Am. J. Hum. Genet. 56:158–166, 1995

Frameshift Deletions of Exons 3–7 and Revertant Fibers in Duchenne Muscular Dystrophy: Mechanisms of Dystrophin Production

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Summary

Duchenne muscular dystrophy (DMD) patients with mutations that disrupt the translational reading frame produce little or no dystrophin. Two exceptions are the deletion of exons 3–7 and the occurrence of rare dystrophin-positive fibers (revertant fibers) in muscle of DMD patients. Antibodies directed against the amino-terminus and the 5′ end of exon 8 did not detect dystrophin in muscle from patients who have a deletion of exons 3–7. However, in all cases, dystrophin was detected with an antibody directed against the 3′ end of exon 8. The most likely method of dystrophin production in these cases is initiation at a new start codon in exon 8. We also studied two patients who have revertant fibers: one had an inherited duplication of exons 5–7, which, on immunostaining, showed two types of revertant fibers; and the second patient had a 2-bp nonsense mutation in exon 51, which creates a cryptic splice site. An in-frame mRNA that uses this splice site in exon 51 was detected. Immunostaining demonstrated the presence of the 3′ end of exon 51, which is in agreement with the use of this mRNA in revertant fibers. The most likely method of dystrophin production in these fibers is a second mutation that restores the reading frame.

Introduction

Duchenne muscular dystrophy (DMD) is caused by mutations in the 2.3-Mb dystrophin gene (Monaco et al. 1986; Burghes et al. 1987; Koenig et al. 1987, 1989; Monaco and Kunkel 1988; Worton and Burghes 1988; Gillard et al. 1989). The majority of mutations result in translational termination and the production of little or no dystrophin (Hoffman et al. 1988; Bulman et al. 1991; Prior et al. 1993*a*, 1993*b*, 1993*c*; Roberts et al. 1994). In this paper, we focus on two exceptions to the frameshift hypothesis (Monaco et al. 1988). First, the deletion of exons 3–7 results in patients showing the entire range of clinical severity of the disease, from DMD to Becker muscular dystrophy (BMD) (Malhotra et al. 1988; Chelly et al. 1990; Winnard et al. 1993). Despite their out-of-frame mutation, patients who have a deletion of exons 3–7 show production of low amounts of dystrophin (Chelly et al. 1990; Gangopadhyay et al. 1992; Winnard et al. 1993). The second exception to the frameshift hypothesis that we studied was the occurrence of rare dystrophin-positive fibers (revertant fibers) (Shimuzu et al. 1988; Nicholson et al. 1989), which are present in ≥50% of muscle biopsies from DMD patients (Klein et al. 1992), who are typically deficient in dystrophin.

In the first case, Chelly et al. (1990), analyzed two BMD patients who have a deletion of exons 3–7. They reported finding two minor in-frame messages—one containing a deletion of exons 3–9 and one containing a deletion of exons 2–7—which were present both in normal muscle and in the patient muscle. However, we and others (Gangopdhyay et al. 1992; Winnard et al. 1993) have not detected these transcripts, and it is not clear that the reported mRNA level (<1%–2% of normal dystrophin mRNA) would be sufficient to account for the production of dystrophin. Furthermore, protein studies that precisely identify which exons are present in dystrophin from these patients have not been performed.

The occurrence of revertant fibers in DMD patients has been reported by several groups (Shimizu et al. 1988; Nicholson et al. 1989, 1992; Burrow et al. 1991; Klein et al. 1992). These positive fibers occur in ≥50% of patients and have been observed in skeletal and cardiac muscle of the *mdx* mouse (Hoffman et al. 1990). Immunohistochemical studies in DMD patients and in the *mdx* mouse have shown that these fibers stain with a panel of antibodies and have an intact carboxyl-terminus (Hoffman et al. 1990; Klein et al. 1992; Nicholson et al. 1992). In DMD patients with deletions, these fibers did not stain with antibodies corresponding to the deleted epitope (Klein et al. 1992). Similarly, we observed staining of revertant fibers with a

Received June 16, 1994; accepted for publication September 29, 1994.

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0002-9297/95/5601-0020$02.00

carboxyl-terminal antibody but not with an antibody corresponding to amino acids 2490–2507 in a patient who has a 2-bp substitution (Klein et al. 1992; Winnard et al. 1992). Therefore, dystrophin production in revertant fibers is probably due to a secondary somatic mutation, which produces an in-frame mRNA that has deleted the original mutation.

In this study, we have examined a series of patients who have a deletion of exons 3–7 and determined that the most likely mode of dystrophin production is initiation at an ATG in exon 8. In addition, we have demonstrated that the most likely mechanism of dystrophin production in some revertant fibers is a secondary mutation. In one case of a DMD patient with revertant fibers, we have determined the presence of an in-frame mRNA that splices exon 49 to a cryptic splice site in exon 51, skipping exon 50. Antibody staining indicates that this is the most likely mRNA species responsible for dystrophin production in these revertant fibers and that it is probably caused by a somatic mutation that disrupts the splicing of exon 50. Last, we have demonstrated that more than one event can give rise to revertant fibers in a single muscle.

Patients and Methods

Patients

Nine patients with a deletion in the 5′ end of dystrophin were studied. Three of these patients with a deletion of exons 3–7 (patients 300, 218, and 63) have been reported by us elsewhere (Winnard et al. 1993). Two patients with a deletion of exons 3–7 (patients B4 and B6) previously reported by Beggs et al. (1991) were also analyzed. We identified four additional patients who have a deletion of exons 3–7 (patients 59.1, 245, 244, and 503) who had not been previously reported. In addition to the cases that had a deletion of exons 3–7, we analyzed one case that had a deletion of exons 2–7 (patient B2) and whose deletion had been reported by Beggs et al. (1991).

Two additional patients were analyzed for the revertant-fiber studies. We have previously reported the mutations in these patients. Patient 71 has a 2-bp nonsense mutation (Winnard et al. 1992), and patient 67 has a duplication of exons 5–7 (Winnard et al. 1993). In all cases, the mutation has been confirmed by PCR amplification of mRNA isolated from the muscle biopsy, followed by sequence analysis.

Mutation Analysis

Genomic DNA was isolated from leukocytes and deletions determined by Southern blotting and multiplex PCR as described elsewhere (Ray et al. 1985; Chamberlain et al. 1988; Beggs et al. 1990). RNA isolation and PCR amplification conditions for reverse transcription–PCR (RT-PCR) products were as described elsewhere, as were sequencing procedures (Winnard et al. 1992, 1993). Analysis of mRNA from patient 71 was performed using primers 5′ CCA GCC ACT CAG CCA GTG AAG GTA3′ (sense) and 5′ CTG GTC TTG TTT TTC AAA TTT TGG GC 3′ (antisense). The PCR products were separated on a 2% agarose gel and were transferred to Hybond N^+ by using Southern blot transfer. The PCR products were hybridized with a ^{32}P-end-labeled primer specific to this junction (5′ CT CAG CCA GTG AAG/GTA CCT GCT CTG 3′: exon 49/middle exon 51).

Carrier detection of the duplication case was performed both by densitometric scanning of Southern blots and by probing of multiplex PCR products obtained under limited PCR cycling followed by scanning, as described elsewhere (Prior et al. 1990). Exons external to the duplication were used as control bands for dosage determinations.

Antibodies

The antibodies Dys 1 and Dys 2 (Nova-Castra Labs) are available commercially and were used according to the manufacturer's instructions, as described elsewhere (Klein et al. 1992). The amino-terminal polyclonal antibody NH_2-Dys, directed against the first 15 amino acids of dystrophin (MLWWEEVEDCYERED), was raised in rabbits and has been described elsewhere (Ervasti et al. 1991) (This antibody was a gift from K. Campbell and K. Matsumura). A second amino-terminal antibody, corresponding to the amino acids encoded in exon 2, as well as two antibodies in exon 8 and one antibody in exon 51, were raised for this study. Antibody Ab1 corresponds to exon 2, amino acids 12–29 (for details of dystrophin sequence, see Koenig et al. 1988); antibody Ab2 corresponds to the 5′ end of exon 8, amino acids 235–252; and antibody Ab3 corresponds to the 3′ end of exon 8, amino acids 256–273. The positions of the antibodies relative to the amino-terminal exons are shown in figure 1. Antibody 4 corresponds to the 3′ end of exon 51, amino acids 2468–2507. The peptide sequences used for injection are listed in table 1. Peptides were synthesized to correspond to specific regions of dystrophin. Then, a peptide that corresponds to a measles-specific viral sequence was coupled to each peptide at the carboxyl end, in order to increase antigenicity (Partidos and Steward 1990; Kaumaya et al. 1993, 1994). The antibodies were generated on the basis of procedures described by Harlow and Lane (1988). Two milligrams of dry peptide was dissolved in ~400 μl of 10% glacial acetic acid, and the pH was raised to approximately pH 5 with 50% NH_2OH. This solution was mixed 1:1 with 1 × PBS. This was then mixed 1:1 with an adjuvant and was injected subcutaneously. Injections of 1–2 mg of solubilized peptide in PBS were repeated every 2–4 wk, until titers were of a sufficient level. Titers were determined by enzyme-linked immunosorbent-assay (ELISA) analysis using fusion proteins corresponding to various regions of dystrophin (Zubrzycka-Gaarn et al. 1988; Bulman et al. 1991; Klein et al. 1992).

For use in western blots or immunocytochemistry, the

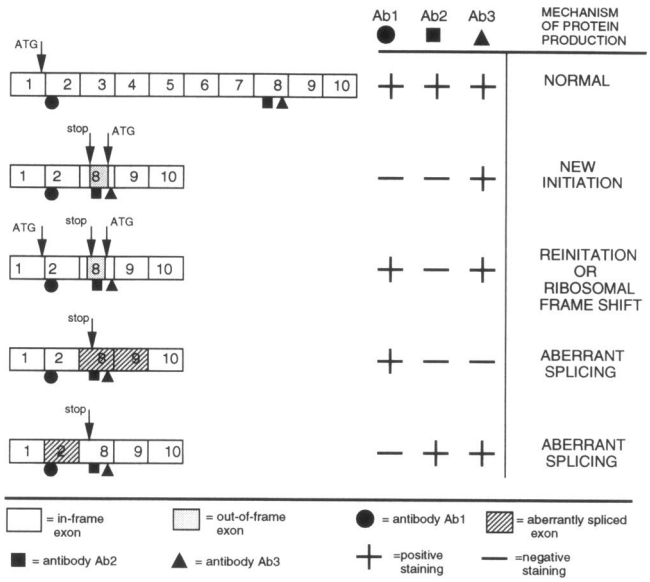

Figure 1 Schematic representation of how antibodies Ab1 or NH_2-Dys, Ab2, and Ab3 were used to demonstrate the mechanism of dystrophin production in patients who have a deletion of exons 3–7. The position of the stop codon and proposed new start site are indicated by arrows. The circle, square, and triangle indicate the position of the epitopes recognized by antibodies 1, 2, and 3, respectively. The table on the right lists the possible outcomes of antibody staining. The possible mechanisms that could give rise to dystrophin production in cases that had a deletion of exons 3–7 are shown on the right-hand side of the table.

antibodies were purified from the rabbit serum by using the methods described by Pringle et al. (1991). Specifically, a saturating concentration of the corresponding gel-purified fusion protein (~10 mg) was layered onto a 2.5–5-cm nitrocellulose square and incubated overnight at 4°C. For antibody Ab1, the fusion protein used for purification corresponded to the amino-terminus of dystrophin (a gift from J. Chamberlain, University of Michigan), while antibodies Ab2–Ab4 were purified using the corresponding fusion proteins described elsewhere (Bulman et al. 1991). The filter was dried and then blocked with 5% dry milk dissolved in PBS and then was washed in PBS. Approximately 300–400 μl of rabbit serum containing antibodies recognizing regions of that fusion protein was incubated with the filter for 2–4 h at room temperature. The filters were then washed in PBS and eluted in 200 ml of elution buffer (0.2 M glycine, 1 mM EGTA, pH 2.3–2.7) for 10 min at room temperature. The solution was then neutralized by addition of 100 μl of 100 mM Tris-base. In order to further reduce nonspecific binding, the procedure was then repeated with the eluted fraction, by using filters that were saturated with either measles-specific peptide or homogenized *mdx* mouse muscle, except that the unbound fraction was retained and used as the purified antibody. The purified antibodies were diluted ~1:1 in PBS, for use in immunocytochemistry.

Staining of sections was done simultaneously with different antibodies. The conditions for immunocytochemistry have been described elsewhere (Klein et al. 1992; Winnard et al. 1992). In brief, immunocytochemistry was carried out on 10-μm cross sections of frozen skeletal muscle from open-limb biopsies. The sections were placed on Super Frost Glass Plus slides (Fisher Scientific), were fixed in cold acetone for 7 min, air-dried for 30 min, and then rehydrated in 1 × PBS for 5 min. The sections were then incubated in a moist chamber with the primary antibodies for >1 h at room temperature. Purified antibodies were diluted as follows: Ab2 (1:1), Ab 3 (1:1), NH_2-Dys (no dilution), and Dys 2 (1:3). The secondary antibodies were biotinylated goat anti-rabbit or anti-mouse (Jackson ImmunoResearch Labs) at a dilution of 1:200. The sections were then washed three times in 1 × PBS and incubated for 1 h at room temperature with Extra/Avidin-fluorescein–conjugated avidin (Sigma Chemical) at a dilution of 1:50. The sections were then washed three times with 1 × PBS and were coverslipped with 90% glycerol, 10% PBS, pH 9.0. Immunostaining was visualized by fluorescence microscopy and was photographed by using Kodak Tmax 400 black-and-white film.

Table 1

Amino Acid Sequences of Synthetic Peptides Used to Raise Antibodies Ab1–A4

Amino Acid Sequence	Dystrophin Exon	Dystrophin Amino Acids
NH-EREDVQKKTFTKWVNAQF-COOH	Exon 2	12–29
NH-SLFQVLPQQVSIEAIQEV-COOH	Exon 8 before ATG	235–252
NH-PRPPKVTKEEHFQLHHQM-COOH	Exon 8 after ATG	256–273
NH-DQVIKSQRVMVGDLEDIN-COOH	Exon 51	2490–2507
NH-GPSLKLLSLIKGVIVHRLEGVE-COOH	Measles-specific sequence	

NOTE.—Each peptide was 40 amino acids in length; the first 18 amino acids were specific to a unique dystrophin sequence, while the last 22 amino acids were common to all the peptides and correspond to a measles-specific virus sequence. This 22-amino-acid measles peptide was coupled to the carboxyl-terminal end of the dystrophin peptide sequences, in order to increase the antigenic presentation of the peptides in vivo.

Results

Patients with a Deletion of Exons 3–7

A series of patients with a deletion of exons 3–7 were analyzed. Elsewhere we have reported RT-PCR analysis of three of the patients who have a deletion of exons 3–7, using primers in exons 1, 2, 8, and 16 (primer 33, exon 1, 1–25 bp; primer 11, exon 2, 236–265 bp; primer 12, exon 8, 977–1,003 bp; primer 13, exon 8, 938–961 bp; and primer 14, exon 16, 2,054–2,077 bp; Winnard et al. 1993). Using these same primers, we analyzed the additional patients who have a deletion of either exons 3–7 or exons 2-7. RT-PCR analysis of all of these patients revealed no alteration of splicing that could account for the production of dystrophin. One patient with a deletion of exons 3–7 produced two additional PCR bands when analyzed by agarose gel electrophoresis. Sequence analysis and restriction analysis of the three bands showed that the lower band had lost exon 9; the upper band contained exon 9, and the middle band was a heteroduplex formed between the lower and upper bands. Neither of these mRNAs corrects the reading frame (data not shown).

Four antibodies were used to test the various models put forward for dystrophin production. The relative positions of the antibodies used and the expected results for various models are shown in figure 1. The antibodies have been characterized as follows: One of the amino-terminal antibodies, NH_2-Dys, has been extensively used in a large number of patients who have various deletions (Matsumura et al. 1993*a*, 1993*b*, 1994). This antibody does not stain most DMD patients; however, it did stain a DMD patient who has a deletion from exon 50 through the entire carboxy-terminal domain but who produces dystrophin (Matsumura et al. 1993*b*). It also stains negatively for all fibers in another DMD patient who has a duplication of exons 5-7, except for one cluster of revertant fibers, which stains positive. In addition, the NH_2-Dys shows positive staining in patients who have a deletion of either exons 13-48 or exons 3-41 and who produce dystrophin (fig. 2; Matsumura et al. 1994). Both NH2-Dys and Ab1 recognize a 427-kD muscle protein on western blots. The second amino-terminal antibody (Ab1) is positive for the 427-kD band on western blots, specifically recognizes a fusion protein encompassing the 5′ end of dystrophin, shows both classical positive staining in normal muscle and absence of staining in dystrophin-deficient DMD fibers, and is positive in the patient whose mRNA is deleted for exons 3–41 (patient 113; fig. 2). The exon 8 antibodies, Ab2 and Ab3, detect the expected 427-kD protein on western blots, recognize corresponding fusion proteins of dystrophin by ELISA and western blots, and do not detect dystrophin in all DMD patients tested, including the DMD patient who produces dystrophin but who has an mRNA deletion of exons 3–41 (patient 113; fig. 2). These antibodies are also positive for one group of fibers in the patient who has a duplication of exons 5–7 (patient 67). This body of data demonstrates that these antibodies are specific for the exons that they are raised against.

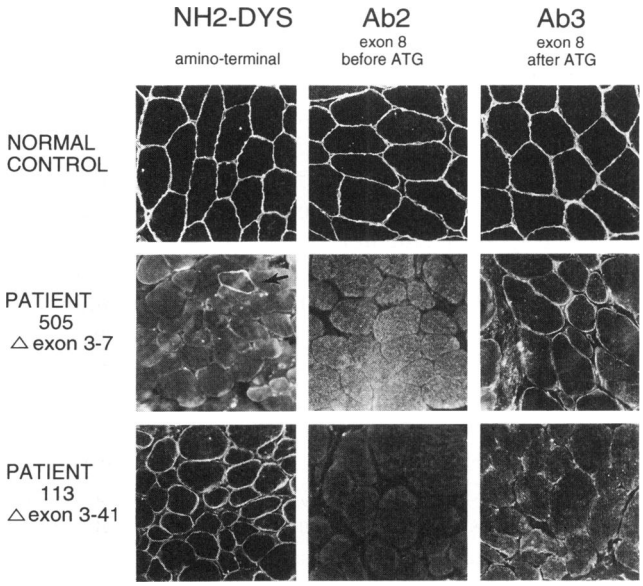

Figure 2 Immunocytochemical staining of muscle tissue from patients who had a deletion of exons 3–7 and from controls. Vertical rows show staining with the same antibody: NH_2-Dys (amino-terminal), Ab2 (exon 8, after the stop but before the ATG), or Ab3 (exon 8, after the ATG). Horizontal rows show serial sections from the same tissue. The patient who had a deletion of exons 3–7 (patient 505) shows no staining with NH_2-Dys or Ab2, except for the presence of the revertant fiber that stains with the amino-terminal antibody (*arrow*). This patient does, however, show positive staining with Ab3, at a level that is consistent with the level seen when the carboxyl-terminal antibody, Dys2, is used. Patient 113 shows positive staining with NH_2-Dys but is negative for staining with Ab2 and Ab3, as would be predicted from the mRNA deletion of exons 3–41.

Tissue sections from nine patients (eight patients with a deletion of exons 3–7 and one patient with a deletion of exons 2-7) were studied, and in all cases the results were the same. Figure 2 shows a typical example of the results obtained. The amino-terminal antibodies, NH_2-Dys (Ervasti et al. 1991) (amino acids 1–15) and Ab 1 (amino acids 12–29), showed no dystrophin staining in any of cases that had a deletion of exons 3–7, with the exception of revertant fibers. Antibody Ab2 recognizes the 5′ end of exon 8 (amino acids 235–252), which occurs before a potential initiation codon in exon 8 but after a stop codon created by the deletion of exons 3–7. Ab2 did not stain the muscle sections of these patients. Antibody Ab3 (amino acids 256–273), which occurs after the initiation codon in exon 8, showed clear positive staining. Additionally, positive staining was also seen with the antibody Dys2, which recognizes the carboxyl-terminal end of dystrophin (data not shown). Likewise, a patient with a deletion of exons 2–7 gave a similar staining pattern. However, an in-frame DMD patient who produces dystrophin and has an mRNA

deletion of exons 3–41 (Winnard et al. 1993; Matsumura et al. 1994) stained positively with the amino-terminal antibodies but not with the exon 8 antibodies, as would be expected (fig. 2). Western blot data obtained in one case that had a deletion of exons 3–7 was negative with antibodies Ab1 and Ab2 and was positive with antibodies Ab3 and Dys 2, which is consistent with the immunohistochemical staining (data not shown).

In two patients with a deletion of exons 3–7, a few dystrophin-positive fibers were observed with the amino-terminal antibody NH_2-Dys. Figure 3 shows these dystrophin-positive fibers. Serial sections of the same sample were also stained with the carboxyl-terminal antibody Dys2. The same fibers that were positive with NH_2-Dys appear as brighter-staining fibers among the positive-staining fibers, which stain with Dys2 (patients with a deletion of exons 3–7 stain positive with Dys2) (data not shown). This would indicate that these antibodies are capable of recognizing the 5′ end of dystrophin even though the structure may have been altered by the deletion of exons 3–7. This also demonstrates revertants in patients producing low levels of dystrophin.

Revertant Fibers

In order to investigate the mechanism of revertant fibers, we analyzed RNA and tissue sections from a patient (patient 71), results of which we have reported elsewhere (Winnard et al. 1992). This patient has a 2-bp point mutation that generates a cryptic splice site in exon 51. To determine whether this splice site was used in the generation of revertant fibers, we isolated RNA from serially cut muscle sections that had been shown by immunocytochemistry to contain revertant fibers. This RNA was examined by RT-PCR to determine whether the new cryptic splice site in this patient was used to produce an in-frame mRNA. The sense primer (5′ CCA GCC ACT CAG CCA GTG AAG GTA 3′) was specifically designed to amplify if exon 49 was cojoined to the cryptic splice site in exon 51, which results in an in-frame mRNA. A PCR product of the correct size is seen only in the patient sample, and not in the control sample, when the PCR products are analyzed. The specificity of this product was demonstrated by hybridization with an oligo that is specific to the exon 49/51 junction (fig. 4). Patient 71, but not the control, was positive for the expected 244-bp fragment, indicating utilization of the cryptic splice site. Even on overexposure, this PCR product cannot be seen in the control lane. The identity of this PCR product was confirmed by direct sequencing.

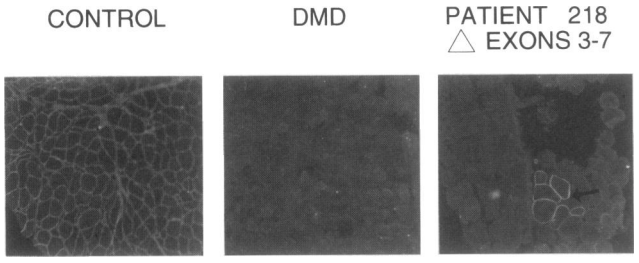

Figure 3 Immunocytochemical staining of serially sectioned muscle tissue from a patient who had a deletion of exons 3–7 (patient 218) and from controls. Tissue sections were stained with the amino-terminal antibody, NH-Dys. Blackened arrow indicates the position of the revertant fibers in the patient who had a deletion of exons 3–7.

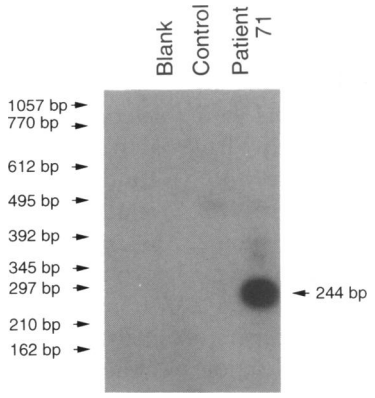

Figure 4 Analysis of mRNA from patient 71. RNA was isolated, reverse transcribed, and amplified with primers specific to the exons 49–51 cryptic splice-site junction. The PCR products were then Southern-blot transferred and hybridized with a probe specific for the exon 49/51 junction. Numbers listed on the left of the figure indicate the size (in bp) of the marker bands. Labels across the top of the figure refer to the sample loaded in that lane. Blank = PCR control, without sample; Control = PCR-amplified product from normal muscle RNA; and Patient = PCR-amplified product from patient 71.

To demonstrate that this mRNA is capable of correcting the reading frame in these revertant fibers, we developed an antibody that was specific to exon 51 downstream of the cryptic splice site. Staining with this antibody (Ab4) (fig. 5) indicated that at least some revertant fibers in this patient stained with exon 51 antibody, indicating that this cryptic splice site is utilized to produce dystrophin-positive fibers.

We identified a series of clusters of revertant fibers in a DMD patient who has an out-of-frame duplication of exons 5–7. Both the patient's mother and the patient's sister were carriers, indicating that the patient inherited the mutation. The carrier determination was done by using Southern blots and PCR along with scanning densitometry (data not shown). This indicates that this patient is not a somatic mosaic. Immunostaining of serially sectioned muscle tissue from patient 67 by a panel of antibodies—NH_2-Dys, Ab1, Ab2, Ab3, Dys1, and Dys2—exhibited two groups of revertant fibers (fig. 6). The first group stained with all the antibodies. The second group stained only with antibodies Dys1 and Dys2. This dual pattern of staining was seen through several serial sections, demonstrating that we had not sectioned through the revertant-fiber cluster. The individual staining pattern indicates that

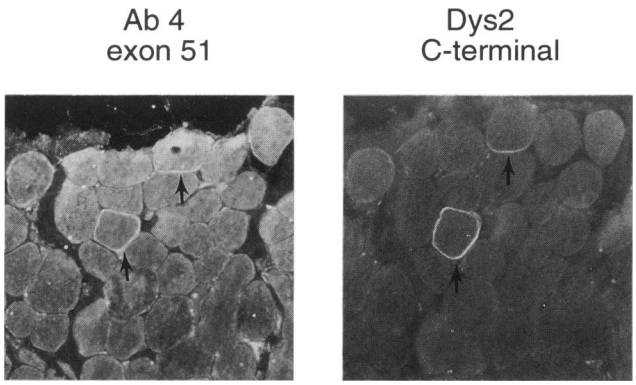

Figure 5 Immunostaining of muscle tissue from patient 71. Serial sections of muscle were stained with an antibody that recognizes exon 51 (Ab4) and with an antibody that recognizes the carboxyl-terminus (Dys2). Arrows indicate the position of the revertant fibers. The same fibers stain positively with both antibodies, at approximately the same intensity.

a separate mechanism was responsible for the restoration of the dystrophin reading frame in these neighboring clusters.

Discussion

In this paper, we have characterized the dystrophin produced by DMD and BMD cases that have a deletion of exons 3–7. These cases are known to produce dystrophin, despite having a mutation that disrupts the translational reading frame. Studies at the mRNA level that use PCR have resulted in conflicting results. We (Winnard et al. 1993) and Gangopadhyay et al. (1992) did not detect any transcripts that resulted in the correction of reading frame. Chelly et al. (1990) reported the presence of two minor transcripts: one that spliced exons 1–8 and one that spliced exons 2–10. Both of these mRNAs were present in normal individuals and in cases that had a deletion of exons 3–7. It can be argued that these differences in mRNA detection arose because of technical differences in the RT-PCR. However, Gangopadhyay et al. (1992) controlled for the level of amplification and still could not detect these mRNA species. The conditions used by Chelly et al. (1990) to detect these transcripts are similar to those reported by them for the detection of illegitimate transcript in lymphocytes (Chelly et al. 1988). Since there is no detectable dystrophin protein in lymphocytes, and since the mRNA is present at less than one copy per cell, this has been termed *illegitimate transcription* and is not believed to give rise to protein. Therefore, although this mRNA species may exist in some patients, it is unclear whether these mRNAs are responsible for the dystrophin production in these cases. In this paper, we have shown that the dystrophin produced in cases that had a deletion of exons 3–7 does not stain either with two amino-terminal antibodies or with an antibody recognizing the first half of exon 8, before a potential ATG initiation site. This latter observation is in agreement with Arahata et al. (1991), who have previously reported that cases that they studied that have a deletion of exons 3–7 do not stain with an antibody directed against amino acids 215–264. Therefore, the negative staining pattern at both positions has been confirmed by two independent antibodies. The antibody that recognizes the later half of exon 8, after the ATG, stained positively, indicating that the dystrophin molecule produced in exons 3–7 cases does not contain the amino-terminal and starts at the ATG in exon 8. It is difficult to reconcile this dystrophin molecule with the expected dystrophin products from the mRNA species reported by Chelly et al. (1990). However, it is possible that these mRNA species would also use the ATG in exon 8.

Last, Thanh et al. (1993) have reported a BMD patient who has a deletion of exons 3–7 that stained with an antibody directed against amino acids in exon 1. At present, we have no clear explanation for this result. Interestingly,

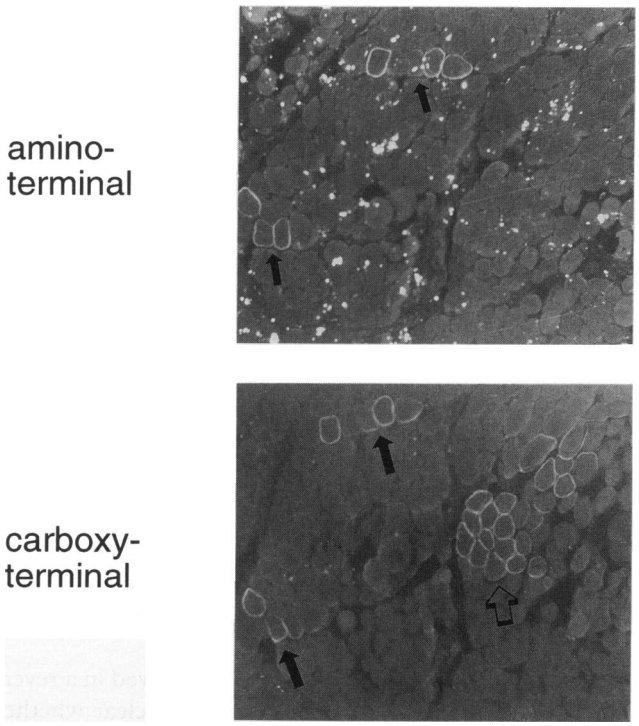

Figure 6 Immunostaining of serially sectioned muscle tissue from patient 67. Section 1 was stained with an amino-terminal antibody (Ab1). Section 2 was stained with the carboxyl-terminus antibody (Dys2). Several serial sections were taken and were stained with a panel of dystrophin antibodies: Ab1, Ab2, Ab3, Dys1 (amino acids 1181–1388), and Dys2 (data not shown). The blackened arrows indicate clusters of revertant fibers that stain with all antibodies tested. The unblackened arrow indicates the cluster of revertant fibers that stain positive with only the more carboxyl-terminal antibodies, Dys1 and Dys 2. The presence of the group of fibers was followed through several serial sections to ensure that we had not sectioned through that fiber group.

these authors were not able to detect dystrophin on western blots using that antibody; however, we were able to detect dystrophin of the predicted size (from exon 8 to the carboxyl-terminus) by western blot using antibody Ab3 (3′ end of exon 8) in one of our patients who have a deletion of exons 3–7 (data not shown). The simplest interpretation of our own data is that dystrophin in the cases that had a deletion of exons 3–7 is produced by initiation at the ATG in exon 8.

Rare dystrophin-positive fibers have been observed in ≥50% of DMD cases (Nicholson et al. 1988, 1993; Shimizu et al. 1988; Hoffman et al. 1990; Burrow et al. 1991; Klein et al. 1992). These dystrophin-positive fibers can occur as a single fiber or a cluster. Elsewhere we have shown that these fibers stain with a panel of antibodies to dystrophin and that somatic mosaicism was ruled out in several cases (Klein et al. 1992). Two explanations have been put forward for these revertant fibers: First, dystrophin could be produced in these fibers because of a somatic mutation. This second site somatic mutation would act to correct the reading frame in these fibers. Second, Sherratt et al. (1993) have suggested that low-level in-frame illegitimate transcripts could account for dystrophin production in revertant fibers. These authors detected in-frame transcripts by amplifying mRNA by RT-PCR followed by sequencing of minor bands. In some instances, no extra bands were observed. When this was the case, the region below the band was excised and reamplified in order to detect in-frame transcripts. In all cases, the patients had a deletion. These alternative mRNA species were also observed in normal muscle. Although it is tempting to relate the observation of these illegitimate transcripts to revertant fibers, it is not clear that this is valid. First, it is not clear why illegitimate mRNA or exon skipping would occur in one fiber versus another fiber in the muscle section. In our experience, revertant fibers appear clonal in nature, as opposed to diffuse overall staining or the checkerboard type of staining sometimes seen in carriers. This clonal staining implies that a specific event has occurred in those fibers to restore the ability to produce dystrophin. This event could be a mutation in a splice consensus sequence, deletion of an exon, or alteration in the way in which splice sites are selected.

It is important to relate the protein observed in a revertant fiber to the mRNA species, as it is unclear whether there is any functional role of low-level illegitimate transcription. In this paper, we have investigated a patient (patient 71) whom we elsewhere had shown did not stain with the antibody 9218 directed against amino acids 2305–2554, exons 48–52 (Klein et al. 1992). This patient has a 2-bp mutation that creates a new splice site in exon 51 (Winnard et al. 1992). This site appears to be used by ~70% of the mRNA species and results in an out-of-frame mRNA. Analysis of exon boundaries surrounding this region indicates that the splicing of exons 49–51 would correct the reading frame (table 2 lists possibilities). It can be seen that, if this splice site is used, it would be specific to this patient and that the latter half of exon 51 would be included in the mRNA encoding the dystrophin. mRNAs that splice out exon 51 would not result in the presence of the epitope at the 3′ end of exon 51 recognized by antibody Ab4 (table 2). Staining of this patient's muscle showed that revertant fibers did indeed stain with Ab4 and that this mRNA was specific to this patient. We suggest that, in this patient, revertant fibers result from the loss of exon 50 and the 5′ end of exon 51, created by the splicing of exon 49 to the new splice site of exon 51. This could result either from a somatic mutation that disrupts the splicing of exon 50 or from a deletion of exon 50. Indeed, somatic reversion events, which are more complicated than illegitimate-exon deletions, are indicated in the cases that had a deletion of exons 3–7. For example, in the patients who had a deletion of exons 3–7, revertants were observed in two cases (figs. 2 and 3). These fibers stained with amino-terminal antibodies as well as with antibodies in exon 8 (data not shown). This would indicate that the secondary mutation is not due to the deletion of an entire exon, but rather is due to a very small deletion or point mutation that corrects the frame.

Table 2

Splicing Events That Could Correct Reading Frame in Patient 71

Splicing (5′ exon–3′ exon)[a]	Deleted Exons	Protein Recognizable by Exon 51 Antibody?
50 to 53	51–52	No
49 to 52	50–51	No
48 to 52	49–51	No
49 to 54	50–53	No
49 to CSS	50, ½ 51	Yes
48 to CSS	49, 50, ½ 51	Yes
47 to CSS	48, 49, 50, ½ 51	Yes
46 to CSS	47, 48, 49, 50, ½ 51	Yes

NOTE.—The exon borders were as reported by Roberts et al. (1993).
[a] CSS = cryptic splice site.

The last observation that we have made is that, somewhat surprisingly, more than one reversion event can be observed in a single section of muscle. Indeed, Wallgren-Pettersson et al. (1993) also observed a dual pattern of revertant-fiber staining in a DMD patient who had a deletion of exons 35–43. In the present paper we have investigated a DMD patient who has a duplication mutation of exons 5–7 inherited from his mother, thereby eliminating the possibility that the revertants are due to somatic mosaicism. A series of clusters of revertant fibers were observed that showed different staining patterns. Given that the most likely current explanation is a second-site somatic mutation, the chance of observing two somatic mutations in a single section would be expected to be very low. This

may indicate either that the frequency of somatic reversions is much higher than expected or that the mechanism is more complicated than straightforward somatic mutation. Further work at the DNA level, using clonal isolates of cells derived either from the *mdx* mouse or from patients will be required to further elucidate the exact events responsible for revertants.

In conclusion, analysis of the translational frame rule–exception patients presented in this study gives further insight into various methods of dystrophin production. In addition, results from the analysis of revertant fibers support the theory that these fibers can be produced by a secondary mutation that removes the original mutation.

Acknowledgments

We would like to thank Drs. J. Chamberlain and K. Corrado for providing a fusion protein encoding the amino-terminus of dystrophin, Drs. K. Campbell and K. Matsumura for providing NH_2-Dys, and Dr. Alan Beggs for tissue from two patients who have a deletion of exons 3–7 and from one patient who has a deletion of exons 2-7. We would also like to thank S. Ingraham for her assistance in preparation of the manuscript. This work was supported by NIH grant R29AR40015 and a Muscular Dystrophy Association grant.

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