Massive Idiosyncratic Exon Skipping Corrects the Nonsense Mutation in Dystrophic Mouse Muscle and Produces Functional Revertant Fibers by Clonal Expansion

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Abstract. Conventionally, nonsense mutations within a gene preclude synthesis of a full-length functional protein. Obviation of such a blockage is seen in the *mdx* mouse, where despite a nonsense mutation in exon 23 of the dystrophin gene, occasional so-called revertant muscle fibers are seen to contain near-normal levels of its protein product. Here, we show that reversion of dystrophin expression in *mdx* mice muscle involves unprecedented massive loss of up to 30 exons. We detected several alternatively processed transcripts that could account for some of the revertant dystrophins and could not detect genomic deletion from the region commonly skipped in revertant dystrophin. This, together with exon skipping in two noncontiguous regions, favors aberrant splicing as the mechanism for the restoration of dystrophin, but is hard to reconcile with the clonal idiosyncrasy of revertant dystrophins. Revertant dystrophins retain functional domains and mediate plasmalemmal assembly of the dystrophin-associated glycoprotein complex. Physiological function of revertant fibers is demonstrated by the clonal growth of revertant clusters with age, suggesting that revertant dystrophin could be used as a guide to the construction of dystrophin expression vectors for individual gene therapy. The dystrophin gene in the *mdx* mouse provides a favored system for study of exon skipping associated with nonsense mutations.

Key words: reversion • dystrophin • nonsense mutation • splicing • exon mapping

Introduction

Duchenne muscular dystrophy (DMD)[1] is an X-linked fatal muscular disease, characterized by the lack of dystrophin expression. The underlying genetic events are frameshift mutations in the dystrophin gene, which in man comprises 79 exons spanning >2.4 million bp (Koenig et al., 1987; Amalfitano et al., 1997). It encodes a 3685–amino acid protein (427 kD) in skeletal muscles that can be divided into NH$_2$-terminal, rod, cysteine-rich, and COOH-terminal domains. The NH$_2$-terminal domain binds to cytoplasmic actin filaments and the cysteine-rich domain to dystrophin-associated protein (DAP) complexes, including dystroglycans, sarcoglycans, and syntrophins, through which dystrophin connects itself to extracellular matrix components. It has been suggested that dystrophin is involved in force transmission from subsarcolemmal actin to the extracellular matrix and protects fiber from contraction-related muscle damage (Winder et al., 1997). The *mdx* mouse is a homologue of DMD and caused by a nonsense point mutation in exon 23 of the gene (Bulfield et al., 1984; Sicinski et al., 1989). Lack of dystrophin expression in both DMD patients and *mdx* mouse results in chronic degeneration and regeneration of skeletal muscles.

Surprisingly, individual dystrophin-positive muscle fibers, called revertant fibers (RFs), have been observed in otherwise dystrophin-negative backgrounds of both DMD patients and *mdx* mouse. Revertant dystrophin, like normal dystrophin protein, shows a membrane localization, suggesting that it may be functional. The incidence of RF in muscles of DMD patients ranges from 0–70% (Burrow et al., 1991; Klein et al., 1992; Fanin et al., 1995; Uchino

et al., 1995), and comprises <1% of fibers in the *mdx* mouse (Hoffman et al., 1990; Nicholson et al., 1993). The biological significance of the RF is not clear. Correlation between the number of RFs in muscles and the clinical prognosis of DMD patients has been inconclusive (Burrow et al., 1991; Nicholson et al., 1993; Fanin et al., 1995).

The mechanisms by which an individual dystrophic muscle fiber acquires its ability to produce dystrophin from the gene with out-of-frame mutations has yet to be determined. Exon skipping in association with nonsense mutations has been reported in genes such as the factor VIII gene in hemophilia A (Naylor et al., 1993), Fanconi anemia group C genes (Gibson et al., 1993), fibrillin (FBN1) gene in Marfan syndrome and in the ornithine δ-aminotransferase (OAT) gene in gyrate atrophy (Dietz et al., 1993), transacylase (E2) gene of the human branched-chain α-keto acid dehydrogenase (BAKAD) complex in maple syrup urine disease (MSUD) (Fisher et al., 1993), and more recently in the 3-hydroxy-3-methylglutaryl-CoA lyase gene (Pie et al., 1997). In the dystrophin gene, exon skipping around point mutations has also been reported, resulting in in-frame transcripts and shortened dystrophin proteins (Shiga et al., 1997; Melis et al., 1998). These particular nonsense point mutations, which were not at the consensus donor or acceptor splice sites, had presumably disrupted the normal splicing by interfering with the splice site recognition sequences.

We had previously identified several alternatively processed dystrophin transcripts that skipped 5 to 11 exons, including the mutated exon 23 in *mdx* mouse muscle (Wilton et al., 1997a). However, it is difficult to determine whether these mRNA transcripts detected by reverse transcription (RT)-PCR from whole muscle tissue are relevant to the production of dystrophin in RFs, which always form a distinctive cluster (Hoffman et al., 1990). In the absence of data relating them directly to RFs, these transcripts could simply be the result of low-level random splicing events.

To address these questions, we examined the dystrophin in RFs of the *mdx* mouse at the protein, RNA, and DNA levels. Serial muscle sections were examined with a panel of exon-specific monoclonal and polyclonal antibodies (Abs) by immunohistochemistry (Thanh et al., 1995). This method enables us to analyze the patterns of exon composition of the revertant dystrophin within individual RFs. We found that reversion of dystrophin expression in *mdx* mice muscle utilizes mechanism(s) involving unprecedented massive exon skipping. The number of missing exons varied from a few to up to 30 in different RF clusters, and several alternatively processed transcripts that are consistent with the most common species of the shortened proteins have also been detected. RFs appear to grow in a clonal fashion, each cluster characterized by its individual species of dystrophin. Revertant dystrophins are at least partially functional, in that they protect the muscle fiber from degeneration.

Materials and Methods

Tissues and Section Preparation

A total of 36 muscle samples from 26 male *mdx* mice aged from new born to 20 mo were examined. Muscles of tibialis anterior and extensor digitorum longus (TE), quadriceps (QU), and posterior compartment (PC) of hind legs were dissected and snap-frozen immediately after the animals had been killed. Serial cross sections of 6 μm were cut onto 3-aminopropyltriethxysilane (Sigma Chemical Co.) coated glass slides, numbered, and stored at −70°C. Sections of TE muscle from *C57BL/10* mouse were used as controls.

Antibodies

A panel of 12 monoclonal and 2 polyclonal Abs against dystrophin were used. These Abs recognize exons flanking the mutated exon 23 of the rod region and in both NH_2- and COOH-terminal domains of dystrophin. The specificity, working dilution, and sources of the Abs are listed in Table I. Titres of all antibodies were adjusted such that they produced approximately equal intensities of staining to each other on normal mouse muscle and no staining on the bulk of fibers in *mdx* muscle, thus permitting broad comparison of differences in intensity between individual revertant clusters. mAb to β-dystroglycan (43DAG/8D5; kind gift from Dr. L. Anderson, Muscular Dystrophy Unit, Newcastle, UK), polyclonal goat anti–α-sarcoglycan, anti–β-sarcoglycan, and anti–α-syntrophin (kind gifts from K.P. Campbell, Department of Physiology and Biophysics, Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City, IA) were also used.

Table I. Antibodies to Mouse Dystrophin

Names	Clone	Species	Exon specificity	Working dilution	Sources and references
MANEX1A	4C7	M*/IgG1	1	1:3	Morris et al., 1995
α60	Serum	Sheep	10–18	1:500	Hoffman et al., 1987
MANDYS19	8F6	M/IgG1	20–21	1:4	Nguyen et al., 1990
MANDYS18	5H9	M/IgG2a	26	1:6	Nguyen et al., 1990
MANDYS16	1B12	M/IgG2b	27–28	1:4	Nguyen et al., 1990
NCL-DYS1	Dy4/6D3	M/IgG2a	26–29	1:3	Nicholson et al., 1989
MANDYS8	Ascites	M/IgG2b	32	1:100	Sigma; Sedgwick et al., 1991
MANDYS110	3H10	M/IgG1	38–39	1:5	Nguyen and Morris, 1993
MANDYS101	7D12	M/IgG2b	40–41	1:4	Nguyen and Morris, 1993
MANEX45A	8F10	M/IgG1	45	1:4	Thanh et al., 1995
MANEX46B	7G1	M/IgG1	46	1:3	Thanh et al., 1995
MANEX50	6A9	M/IgG1	50	1:4	Thanh et al., 1995
p6	Serum	Rabbit	57–60	1:500	Sherratt et al., 1992
MANDRA1	Ascites	M/IgG1	77	1:100	Sigma; Ellis et al., 1990

*M, mouse.

Nonspecific Background Blocking for the Use of mAb

A new blocking method was applied to prevent the binding of secondary anti–mouse Ab to the endogenous mouse Igs or other mouse tissue components when mAb was used (Lu and Partridge 1998). In brief, unlabeled rabbit anti–mouse Igs (react with all mouse IgG subclasses, IgA, and IgM; DAKO) and goat anti–mouse Igs (react with mouse IgG, IgA, and IgM; DAKO) were digested with papain (5% of Igs, wt/wt; Sigma Chemical Co.) to obtain monovalent Fab and Fc. The Fab-Fc was applied onto the sections for 60 min before the primary Abs. Composite blocks of muscle samples from *C57BL/10* and *mdx* mouse were used for optimizing the condition with individual Abs.

Immunohistochemistry

Sections were air-dried, hydrated in PBS, and then subjected to either immunoperoxidase staining to produce permanent preparations, or immunofluorescence method which permits better image recording and double labeling. In brief, sections were incubated for 30 min with normal serum (1:20) of the animals from which the second layer Abs was raised. For the three-step immunoperoxidase method, sections were then incubated with primary Ab for 1 h followed by biotinylated second layer Ab (rabbit anti–mouse Igs, 1:400 dilution [DAKO]; or swine anti–rabbit Igs, 1:500 [DAKO]; or donkey anti–sheep/goat Igs, 1:500 dilution [Amersham Life Science]) for 45 min. Endogenous peroxidase activity was blocked with 1% H_2O_2 in PBS for 10 min. Sections were then incubated with HRP-conjugated streptavidin (1:200; DAKO) for 45 min. Enzyme activity was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB, 1 mg/ml PBS; Sigma Chemical Co.) and 0.1% H_2O_2 for 4 min. Finally, the sections were counterstained with hematoxylin. For immunofluorescent staining, sections after the secondary Ab were incubated with Texas red–conjugated streptavidin (1:200; DAKO) for 45 min and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.). All Abs were diluted in 3% BSA in PBS unless otherwise stated, and intervening washes carried out with PBS for 5 min twice. Negative controls were carried out by replacing the primary Abs with 3% BSA in PBS or by mAb NCL-DYS3 (Novocastro), which is specific for human dystrophin. mAb NCL-spect2 (Novocastro) to β-spectrin was used as a positive control for monitoring integrity of the sarcolemma membrane. Composite sections of *C57BL/10* and *mdx* muscle with known RF were used for monitoring positive and background staining.

For immunofluorescence double staining, sections were incubated with both rabbit anti-dystrophin Ab p6, and one of the mAbs simultaneously for 1 h, followed by Texas red–conjugated donkey anti–rabbit Igs (1:200; Amersham Life Science) and FITC-conjugated goat anti–mouse Igs (1:50; DAKO) for 45 min.

Detection of Alternatively Processed Dystrophin Gene Transcripts

Total RNA was extracted from muscle of *mdx* mouse using an acid phenol extraction as described by the manufacturer (RNAzol; Tel-Test Inc.). Approximately 50–100 ng of total RNA was used in a 12.5-μl single step cDNA synthesis and primary DNA amplification reaction using Titan Expand (Boehringer Mannheim). The primary DNA amplification reaction was carried out with the appropriate outer primers (Table II) for 40 cycles of 90 (30 s), 55 (1 min), and 72°C (6 min). 1 μl was removed and reamplified using the inner primers for 40 cycles of 90 (30 s), 55 (1 min), and 72°C (6 min). The amplification products were fractionated on a 2% agarose gel in Tris-acetate/EDTA buffer. RT-PCR products smaller than expected were reamplified using bandstab (Wilton et al., 1997b) and purified using Quiaquick PCR spin columns (Qiagen). Direct DNA sequencing was carried out using the Big Dye chain terminator chemistry (PE Applied Bio-Tech), where the products were fractionated and analyzed on an ABI 373 DNA sequencer.

Double Labeling of Immunohistochemistry and DNA In Situ Hybridization

Serial sections (10 mm) were cut from 18-mo-old male *mdx* mice and stained first with anti-dystrophin Ab p6 followed by peroxidase-conjugated swine anti–rabbit Igs. Enzyme activity was developed with DAB and 0.1% H_2O_2 for 4 min. Sections were then washed with PBS, digested with RNase A (100 μg/ml for 1 h at 37°C; Sigma Chemical Co.), washed with PBS, and dehydrated through graded ethanols.

Table II.

Primer	Sequence 5′ to 3′
13F outer	GCT TCA AGA AGA TCT AGA ACA GGA GC
13F inner	CTC GCT CAC TCA CAT GGT AGT AGT G
35R outer	GGT GAC AGC TAT CCA GTT ACT GTT CAG
35R inner	GCC CAA CAC CAT TTT CAA AGA CTC TC
50R outer	CCA GTA GTG CTC AGT CCA GGG
50R inner	GGT TTA CAG CCT CCC ACT CAG

Primers used in the detection and characterization of alternatively processed dystrophin gene transcripts. Other primers have been described in a previous publication (Wilton et al., 1997a).

A fragment of 7.2-kb mouse genomic DNA, from intron 21 to 5′ sequence of exon 25 of the dystrophin gene, was created and used as a probe (sequence data available from EMBL/GenBank/DDBJ under accession no. AF095738). Mouse genomic sequence of XIST gene and Y chromosome specific sequence Y1 were also used as positive control probes (Nishioka, 1988). Probes were labeled with digoxigenin by nick translation using Nick Translation System (Boehringer Mannheim). pBR322 DNA was used as a negative control probe.

The hybridization protocol was essentially the same as described previously (Lu and Dover, 1993). The hybridization mixture consisted of deionized formamide (50%), dextran sulphate (5%), 2× SSC (1× SSC = 0.15 M sodium chloride, 15 mM sodium citrate), sheared herring sperm DNA (100 μg/ml), sodium dodecyl sulphate (0.1%), and the labeled probe (1 μg/ml). 10–30 μl of hybridization mixture was applied on to immunohistochemically stained sections, which were then covered and edge-sealed with rubber solution. Probe and target DNA were denatured simultaneously for 8 min at 90°C, and hybridization was carried out at 42°C overnight. Washes were carried out with 2× SSC for 30 min at room temperature and 0.2× SSC for 20 min twice at 40°C. Digoxigenin-labeled probes were detected with FITC-conjugated sheep anti-digoxigenin Fab (1:20; Boehringer Mannheim), followed by biotinylated goat anti-FITC Igs (1:200; Vector Laboratory) and streptavidin/FITC (1:200; Amersham Life Science). Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Hybridization signals in 1,000 myonuclei in both RFs and adjacent non-RFs were counted.

Assessment

Muscle fibers were regarded as dystrophin-positive only when more than half the membrane circumference was stained in cross sections. RFs adjacent to each other were characterized as a single cluster. Exon mapping of revertant dystrophin was mainly carried out with three-step streptavidin immunoperoxidase method on serial sections counterstained with hematoxylin, as they provide strong and permanent staining as well as morphological structure for subsequent time-consuming comparison. Double labelings were also carried out with one polyclonal Ab p6 and one of the mAbs for confirmation. To avoid possible false negative results due to the limited territory of dystrophin distribution along the axis of fiber, revertant clusters were included in the exon mapping analysis only when positive staining was observed on both ends of a series of sections. For closer comparison of RFs in mice of different age groups, 100 serial sections (10-μm thick) of TE muscle from mice aged 0 (new born), 2 wk, 4 wk, 2 mo, 4 mo, and 18 mo (three mice per group) were stained with polyclonal Ab p6 to exons 57–60. The number of revertant clusters, the number of RFs in each clusters, and the length of RFs were counted. The total number of RFs counted in all 100 sections was added and referred to as RF sections.

Results

RFs Are Observed in mdx Mouse Muscles of All Ages and Increase in Number, Size, and Length with Age

Dystrophin-positive fibers were seen in all muscles of new born mice. Almost all RFs were isolated individual fibers; even where a few RFs were concentrated in a small area, they were not adjacent one to another (Fig. 1 A). These RFs contained either centrally or peripherally localized
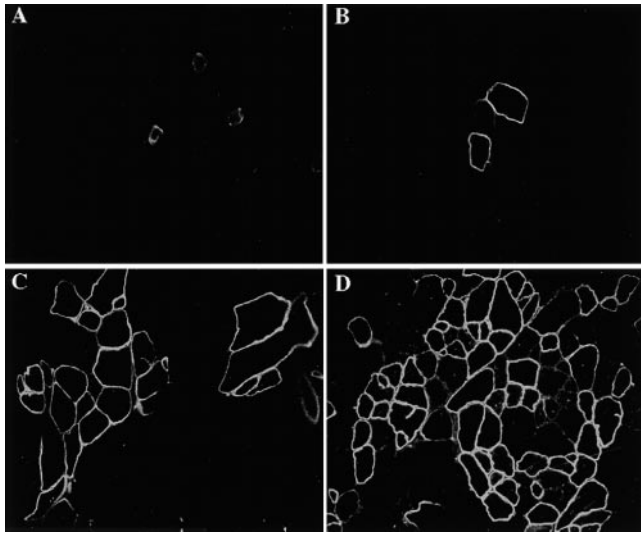
Figure 1. Age-related expansion of RF in *mdx* mouse muscles. (A) New born, (B) 4 wk, (C) 4 mo, and (D) 18 mo. The size of RF clusters increases from isolated single fibers in the new born mouse to a huge cluster containing >60 RFs in the 18-mo-old mouse. Variation of RFs in size and of staining intensity within a cluster is seen in mice aged 4 wk or older. Sections are stained for dystrophin with polyclonal antibody p6.

Figure 2. An overall age-related increase of RFs in TE muscle of *mdx* mouse. The number and length of RF clusters and maximum number of RFs within one cluster increase steadily, resulting in a huge increase in the number of RF sections within 1,000 μm of TE muscles. NB, new born mice; W, weeks; M, months; and Maxi No, maximum number. Vertical bars represent standard deviation. The total number of RFs counted in all 100 sections was referred to as RF sections.

nuclei. The dystrophin staining was generally weaker than that seen in normal mouse muscle of same age, or RFs in older mice.

At 2-wk-of-age, all fibers of *mdx* muscle contained peripheral nuclei, and showed no sign of degeneration. The number of RFs increased significantly, largely reflecting the increase of cluster size with up to five RFs per cluster.

By 4-wk-of-age, *mdx* mouse muscle was undergoing a continuous process of degeneration and regeneration characterized by the presence of necrotic fibers and centrally localized nuclei. This was observed in all groups of older mice. The overall number of RFs and the size of RF clusters increased steadily over the lifetime of the mouse in TE, quadriceps, and posterior compartment muscles. The maximum number of RFs within one cluster was 11, 24, 34, and >100 in mice aged 4 wk, 2 mo, 4 mo, and 18 mo or older, respectively (Fig. 1, B–D).

The growth of RF with age is best illustrated by the results of 100 serial sections (10-μm thick) of TE muscles as summarized in Fig. 2. The total number of RF clusters ranged from 41–48 (all single fibers) in new born mice, to 81 (maximum 40 RFs in one cluster) in 18-mo mice. Similarly, the median length of RFs increased from 10 μm in new born, to 510 μm in 18-mo mice. No RF cluster exceeded the span of sample length of 1 mm at 4 wk or younger, but >10% surpass this length at 4 mo and 30% at 18 mo. Most significantly, the average number of RFs in each section was more than doubled between each age group, from 0.73 RFs per section in new born mice to 141.1 per section in 18-mo mice. The increase in the number of RF clusters with age was relatively small. This could be due partly to the fact that the increased number of RFs within an area made it difficult to distinguish each individual cluster.
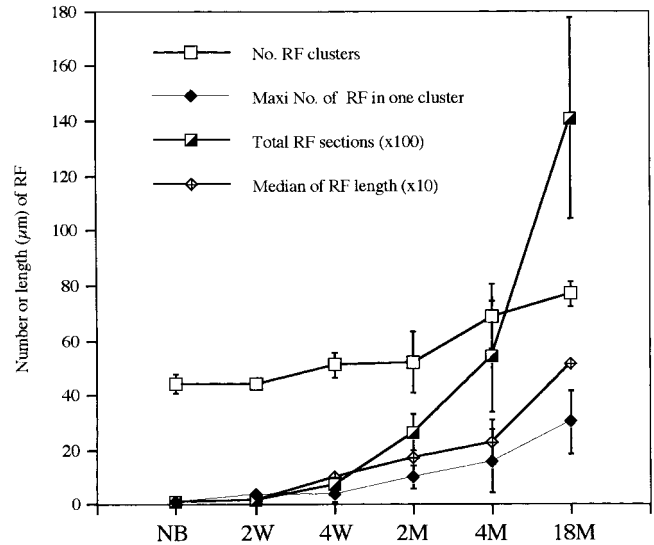
RFs in mice aged 4 wk or older varied in size from hypertrophic to small caliber myotubes seen in new born mice, with mixed peripheral and centrally localized nuclei (Fig. 1). RFs of small caliber often showed limited length (<50 μm) despite the overall increase in the length of RF and the size of revertant cluster. However, the intensity of the staining was generally at similar levels within individual RFs except towards the ends of RF segments, which showed weaker staining in serial sections.

Restoration of DAPs in RF

Protection of muscle fibers from degeneration depends on restoration of DAPs by dystrophin. In *mdx* mice with a functional dystrophin transgene, the level of sarcolemma-bound DAPs was restored in skeletal muscles in association with alleviation of muscular dystrophy (Dunckley et al., 1993). Therefore, we examined the expression of DAPs in RFs of *mdx* muscle. The majority of *mdx* muscle fibers showed a greatly reduced level of β-dystroglycans, α- and β-sarcoglycans, and α-syntrophin when compared with the muscle fibers from normal C57 mouse. However, all four proteins were clearly demonstrated in RFs at levels comparable to that observed in normal muscle of C57 mice (Fig. 3). Variation in staining intensity with the four Abs was seen within clusters of RFs, and appeared to be correlated with the staining intensity of dystrophin. The restoration of DAPs in RFs was seen in revertant clusters of all sizes.

Dystrophin in RF Contains Functional Domains Despite Massive and Noncontiguous Exon Losses

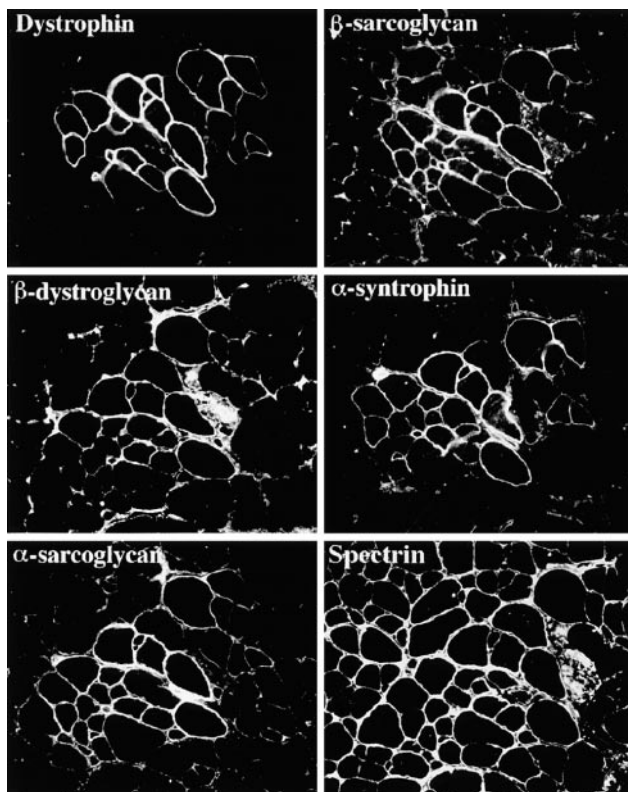Previous studies have shown that two domains of dystro-

Figure 3. Expression of dystrophin and dystrophin-associated glycoprotein complex in RFs. A group of fibers are stained with p6 for dystrophin. The same group of fibers are clearly stained for β-dystroglycan, α- and β-sarcoglycan, and α-syntrophin and the staining intensity is correlated to that of dystrophin. The remaining fibers are only weakly or not stained for the Abs. All fibers are stained for spectrin (bottom left panel). Serial sections of TE muscle from 18-mo-old *mdx* mouse.

phin are important for its function: the NH_2-terminal domain and the cysteine-rich domain (Winder et al., 1997). We examined revertant dystrophin with Abs to epitopes flanking and within these two regions: Abs MANEX1A (against exon 1) and α60 (against exons 10–18) in the NH_2-terminal domain; and p6 (against exons 57–60) and MANDRA1 (against exon 77) to the cysteine-rich COOH-terminal domains. Dystrophin in nearly all RFs was stained with these Abs and this was consistent in mice of all ages. Only a few fibers (4%) lost the epitope in exon 77 recognized by MANDRA1. These fibers were stained with MANEX1A, α60, and P6 (Fig. 4). This result is consistent with the membrane localization of revertant dystrophin in muscles of *mdx* mice of all ages.

Antibodies to the rod and hinge regions of dystrophin produced surprisingly different results from those to the NH_2-terminal and cysteine-rich domains. Of a total of 279 RF clusters recognized by Abs to NH_2-terminal and exons flanking cysteine-rich domains, <4% of RF clusters were stained with the mAb MANDYS19 to exons 20 and 21, two exons upstream of the mutated exon 23. Similarly, only 7.9% of the total RFs were stained with Ab MANDYS18 to exon 26, three exons downstream of the mutated exon 23. Increasing numbers of RFs were recognized by Abs to exons towards the COOH-terminal rod domain. RFs stained with p6 were nearly all recognized by Ab MANEX50 to exon 50 (Fig. 5). Variations in exon loss between different revertant clusters were observed within each individual muscle as well as between different muscle samples.

Although the majority of RFs expressed dystrophin missing exons in one region adjacent to the point mutation, four revertant clusters were found to have an exon loss in a second region around the exons 32–45 (Fig. 5). The frequency of exon loss involving two noncontiguous regions is likely to be an underestimate, as the epitopes examined only covered a small number of exons.

Variation in intensity of dystrophin staining was also observed. Weak positivity was often seen with Abs MANDYS18, MANDYS16, and NCL-DYS1 to exons 26, 27 and 28, and 26–29, respectively, all of them recognizing exons flanking the mutated exon 23 (Fig. 4). This signal variation was not attributable to differences in the process of immunohistochemistry, since other RFs with strong
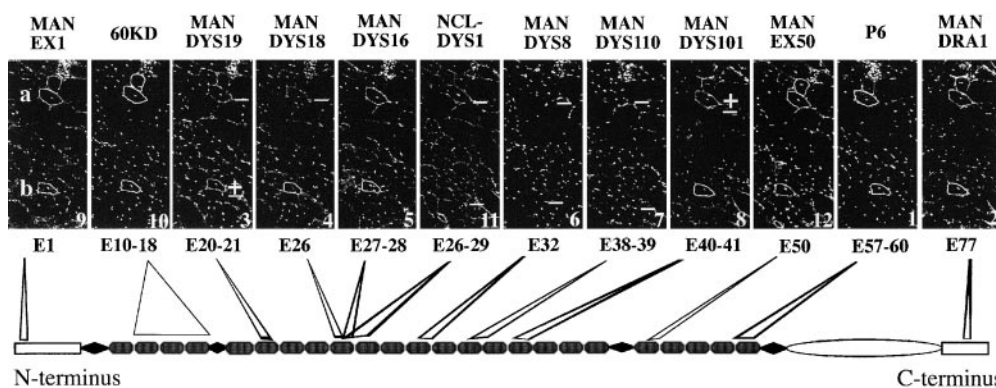

Figure 4. Exon mapping of RF. Staining of serial sections with a panel of 12 Abs (name above and exon specificity below the pictures). Sections are arranged in such an order from left to right with Ab recognizing exons from NH_2-terminal to COOH-terminal. The diagram below illustrates the position of the epitope(s) recognized by the Abs on the dystrophin molecule. Serial number of sections appears at the bottom right corners.

Two isolated fiber clusters marked a and b with two and one fibers, respectively. Fibers a are recognized by Abs to NH_2-terminal exons up to 18, but negative with Abs to exons 20–26. These fibers are positive with Ab to exons 27–28, but negative again with Abs to exons from 26–29 to 38–39 followed by relatively weak staining with Ab to exons 40–41. Fiber b is recognized by Abs to NH_2-terminal exons up to 27–28, with relatively weak staining with Abs to exons 20–21. This fiber is not recognized with Ab against exons 26–29 to 38–39. Another revertant fiber of small caliber stained with Abs to exons 26–29 and 50 appears at the bottom of the sections, which are at the end of this serial sections. The primary Abs were detected with peroxidase method and the pictures are presented as negative image to enhance contrast. Nuclei appear positive due to the haematoxylin counterstaining.
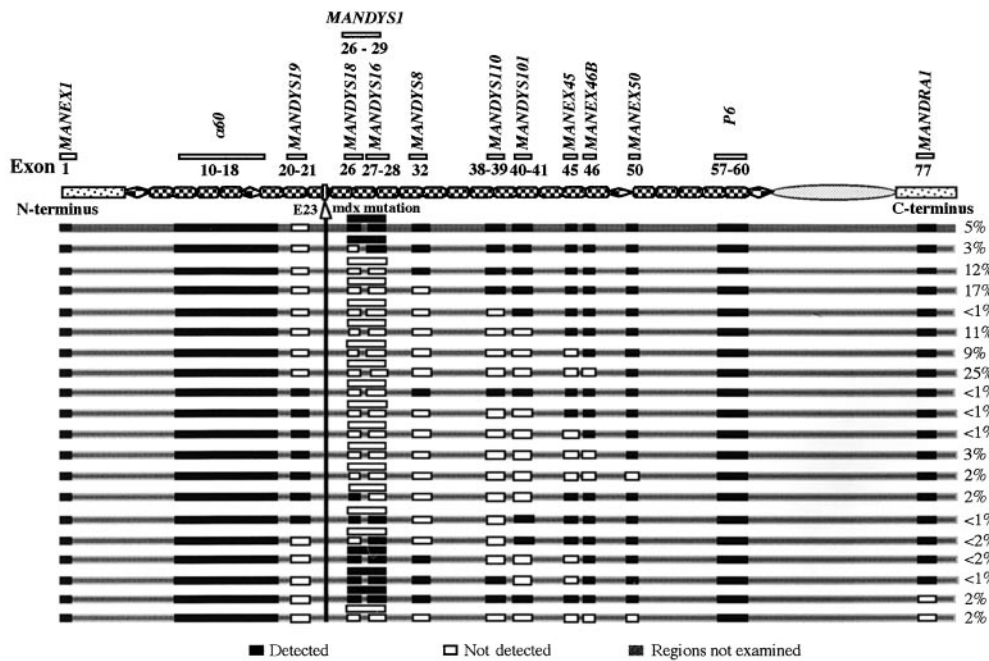
Figure 5. Patterns of epitope loss in RF. Total of 279 RF clusters were examined with 14 Abs to dystrophin by three-step immunoperoxidase staining. Loss of 20 exons or more was found in >65% of RF clusters.

staining were sometimes seen in the same microscopic field.

Spliced mRNAs Predicted from Exon Mapping Are Readily Detected by RT-PCR

In an earlier study, alternatively processed dystrophin gene transcripts were examined between exons 18 and 30 (Wilton et al., 1997a). Several species of transcripts with exon skipping were detected in muscle of both *mdx* and normal mouse, and some of them correlated with the exon arrangement of the dystrophin protein in RFs. These transcripts where exon 18 was spliced to exon 26 or 30 (in-frame) could account for 15% of the dystrophin-positive fibers observed by the exon mapping. However, these transcripts did not reflect the most common isoforms of revertant dystrophin predicted from the exon mapping results, many of which lack 20 or more exons. Therefore, new exonic primers were selected to look for exon skipping between exons 13 to 50 by Nested PCR. In addition to the expected full-length product, shorter dystrophin transcripts were also observed. One of the most commonly encountered alternative transcripts was an exon 18–35 splicing, as confirmed by sequence analysis (Fig. 6 A). This particular exonic rearrangement corresponds to 17% of the RFs identified by the exon mapping. Another of the shorter transcripts was found to have risen from exon 13, being joined to exon 48 (Fig. 6 B). This transcript could have directed synthesis of the most commonly encountered (25%) revertant dystrophin isoforms.

Detection of Genomic Sequence (Exons 22–25) in Nuclei of RF

More than 80% of RFs examined showed a loss of epitopes between exons 21 and 30. We sought to determine whether deletion at the genomic level underlies the massive exon skipping at the mRNA and protein levels. How-

ever, examination of DNA extracted from muscle tissue does not provide useful information, as the RFs account for a very small proportion of muscle fibers. Attempts to isolate possible revertant myoblasts from muscle culture of *mdx* mouse has so far failed to establish any clones producing revertant dystrophin (unpublished data). We therefore examined the serial sections from 18-mo-old mice containing large RF clusters by combined immunohistochemistry and DNA in situ hybridization. The revertant dystrophins in these RFs were all negative with Abs MANDYS19 (to exons 20 and 21) and MANDYS 18 (to exon 26). Simultaneous detection of revertant dystrophin protein and genomic sequence of intron 21 to exon 25 within individual RFs, would appear to rule out a genomic deletion mechanism and imply a splicing-based exon skipping. The results showed that the majority of nuclei in muscles from both normal and *mdx* mice (84%) demonstrated positive nuclear signal with the probe. A similar proportion of nuclei within RFs (81%) was also found positive with the probe, indicating the presence of this region at genomic level (Fig. 7). The difference in proportion of hybridization-positive nuclei between RFs and non-RFs was not statistically significant ($P > 0.16$), and there was no difference in the intensity of signal between the two populations. The lack of positive signals in a small proportion of myonuclei in both RFs and non-RFs as well as nuclei of mesenchymal cells is mainly due to sectioning, since the control XIST and Y1 probes labeled a similar proportion of nuclei (data not shown).

Discussion

Revertant Dystrophin Is Functional

Mutations in the dystrophin gene can result in a spectrum of phenotypes, ranging from almost asymptomatic to severe DMD (Hoffman, 1993). Generally, the milder forms
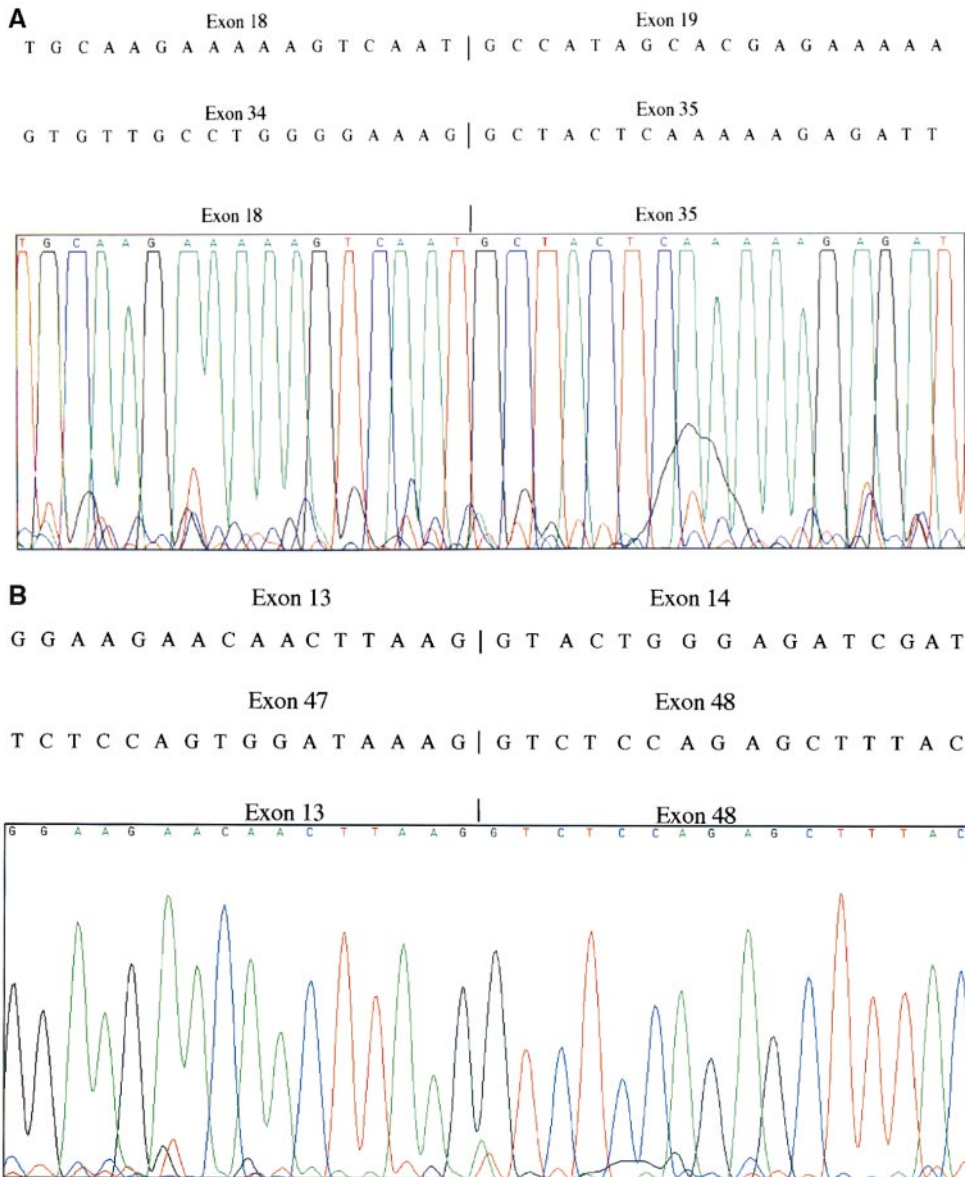
Figure 6. Sequence profile of the alternative dystrophin transcript across exons 18–35 (A) and exons 13–48 (B) detected by RT-PCR. The joining sequences between exons 18 to 19, exons 34 to 35, exons 13 to 14, and exons 47 to 48 are shown above the graphs. Both 3′ of exons 13 and 18, and 5′ of exons 35 and 48 end with complete codon (AAT, AAG and GCT, GTC respectively), and the resulting transcripts are in-frame.

of the muscular dystrophy are associated with in-frame deletions that permit the synthesis of shortened dystrophin proteins with functional NH₂- and COOH-terminal domains. In some cases, massive genomic deletion, such as from exons 17–48, 13–48, and 17–51, have been associated with a mild form of the disease Becker muscular dystrophy (BMD) (Love et al., 1991; Winnard et al., 1993; Mirabella et al., 1998). Here, we show that in the *mdx* mouse, a variety of exon skipping events have created revertant dystrophins which bypass the original nonsense mutation. Exon mapping indicated that the most common RFs arise from the skipping of 10 or more exons, most frequently from around exons 20 and 21 to around exon 32 (17%) or exon 46 (25%). We subsequently detected the predicted alternatively processed mRNA where exon 18 was joined to exon 35, and 13 joined to exon 48. Although it is not possible to establish a causal link between any alternatively processed mRNA and a particular RF, the exon mapping

result correlates well with some of the detected transcripts.

Despite considerable variation in the extent of exon skipping between individual RFs, all revertant dystrophins contained the intact NH₂-terminal and cysteine-rich domains, resulting in the reassembly of DAP complexes at the sarcolemmal membrane. Interestingly, the most commonly encountered (25%) revertant dystrophin proteins correspond to a massive human deletion that produced only a mild Becker muscular dystrophy phenotype (Mirabella et al., 1998).

More direct evidence for function of the revertant dystrophins is the increase in size of RF clusters with increasing age. Muscle repair in the *mdx* mouse is accomplished by proliferation of myoblasts and fusion of these cells into damaged segments of muscle fibers (Blaveri et al., 1999). However, since dystrophin is not expressed in myogenic cells, it can confer no advantage to them. This implies that the increase in size of revertant clusters arises only by
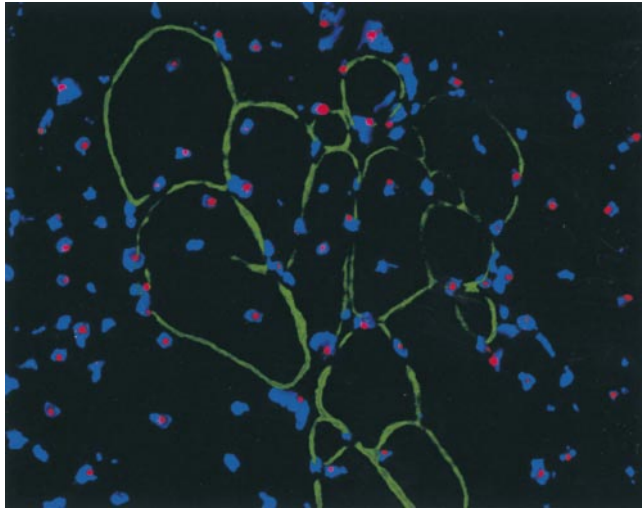
Figure 7. Combined immunohistochemistry and in situ hybridization for detection of dystrophin protein and genomic sequence of intron 21 to exon 25. Dystrophin protein in RFs is stained with immunoperoxidase method and the positive staining is shown as false green color. Hybridization signals for the genomic region of dystrophin sequences are shown as false red color. Nuclei are counterstained with 4,6-diamidino-2-phenylindole (DAPI), showing as blue. Sections are first stained with antibody p6 for dystrophin on unfixed sections followed by in situ hybridization.

preferential survival of muscle fiber segments which express revertant dystrophin.

Mechanisms Involved in the Restoration of Dystrophin Expression in RF

Since their discovery in dystrophic man and mouse, the nature of revertant dystrophins in otherwise dystrophin-negative muscle has been an abiding mystery. Elucidation of the underlying mechanisms responsible for the production of revertant dystrophin has been severely hampered by the combination of their low frequency, the multinuclear nature of the muscle fiber, and sheer size of the dystrophin gene (Burrow et al., 1991; Klein et al., 1992; Fanin et al., 1995). Based on limited immunohistochemical analysis and results from other systems, several theories have been proposed, including somatic suppression, use of developmentally regulated alternative promoters, alternative splicing, and somatic mutations (Hoffman et al., 1990; Nicholson et al., 1993; Fanin et al., 1995; Winnard et al., 1995; Darling et al., 1999). Our demonstration that almost all revertant dystrophins in muscles of *mdx* mice lack partial sequences around exon 23 argues strongly against somatic suppression as a cause of the revertant phenotype as this mechanism would produce dystrophin of full length. Similarly, all revertant dystrophins contained an intact NH_2-domain, showing that alternatively regulated known promoters downstream of mutated exon 23 have not been used. This leaves somatic mutations and alternative splicing as the most plausible mechanisms for the generation of in-frame transcripts from which shortened revertant dystrophins are translated.

Is Revertant Dystrophin a Result of Secondary Deletional Mutation?

The dystrophin gene appears to be prone to deletional mutation, which accounts for up to 80% of BMD and 64% of DMD cases. Deletions involving as many as 30 exons, although extremely rare, have been reported (Chelly et al., 1990; Love et al., 1991; Winnard et al., 1993). Clearly then, deletional mutation must be considered as a potential cause of the dystrophin expression in RF (Hoffman et al., 1990; Fanin et al., 1995; Winnard et al., 1995; Thanh et al., 1995). This would be consistent with the feature of clonal growth and expansion of revertant clusters. The frequency of RF and the rate of mutation in the human dystrophin gene appear to be in approximate agreement too. Our best estimate of the incidence of revertant foci comes from serial sections spanning a 1-mm length of the belly portion of TE muscles of new born *mdx* mice. Some 40–50 short (10–20 μm) segments of single RF were identified in such samples, each involving only 1 or 2 myofiber nuclei, and presumably representing the differentiated product of a single revertant myogenic cell lying nearby. The muscle cross section comprises some 3,000–4,000 fibers, each containing ~100 nuclei/mm, giving an incidence of the reversion event of $1:10^3$–$1:10^4$, which is slightly higher than the frequency of new mutations in the DMD locus (Amalfitano et al., 1997) during gametogenesis but not wildly disparate.

However, it is difficult to square the pattern of RF with a standard model of deletional mutation. Mutations are generally proliferation-related random events, and therefore would most likely occur during the many divisions of prenatal muscle development. In this instance, one might expect to find big clusters of RFs in new born or young mice as a result of prenatal clonal expansion. However, this has not been observed in our survey of a large number of mice younger than 6 wk.

Empirically, our failure to detect loss of corresponding genomic sequences in the myonuclei of RFs by a combination of immunohistochemistry and in situ hybridization is strong evidence against an explanation based on genomic deletion. The detection rate with the probe of ~80% in the nuclei of both revertant and nonrevertant muscle fibers is a common finding with probes to punctate markers on sectioned material and implies that 100% of nuclei, or very close to it, actually carry this region of the dystrophin gene (Partridge et al., 1992). Thus, the majority, at least, of nuclei in RF are not clonally derived from a myoblast with large genomic deletion. One might conjecture that an undetectably small proportion of nuclei carrying deletions of the gene could give rise to revertant dystrophin that become dispersed over a large territory within the fibers. However, the expansion of the myonuclear domain and dispersion of dystrophin within a fiber are limited as discussed in a review by Allen et al. (1999) and shown in *mdx* muscle fibers made mosaic by transplantation of normal myoblast (Blaveri et al., 1999). We would expect to detect a significant proportion of myonuclei with a genomic deletion if this mechanism is responsible for producing the near-normal amount of revertant dystrophin over large territories in the majority of RFs. However, it should be pointed out that mutations such as deletional mutations or

point mutations affecting splicing may well be involved in the production of revertant dystrophins, although such events may be rare.

Is RNA Splicing Likely To Be the Principal Mechanism?

Regulated alternative splicing of many genes is a feature of muscle differentiation (Reed and Maniatis 1985; Strehler et al., 1985; Breitbart and Nadal-Ginard 1987; Wieczorek et al., 1988; Smith et al., 1989), and has also been demonstrated in the expression of dystrophin, with exon 9 frequently being skipped from the mature mRNA in nonmuscle tissues (Reiss and Rininsland, 1994). At least three distinct COOH-terminal sites, exons 68, 71–74, and 78, show differential splicing (Feener et al., 1989; Bies et al., 1992). To get direct evidence for the involvement of RNA splicing in exon skipping of revertant dystrophin would require isolation of a cultured cell line that consistently expresses revertant dystrophin. This has not proven possible so far either in our laboratory or in another laboratory where it has been attempted (Gussoni, E., personal communication). Nonetheless, there is circumstantial support for this mechanism. Firstly, aberrant splicing is the simplest explanation of the finding of several transcripts with variable numbers of skipped exons in muscles of both the normal and the *mdx* mouse demonstrated here and previously (Wilton et al., 1997a). Secondly, although atypical mutations involving two noncontiguous regions in the same dystrophin gene have been reported (Zatz et al., 1998), the probability of an in-frame transcript created by two independent mutations should be <0.01% when the rate of single mutation is 1:10^3. It is therefore highly unlikely that the high frequency (at least 10%) of two noncontiguous exon skips could arise without involvement of alteration in RNA splicing. Thirdly, exon 77 at dystrophin COOH-terminal, which is often differentially spliced in normal muscle (Feener et al., 1989), is missing in a proportion of RFs, suggesting that alternative splicing of the gene is operating in the RFs. In fact, this is the first demonstration of a dystrophin protein lacking exon 77, probably because the clonal nature of RF clusters makes it detectable against a dystrophin-free background.

Lastly, the fact that RFs in new born mice are always single and of limited length, rather than in clusters, suggests that the revertant event is triggered at the stage of myotube formation. Thus, the onset of the revertant phenotype corresponds well to that of muscle differentiation at the time that imprinting of splicing machinery appears to take place and regulated alternative splicing becomes established. Such a splicing mechanism is consistent with the presence of genomic regions that are skipped in the protein of the revertant dystrophins.

Against the idea of splicing as the mechanism behind production of revertant dystrophin is the apparent clonal nature of expansion of RF clusters. To explain this in terms of splicing, one would have to postulate a mechanism by which each of at least 20 variants of splicing pattern generated by random events could establish itself as a predominant, efficient process and spread within a fiber and to adjacent fibers. Although such mechanisms have not been demonstrated, some observations suggest that

they may well exist in muscle. Thus, in *Drosophila* muscle development, one particular myogenic cell can behave as a founder to shape that character of the fiber by setting the pattern of gene expression of the myonuclei within the fiber (Rushton et al., 1995; Baylies et al., 1998). Secondly, the skeletal muscles are known to be versatile for conversion between different fiber types under conditions such as stress and electric stimulation. This process is associated with the use of alternative splicing in genes such as myosins and TnT genes (Kelly and Rubinstein, 1994). By extrapolation, one might conjecture that a particular set of microenvironments might shape the pattern of gene expression by changing splicing pattern as well as switching on/off different genes (Allen et al., 1999). The age-related clonal expansion of RF in *mdx* mice suggests that one such microenvironmental factor might be the process of local muscle degeneration and regeneration. This is supported by our unpublished observation that revertant dystrophins could not be detected by immunohistochemistry in muscle of normal mice, despite the presence of dystrophin mRNA with a variety of exon skipping, though less abundant than in *mdx* muscle (Wilton et al., 1997a). Also consistent with this notion is RFs in utrophin transgenic *mdx* mice. The expression of utrophin greatly diminishes muscle degeneration (Tinsley et al., 1998), and the size and number of revertant clusters are much smaller in these mice than those in *mdx* mice (unpublished observation). Thus, an aberrant splicing pattern that produces a functional product might be extended by selection as a predominant form to other domains of the fiber and adjacent fibers in the process of muscle degeneration and regeneration, but not in the normal muscles. This process would be enhanced if the functional product is able to protect the fiber from degeneration. The expansion of revertant dystrophin to a group of fibers may also be accelerated by sharing a common cytoplasmic environment through fiber branching. The growth in size of revertant cluster appears to be correlated well with the extent of fiber branching, being most extensive in the older *mdx* mice (Bockhold et al., 1998). The idea that functional selection (imprinting) preserves the pattern of random splicing certainly needs to be critically examined.

Clinical Significance

Understanding the exon composition of functional revertant dystrophins is of clinical significance even though the underlying mechanism is not clear. The lack of at least a few exons in revertant dystrophins raises a question that restoration of full-length dystrophin by gene therapy could induce problems of immunogenicity. Therefore, it may be important to characterize the commonly encountered revertant isoforms so that a panel of functional dystrophin transgenes can be designed for individual patients such as to avoid immune rejection. We are also exploring the possibility of inducing specific exon skipping in the dystrophin gene by using 2-0 methyl antisense oligonucleotides directed at exon–intron junctions. Redirecting the splicing process has allowed the skipping of exon 23 from the mdx dystrophin mRNA (Wilton et al., 1999). However, this shortened form of transcript only occurred at low levels, with no demonstration of its protein and function. It may therefore be necessary to target multiple exon–intron

junctions to induce multiple exon splicing as observed in revertant dystrophins.

A Model for Study of Functional Reversion of Nonsense Mutations

Reports of evasion of nonsense mutations in other systems are mainly based on the demonstration of RT-PCR–elicited transcripts lacking the mutated exon and simply explained by RNA splicing (Dietz et al., 1993; Fisher et al., 1993; Gibson et al., 1993; Naylor et al., 1993; Endo et al., 1995; Lin et al., 1998). However, such PCR-based investigations give no information on the spatial distribution of the molecular events that generate these transcripts. In the *mdx* and normal mouse, some readily demonstrable shortened in-frame transcripts (Wilton et al., 1997a) did not appear relevant to the protein products we found in RFs. Conversely, transcripts responsible for expression of functional dystrophin in RF, but produced by only a limited number of cells, are likely to be concealed in a background rich in various transcripts. In this light, no functional significance can be attributed to in-frame transcripts generated by RT-PCR in the absence of protein products.

The nature of revertant dystrophin is unprecedented for its sheer scale and complexity of exon skipping and its clonal growth. This combination of properties presents the conundrum of not being fully reconcilable with current models of the mechanisms of gene transcription and translation and provides a unique model for elucidating the complexity of exon skipping. This is attributable partly to the large size of the dystrophin gene and the fact that a high proportion of exons is bounded by complete codons, a combination that gives a high probability of in-frame transcripts from random exon skipping. The resulting functional protein can localize to the membranes and protect the muscle fiber segments in which they occur, tending to amplify their signal, highlighting them as rare events in the null-background of dystrophic muscle. Few other genes possess so extensive a range of properties that conspire to make their aberrant products conspicuous. The dystrophin gene in the *mdx* mouse thus can be seen as an especially favored system for detecting and studying such events, but there is no special reason to think that the mechanism underlying RFs is restricted to the dystrophin gene.

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