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Somatic Reversion/Suppression in Duchenne Muscular Dystrophy (DMD): Evidence Supporting a Frame-restoring Mechanism in Rare Dystrophin-positive Fibers

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Summary

Many Duchenne muscular dystrophy (DMD) patients are known to have rare staining dystrophin-positive fibers, termed "revertants." The precise etiology of these rare fibers is unknown. The most likely explanation, however, is somatic mosaicism or somatic reversion/suppression. Immunocytochemistry was performed on serial sections from deleted and nondeleted patients, with a panel of antibodies—9219, 1377, 9218, and Dys-2—that span dystrophin. Both familial and nonfamilial patients possessed revertants. Either the same clusters or individual revertant fibers stained with amino- and carboxyl-terminal antibodies in all 14 DMD patients. In patients with deletions, revertants did not stain with antibodies raised to polypeptide sequences within the deletion. These results indicate that positively staining fibers are not the result of somatic mosaicism in deleted patients. Five of 10 patients without deletions had revertant fibers. In two of these patients, the revertant fibers did not stain with antibody 9218, which was generated against amino acids 2305–2554 and which corresponds to exons 48–52. The remaining antibodies from the panel stained the same fibers on separate serial sections in these two patients. The most likely mechanism giving rise to these positively staining fibers is a second site in-frame deletion. Antibodies generated to polypeptide sequences within deletions can be used to control for the natural occurrence of revertant fibers in myoblast transfer studies and may be useful in the detection of point mutations.

Introduction

The cloning of the Duchenne muscular dystrophy (DMD) gene (Monaco et al. 1986; Burghes et al. 1987; Koenig et al. 1987) led directly to the identification of the protein product, dystrophin, which is absent in boys with DMD (Hoffman et al. 1987). Deletion/duplication mutations occur in 65% of patients (Den Dunnen et al. 1987; Gillard et al. 1989; Koenig et al. 1989) and, in most DMD patients, these deletions disrupt the translational frame of dystrophin (Malhotra et al. 1988; Monaco et al. 1988; Koenig et al. 1989).

The mdx mouse (Bulfield et al. 1984) is an authentic model of DMD in mice. The mdx mouse is dystrophin deficient and has a point mutation that introduces a translational stop codon at bp 3185 (Sicinski et al. 1989).

Immunocytochemical studies have localized dystrophin to the muscle sarcolemma (Arahata et al. 1988; Zubrzycka-Gaarn et al. 1988). Electron-microscopic studies using immunogold staining demonstrated that dystrophin is localized on the inside surface of the sarcolemma (Watkins et al. 1988; Carpenter et al. 1990; Cullen et al. 1990).

Immunofluorescence studies of DMD patients and mdx mice, using dystrophin antibodies, show that the majority of muscle fibers do not stain. However, we and others have observed a low frequency of dystro-

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phin-positive fibers in DMD patients and in the mdx mouse (Shimizu et al. 1988; Nicholson et al. 1989; Hoffman et al. 1990; Burrow et al. 1991).

These dystrophin-positive fibers are termed "revertants" and have been most extensively studied in the mdx mouse. These murine fibers stain with a panel of antibodies spanning the dystrophin molecule. These dystrophin-positive cells occur in heart and skeletal muscle. The frequency of dystrophin-positive fibers increases with mutagenic doses of X-rays (Hoffman et al. 1990). These experiments in the mdx mouse provide strong evidence that these fibers are not an artifact or due to alternative or illegitimate splicing (Hoffman et al. 1990). Hoffman et al. suggest that the most likely mechanism is a second site reversion mutation or a tRNA suppressor mutation.

We have observed these fibers in deletion and nondeletion DMD patients and patients with a family history of DMD (Burrow et al. 1991). One explanation for these positively staining fibers is the occurrence of a second site deletion that restores the dystrophin reading frame in these cells. In the present paper, we present the first data to support this hypothesis.

We examined these rare dystrophin-positive fibers in DMD patients by using a panel of antibodies that span the dystrophin molecule. These positive fibers are present in both familial and nonfamilial cases. In patients with deletions, the positive fibers stained only with antibodies raised to polypeptide sequences flanking the deletion. Antibodies raised to sequences within the deletion invariably failed to recognize revertant fibers. These data indicate that, in deleted cases, these dystrophin-positive fibers do not represent somatic mosaicism, and they suggest the likelihood of a reversion-mutation mechanism. Furthermore, of five nondeleted patients who had rare dystrophin-positive fibers, two were unique because staining was absent for only one of the antibodies of the panel. These data suggest that a second site deletion was likely to have removed the mutation and to have restored the translational frame in these rare dystrophin-positive fibers.

Patients and Methods

Patient Population

Muscle biopsies from 31 patients previously analyzed for dystrophin and DNA mutations were examined (Burrow et al. 1991; Klein et al., submitted). Twenty-one of these had deletions. None of these patients were positive for dystrophin on western analysis, except for one intermediate patient (67) (Brooke et al. 1983), who had extremely low levels of dystrophin.

Immunostaining and Antibody Preparation

Four antibody preparations were used: 9219, 1377, 9218, and Dys-2. Antiserum 9219 was produced by immunization of sheep by a TrpE-DMD fusion protein and reacts to the dystrophin amino-terminus corresponding to exons 4–16, amino acids 67–667 (Bulman et al. 1991*b*). Antiserum 9218 was similarly produced in sheep and was raised against the antigenic region corresponding to exons 48–52, amino acids 2305–2554. Construction, induction, and antigen purification were performed as described elsewhere (Zubrzycka-Gaarn et al. 1988; Bulman et al. 1991*b*). Antiserum 1377 was produced by immunization of rabbits by a protein-A DMD fusion protein produced by subcloning exons 34–47 into the PRIT-2T vector and corresponds to amino acids 1559–2304. The antigen was denatured prior to immunization. Antibodies 9219, 1377, and 9218 were further purified by passage over an mdx mouse muscle column as described by Burrow et al. (1991). In some instances, the antibody was purified on an affinity column that contained the fusion protein, according to the method described by Hoffman et al. (1990). The monoclonal antibody Dys-2 was raised against the last 17 amino acids of the carboxy-terminus of dystrophin (Nova-Castra Labs, Newcastle upon Tyne, UK).

Immunostaining was carried out on 8–12-μm cross sections of skeletal muscle from open-limb biopsies. Immediately after the biopsy, tissue was mounted on wooden chucks in gum tregacanth, and the muscle tissue was frozen in isopentane that was cooled in liquid nitrogen and stored at −70°C. Fresh serial sections were placed on Super Frost Glass Plus slides (Fischer Scientific, Pittsburgh), were fixed in acetone, and were stained with the panel of antibodies. Purified antibodies were diluted in PBS (pH 7.3) as follows: 9219 (2.66 μg/μl) (1:6); 1377 (0.58 μg/μl) (1:8); and 9218 (0.86 μg/μl) (1:8). Dys-2 was obtained as a tissue-culture supernatant from the manufacturer and was used at a 1:10 dilution according to their instructions.

The tissue sections were incubated with the primary antisera for 1 h in a moist chamber and then were washed three times with PBS. The secondary biotinylated IgG antibody—anti-sheep, -rabbit, or -mouse (Jackson Immunoresearch Labs, West Grove, PA)—was then added for 1 h at a 1:200 dilution. Finally, the sections were incubated with a 1:200 dilution of

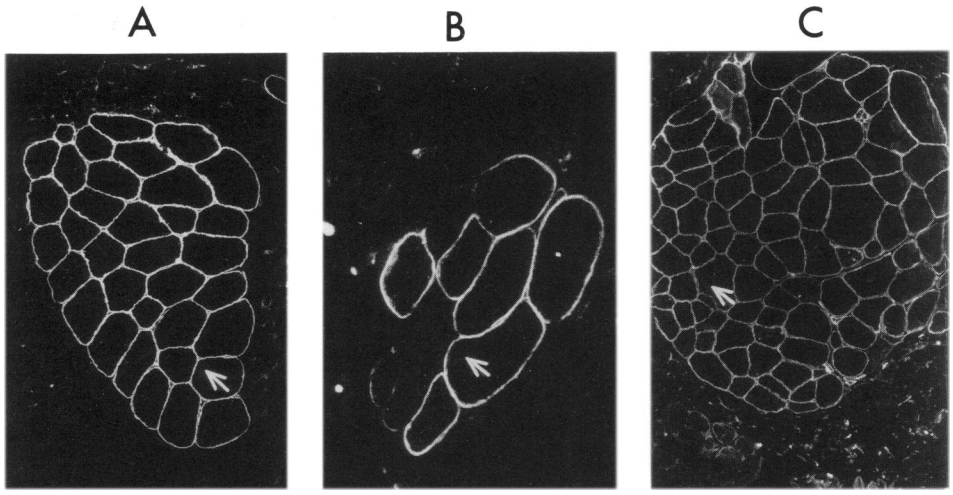


Figure 1 Examples of revertant clusters at reduced magnification and stained with the Dys-2 antibody (magnification ×43). Panels *A, B,* and *C* correspond to fibers from 169, 14, and 15, respectively. White arrows indicate the same fibers as shown in figs. 3 and 5 at magnification ×250.

fluorescein-conjugated avidin (E.Y. Labs, San Mateo, CA) coverslipped with 90% glycerol, 10% PBS (pH 9.0) and were examined under fluorescent microscopy. Control sections were incubated with either sheep, rabbit, or mouse preimmune sera which were matched for protein content. Normal skeletal muscle tissue was simultaneously stained with the panel of antibodies. Samples were photographed with Kodak Ecktachrome P1600 or Tmax P3200 film at an ASA setting of 1600/3200.

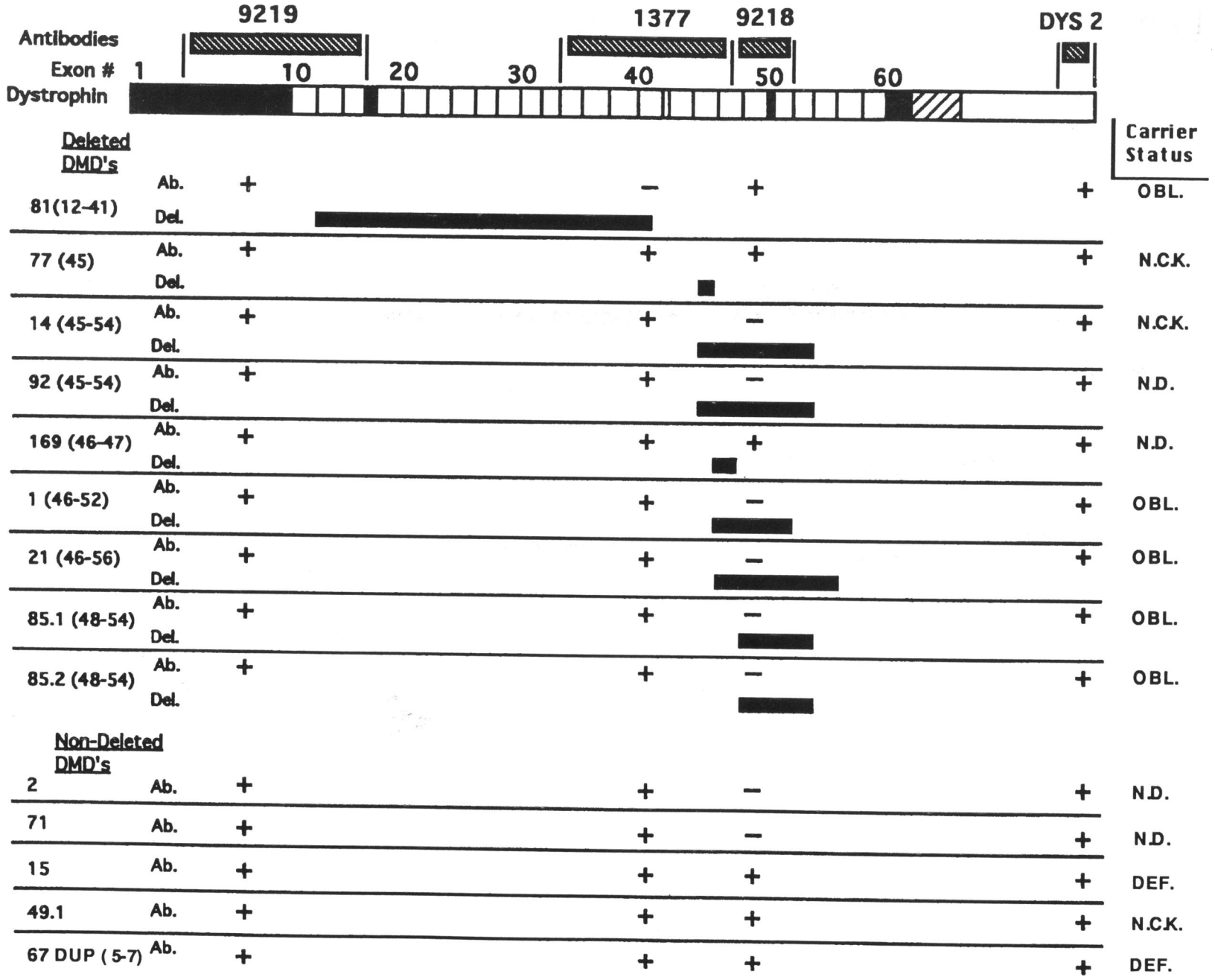
Results

Thirty-one patients were analyzed for dystrophin-positive fibers. Twenty-one patients had a deletion, and nine of these had dystrophin-positive fibers. There were 10 nondeletion patients, one of whom had a duplication; five of these cases, including the duplication case, had dystrophin-positive fibers. Therefore, in both deletion and nondeletion cases, approximately 50% of patients have dystrophin-positive fibers. In all deletions studied, the mutation disrupted translational frame. The dystrophin-positive fibers were observed as clusters or as single fibers. The staining of these fibers is clearly positive, and they stain with reasonable intensity (fig. 1).

Figure 2 summarizes the results obtained by staining deletion patients with the antibody panel. The regions of the transcript to which these antibodies were raised is also indicated in figure 2. In all cases, the amino- and carboxy-terminal antibodies stained these dystrophin-positive fibers.

Muscle biopsies from five patients with deletions confined to the 5′ end, from five with deletions in the deletion-prone region, from one with a deletion of exons 12–28, and from one with a deletion of exon

Figure 2 Summary of the data obtained from staining revertant fibers in DMD patients with a panel of antibodies that span dystrophin. The top line (hatched boxes) indicates the region of dystrophin used to generate the antibodies. Dys-2 was raised against the last 17 amino acids and, therefore, has no exon designation. The line below dystrophin is a diagram of dystrophin, indicating the exon number and domains of dystrophin. Patients are listed vertically on the left, along with the exons that are deleted or duplicated. The carrier status of the mother is indicated to the right. OBL. = obligate carrier (defined as either a mother with a high CK on three separate assays, the presence of the deletion in the mother by dosage analysis, or direct indication from other affected individuals in the pedigree). N.D. = not determined; N.C.K. = normal CK levels in mother; and DEF. = definite carrier (defined as affected boy with a sister with high CK). The deletion in each patient is denoted by a black bar. The duplication in patient 67 is not indicated diagrammatically. The staining status of a revertant fiber or cluster is indicated by a plus (+) sign or minus (−) sign. AB. = antibody staining results. Del. = deletion result. All immunocytochemistry results were obtained on serial sections that were stained, from amino- to carboxyl-terminus, with the antibody panel.


9219
1377
9218
DYS 2
Antibodies
Exon #
1
10
20
30
40
50
60
Dystrophin
Carrier Status
Deleted DMD's
Ab.
Del.
81(12-41)
OBL.
77 (45)
N.C.K.
14 (45-54)
N.C.K.
92 (45-54)
N.D.
169 (46-47)
N.D.
1 (46-52)
OBL.
21 (46-56)
OBL.
85.1 (48-54)
OBL.
85.2 (48-54)
OBL.
Non-Deleted DMD's
2
N.D.
71
N.D.
15
DEF.
49.1
N.C.K.
67 DUP (5-7)
DEF.

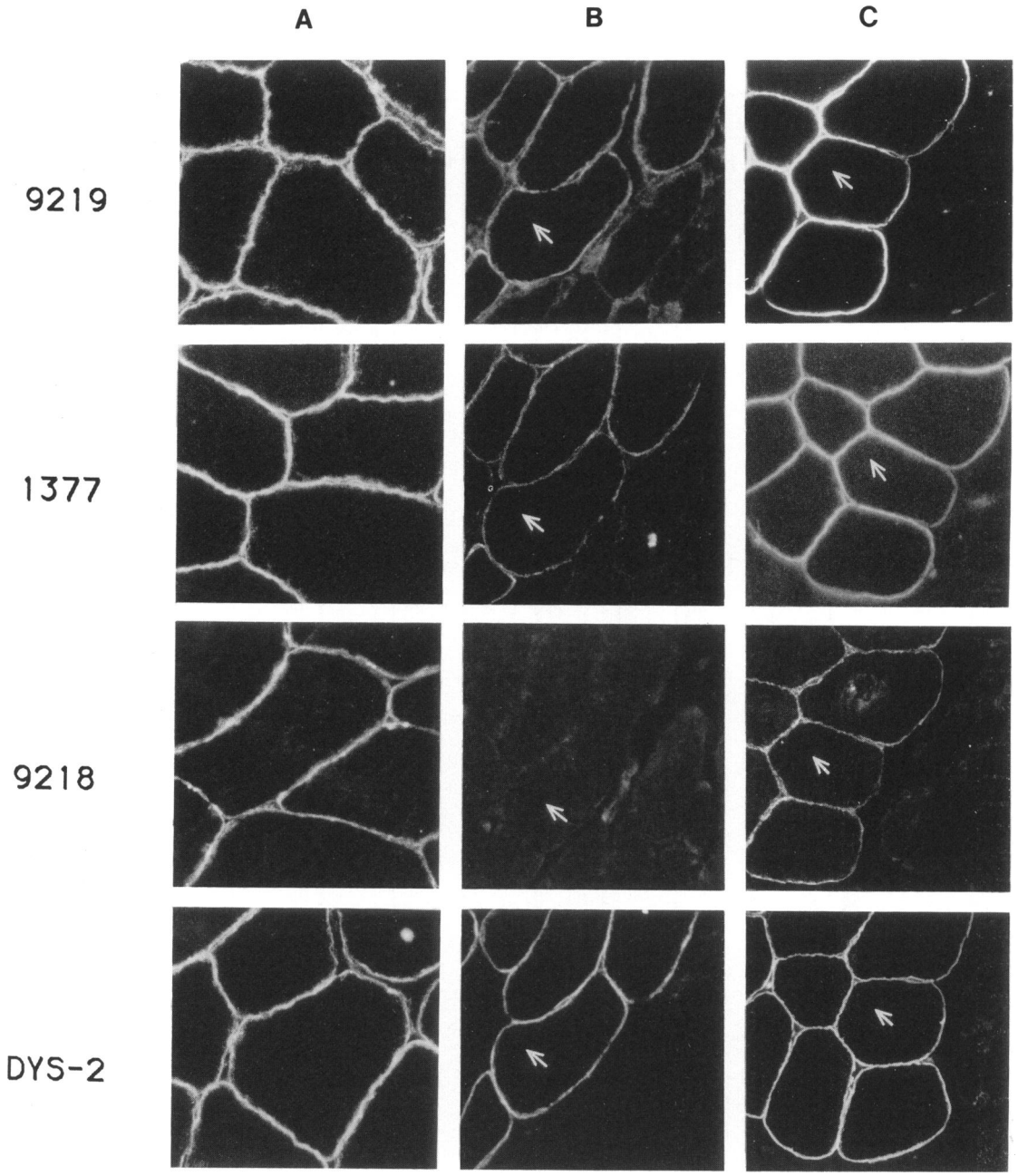


Figure 3 Antibody staining of revertant fibers in DMD patients with DNA deletions. The dystrophin antibodies used are on the left. Arrows indicate the same fiber on separate serial sections. Panel *A* (magnification ×310) shows a normal control stained with each antibody of the panel: 9219, 1377, 9218, and Dys-2. Panel *B* (magnification ×250) shows that DMD patient 14 has a deletion of exons (45–54), thereby removing the antigenic region of 9218. Panel *C* (magnification ×250) shows patient 169 stained with each antibody of the panel. This patient has exons (46–47) deleted, thus removing only 14% of 1377's antigenic region.

41 through the entire 3′ end of dystrophin showed no dystrophin-positive fibers. In all deletion patients with dystrophin-positive fibers, the antibodies stained 5′ and 3′ of the deletion but did not stain within the antigenic region that was deleted. The panel of antibodies stained the same group of fibers on serial sections.

Figure 3*B* shows the panel of antibodies staining

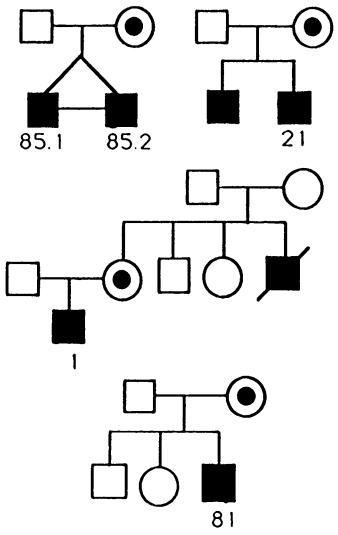


Figure 4 Pedigrees from four families with rare positive fibers. Unblackened squares denote unaffected males; unblackened circles denote unaffected females; blackened squares denote affected males; circles with a dot in the center denote carrier females; a slash (/) through the symbol indicates that the individual is deceased. Patients 85.1 and 85.2 are MZ twins whose mother had a high CK level. Patient 21 has an affected brother. Patient 1 had an affected uncle. Patient 81 has a mother with a verified deletion and high CK levels.

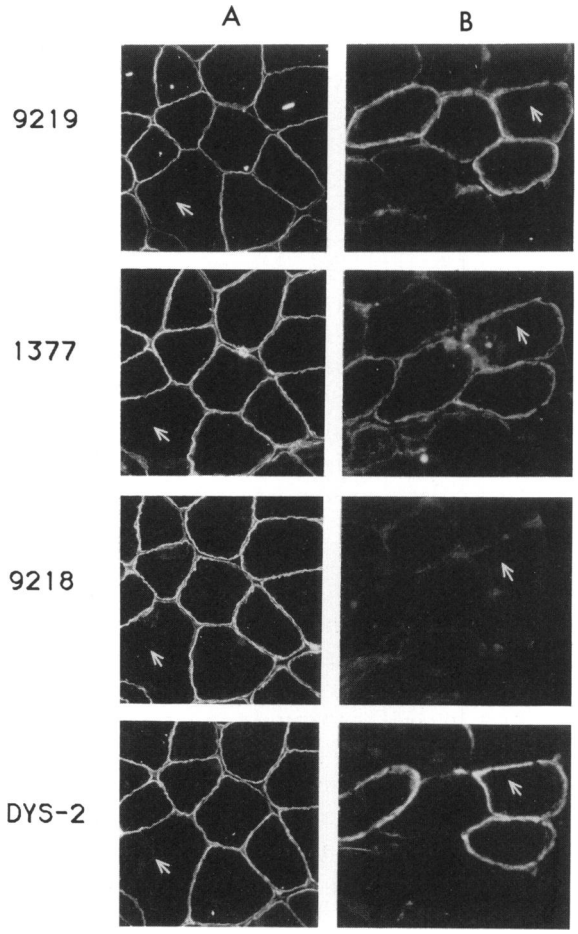


Figure 5 Staining of revertant fibers in DMD patients without deletions. The antibodies used are indicated on left. The magnification in all cases is ×250. Arrows indicate the same fiber on separate sections of the same biopsy. Panel *A* shows patient 15 stained with the antibody series. With all antibodies, this patient has the same positive fibers. Panel *B* shows patient 71 stained with the antibody panel. Patient 71 had revertant fibers that stained with antibodies 9219, 1377, and Dys-2 but not with antibody 9218.

patient 14, who had a deletion of exons 45–54, thus removing staining with antibody 9218. This patient and others had deletions that extend several exons into 1377's antigenic region, without eliminating staining with this antibody. In particular, this patient had 21% of the 1377 antigenic region removed. In contrast, patient 81 was deleted for 55% of 1377's antigenic site while completely maintaining 9218's antigenic region. This patient demonstrated dystrophin-positive fibers with 9218 but not with 1377. In addition, patient 169, who was deleted for the last two exons of 1377's antigenic site, showed dystrophin-positive fibers with this antibody and with all others in the panel (fig. 3*C*). This was also true of one intermediate patient (77) who had only one exon missing from 1377's antigenic domain and still stained positively with this antibody.

Some of the cases with rare positive fibers and deletions have family histories. The pedigrees are shown in figure 4. Patient 81 had a mother with a confirmed deletion and high creatine kinase (CK) levels, as indicated in figure 2. In addition, patients 92 and 14, who had no family history, showed an absence of staining with antibody raised against the deleted region.

We also studied 10 nondeletion cases, five of which had dystrophin-positive fibers. These are summarized in figure 2. Revertant fibers were demonstrated with the full panel of antibodies in patients 15 (fig. 5*A*), 49.1, and 67 (duplication of exons 5–7). However, in two cases—i.e., 71 (fig. 5*B*) and 2—antibody 9218 did not identify dystrophin-positive fibers, although these patients did show dystrophin-positive fibers with the remaining antibodies. Simultaneous controls included positive fiber staining with 9218 in normal muscle and in other nondeleted patients.

Patient 67 was also of particular interest. In this intermediate patient, only muscle fibers belonging to six clusters were positive for dystrophin. The sizes of these clusters were 1–30 fibers. Western blot analysis

of this patient, by using tissue from the same block that was used for immunocytochemistry, showed an extremely faint band at the position expected for dystrophin. No other patients had dystrophin by western blot analysis.

Discussion

In patients with the typical DMD phenotype, rare staining dystrophin fibers (i.e., revertants) are observed despite the absence of dystrophin on western blot analysis (Shimizu et al. 1988; Nicholson et al. 1989; Hoffman et al. 1990; Burrow et al. 1991). Five general mechanisms could be suggested to explain these dystrophin-positive fibers: (1) normal developmentally regulated alternative splicing, (2) low-level illegitimate splicing, (3) somatic reversions, (4) somatic suppression, and (5) somatic mosaicism.

Hoffman et al. (1990) have studied these dystrophin-positive fibers in the mdx mouse by using a panel of antibodies covering dystrophin. These authors showed that these fibers stained with all dystrophin antibodies. In DMD patients, by using a distinct panel of antibodies described in the present paper, we also observed that dystrophin-positive fibers stain with a panel of antibodies. Furthermore, in patients with a deletion that removed a particular epitope, the antibody corresponding to that epitope did not identify the revertant fibers. This is true despite revertant staining by antibodies that flank the deletion. These findings argue strongly that these rare fibers have dystrophin of restored reading frame and not a cross-reactive protein detected by the antibodies.

Alternative or illegitimate splicing could be suggested as a mechanism by which these dystrophin fibers bypass the mutation. A number of arguments against this possibility have been put forward by Hoffman et al. (1990). The observations that led to this conclusion are as follows: (*a*) alternative splicing has not been observed in the region of the mdx mouse mutation (Feener et al. 1989); (*b*) there was no correlation to muscle fiber type; (*c*) illegitimate splicing would result in low-level expression of dystrophin in all fibers; and (*d*) these occur at a low level and appear clonal in nature. Furthermore, we have observed that these dystrophin-positive fibers are found in patients with heterogeneity in site, size, and location of mutation. Alternative splicing has not been reported to occur at such a heterogeneous number of locations, but only at the amino- and carboxy-termini of dystrophin (Feener et al. 1989; Boyce et al. 1991).

Amplification of mRNA in either deleted or nondeleted patients has not indicated any alternative splicing that could account for these dystrophin-positive fibers (A. V. Winnard and A. H. M. Burghes, unpublished data). If alternative or illegitimate splicing accounted for these fibers, then this event would only occur in a few cells and in the nondeletion patients, 17 and 2, and it would remove the entire domain responsible for staining with antibody 9218, exons 48–52, amino acids 2305–2554. In our opinion, illegitimate or alternative splicing is an unlikely explanation to account for these so-called revertant fibers.

Somatic mosaicism, however, could account for these dystrophin-positive fibers. Somatic mosaicism has been reported in hemophilia B (Taylor et al. 1990) and in osteogenesis imperfecta (Cohn et al. 1990; Constantinou et al. 1990; Wallis et al. 1990). The possibility of somatic mosaicism occurring in DMD seems likely, given that 7%–14% of DMD carriers are germ-line mosaics (Bakker et al. 1987, 1989; Darras and Francke 1987). However, the occurrence of dystrophin-positive fibers in the mdx mouse (Hoffman et al. 1990) would argue against somatic mosaicism as an explanation.

In DMD patients, two predictions can be made if these dystrophin-positive fibers are due to somatic mosaicism. First, they should not occur in familial cases, and, second, in deletion cases, the fibers should stain with antibodies that lie within the deletion. We have found familial cases that have dystrophin-positive fibers (Burrow et al. 1991) (fig. 2), and, in deletion patients in whom the antigenic site has been entirely removed, the revertant fiber did not stain with the corresponding antibody (fig. 3*B*). This makes somatic mosaicism an unlikely explanation to account for dystrophin-positive fibers in our familial and deletion cases.

The most likely mechanism responsible for the generation of these dystrophin-positive fibers is somatic reversion or suppression. In order to obtain antibody staining at the amino- and carboxy-termini of dystrophin, any suppressor mutation must correct the reading frame. Thus, the suppression occurs at the genetic level. One class of suppressor mutation that could do this is the classical tRNA suppressor mutation originally described in prokaryotes.

Suppressor tRNA mutations would be predicted to be somatic mutations that occur within a few cells and that are capable either of correcting reading frame by reading a four-base codon, as in the *suf A, B,* and *C* genes, or by inserting an amino acid instead of recog-

nizing a termination codon, as in the amber, ochre, and opal suppressor mutations (Roth 1974). However, given that there are 1,300 copies of tRNA in the human haploid genome (Hatlen and Attardi 1971), and that only one copy is likely to correct the mutation, staining may be predicted to be less intense than that observed in these rare positive fibers.

Although it is feasible for a tRNA to recognize a four-base or two-base codon to correct the frame in a deletion case, we consider this to be an unlikely mechanism. In point mutation cases, a tRNA suppressor is a possible explanation; however, it is not likely to be the sole mechanism. We have observed two patients without detectable deletions, whose revertant fibers appear to have removed the epitope recognized by antibody 9218.

We have observed a dystrophin fiber frequency of 2.0×10^{-2} (Burrow et al. 1991). If all clusters or single fibers in a section are considered to arise from the same genetic event, then the frequency of reversion would be 3.47×10^{-4} in DMD patients (Burrow et al. 1991). In the mdx mouse, the frequency of revertants in muscle was 10^{-2} in American mice and 2×10^{-4} in British mdx mice. Therefore, the frequency of dystrophin-positive fibers in DMD and mdx mouse skeletal muscle is similar.

On the other hand, the rate of dystrophin-positive fibers in mdx cardiocytes is 2.0×10^{-5} (Hoffman et al. 1990). The difference between heart and skeletal muscle reversion rates has been accounted for in the mdx mouse. First, a single satellite cell with a reversion can supply a large area with dystrophin-positive nuclei. Second, a cluster of dystrophin-positive fibers could belong to a syncitium of split fibers, as demonstrated in the mdx mouse (Watkins et al. 1989). In skeletal muscle, the tissue undergoes regeneration, whereas cardiac tissue does not. Therefore, the opportunity for reversion in skeletal muscle is higher. As indicated elsewhere (Hoffman et al. 1990) for the mdx mouse, these figures are higher than those reported for reversion in other mammalian systems (Greenspan et al. 1988). The unexpectedly high frequencies may imply that the dystrophin gene may inherently have a higher likelihood for reversion. Our data support reversion/suppression as the major mechanism giving rise to these positive fibers.

The most direct evidence comes from two patients without deletions, who possessed rare dystrophin-positive fibers. Patients 2 and 71 had no deletion and demonstrated rare dystrophin-positive fibers, with all antibodies except 9218. We suggest that, in these fibers, a deletion has removed the antigenic site. This deletion would be in frame and would remove the original mutation.

Other patients may well have secondary mutations that delete smaller antigenic regions not detected by these polyclonal antibodies. It is interesting to note that all our deleted patients with rare positive fibers have deletions near the region of high deletion frequency (Den Dunnen et al. 1987; Gillard et al. 1989; Koenig et al. 1989). This may explain the high frequency of revertants observed in DMD.

An intragenic deletion at the region of high deletion frequency is suggested by our data as one likely reversion mutation capable of restoring dystrophin's reading frame in revertant fibers. The deletions that restore translational frame in these fibers would be predicted to remove the original mutation. Therefore, with the aid of a panel of antibodies, the site of the restoring deletion can be identified. These data will confine the original mutation to a defined region of dystrophin which can then be analyzed. The existing method of detecting point mutations in DMD (Bulman et al. 1991*a*) should be complemented by this approach.

Myoblasts have been introduced into mdx mice, demonstrating the possibility of repopulating the muscle with dystrophin-positive fibers (Partridge et al. 1989). This has led to the use of myoblast transfer in clinical trials. In these trials, it is important to control for the naturally occurring dystrophin-positive fibers. Our results demonstrate that antibodies corresponding to deleted regions can be used to control for the natural occurrence of these revertant fibers in myoblast transfer studies.

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