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Characterization of Revertant Muscle Fibers in Duchenne Muscular Dystrophy, Using Exon-Specific Monoclonal Antibodies against Dystrophin

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Summary

Most Duchenne muscular dystrophy (DMD) patients have genetic deletions or point mutations in the dystrophin gene that alter the reading frame of dystrophin mRNA. This causes early termination of translation, and no dystrophin (or, less commonly, a truncated N-terminal dystrophin fragment) is produced. In many DMD patients, however, a small proportion of muscle fibers show strong dystrophin staining, and these "revertant fibers" are thought to arise by a mechanism that restores the reading frame. Exon-specific monoclonal antibodies (mAbs) have now been used to determine, for the first time, which exons are removed, in order to correct the reading frame in individual muscle fibers. Thus, 15 revertant fibers in a DMD patient with a frameshift deletion of exon 45 were shown to correct the frameshift by the additional deletion of exon 44 (or perhaps exon 46 in some fibers) from the dystrophin mRNA, but not by larger deletions. This result was consistent with reverse transciption (RT)–PCR and sequencing of a minor dystrophin mRNA with an exon 43/46 junction in this biopsy. In a DMD patient with a frameshift deletion of exons 42 and 43, however, larger deletions than the minimum necessary were used to correct the frameshift. In this patient, who produces a half-size N-terminal dystrophin fragment in all fibers, we were able to show that the revertant dystrophin *replaces* the truncated dystrophin in revertant-fiber sarcolemma. The results are consistent with somatic mutations in revertant-fiber nuclei, which result in removal of additional exons from dystrophin mRNA. They do not clearly distinguish between additional somatic deletions and somatic effects on dystrophin mRNA splicing, however, and both mechanisms may be operating.

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Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (Becker MD) are caused by genetic defects in the dystrophin gene on the X-chromosome, ~60% of mutations being genetic deletions of single exons or groups of exons (Worton and Thompson 1988). The reading-frame hypothesis (Malhotra et al. 1988; Monaco et al. 1988) suggested that deletions that alter the reading frame of dystrophin mRNA should produce no functional dystrophin and severe DMD, while in-frame deletions might produce a partly functional internally deleted dystrophin and the milder Becker disease. Although exceptions have been observed (Malhotra et al. 1988; Chelly et al. 1990; Le Thiet Thanh et al. 1993; Winnard et al. 1993), the reading-frame rule has been confirmed experimentally in >90% of cases.

Although the great majority of muscle fibers in DMD patients are dystrophin negative, using antibodies to the C-terminal region, a small proportion of dystrophin-positive fibers are found in >50% of all DMD patients (Nicholson et al. 1989) and also in the *mdx* mouse (Hoffman et al. 1990). DMD patients with larger numbers of these dystrophin-positive "revertant" fibers may have less severe muscle disease (Nicholson et al. 1993), though others have failed to observe this correlation (Fanin et al. 1992). It has been established that revertant fibers produce a dystrophin that lacks the region encoded by the genetically deleted exons but that does have a normal C-terminus, indicating that the reading frame has been restored (Hoffman et al. 1990; Klein et al. 1992). Frame-restoring mechanisms that have been suggested include additional somatic deletions (Klein et al. 1992) and alternative splicing of dystrophin mRNA (Sherratt et al. 1993). Although additional regions of dystrophin flanking the genomic mutation have been shown to be missing in revertant fibers (Klein et al. 1992; Wallgren-Pettersson et al. 1993), it has not been possible to deduce precisely which exons are missing from the dystrophin mRNA. Direct reverse transcription (RT)–PCR studies of dystrophin mRNA (Chelly et al. 1990; Roberts et al. 1991) are inconclusive, because it is not possible to correlate minor mRNA species in a whole muscle biopsy with individual revertant fibers in that biopsy. For example, a 1% level of a minor dystrophin mRNA could mean either

that 1% of the fiber nuclei express only this mRNA or that all fiber nuclei express it at low levels (alternative splicing). The latter process may also be seen in lymphocytes where a small proportion of the "illegitimate" dystrophin mRNA transcripts are thought to be alternatively spliced (Roberts et al. 1991). We therefore set out to determine exactly which dystrophin exons are expressed in revertant fibers by using a panel of exon-specific monoclonal antibodies (mAbs) that includes some newly prepared mAbs against a "deletion-hotspot" region of dystrophin.

Methods

Monoclonal Antibodies

The following mAbs have been described elsewhere: exon 1—MANEX1A (Le Thiet Thanh et al. 1993); exons 31 and 32—MANDYS1 (Nguyen thi Man and Morris 1993); exons 40 and 41—MANDYS101; exon 43—MANDYS106 (Nguyen thi Man et al. 1992; Nguyen thi Man and Morris 1993); and exons 75–79—MANDRA1 (Nguyen thi Man et al. 1992). Exon boundaries are from Koenig et al. (1989) and Roberts et al. (1993).

New mAbs were prepared against exons 46 (MANEX46B) and exons 47 and 48 (MANEX4748A). The preparation and mapping of these mAbs will be described in detail elsewhere (Le Thiet Thanh et al., in press). Briefly, dystrophin cDNA fragments corresponding to exons 45 and 46 and exons 45–50 were amplified from total human muscle RNA by RT-PCR (see below) and were cloned in-frame into the pEX2 expression vector, to obtain recombinant fusion proteins. A panel of 20 mAbs was produced from these fusion protein immunogens, as described elsewhere (Nguyen thi Man et al. 1990, 1992). Epitope mapping was performed with recombinant subfragments produced in the same way and with Becker MD patients with known genetic deletions. Thus, MANEX46B binds to dystrophin fragments containing the exon 46 product and fails to bind to dystrophin from Becker MD patients lacking exon 46. Similarly, MANEX4748A requires that the products of both exons 47 and 48 be present for binding and fails to bind to dystrophin from Becker MD patients lacking either exon 47 or exon 48.

Patients

The exon-42/43 and exon-45 deletion DMD biopsies have been described elsewhere (Helliwell et al. 1992, 1994).

RT-PCR and DNA Sequencing

Total RNA was prepared from 5–10 mg of normal human muscle tissue by using a guanidine hydrochloride and SDS/phenol method (Wilton et al. 1993). For cDNA, total RNA was incubated at 37°C for 1 h with 100 pmol of random hexamer primers (Life Technologies) and 200 U Moloney Murine leukemia virus RT (Life Technologies) in a 20-μl reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, and 18 U of ribonuclease inhibitor (U.S. Biochemicals). The reaction was terminated by heating to 94°C for 5 min, and 5 μl were used for PCR.

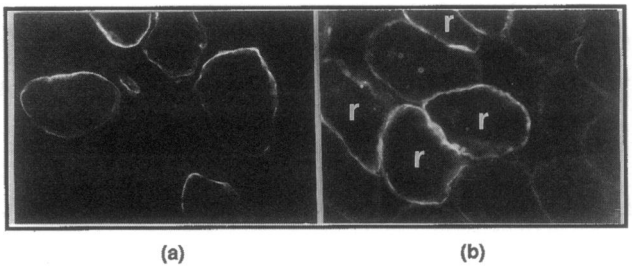

Figure 1 Revertant fibers in two DMD patients. Sections were stained with the mAb MANDYS1 against exons 31/32 of dystrophin (Nguyen thi Man and Morris 1993). In *a*, an exon 45–deletion patient produces a dystrophin in the revertant fibers only. In *b*, an exon 42/43–deletion patient produces a truncated dystrophin encoded by exons 1–41 in all nonrevertant fibers (Helliwell et al. 1992). Revertant fibers (labeled "r") are more brightly stained.

PCR amplification was carried out in a final volume of 50–100 μl containing 100 nM of forward and reverse primers and 50–200 μM of dNTPs, in PCR buffer (1.5–3.0 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, and 0.01% gelatin). After pretreatment at 94°C for 4 min, 1 U of Taq polymerase was added, and the mixture was subjected to 30 cycles (0.5 min at 55°C, 1 min at 72°C, and 0.5 min at 94°C) followed by 5 min at 72°C.

The oligonucleotide primers were in exons 43 (forward; 5′-GCTCTCTCCCAGCTTGATTTCCAA-3′) and 50 (reverse; 5′-GGCTGCAGTAGGAGAGGCTCCAATAGT-3′) of the dystrophin gene. PCR products were recovered from 1% agarose gels by using the QIAEX gel extraction kit (Qiagen) and were cloned into the pT7Blue T-tailed vector according to the supplier's instructions (Novagen). DNA sequencing was done with Sequenase v2.0, ^{35}S-dATP, and the exon 43 forward primer.

Immunohistochemistry

Immunohistochemistry of frozen sections of human muscle by using FITC-labeled anti-(mouse Ig) as a second antibody has been described elsewhere (Nguyen thi Man et al. 1992).

Results

Figure 1 shows revertant fibers in the two DMD patients studied. A patient with a deletion of exon 45 only is shown in figure 1*a;* only the revertant fibers in this biopsy show membrane staining with antidystrophin mAbs. Figure 1*b* shows an unusual DMD patient, with a deletion of exons 42 and 43, who produces a truncated dystrophin corresponding to the N-terminal half of dystrophin before the

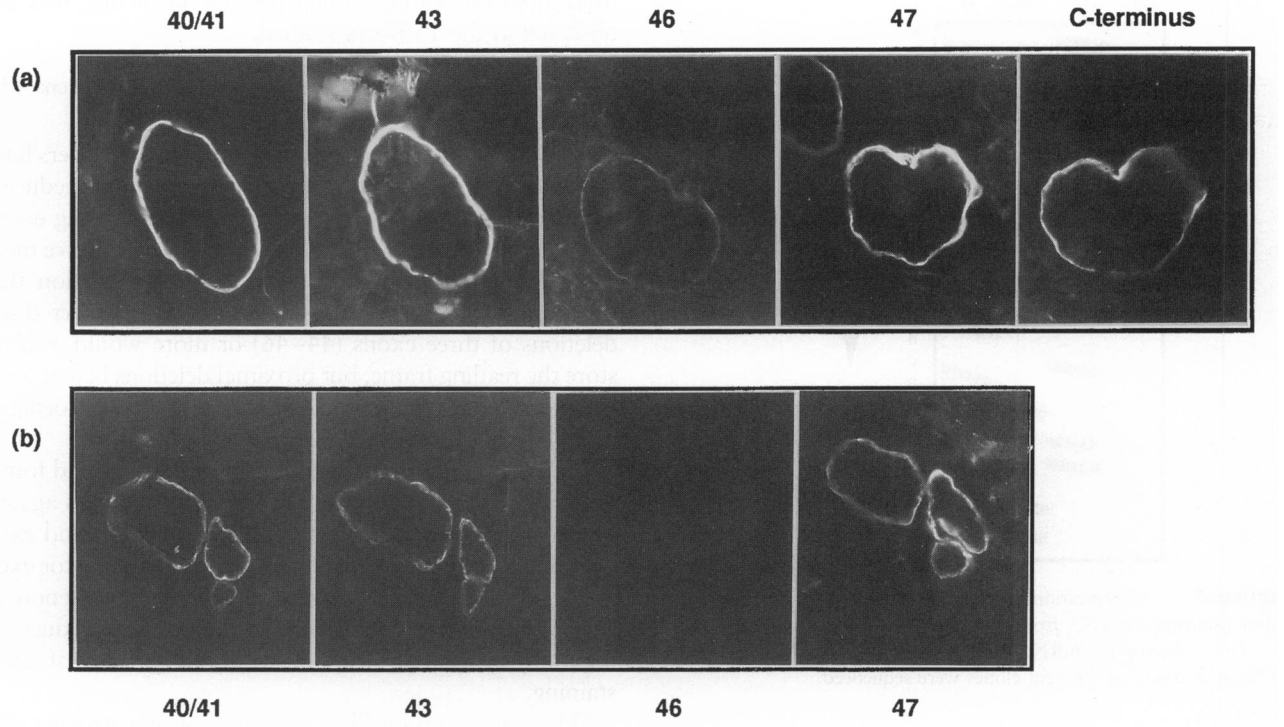

Figure 2 Analysis of revertant fibers in the exon 45 deletion patient, using a panel of exon-specific mAbs. The exon specificity of each mAb used is shown by each serial section. In *a*, some revertants express exon 46 of the dystrophin gene, but exon 46 is not detectably expressed in others, as in *b*. All revertants express exons 43 and 47. The mAbs were MANDYS 101 (40/41), MANDYS106 (43) (Nguyen thi Man and Morris 1993), MANEX46B, MANEX4748A, and MANDRA1 (Nguyen thi Man et al. 1992).

deletion (Helliwell et al. 1992). All fibers show positive dystrophin staining, using a mAb that binds before the deletion, but revertant fibers in figure 1*b* are more brightly stained. This patient was of particular interest because it might be possible to determine whether more than one type of dystrophin molecule is expressed in the same fiber.

Revertant Fibers in a DMD Patient with an Exon 45 Deletion

We examined 15 revertant fibers that stained strongly for dystrophin with C-terminal mAbs in serial sections of a muscle biopsy, using a panel of mAbs spanning the area surrounding the exon 45 deletion. Eight of 15 were negative with exon 46 mAbs (fig. 2*b*), suggesting that the frameshift caused by removal of exon 45 has been corrected by additional removal of exon 46. Seven of 15 were positive with exon 46 mAbs, though staining was much weaker than normal (fig. 2*a*); this suggests that removal of exon 44 has corrected the frameshift in these fibers, though we were not able to test this directly, since mAbs against exon 44 are not yet available. All 15 fibers gave positive dystrophin staining with mAbs against both the N-terminal and the C-terminal sides of the deletion, including mAbs against exon 43 and exon 47/48 (fig. 2a), so there is no evidence for the removal of more than a single additional exon in any of the 15 fibers.

RT-PCR was performed on total RNA extracted from 5 mg of the biopsy, to detect the minor dystrophin mRNAs predicted for these revertant fibers. Primers in exons 43 and 50 produced a single PCR product of 919 bp, corresponding to the main, out-of-frame mRNA (fig. 3). A dilution experiment (not shown) suggested that mRNAs present at <1% of the main band would not be detected by ethidium bromide staining, so the region of the agarose gel

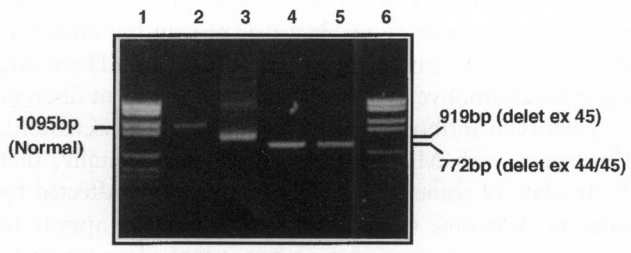

Figure 3 RT-PCR amplification of minor dystrophin mRNAs in a DMD muscle biopsy with an exon 45 deletion. Primers in exons 43 and 50 amplify a DNA of 1095bp in normal muscle (lane 2) and 919 bp in the exon 45–deleted DMD muscle (lane 3). When DNA in the region immediately below 919 bp in lane 3 was excised, purified, and reamplified, a smaller DNA band was obtained (lane 4). Plasmid clones from this band were shown by DNA sequencing to lack exons 44 and 45 (fig. 4). Lane 5, PCR amplification of one of the plasmid clones sequenced are shown, and lanes 1 and 6 are Mr markers (Boehringer Type VI).

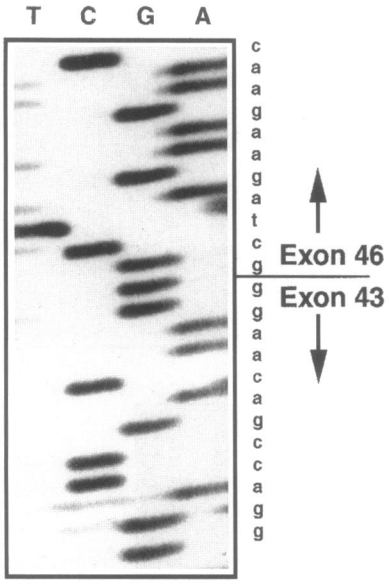

Figure 4 Identification of an in-frame exon 43–46 junction in the minor dystrophin mRNA from fig. 3. The PCR product in fig. 3, from the minor dystrophin mRNA, was cloned into the T-tailed vector, pT7Blue, and several different clones were sequenced.

below the main band was extracted, purified, and reamplified with the same primers. A single smaller PCR product was obtained, the size of which would be consistent with removal of either exon 44 or exon 46 (772 or 771 bp, respectively; fig. 3). This band was cloned into a "T-overhang" plasmid for DNA sequencing, and four separate clones were found to have the exon 43–46 junction (fig. 4), indicating removal of exon 44 only. Twenty clones were also screened for the presence of exon 44, by PCR, but no positives were found. By suggesting that only exon 44 is removed, these data appear to conflict with the observation that some fibers fail to stain with exon 46 mAbs. However, the binding of MANEX46 appears to be greatly weakened by the abnormal exon 43–46 junction (fig. 2*a*), so the possibility that exon 46 product was present but undetectable in figure 2*b* cannot be ruled out entirely. There are, however, alternative explanations for the apparent discrepancy between antibody and RT-PCR data (see Discussion). This result with MANEX46 confirms, incidentally, that the binding of some dystrophin mAbs may be affected by adjacent deletions, though the effect does not appear to extend over more than one or two exons. The extent to which this occurs is likely to depend on the conformation dependence of each mAb, with linear determinants being less affected.

We conclude that many revertant fibers in this particular DMD patient have corrected the frameshift deletion of exon 45 by an additional deletion of exon 44, while the antibody data suggest that exon 46 is deleted instead in some fibers. Correction of the frameshift by deletion of more than one exon, though possible in theory, was not observed in the 15 fibers studied.

Revertant Fibers in a DMD Patient with a Deletion of Exons 42 and 43

This biopsy was of added interest, because all fibers have a truncated dystrophin and show positive, though reduced, dystrophin staining with mAbs up to and including exons 40/41 (Helliwell et al. 1992), but revertant fibers have more dystrophin (fig. 1*b*). The only small extra deletion that would correct the frameshift is of exon 44. Larger distal deletions of three exons (44–46) or more would also restore the reading frame, but proximal deletions before exon 42 would not (exon boundaries are taken from Koenig et al. [1988, 1989]).

We studied 19 revertants fibers in this biopsy and found only 1 isolated fiber that was recognized by mAbs against both exons 40/41, before the genomic deletion, and exon 46, after the deletion (fig. 5). MAbs to test directly for exon 44 are not yet available, but only an extra deletion of exon 44 from the dystrophin mRNA would produce an internally deleted dystrophin with this pattern of mAb staining.

While the revertant fiber in figure 5 shows stronger dystrophin staining than the surrounding fibers, the revertant fiber in figure 6*a* is clearly less strongly stained than the surrounding fibers with exon 40/41 mAb, but it stains strongly with mAbs against exons 31/32 or exon 47 (and exon 46; not shown). Four fibers were found with this staining pattern, which shows that some exons between 33 and 41 are missing from the mRNA, *in addition* to exon 44. The result also suggests that the revertant dystrophin is being produced *instead of*, rather than in addition to, the truncated dystrophin found in other fibers. In six other fibers, for technical reasons, it was difficult to determine whether the exon 40/41 mAb was staining as strongly as, or less strongly than, surrounding fibers (a significant part of the membrane has to be separate from adjacent fiber membranes, as in fig. 6*a*). The last eight revertant fibers were dystrophin negative with exon 46 mAbs, which shows

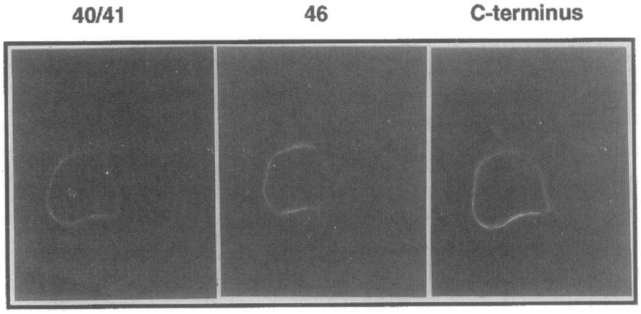

Figure 5 A revertant fiber produced by removal of exon 44 from the dystrophin mRNA of an exon 42/43–deletion patient only. The mAbs were MANDYS101, MANEX46B, and MANDRA1.

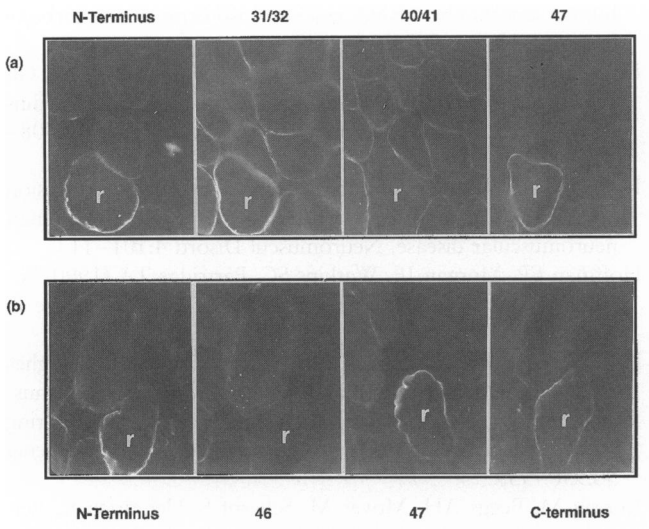

Figure 6 Revertant fibers produced by removal of exon 44 and additional exons from the dystrophin mRNA of an exon 42/43–deletion patient. All fibers in this patient show moderate staining with mAbs up to and including that against exons 40/41. The more brightly stained revertant fibers are labeled "r." The mAbs were (*a*) MANEX1A (Le Thiet Thanh et al. 1993), MANDYS1, MANDYS101, AND MANEX4748A; and (*b*) MANEX1A, MANEX46B, MANEX4748A, and MANDRA1.

that exons 45 and 46 must have been removed from these fibers, in addition to exon 44, in order to correct the frameshift.

Discussion

Using a panel of mAbs spanning exons 40–48 of dystrophin, we have studied 15 strongly dystrophin-positive revertant fibers from a DMD patient with a frameshift deletion in the dystrophin gene of exon 45 and 19 strongly dystrophin-positive revertant fibers in a patient with a frameshift deletion of exons 42 and 43. We interpret the results as showing that the frameshifted dystrophin mRNA has been replaced in these fibers by an in-frame dystrophin mRNA, as a result of a somatic mutation that results in the removal of additional exons from the mRNA. In the first biopsy, all corrections of the frameshift were achieved by removal of a single exon. In the second biopsy, a single-exon correction also occurred, but larger mRNA deletions, including removal of exons on both sides of the germ-line deletion, were more common.

A somatic event of some kind is the only mechanism likely to cause such a dramatic change in gene expression in only a few individual fibers, and the increase in revertant fibers after irradiation of *mdx* mice (Hoffman et al. 1990) clearly implies a mutation event. This could be a mutation that removes extra exons from the dystrophin gene (somatic deletion) or one that results in splicing-out extra exons from the dystrophin mRNA (e.g., a splice-site mutation such as that demonstrated in a dystrophic dog [Sharp et al. 1992]). These alternatives could be distinguished by examining the genomic DNA in a single revertant-fiber nucleus, but this is technically difficult. The frequency of revertant fibers is higher than might be expected for somatic mutations (Klein et al. 1992). It is possible, however, that there is some selection in favor of survival of revertant fibers, because they express a "Becker-type" dystrophin, since Danko et al. (1992) have shown that expression of either full-length or Becker-like dystrophins in *mdx* muscle fibers increases their survival. The observation that revertant fibers are more frequent in older DMD patients (Fanin et al. 1992) would also be consistent with a somatic mutation and selection hypothesis.

Somatic mutations must occur in individual nuclei, although, if the mutation occurred in a dividing, prefusion myoblast, the same mutation could be carried by a number of nuclei within the same multinucleate muscle fiber; too little is known about muscle development and turnover to predict how many. The effects of a somatic mutation need not be diluted out by other nuclei in the myofiber, however, because large mRNAs and proteins remain close to their nucleus of origin rather than diffusing evenly throughout the muscle fiber (Ono et al. 1994). This appears to be the case for dystrophin, since the distribution of revertant dystrophin along the muscle fiber in *mdx* mice is patchy, which reflects the distribution of revertant and nonrevertant nuclei (Hoffman et al. 1990). This may also explain occasional revertant fibers that show strong dystrophin staining on one side and little or no staining on the other (results not shown). One would also expect the revertant dystrophin mRNA and dystrophin to *replace* the frameshifted mRNA and its product, if any, in that part of the fiber under the control of revertant nuclei (rather than being coproduced), and this is precisely what we found when a DMD patient, making a truncated dystrophin in all fibers, provided a rare opportunity to test this experimentally (fig. 6*a*). We cannot rule out the possibility that both dystrophins are made, but the revertant dystrophin prevents the truncated dystrophin from attaching to the muscle membrane. However, the dystrophin C-terminus attaches to the sarcolemma through dystroglycan (Matsumura and Campbell 1994; Suzuki et al. 1994), which is greatly increased in revertant fibers (Helliwell et al. 1994), whereas the truncated dystrophin is likely to associate with the membrane through a different mediator. Competition between the two forms for membrane binding would therefore only occur if this unknown mediator were both limiting and shared by the two forms.

Only the most strongly dystrophin-positive fibers were selected for this work because weakly positive fibers would be difficult to study using mAbs of various avidities (the strongest mAbs reveal a larger number of positive fibers). However, weakly positive fibers were present in significant numbers (figs. 2 and 6), as observed by others (Nicholson et al. 1993). If this does represent genuine dystrophin ex-

pression (and we have no reason to doubt this), and if the biopsy samples are representative of the whole muscle, then the high frequency of revertants could present problems for a somatic deletion hypothesis. It has been suggested that two different mechanisms may be operating, a nuclear event in the dystrophin gene for the strong revertants and an indirect effect on splicing for the more weakly staining fibers (Nicholson et al. 1993). The presence of weakly positive fibers means that great care must be taken in making correlations between dystrophin mRNA analysis of the whole biopsy by RT-PCR and antibody analysis of the strong revertants only. It might explain, for example, why exon 44 is more frequently removed from the minor muscle dystrophin mRNA in figure 3 than the antibody data in figure 2 would suggest, since loss of exon 44 is also observed in lymphocytes from DMD patients with exon 45 deletions (Roberts et al. 1991). If small amounts of exon 44–deleted "illegitimate transcripts" were produced in all nuclei, by alternative splicing, this would resolve the discrepancy between RT-PCR and antibody data in favor of the antibody evidence that removal of either exon 46 or exon 44 can contribute to frameshift correction in the strong revertants. The possibility that alternative splicing without somatic mutation produces all revertant fibers has been raised (Nicholson et al. 1992; Sherratt et al. 1993), but in all known examples of this, every nucleus—and not just a few—appears to be affected. Examples include tissue-specific alternative splicing of dystrophin mRNA (Feener et al. 1989; Bies et al. 1992; Lederfein et al. 1992) and cases of DMD caused by splicing out abnormal exons (Matsuo et al. 1991; Narita et al. 1993).

Acknowledgments

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