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Prevention of dystrophic pathology in *mdx* mice by a truncated dystrophin isoform

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The C-terminal domain of dystrophin is alternatively spliced to produce a variety of tissue and developmental stage-specific isoforms. Recent studies suggest that the C-terminal domain binds to the dystrophin-associated glycoprotein complex (DGC) in muscle, but little is known about the functional significance of the alternative splicing or what role individual isoforms may play in specific tissues. The major dystrophin transcript in brain lacks exons 71 – 74, and encodes an isoform not observed in skeletal muscle. To explore the capacity of this truncated isoform to function in muscle, we have generated transgenic mice expressing a murine dystrophin mini-gene missing exons 71 – 74. Uniform expression of this construct on a mutant *mdx* mouse background results in normal muscle morphology and physiology, and prevents the development of muscular dystrophy. These mice also display normal expression and localization of the DGC, suggesting that the alternatively spliced exons are not required for dystrophin function in skeletal muscle. An additional line of mice was analyzed that had a mosaic pattern of expression. These mice display a markedly milder phenotype than *mdx* mice, despite the expression of dystrophin in only half the muscle fibers. These results indicate that viral delivery of dystrophin to a simple majority of fibers in a muscle group would greatly reduce the dystrophic pathology associated with Duchenne muscular dystrophy.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting 1 in 3500 newborn males (1). DMD is characterized by progressive muscle weakness associated with necrosis of muscle fibers and fibrosis of muscle tissue. Patients are usually wheelchair bound by age 8 – 11 years and generally die of respiratory failure by their early twenties. DMD and the less severe Becker muscular dystrophy (BMD) are caused by defects in the dystrophin gene, a complex locus at Xp21 that spans 2.4 Mb and contains at least 83 exons including five promoters (see Ahn and Kunkel in reference 2 for review). Mutations in the murine dystrophin gene have been found in several strains of *mdx* mice, which are animal models for DMD (3 – 5). The dystrophin gene encodes multiple tissue-specific isoforms that are expressed in a variety of human and mouse tissues. The major muscle isoforms of dystrophin are encoded on an approximately 14 kb mRNA that displays differential splicing patterns (6,7). C-terminal isoforms are also generated by the use of alternative promoters located upstream of exons 56 and 63 (2). Dystrophin is localized to the sarcolemmal membrane and is composed of four distinct structural domains (2). The amino-terminal domain displays sequence similarity with a family of F-actin-binding proteins including α-actinin and β-spectrin. This domain is followed by a series of 24 spectrin-like repeats each with an average size of 109 amino acids, that is hypothesized to form a triple-helical coiled coil interrupted by four hinge regions. The C-terminal portion of dystrophin contains a cysteine-rich domain followed by a highly conserved region that is homologous with the dystrophin-related protein (DRP), which is localized in skeletal muscle at the neuromuscular junctions (8,9).

Five exons located in the C-terminal domain of dystrophin display a complex pattern of alternative splicing (6,7). Exon 78, the penultimate exon, is present in adult skeletal muscle transcripts, but is spliced from the mature mRNA in fetal tissues and some adult tissues which use the non-muscle promoters of the dystrophin gene. Exons 71 through 74 are also alternatively spliced to generate multiple tissue-specific isoforms (6,7). These four exons, which span a 330 bp region of the mRNA, are absent from an isoform of dystrophin normally present in brain tissue (6). The dystrophin mRNAs produced in mouse skeletal muscle either contain all four of those exons or are missing the 159 bp exon 74 (7). The function of these alternatively spliced exons is unknown, but several hypotheses exist. This region is a candidate for direct interaction with, or regulation of, one or more of the dystrophin-associated proteins (DAPs). The dystrophin glycoprotein complex (DGC) consists of at least six proteins and glycoproteins that link dystrophin to the sarcolemma (10). These

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proteins are present at greatly reduced levels in DMD and *mdx* muscle, and are thought to be stabilized by an interaction with dystrophin. Consequently, different isoforms of dystrophin could interact with different isoforms of the DAPs. It is also possible that the alternative splicing is important primarily for the non-muscle dystrophin transcripts produced in many mouse and human tissues, allowing these truncated proteins, whose functions are unknown, to act in a different cellular environment than that found in skeletal muscle. Another possibility is that the alternatively spliced isoforms found in skeletal muscle are artifacts of developmental regulation or evolution and do not function differently from the full length protein.

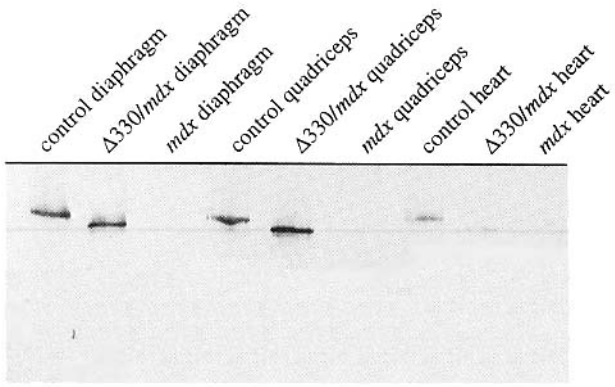

Figure 1. Immunoblot analysis of Δ330 transgenic *mdx* mice. An affinity-purified antibody raised against the C-terminus of dystrophin detects the 427 kDa protein in control (C57B1/10) diaphragm, quadriceps and heart protein extracts, and the 415 kDa protein expressed from the transgene in similar extracts from Δ330/ *mdx* mice. No dystrophin is detected in extracts from *mdx* mice. 150 μg of total protein were analyzed for each sample.

We have previously demonstrated that high level expression in muscle of a full-length copy of the dystrophin cDNA prevented the development of dystrophic symptoms in *mdx* mice (11). To test the functional role of exons 71 through 74 in skeletal muscle, we generated transgenic-*mdx* mice expressing a dystrophin construct deleted for these exons (Δ330). One transgenic line expressed the Δ330 isoform in diaphragm muscle at levels similar to that of control mice, and expressed the transgene at two to five times control levels in quadriceps muscle. Two other lines of transgenic- *mdx* mice were analyzed that displayed a mosaic expression pattern of the Δ330 transgene in quadriceps. Our results indicate that the complete C-terminal domain of dystrophin is not required for prevention of dystrophic changes in skeletal muscle and that viral delivery protocols that result in transduction of less than 100% of the target muscle fibers could still provide an ameliorative effect on the dystrophic phenotype.

RESULTS

Prevention of the *mdx* phenotype by uniform expression of the Δ330 dystrophin isoform

To study the function in skeletal muscle of the alternatively spliced exons in the C-terminus of dystrophin, transgenic mice were

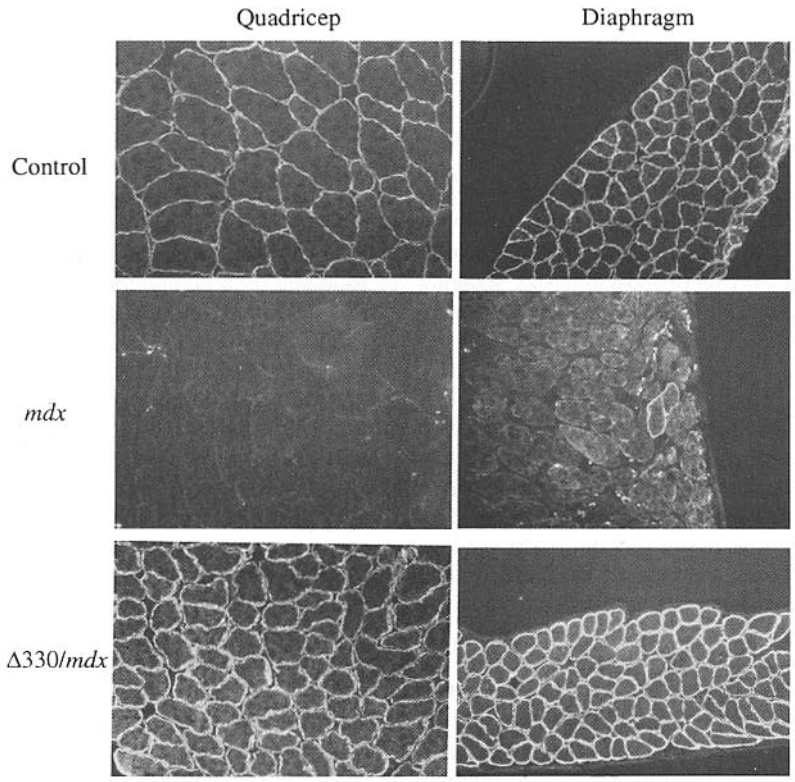

Figure 2. Immunostaining of 3 month old control (C57B1/10), *mdx*, and Δ330/ *mdx* quadriceps and diaphragm unfixed-frozen 7 /μm sections. Staining with antisera against the dystrophin N-terminus shows the presence of the Δ330 isoform on the sarcolemma. Note the presence of two dystrophin-positive 'revertant' fibers in *mdx* diaphragm. All photographs were taken at the same exposure and magnification.

generated that express a murine dystrophin cDNA construct missing exons 71 through 74. Male F_0 mice positive for the transgene were used to generate lines by breeding them on to an *mdx* genetic background so that expression of the transgene-encoded protein could be analyzed in the absence of endogenous dystrophin (Fig. 1). Line 330-66 was observed by Western analysis to express the transgene-encoded dystrophin isoform in quadriceps muscle at two to five times the level at which the wild-type gene is expressed in C57B1/10 control mice. Expression of the transgene in diaphragm was similar to the dystrophin levels in diaphragm muscles of control mice. This truncated isoform is approximately 12.1 kDa smaller than the full-length protein and can be resolved from endogenous dystrophin on 6% SDS-PAGE gels (Fig. 1).

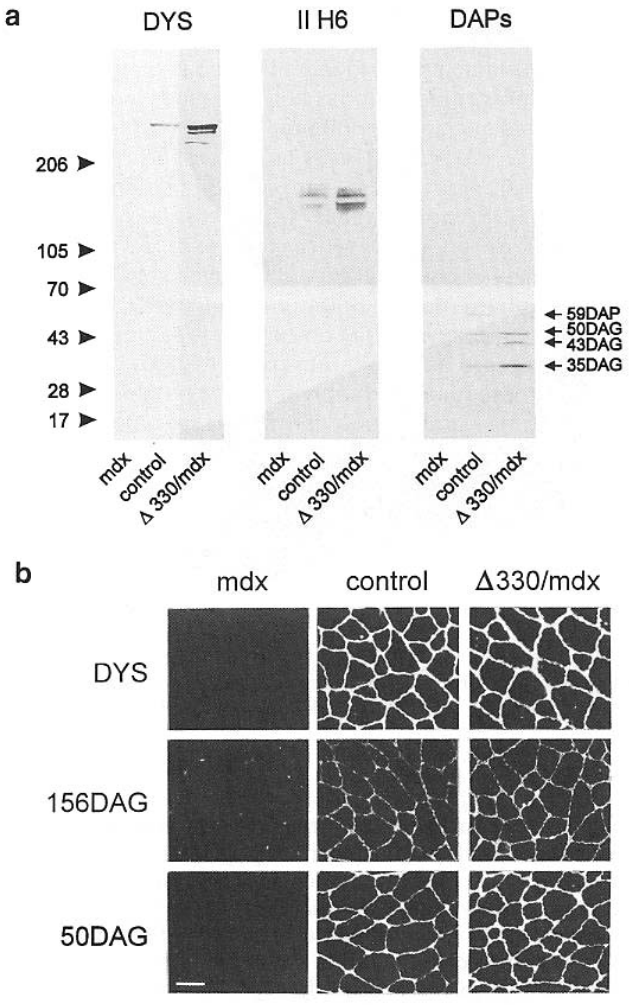

Figure 3. (a) Immunoblot analysis of dystrophin (DYS) and the dystrophin-associated proteins (DAPs) in skeletal muscles of control, *mdx*, and Δ330/*mdx* uniformly expressing transgenic mice. A monoclonal antibody to α-dystroglycan, II H6, detects two to five times more of the 156 DAG in protein extract from Δ330/*mdx* mouse skeletal muscle membranes than in control skeletal muscle membranes. A cocktail of anti-DAPs antibodies shows the presence of all of the DAPs in Δ330/*mdx* skeletal muscle at levels similar to, or slightly higher than those in control muscle, **(b)** Immunostaining of skeletal muscle sections with antibodies against dystrophin, 156DAG, and 50DAG. This staining shows the presence of the 156DAG and 50DAG in skeletal muscle from the Δ330/*mdx* transgenic mice. Bar indicates 50 μm.

To determine the effect of the Δ330 deletion on dystrophin localization, Δ330/*mdx* transgenic mice were analyzed by immunohistochemistry. Diaphragm and quadriceps unfixed frozen sections from these mice were stained with an affinity-purified polyclonal antibody specific for the amino-terminus of dystrophin. As in C57B1/10 control animals, the Δ330 isoform was observed in all muscle fibers and was localized exclusively to the sarcolemmal membrane (Fig. 2). This result indicates that the 110 amino acids absent from this dystrophin isoform are not necessary for localization of dystrophin to the sarcolemma.

The DGC consists of integral and peripheral membrane proteins and glycoproteins which together link dystrophin to the extracellular matrix. In order to test whether the Δ330 isoform is able to form a complex with the DAPs and stabilize these proteins, Δ330/*mdx* mice were analyzed with antisera against the DAPs. A monoclonal antibody to a-dystroglycan, the 156 kDa dystrophin-associated glycoprotein, and a mixture of antibodies to the DAPs were used for Western analysis of microsomal proteins isolated from control, mdx, and Δ330/*mdx* transgenic mice (Fig. 3a; 12). In addition, antibodies against α-dystroglycan, adhalin (50DAG), and the 59DAP were used for immunofluorescence analysis of control, mdx and Δ330/*mdx* quadriceps sections (Fig. 3b and data not shown). The Δ330 transgene-encoded dystrophin was found to restore the DAPs to levels equal to or slightly above those found in control animals, suggesting that the 110 amino acids encoded by these alternatively spliced exons are not required for formation of the DGC in skeletal muscle (Fig. 3).

While these results indicate that the DAPs are stabilized by the presence of the truncated dystrophin isoform, they do not indicate whether the complex formed is fully functional. Since both dystrophin and the DAPs are virtually absent from DMD muscle, and since absence of adhalin, the 50 kDa DAG by itself leads to an autosomal recessive muscular dystrophy with DMD-like symptoms (13), prevention of muscular dystrophy would likely require a functional interaction between dystrophin and the DAPs. To explore the effectiveness of the DGC formed in the Δ330/*mdx* transgenic mice, we analyzed the morphology of muscles from these mice. The absence of dystrophin in *mdx* mice leads to necrosis of skeletal muscle tissue and replacement of muscle cells with connective and adipose tissue (14,15). However, analysis of diaphragm and quadriceps muscle histological sections from Δ330/*mdx* transgenic mice revealed a normal morphology that was indistinguishable from C57B1/10 control animals (Fig. 4). These results indicate that the Δ330 truncated dystrophin isoform prevents development of morphological abnormalities associated with a defective dystrophin—DGC interaction.

We have demonstrated previously that diaphragm muscles from mdx mice display a significant functional deficit manifested by dramatic reductions in both the force and power capacities of the muscles (11). A fully functional interaction between dystrophin and the DAPs should prevent the development of force deficits, so we measured the maximum isometric force produced by the diaphragms of the Δ330/*mdx* mice. Four-month-old wild-type C57B1/10 mice generate a force of 222 kN/m^2, whereas *mdx* mice only produce a maximum force of 117 kN/m^2 (Fig. 5). The Δ330/*mdx* mice that were uniformly expressing the Δ330 transgene generate a specific force of 211 kN/m^2, which is not

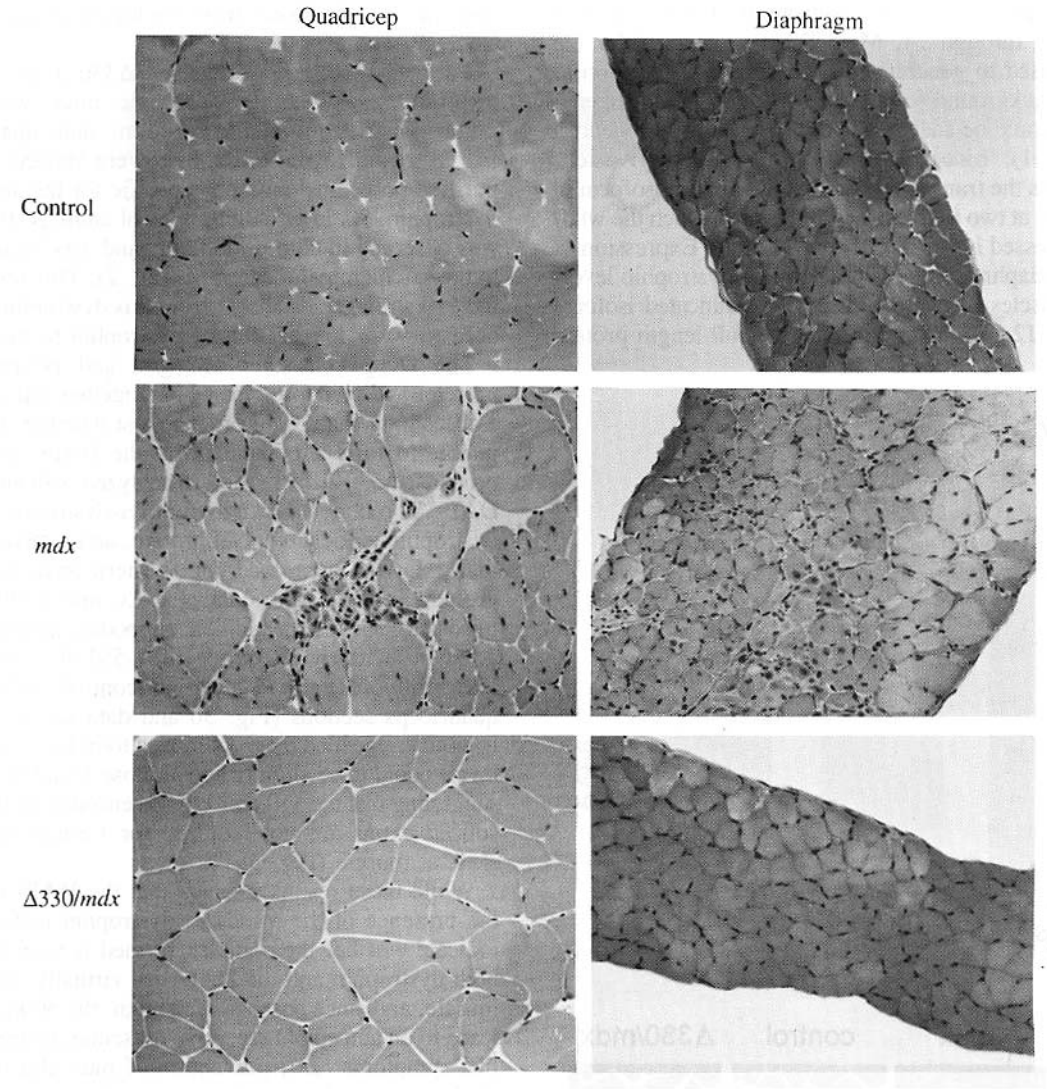

Figure 4. Morphology of quadriceps and diaphragm sections from control, *mdx*, and Δ330/*mdx* mice. Haematoxylin and eosin staining of 4 μm sections fixed in Kamovsky's fixative (2% glutaraldehyde, 2% formaldehyde) show the absence of dystrophic pathology in uniformly expressing transgenic mice. All photographs were taken at the same exposure and magnification.

significantly different from the values for the control animals (Fig. 5). These data demonstrate that this truncated isoform of dystrophin restores the integrity of the sarcolemma and permits maintenance of control values for force.

Analysis of mosaic expression of the Δ330 transgene

Two additional lines of transgenic mice from independent founder animals were analyzed by immunohistochemistry and found to express varying amounts of dystrophin on a fiber by fiber basis (Fig. 6). These lines were observed by Western analysis to have overall levels of transgene expression comparable with that produced by control animals (data not shown). However, individual fibers produced levels of dystrophin that varied between undetectable levels to levels greater than that in control mice. This phenotype was observed in multiple generations of animals and was therefore not due to a mosaic retention of the transgene in somatic cells. Fixed frozen sections of quadriceps muscles from animals ranging in age from 5.5 weeks to 4 months were stained with an affinity-purified polyclonal antibody specific for the amino-terminus of dystrophin. These same sections were counterstained with the DNA stain DAPI to assay for the presence of central nuclei which are indicative of muscle fiber regeneration and are extremely abundant in mdx quadriceps muscle. There were no large patches of either dystrophin-positive or dystrophin-negative fibers observed in any of these transgenic animals regardless of age, and there appeared to be no correlation between dystrophin expression in a fiber and the presence of central nuclei (Fig. 6). Nine hundred fibers were scored for the presence of central nuclei which were found to be present in 23% of dystrophin expressing fibers and 32% of dystrophin non-expressing fibers, and many non-expressing fibers appeared morphologically to be quite normal (Fig. 6 and data not shown). About 50% of the muscle fibers appeared to be expressing detectable amounts of dystrophin from the transgene and there

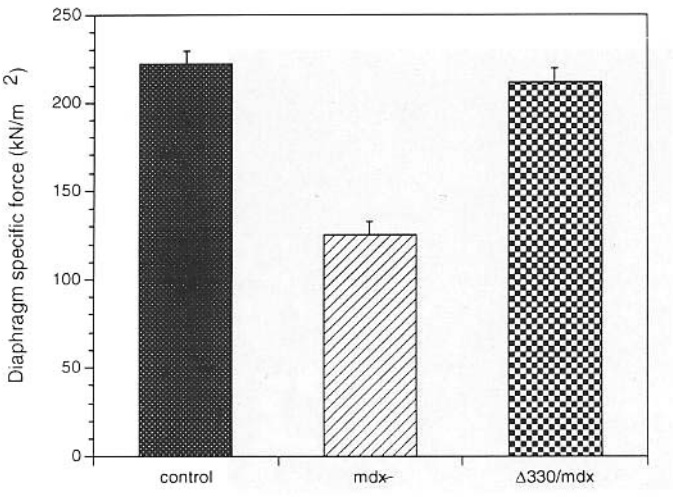

Figure 5. Measurements of diaphragm specific force in 4 month old control, *mdx*, and Δ330/*mdx* mice. The forces generated by diaphragm muscles from C57B1/10, *mdx*, and Δ330/*mdx* mice were 222 kN/m^2 (SD 8.8), 117 kN/m^2 (SD 9.2), and 211 kN/m^2 (SD 7). The specific force developed by the diaphragm muscles of *mdx* was 53% of the values for diaphragm muscles of control 4 month old mice. The loss in force development is completely prevented by uniform expression of the Δ330 transgene.

was no increase in this percentage over age, suggesting that dystrophin-negative fibers were not being systematically replaced with dystrophin-positive fibers over the time period analyzed.

Histological sections of quadriceps from mosaic transgenic mice were observed to contain less fibrosis and more patches of healthy fibers than similar sections from age-matched *mdx* mice (Fig. 7). Quadriceps sections from 3 month old *mdx* mice contain extensive regions of muscle deterioration, and this was never observed in the mice expressing dystrophin in approximately one-half of muscle fibers. Similarly, although there are very few fibers in *mdx* quadriceps cross-sections from 3 month old mice that do not appear to contain central nuclei, there are many fibers that do not appear to have undergone a cycle of degeneration and regeneration in the transgenic mice with mosaic expression.

Diaphragm sections from selected mosaic animals were also analyzed by immunohistochemistry but were observed to express dystrophin from the transgene in only 10–20% of muscle fibers. The morphology of diaphragm muscle from these mice appeared indistinguishable from *mdx* mice, and therefore further analysis was not pursued.

DISCUSSION

We have analyzed several lines of transgenic mice that display either uniform or mosaic expression of a dystrophin mini-gene construct deleted for exons 71 through 74. These exons are alternatively spliced to generate tissue- and developmental-stage-specific dystrophin isoforms in humans and mice, but little information is available as to the role of alternative splicing or the functional significance, if any, of these differentially utilized protein coding regions. Our data have shown that this region is not critical for the function of dystrophin in skeletal muscle. Uniform expression of the Δ330 transgene was observed to prevent completely the development of dystrophic symptoms in *mdx* mouse muscle. The Δ330 dystrophin protein displayed proper localization to the sarcolemma and led to DAP expression and localization indistinguishable from that in control mice. These results demonstrate that either: (i) the dystrophin domain encoded by exons 71–74 is not directly involved in binding to the DAPs, or (ii) that a functional DGC can be assembled even without the binding of one or more DAP to this domain. Histological sections of muscle from these transgenic animals displayed normal morphology and diaphragm force was indistinguishable from that in control mice.

A variety of studies implicate the C-terminal region as a critical functional domain of dystrophin. Patients with frameshifting mutations that disrupt expression of the C-terminus almost invariably have a DMD, rather than a milder BMD, phenotype (16,17). The major exception to this correlation is patient C.M., whose large genomic deletion was associated with a mild form of muscular dystrophy together with mental retardation, glycerol kinase deficiency, and congenital adrenal hypoplasia (18). While the precise 5' deletion breakpoint in that patient's dystrophin gene has not been determined, it maps in close proximity to the upstream boundary of the alternatively spliced domain deleted from the mini-gene used in this study (reference 18 and J.S.C., unpublished observations), suggesting that sequences deleted in this patient may be less important for normal dystrophin function than upstream C-terminal sequences. Suzuki *et al.* (19) have presented biochemical evidence that the C-terminus of dystrophin binds directly to the DAPs. Since the DGC is destabilized in *mdx* mice, yet appears completely stable in Δ330/*mdx* mice, any association normally occurring between the DAPs and the deleted region of dystrophin is apparently not critical for stabilization of the complex. Together these results indicate that the amino acids encoded on exons 71–74 are less critical for dystrophin function than C-terminal sequences located further 5'.

While the Δ330 isoform of dystrophin has not been observed in adult skeletal muscles, our results indicate that it is capable of normal function in these tissues (7). The only alternatively spliced forms observed in adult skeletal muscle are a Δ159 isoform, a Δ105 isoform, and a A66 isoform observed in mouse, human, and chicken tissues, respectively (6,7,20). These arise from the skipping of exons 74 (Δ159), exons 71–72 (Δ105), and exons 72 (Δ66) (6,7,20). As these exons can be eliminated from the dystrophin transcript without obvious effect on muscle morphology or physiology (Figs 3 -5), it is not clear what role, if any, these truncated isoforms may play in skeletal muscle. Alternative splicing in muscle may simply reflect conservation of splicing machinery for a function which no longer exists, or these isoforms may only be expressed from a small subset of nuclei in skeletal muscle and may play a subtle role that is not yet apparent. In contrast, alternative splicing appears to be far more prevalent, and could be more functionally relevant, in tissues other than skeletal muscle. The C-terminus of dystrophin is expressed from internal promoters within the gene, and these truncated isoforms are likely to play a role distinct from that of the full-length protein (2). The C-terminal isoforms are also alternatively spliced (J.A.R. and J.S.C., unpublished observations), and such splicing may be important either for interaction with DAPs in non-muscle tissues, or with proteins distinct from the DAPs found in muscle (21). Since no functional roles have been described for dystrophin in non-muscle tissues, it will be difficult to test the importance of alternative splicing in such tissues.

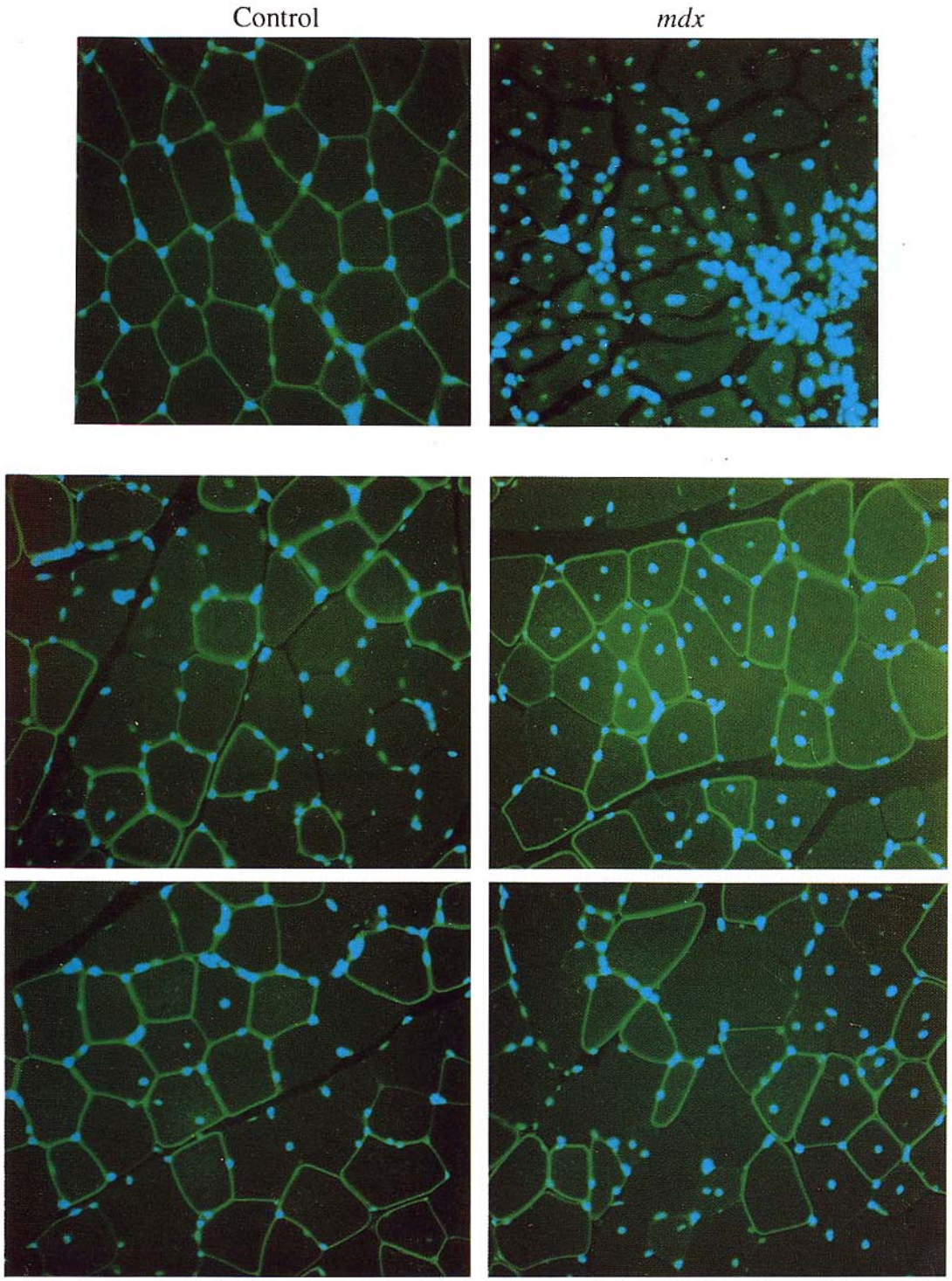

Figure 6. Immunostaining of quadriceps muscle sections from 3 month old Δ330/*mdx* mice that display a mosaic pattern of expression. 7 μm formaldehyde-fixed sections were stained with antisera against the dystrophin N-terminus (green) and counterstained with DAPI (blue). Expression of the transgene can be detected in approximately 50% of quadriceps muscle fibers. Note that there is no correlation between dystrophin expression and presence of central nuclei and that regions with similar amounts of dystrophin positive fibers have varied percentages of central nuclei. All photographs were taken at the same exposure and magnification.

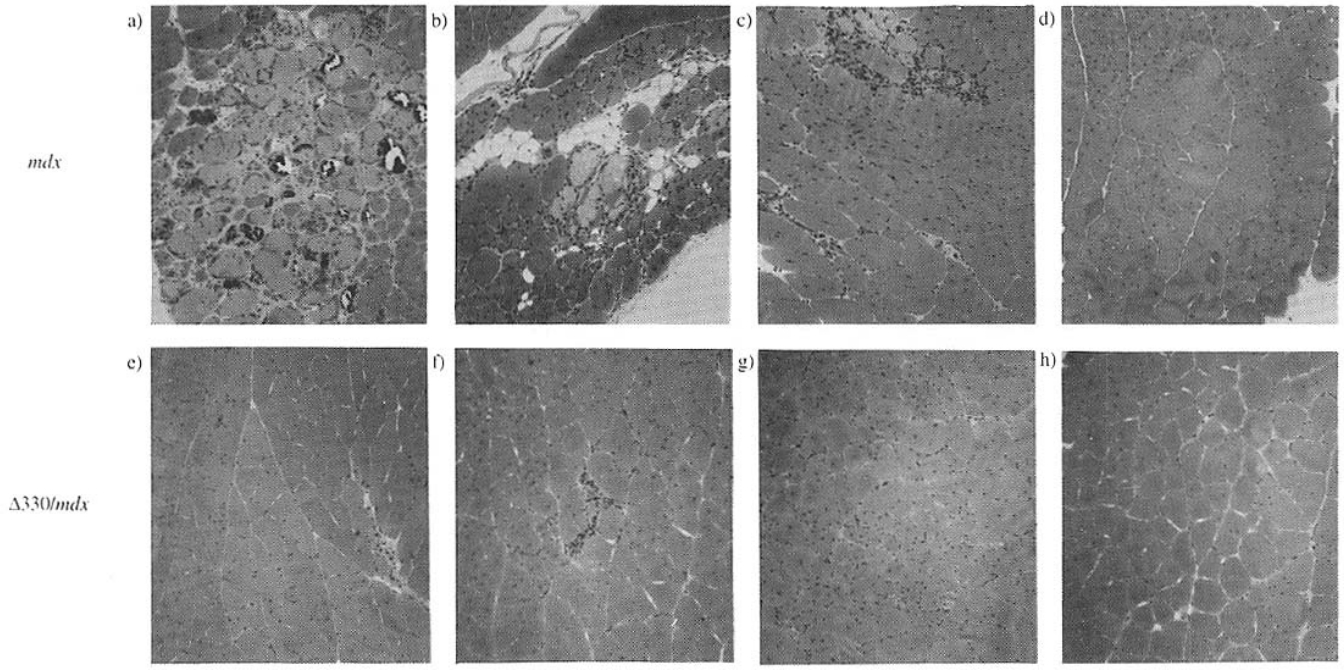

Figure 7. Panels **(a)** and **(b)** display two regions from mdx quadriceps muscle with a moderate to severe dystrophic pathology; these represent the most dystrophic regions typically observed in mdx quadriceps, and reveals a more extensive fibrosis than was observed in Δ330/*mdx* transgenic mice. Panels **(e)** and **(f)** show examples of the most extensive pathology observed in A330/*mdx* mice which display a mosaic pattern of expression. Panels **(c)** and **(d)** show examples of quadriceps muscle sections from mdx mice that display minimal dystrophic alterations, whereas panels **(g)** and **(h)** show sections from Δ330/*mdx* mice with the least amount of morphological abnormalities. In all cases pathological alterations observed in the muscles of mdx mice were more extensive than in those observed in muscles of Δ330/*mdx* mice which express dystrophin in approximately 50% of the fibers.

A requirement for delivery of multiple dystrophin isoforms to muscle would greatly complicate efforts to develop gene therapy for DMD. We have demonstrated in this and an earlier study that expression of a single isoform of dystrophin in skeletal muscle prevents the development of muscular dystrophy in *mdx* mice (11). Viral vectors, such as adenovirus, currently being considered for gene delivery have an upper cloning limit of approximately 8 kb (22). In order to make use of these vectors in a potential treatment for DMD, it may be necessary to design a truncated version of the 14 kb dystrophin cDNA. Development of a maximally functional clone will require an exhaustive structure/function analysis of the protein. Despite the highly conserved nature of the 330 bases deleted from the mini-gene analyzed in this study (only a single nucleotide change exists between the mouse and human mRNAs; 7), our results indicate that this region could be removed from a dystrophin mini-gene with minimal effect on the encoded protein.

An additional concern for gene therapy trials is the question of what percentage of muscle fibers need to be transduced to eliminate dystrophic symptoms. Transgenic mice that exhibit mosaic expression of a dystrophin mini-gene appear much healthier than age-matched *mdx* control animals (Fig. 7). While significant progress has been made recently both in developing truncated dystrophin mini-genes and in delivering them to muscle tissues using adenoviruses (23,24), the delivery efficiencies are still quite low. Although it is unclear why the transgene was differentially expressed between isogenic cells, these mosaic mice provide evidence that delivery of dystrophin to less than 100% of the fibers in a muscle group could still have a greatly beneficial effect on the dystrophic phenotype. Further studies that lead to functionally optimized mini-gene vectors as well as increased viral delivery efficiencies could lead to effective gene therapy protocols for DMD in the near future.

MATERIALS AND METHODS

Construction of transgene

The Δ330 deletion, missing bases 10424–10752 of the murine dystrophin cDNA sequence (GenBank accession no. M68859) was cloned from reverse transcription–PCR products of C57BL/10 brain RNA as described previously (7). An internal *Nru*I–*Sst*I fragment was removed from this clone and used to replace the corresponding fragment in a 4 kb *Bam*HI fragment containing the C-terminal region of dystrophin and the 3'-untranslated region. The 3' *Nru*I-*Not*I fragment was removed from this resulting clone and used to replace the corresponding fragment in the full length mouse dystrophin cDNA. The entire Δ330 cDNA was then excised from the vector with *Eag*I and used to replace the non-deleted dystrophin cDNA in pMDA (11), which contains 6.5 kb of mouse muscle creatine kinase (MCK) regulatory regions and an SV40 polyadenylarion site.

Generation of transgenic mice

Wild-type hybrid embryos from C57B1/6J x SJL/J were injected with the pMDA/Δ330 transgene as described (25). F_0 mice were screened by PCR with primers to the first intron of the MCK promoter construct and to the first exon of the mouse dystrophin cDNA. Positive males were mated with *mdx* females

to generate transgenic/*mdx* F_1 males. These males were mated with *mdx* females and the transgene-positive progeny were used for further analysis.

Western blot analysis

For the detection of dystrophin in Fig. 1, total protein was extracted from the diaphragms, hearts and quadriceps of C57BL/10, *mdx*, and transgenic-*mdx* mice, and the concentration of the proteins was determined using the Coomassie Plus Protein Assay Reagent (Pierce). One hundred and fifty micrograms of each protein sample was electrophoresed for 4.5 h on a 6% polyacrylamide – SDS gel, transferred on to Biotrace Nitrocellulose (Gelman Sciences) using the BioTrans Model B semi-dry blotting apparatus (Gelman Sciences), and hybridized with an affinity-purified polyclonal antibody raised against the C-terminus of dystrophin (G.Cox and J.S.C., submitted). The blot was then washed with Tris-buffered saline (TBS) pH 7.4 + 0.1% Tween-20 and hybridized with a goat antirabbit antibody (Pierce) conjugated to alkaline phosphatase. The blot was again washed and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue tetrazolium) (Sigma) was used for color development. Each lane of the blot shown in Fig. 3a contained 500 μg of total microsomes (12) from control, *mdx*, or Δ330/*mdx* transgenic mouse skeletal muscle, and this blot was stained with antidystrophin, anti-156 kDa dystrophin-associated glycoprotein (DAG) monoclonal antibody (II H6), or a cocktail of anti-dystrophin associated protein (DAP) antibodies (26–29).

Immunohistochemistry

Skeletal muscle was removed from mice of various ages, cut into strips, and either frozen slowly in Tissue-tek OCT media (Miles Inc.), or fixed in 3.7% formaldehyde for 3 h. Fixed sections were soaked in sucrose overnight, embedded in OCT, frozen, and sectioned with a cryostat. These sections were extracted with 0.5% Triton X-100 in Hartman's potassium phosphate-buffered saline (KPBS) for 5 min on ice and then rinsed. Seven micrometer cryosections from fixed and unfixed muscle were blocked with 1% gelatin in KPBS for 15 min, washed, and then incubated for either 2 h (unfixed) or overnight (fixed) with a 1/1300 dilution of an affinity-purified polyclonal antibody raised against the amino-terminus of mouse dystrophin (K.Corrado and J.S.C., manuscript in preparation). Sections were washed three times for 5 min and then incubated for 1 h with biotin-labeled goat antirabbit polyclonal antibodies (Pierce). Sections were again washed and incubated with FITC (fluorescein isothiocyanate) conjugated to streptavidin. After a final wash, Vectashield (Vector Laboratories Inc.) with or without DAPI (4,6-diamidino-2-phenylindole) was applied and sections were photographed through an FITC filter or a dual bandpass filter (Chromatek) under x50 magnification using a Nikon Optiphot-2 microscope. All washes and hybridizations were done in KPBS+0.2% gelatin and 0.1% Tween-20.

For the immunofluorescence analysis in Fig. 3b, 7 μm thick transverse cryosections were immunostained with dys-2 (a monoclonal antibody against C-terminal dystrophin) (Novacastra), affinity-purified anti-α-dystroglycan, or monoclonal antibody IVD31 specific for adhalin at a dilution of 1:200, 1:5, or 1:1, respectively (26–29).

Histology

Skeletal muscle was removed from animals of various ages, cut into strips and fixed in Kamovsky's fixative. Fixed samples were embedded in glycol methacrylate (GMA) and 4 μm sections of this block were cut and stained with hematoxylin and eosin and photographed with X25 (Fig. 4) or X50 (Fig. 7) magnification on a Nikon Optiphot-2 microscope.

Diaphragm force measurements

Diaphragm strips 1– 2 mm wide, including an adjacent section of a single rib and part of the central tendon, were cut from the central region of the lateral costal hemidiaphragm and immersed in an oxygenated bath containing mammalian Ringer solution (pH 7.4) at 25°C. Muscles were stimulated directly by an electric field produced by current pulses transmitted between two platinum electrodes placed longitudinally on either side of the muscle. Square-wave pulses 0.2 ms in duration were amplified to increase current intensity to a sufficient level to produce a maximum isometric response. Muscles were adjusted to optimum length (L_0) for the development of isometric force. Force was determined during maximum isometric tetanic contractions (30). The central tendon and rib bone were trimmed and the muscle was blotted and weighed immediately. The mean cross-sectional area of each diaphragm strip was determined by dividing muscle mass (kg) by the product of L_0 (m) and 1060 kg/m^3, the density of mammalian skeletal muscle. The total muscle cross-sectional area was used to estimate the specific force (kN/m^2).

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