-terminal domains of dystrophin (DYS), a monoclonal antibody against the 50DAG and affinity-purified sheep polyclonal antibodies against the 156DAG, 59DAP, 43DAG and 35DAG. Small clusters of dystrophin-positive muscle fibers (revertants) were observed among the vast majority of dystrophin-negative fibers. Affinity-purified rabbit polyclonal antibodies against the first 15 amino acids of the N-terminus and the last 10 amino acids of the C-terminus of dystrophin also stained the sarcolemma of revertants (not shown). The level of expression of the DAPs was generally higher in the revertants than in the surrounding dystrophin-negative fibers. The apparent intracytoplasmic dystrophin-staining in one revertant is probably due to the staining of the invaginating sarcolemma of a splitting fiber at this particular section. Bar 100 μm.

[19]; (5) all of the DAPs are greatly reduced in dystrophin-deficient muscle fibers, while they are well preserved in dystrophin-positive fibers in a symptomatic DMD carrier [20]; (6) the specific deficiency of the 50 kDa dystrophin-associated glycoprotein (50DAG) alone causes severe childhood autosomal recessive muscular dystrophy with a DMD-like phenotype [21]; (7) the DAPs are expressed abnormally, despite the near-normal presence of dystrophin, in another type of severe childhood muscular dystrophy, Fukuyama-type congenital muscular dystrophy [22].

In DMD patients and mdx mice, a small percentage of muscle fibers are known to show dystrophin-staining along the sarcolemma [23–28]. According to Burrow *et al.* [26] dystrophin-positive muscle fibers (revertants) are found in more than 50% of DMD patients. Recent studies indicate that revertants are the result of a second in-frame deletion rather than the result of somatic mosaicism [27, 28]. On the other hand, the functional significance of revertants expressing internally-deleted dystrophin remains unclear. Recently, the important role of the cysteine-rich and carboxyl-terminal domains of

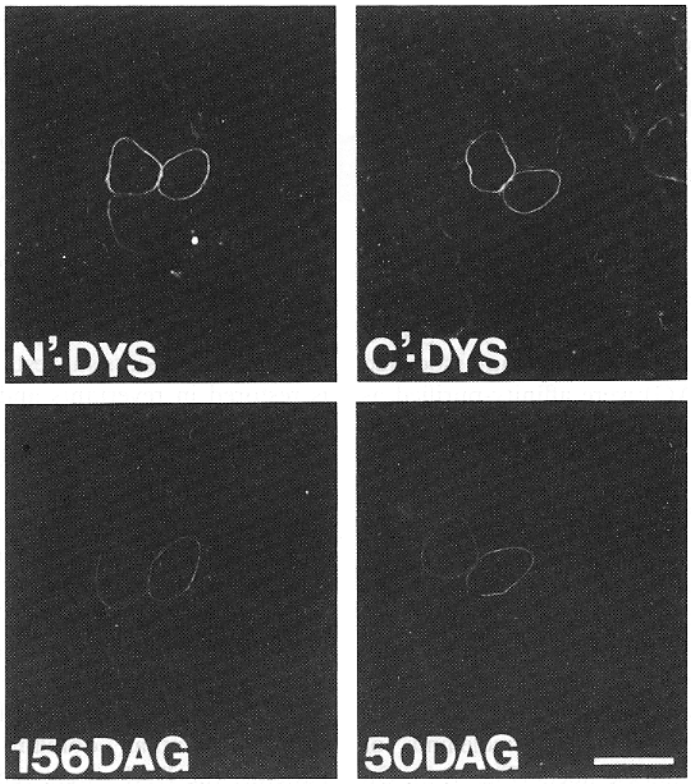

Fig. 2. Immunohistochemical analysis of dystrophin and the DAPs in the skeletal muscle from an 8-yr-old DMD patient who had no detectable abnormalities of the dystrophin gene. Serial transverse cryosections (7 μm) were immunostained with an affinity-purified rabbit polyclonal antibody against the first 15 amino acids of the N-terminus of dystrophin (N'-DYS), an affinity-purified rabbit polyclonal antibody against the last 10 amino acids of the C-terminus of dystrophin (C'-DYS), an affinity-purified sheep polyclonal antibody against the 156DAG and a monoclonal antibody against the 50DAG. Two muscle fibers in this field showed positive staining for antibodies against both the N- and C-termini of dystrophin. The 156DAG and 50DAG were co-distributed with dystrophin in these fibers, while they were greatly reduced in the dystrophin-negative fibers. The 59DAP, 43DAG and 35DAG showed the same distribution as the 156DAG and 50DAG (not shown). Bar 100 μm.

dystrophin in the molecular pathogenesis of DMD [29–35] has been explained by the localization of the DAPs-binding site in these domains [19, 36]. These findings indicate the importance of knowing if the DAPs-binding site is retained in internally-deleted dystrophin expressed in revertants. In the present study, we investigated the status of the DAPs in revertants of DMD skeletal muscle.

MATERIALS AND METHODS

Skeletal muscle biopsy specimens

Skeletal muscle biopsy specimens were obtained from five DMD patients. Diagnosis of DMD was made based on the combination of history of illness, physical examination, family history, electromyography, pathological examination of the biopsied skeletal muscle, immunohistochemical and immunoblot analyses of dystrophin, and analysis of the dystrophin gene. In all five patients, immunohistochemical analysis using several antibodies against distinct domains of dystrophin demonstrated the absence of dystrophin in the vast majority of muscle fibers (described below). Immunoblot analysis performed in two patients did not reveal dystrophin (not shown). Analysis of the dystrophin gene was performed in four out of the five patients reported here, and demonstrated a deletion of exons 12–43 in one patient and no detectable abnormalities in three patients (not shown).

Immunohistochemistry

Indirect immunofluorescence microscopy of 7 μm thick cryosections from skeletal muscle biopsy specimens was performed as described

previously [4, 6, 14, 15, 18, 19]. 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 affinity-purified as described [4, 7]. Monoclonal antibody $VIA4_2$ against the cysteine-rich and the carboxyl- terminal domains of dystrophin and $IVD3_1$ against the 50DAG were characterized previously [4, 6, 7, 19]. Sheep antibodies against the 156 kDa α-dystroglycan (156DAG), 59 kDa dystrophin-associated protein (59DAP), 50DAG, 43 kDa dystroglycan (43DAG) and 35 kDa dystrophin-associated glycoprotein (35DAG) were affinity-purified as described [10, 14, 16, 18–22].

RESULTS AND DISCUSSION

Similar results were obtained from all five DMD patients reported here (representative figures of the results are shown in Figs 1, 2). Dystrophin was absent in the vast majority of muscle fibers in these patients and all of the DAPs were greatly reduced in the sarcolemma of dystrophin-negative muscle fibers (Figs 1, 2). On the other hand, a very small percentage (less than 2%) of the muscle fibers were found to be dystrophin-positive. These fibers were stained with an antibody against the N-terminus, antibodies against the cysteine-rich and carboxyl-terminal domains, and an antibody against the C-terminus of dystrophin (Figs 1, 2). Dystrophin-positive fibers were occasionally found in small clusters, in accordance with previous reports (Fig. 1) [23–28]. The status of the expression of the DAPs in these rare dystrophin-positive fibers was investigated by immunohistochemical analysis of serial transverse cryosections. The results demonstrated that the intensity of the DAPs staining was generally higher in the sarcolemma of these fibers than in the surrounding dystrophin-negative fibers (Figs 1, 2). The intensity and distribution of the staining by different antibodies were not necessarily uniform and appeared to vary slightly among different fibers (Figs 1, 2). This could be due to the variable sensitivities of different antibodies and/or the variable levels of expression of dystrophin and the DAPs in the longitudinal orientation of these fibers. Unfortunately, the rarity of these dystrophin-positive fibers and the large number of antibodies used in this study hindered us from testing the latter hypothesis by immunohistochemical analysis of serial longitudinal cryosections.

Here we have presented data which suggest the co-expression of dystrophin and the DAPs in the sarcolemma of revertants in DMD skeletal muscle. Consistent with previous reports [25, 28], the translational reading frame of the dystrophin gene is considered to be restored in revertants in our patients, since these fibers were stained with antibodies against both the N-terminus and the C-terminus of dystrophin. Furthermore, the finding that all of the DAPs were restored in revertants indicates that the DAPs-binding site was retained in the dystrophin of these fibers. All together, our results suggest that the entire DGC is restored in revertants and, thus, the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix is likely to be restored in these muscle fibers.

Recently, the DAPs-binding site was confined to the cysteine-rich and the first half of the carboxyl-terminal domains, based on the results of limited calpain digestion of the DGC [36]. This is also supported by our recent findings on the status of the DAPs in the unique DMD patients who had dystrophin lacking the cysteine-rich and carboxyl-terminal domains [19]. In these patients, the DAPs were reduced in the sarcolemma as in typical DMD patients, despite the proper localization of truncated dystrophin to the sarcolemmal region [19]. This suggested that the deficiency of the DAPs was the cause of the severe phenotype in these patients [19].

The precise mechanism and site of the restoration of the translational reading frame of the dystrophin gene in revertants remain unclear [25, 28]. In the vast majority of DMD patients, deletions/duplications of the dystrophin gene do not involve the exons which correspond to the cysteine-rich and carboxyl-terminal domains [29]. Thus, the exon(s) which corresponds to the DAPs-binding site in these domains is not deleted in the majority of DMD patients, including those patients confirmed to have revertants [26, 28]. Our results indicate that the restoration of the translational reading frame of the dystrophin gene in revertants should have occurred 5′ of the exon(s) which corresponds to the DAPs-binding site. Recently the skipping of constitutive exons containing nonsense mutations were identified for the fibrillin gene in Marfan syndrome and the gene encoding ornithine δ-aminotransferase in gyrate atrophy [37]. These findings suggest that the restoration of the translational reading frame by intrinsic genetic mechanisms could be a phenomenon which occurs in a number of genes to circumvent the devastating effects of

nonsense mutations.

It was reported that mutagenetic doses of X-irradiation lead to an increase in the number of revertants in mdx mice in which the exon(s) of the dystrophin gene corresponding to the DAPs-binding site remains intact [25, 38]. Furthermore, it was suggested that the presence of revertants could be related to slower progression of DMD [27]. While it is unknown if the DAPs-binding site is actually restored in dystrophin of mdx revertants, these findings, all together, suggest a possibility that therapeutic approaches to increase the number of revertants could be beneficial for DMD.

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REFERENCES

1. Hoffman E P, Brown R H, Kunkel L M. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 1987; **51**: 919–928.
2. Koenig M, Monaco A P, Kunkel L M. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 1988; **53**: 219–228.
3. Campbell K P, Kahl S D. Association of dystrophin and an integral membrane glycoprotein. *Nature* 1989; **338**: 259–262.
4. Ervasti J M, Ohlendieck K, Kahl S D, Gaver M G, Campbell K P. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* 1990; **345**: 315–319.
5. Yoshida M, Ozawa E. Glycoprotein complex anchoring dystrophin to sarcolemma. *J Biochem* 1990; **108**: 748–752.
6. Ohlendieck K, Ervasti J M, Snook J B, Campbell K P. Dystrophin–glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. *J Cell Biol* 1991; **112**: 135–148.
7. Ervasti J M, Kahl S D, Campbell K P. Purification of dystrophin from skeletal muscle. *J Biol Chem* 1991; **266**: 9161–9165.
8. Ohlendieck K, Campbell K P. Dystrophin constitutes 5% of membrane cytoskeleton in skeletal muscle. *FEBS Lett* 1991; **283**: 230–234.
9. Ervasti J M, Campbell K P. Membrane organization of the dystrophin–glycoprotein complex. *Cell* 1991; **66**: 1121–1131.
10. Ibraghimov-Beskrovnaya O, Ervasti J M, Leveille C J, Slaughter C A, Sernett S W, Campbell K P. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* 1992; **355**: 696–702.
11. Hemmings, L, Kuhlmann P A, Critchley D R. Analysis of the actin-binding domain of α-actinin by mutagenesis and demonstration that dystrophin contains a functionally homologous domain. *J Cell Biol* 1992; **116**: 1369–1380.
12. Way M, Pope B, Cross R A, Kendrick-Jones J, Weeds A G. Expression of the N-terminal domain of dystrophin in *E. coli* and demonstration of binding to F-actin. *FEBS Lett* 1992; **301**: 243–245.
13. Matsumura K, Campbell K P. Deficiency of dystrophin-associated proteins: a common mechanism leading to muscle cell necrosis in severe childhood muscular dystrophies. *Neuromusc Disord* 1993; **3**: 109–118.
14. Ohlendieck K, Campbell K P. Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. *J Cell Biol* 1991; **115**: 1685–1694.
15. Ohlendieck K, Ervasti J M, Matsumura K, Kahl S D, Leveille C J, Campbell K P. Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* 1991; **7**: 499–508.
16. Matsumura K, Ervasti J M, Ohlendieck K, Kahl S D, Campbell K P. Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. *Nature* 1992; **360**: 588–591.
17. Tinsley J M, Blake B J, Roche A, *et al.* Primary structure of dystrophin-related protein. *Nature* 1992; **360**: 591–593.
18. Ohlendieck K, Matsumura K, Ionasescu V V, *et al.* Duchenne muscular dystrophy: deficiency of dystrophin-associated proteins in the sarcolemma. *Neurology* 1993; **43**: 795–800.
19. Matsumura K, Tomé F M S, Ionasescu V V, *et al.* Deficiency of dystrophin-associated proteins in Duchenne muscular dystrophy patients lacking C-terminal domains of dystrophin. *J Clin Invest* 1993; **92**: 866–871.
20. Matsumura K, Nonaka I, Arahata K, Campbell K P. Partial deficiency of dystrophin-associated proteins in a young girl with sporadic myopathy and normal karyotype. *Neurology* 1993; **43**: 1267–1268.
21. Matsumura K, Tomé F M S, Collin H, *et al.* Deficiency of the 50 K dystrophin-associated glycoprotein in severe childhood autosomal recessive muscular dystrophy. *Nature* 1992; **359**: 320–322.
22. Matsumura K, Nonaka I, Campbell K P. Abnormal expression of dystrophin-associated proteins in Fukuyama-type congenital muscular dystrophy. *Lancet* 1993; **341**: 521–522.
23. Shimizu T, Matsumura K, Hashimoto K, *et al.* A monoclonal antibody against a synthetic polypeptide fragment of dystrophin (amino acid sequence from position 215 to 264). *Proc Japan Acad B* 1988; **64**: 205–208.
24. Nicholson J V B, Davison K, Johnson M A, *et al.* Immunoreactivity in patients with Xp21 muscular dystrophy. *J Neurol Sci* 1989; **94**: 137–146.
25. Hoffman E P, Morgan J E, Watkins S C, Partridge T A. Somatic reversion/suppression of the mouse mdx phenotype *in vivo*. *J Neurol Sci* 1990; **99**: 9–25.
26. Burrow K, Coovert D D, Klein C J, *et al.* Dystrophin expression and somatic reversion in prednisone-treated and untreated Duchenne dystrophy. *Neurology* 1991; **41**: 662–666.
27. Nicholson L V B, Bushby K M D, Johnson M A, Dunnen J T den, Ginjaar J B, Ommen G J B van. Predicted and observed sizes of dystrophin in some patients with gene deletions that disrupt the open reading frame. *J Med Genet* 1992; **29**: 892–898.
28. Klein C J, Coovert D D, Bulman D E, Ray P N, Mendell J R, Burghes A H M. Somatic reversion/suppression in Duchenne muscular dystrophy (DMD): evidence supporting a frame-restoring mechanism in rare dystrophin-positive fibers. *Am J Hum Genet* 1992; **50**: 950–959.
29. Koenig M, Beggs A H, Moyer M, *et al.* The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am J Hum Genet* 1989; **45**: 498–506.
30. Beggs A H, Hoffman E P, Snyder J R, *et al.* Exploring the molecular basis for variability among patients with Becker muscular dystrophy: dystrophin gene and protein studies. *Am J Hum Genet* 1991; **49**: 54–67.

31. Hoffman E P, Garcia C A, Chamberlain J S, Angelini C, Lupski J R, Fenwick R. Is the carboxyl-terminus of dystrophin required for membrane association? A novel, severe case of Duchenne muscular dystrophy. *Ann Neurol* 1991; **30**: 605–610.
32. Récan D, Chafey P, Leturcq F, *et al.* Are cysteine-rich and COOH-terminal domains of dystrophin critical for sarcolemmal localization? *J Clin Invest* 1992; **89**: 712–716.
33. Helliwell T R, Ellis J M, Mountford R C, Appleton R E, Morris G E. A truncated dystrophin lacking the C-terminal domains is localized at the muscle membrane. *Am J Hum Genet* 1992; **50**: 508–514.
34. Bies R D, Caskey C T, Fenwick R. An intact cysteine-rich domain is required for dystrophin function. *J Clin Invest* 1992; **90**: 666–672.
35. Clemens P R, Ward P A, Caskey C T, Bulman D E, Fenwick R G. Premature termination mutation causing Duchenne muscular dystrophy. *Neurology* 1992; **42**: 1775–1782.
36. Suzuki A, Yoshida M, Yamamoto H, Ozawa E. Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxyl-terminal domain. *FEBS Lett* 1992; **308**: 154–160.
37. Dietz H C, Valle D, Francomano C A, Kendzior R J, Pyeritz R E, Cutting G R. The skipping of constitutive exons *in vivo* induced by nonsense mutations. *Science* 1993; **259**: 680–683.
38. Sicinski P, Geng Y, Ryder-Cook A S, Barnard E A, Darlison M G, Barnard P J. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* 1989; **244**: 1578–1580.