

# Transgenic *mdx* Mice Expressing Dystrophin with a Deletion in the Actin-binding Domain Display a "Mild Becker" Phenotype

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**Abstract.** The functional significance of the actin-binding domain of dystrophin, the protein lacking in patients with Duchenne muscular dystrophy, has remained elusive. Patients with deletions of this domain (domain I) typically express low levels of the truncated protein. Whether the moderate to severe phenotypes associated with such deletions result from loss of an essential function, or from reduced levels of a functional protein, is unclear. To address this question, we have generated transgenic mice that express wild-type levels of a dystrophin deleted for the majority of the actin-binding domain. The transgene derived protein lacks amino acids 45-273, removing 2 of 3 *in vitro* identified actin interacting sites and part of hinge 1. Examination of the effect of this deletion in mice lacking wild-type dystrophin (*mdx*) suggests that a functional domain I is

not essential for prevention of a dystrophic phenotype. However, in contrast to deletions in the central rod domain and to full-length dystrophin, both of which are functional at only 20% of wild-type levels, proteins with a deletion in domain I must be expressed at high levels to prevent a severe dystrophy. These results are also in contrast to the severe dystrophy resulting from truncation of the COOH-terminal domain that links dystrophin to the extracellular matrix. The mild phenotype observed in mice with domain I-deletions indicates that an intact actin-binding domain is not essential, although it does contribute to an important function of dystrophin. These studies also suggest the link between dystrophin and the subsarcolemmal cytoskeleton involves more than a simple attachment of domain I to actin filaments.

**D**UCHENNE muscular dystrophy is a severe X-linked lethal myopathy caused by mutations in the dystrophin gene (Koenig et al., 1987). Patients with mutations in this gene display quantitative and/or qualitative deficiencies in expression of the protein dystrophin. Phenotypically, this defect results in progressive muscle degeneration initially manifested in limb skeletal muscles, while patient death generally occurs from respiratory or cardiac failure (Emery, 1993). The milder Becker muscular dystrophy (BMD)<sup>1</sup> is an allelic disease characterized by a later age of onset and a slower progression of muscle degeneration (Bushby, 1992). Dystrophin is also absent in the *mdx* mouse, an animal model for DMD (Bulfield et al., 1984).

Based on structural similarities with the cytoskeletal proteins,  $\alpha$ -actinin and spectrin, the dystrophin molecule is thought to be composed of four distinct domains: an NH<sub>2</sub>-terminal actin-binding domain (domain I), 24  $\alpha$ -helical repeats that form a coiled-coil central rod domain, and two

globular COOH-terminal domains, the first containing a cysteine-rich region, followed by an alternatively spliced region (Fig. 1 *a*; for reviews see Ahn and Kunkel, 1993; Amalfitano et al., 1996). NH<sub>2</sub>-terminal fragments of dystrophin bind actin *in vitro* and localize to the sarcolemma of muscle fibers (Hoffman et al., 1991; Way et al., 1992; Helliwell et al., 1992; Corrado et al., 1994; Dunckley et al., 1994; Jarrett and Foster, 1995). The COOH-terminal domain binds to a transmembrane glycoprotein complex that attaches to laminin in the extracellular matrix (Ervasti and Campbell, 1991; Dickson et al., 1992; Ervasti and Campbell, 1993). These observations have led to the hypothesis that dystrophin forms a structural link between the sarcolemmal cytoskeleton and the extracellular matrix that helps to protect the sarcolemma from damage during contraction.

Ultrastructurally, dystrophin is localized to the cytoplasmic surface of the sarcolemma (Zubrzycka-Gaarn et al., 1988). Dystrophin is concentrated at distinct regions termed costameres which form transverse associations between the sarcolemma and the contractile elements of the myofiber, suggesting that dystrophin may interact with a number of other cytoskeletal proteins (Minnetti et al.,

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1992; Porter et al., 1992; Masuda et al., 1992; Straub et al., 1992). Dystrophin associates with aciculin in cultured muscle cells and binds talin in vitro (Belkin and Burrige 1995; Senter et al., 1993). Several groups have observed dystrophin at internal regions of the myofiber. In particular, dystrophin has been localized within the skeletal muscle sarcomere as a pair of thicker lines overlying the I band and at a region overlying the M lines (Masuda et al., 1992; Porter et al., 1992). Taken together, these observations suggest that dystrophin plays a complex role in the mature myofiber. However, the specific function(s) of dystrophin in different cellular compartments is not known.

Correlation of mutations with severity of phenotype has allowed inferences as to the functional importance of certain domains of dystrophin (Amalfitano et al., 1996). In general, mutations that disrupt the normal reading frame lead to more severe symptoms associated with DMD, whereas mutations that cause in-frame deletions lead to the less severe BMD (Monaco et al., 1988). However, a number of exceptions to this rule have been observed, the majority of which involve deletions in domain I. One class of exceptions involving domain I are patients with a deletion of exons 3-7. This deletion is an mRNA frame-shifting mutation yet these patients display a range of phenotypes including BMD (Malhotra et al., 1988; Baumbach et al., 1989; Koenig et al., 1989; Winnard et al., 1993). These patients frequently express low levels of a dystrophin generated either by exon skipping or by translational initiation at an AUG in exon 8 (Chelly et al., 1990; Thanh et al., 1993; Winnard et al., 1995). The fact that some patients expressing a dystrophin lacking exons 1-7 display a Becker phenotype suggests that the actin-binding domain is not critical (Winnard et al., 1995). Other exceptions to the frame-shift rule have been observed in patients with small in-frame deletions in domain I. These patients display great phenotypic variability, often involving a more severe phenotype than expected and a faster rate of progression of muscle breakdown (Koenig et al., 1989; Beggs et al., 1991; Comi et al., 1994; Muntoni et al., 1994). It has also been reported that dystrophin proteins deleted for portions of domain I are consistently found at levels well below those in normal control muscle (Beggs et al., 1991; Winnard et al., 1993; Muntoni et al., 1994).

The structural model suggests that dystrophin binding to actin at the NH<sub>2</sub> terminus and to the dystrophin-glycoprotein complex (DGC) at the COOH terminus forms a direct link between the intracellular cytoskeleton and the extracellular matrix that is critical for maintaining the structural integrity of the sarcolemma (Campbell, 1995). In concordance with this model, mutations of various DGC members, laminin, or the COOH-terminal domain of dystrophin all lead to a severe muscular dystrophy (Matsumura et al., 1993; Roberds et al., 1994; Tome et al., 1994; Noguchi et al., 1995; Bönnemann et al., 1995; Lim et al., 1995). In contrast, the variable phenotypes observed in patients with NH<sub>2</sub>-terminal deletions of dystrophin has made correlations of structure and function in this domain less clear. Specifically, it is unclear whether the phenotypes associated with deletions in domain I are due to mutations that cause a loss of cytoskeletal attachment, or from destabilization of the protein resulting in low levels of a functional molecule. We addressed this question by monitoring

the effects of a deletion in domain I in transgenic *mdx* mice. By overexpressing an internally truncated dystrophin, we were able to generate higher levels of dystrophin than are found in patients with similar mutations. Our results indicate that the actin-binding domain I is not essential for dystrophin function, and that normal levels of dystrophin containing a deletion in this domain lead to a very mild dystrophic phenotype.

## Materials and Methods

### Construction of Plasmids for Embryo Microinjection

Plasmid p $\Delta$ abd1 was constructed from pCAA, a plasmid containing the -3,300-bp mouse muscle creatine kinase (MCK) gene promoter plus enhancer (Johnson et al., 1989), the 13,815-bp murine dystrophin cDNA, and the SV40 polyadenylation signal (Phelps et al., 1995). The extreme 5' end of the dystrophin cDNA was excised as a 1.4-kb NotI-SpeI fragment and ligated into pBluescript II SK<sup>-</sup> (Stratagene, La Jolla, CA) from which the PstI site had been destroyed. A PstI-NsiI fragment that spanned bases 355-1041 of the dystrophin cDNA (Lee et al., 1991; Chamberlain et al., 1991) was excised from the latter plasmid, which was then self-ligated to create a 687-bp deletion. This truncated fragment was then excised as a NotI-SpeI fragment and religated into the original NotI-SpeI sites of pCAA to create a dystrophin expression vector deleted for the actin-binding domain (Fig. 1 a). The correct construction of this clone was verified by restriction enzyme analysis, the polymerase chain reaction (PCR) using primers outside of the deletion, and DNA sequencing through the deleted region. Plasmid p $\Delta$ abd2 is similar to p $\Delta$ abd1 except that it contains the SV40 VP1 intron inserted between the MCK promoter and dystrophin (Phelps et al., 1995).

### Generation of Transgenic Mice

Transgenic mice were generated by microinjection of purified p $\Delta$ abd1 or p $\Delta$ abd2 insert into F2 hybrid zygotes from C57BL/6J  $\times$  SJL/J parents as described (Hogan et al., 1986). Positive transgenic mice were identified by PCR analysis of tail DNA using primers specific for the 3' end of the murine dystrophin cDNA and the SV40 polyadenylation site. The presence of the deletion within the cDNA was verified by PCR using a primer within the MCK promoter and a primer within the dystrophin cDNA. Positive F<sub>0</sub> mice were also verified by hybridization of genomic tail DNA with a probe internal to the dystrophin cDNA. Transgenic mice on the *mdx* background were produced by crossing F<sub>0</sub> transgenic male mice with C57BL/10*mdx* females. Transgenic F<sub>1</sub> males were then bred with C57BL/10*mdx* females to produce both male and female offspring on the *mdx* background.

### Histological Analysis

Histological 4- $\mu$ m sections were prepared from muscle tissues fixed in 4% paraformaldehyde and 1% glutaraldehyde, embedded in glycol methacrylate, and stained with hematoxylin and eosin. For central nuclei counts, histological sections were photographed and the percentage of centrally nucleated myofibers was determined by dividing the number of myofibers containing one or more centrally located nuclei by the total number of nucleated fibers. Myofibers with no nuclei in the plane of section were not counted.

### Immunofluorescent Localizations with 7- $\mu$ m Transverse Cross-Sections

Skeletal muscle was removed from mice, cut into strips, and frozen slowly in tissue-tek OCT media. Unfixed  $\sim$ 7- $\mu$ m muscle cryosections were stained as described previously with an affinity-purified rabbit polyclonal antibody against a fusion protein with the last 315 amino acids of murine dystrophin, which was preadsorbed to eliminate cross reactivity with utrophin (Cox et al., 1994).

### Immunofluorescent Localizations with Thin Sections

Freshly dissected mouse quadriceps muscle was cut into small pieces ( $\sim$ 1 mm  $\times$  1 mm) and fixed in 1.2% formaldehyde/PBS for 1 h on ice. The tis-

sues were then infused with 1.6 M sucrose, 0.5% formaldehyde/PBS for 24 h at 4°C. Longitudinally oriented mounted tissue blocks were quick frozen in liquid nitrogen. 0.5–1.0  $\mu\text{m}$  sections were cut at  $-80^\circ\text{C}$  using an Ultracut E, FC4 cryomicrotome (Riechert-Jung, Vienna). Tissue sections were blocked for 2 h at 4°C with 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) in PBS, incubated overnight with primary antibodies, washed, and incubated for 4 h at 4°C with either fluorescein- or rhodamine-conjugated secondary antibodies. All washes were done in PBST (0.5% Tween-20) and antibody dilutions were in PBS plus 1% normal goat serum. Rhodamine-conjugated phalloidin (Sigma Chem. Co., St. Louis, MO) was used at a concentration of 10 nM and anti- $\alpha$ -actinin ascites fluid (mAb1682, Chemicon, Temecula, CA) was diluted 1:500. Immunofluorescence was observed on a Zeiss Axioscope equipped with a Canon EDS63 camera. All photography was performed with TMAX 400 film (Eastman-Kodak, Rochester, NY).

### Western Blot Analysis

Total muscle protein homogenates were prepared from either quadriceps or diaphragm tissues frozen in liquid nitrogen. Tissues were ground to a fine powder under liquid  $\text{N}_2$ , suspended in homogenate buffer (1% SDS, 5 mM EGTA, 1 mM PMSEF, 1 mM benzamide, 0.5 mg/ml leupeptin, and 0.2 U/ml aprotinin), vortexed briefly, and heated to  $100^\circ\text{C}$  for 2 min. Insoluble material was removed by centrifugation (5 min, 12,000 rpm at 4°C) and the supernatant was stored at  $-80^\circ\text{C}$ . Proteins separated on 2–12% agarose-acrylamide composite gradient gels were transferred electrophoretically onto nitrocellulose for 1 h at 2 mA/cm<sup>2</sup> using a semidry blotter (Khyse-Anderson, 1984; Wang et al., 1989). Purified rabbit myofibrillar proteins were used as molecular weight standards and were stained with India ink after transfer. Membranes were blocked in TBST (1% Tween-20) containing 5% wt/vol nonfat instant dry milk powder. Antibodies were diluted in TBST (primary antibodies) or TBST plus 0.5%

normal goat serum (secondary antibodies). Bound alkaline phosphatase-conjugated secondary antibodies were visualized using NBT and BCIP (Sigma Chem. Co.). Dystrophin expression levels in transgenic mice were estimated by comparison with extracts from control mice on multiple independent blots, an example of which is shown in Fig. 1 b.

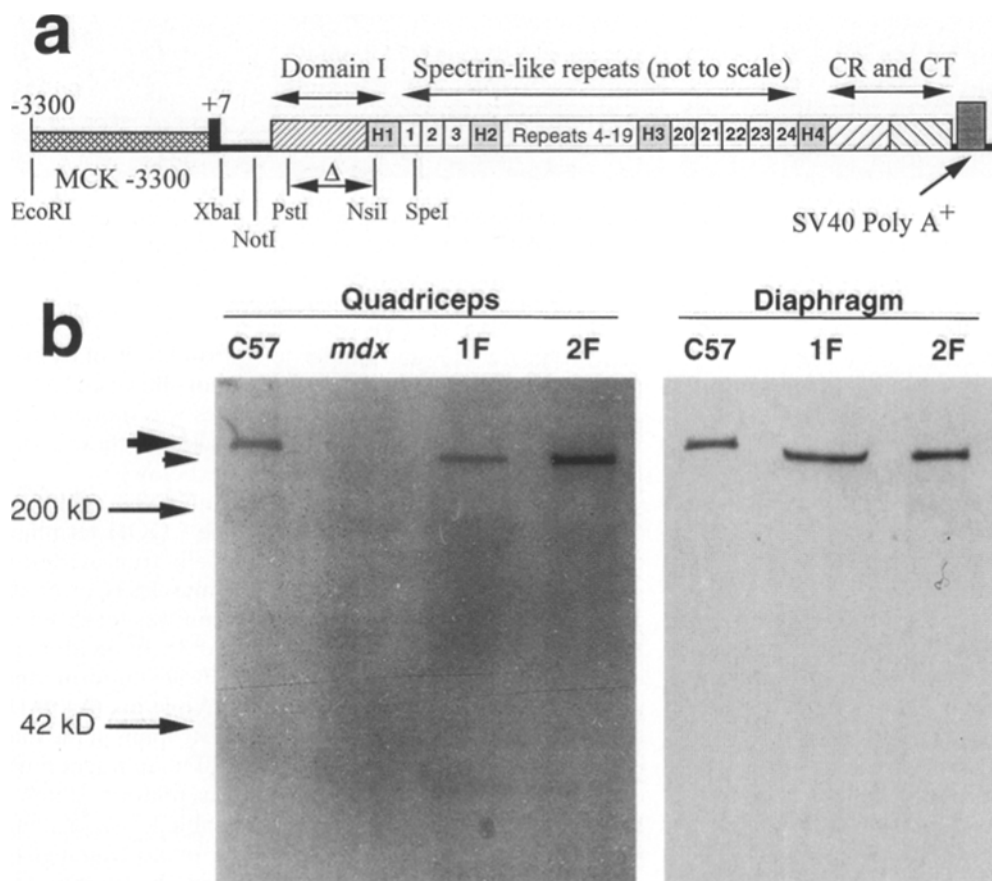
### Measurement of Muscle Mechanical Properties

Small bundles of intact fibers were removed from the diaphragm muscles of 6–7-month old mice. Specific force (kN/m<sup>2</sup>) was determined during maximum isometric tetanic contraction in vitro at 25°C, and then normalized to total cross-sectional area. Power output (W) was determined by isovelocity shortenings from 100%  $L_0$  to 90%  $L_0$  during maximum muscle activation, and was normalized by muscle mass (McCully and Faulkner, 1984; Cox et al., 1993).

## Results

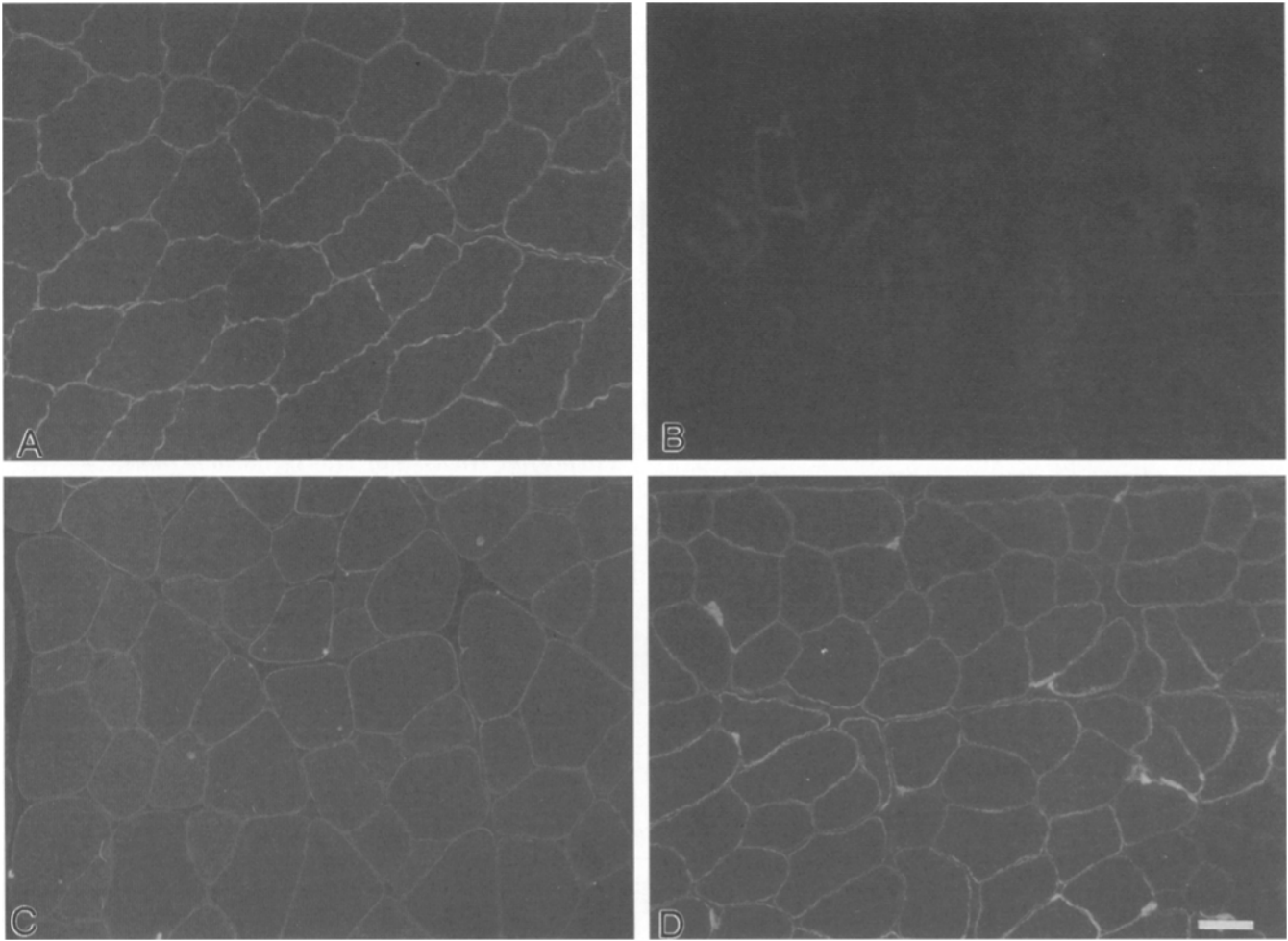
### Phenotypic Analysis of Transgenic *mdx* Mice

To study the function of the actin-binding domain of dystrophin, transgenic mice were generated that express a murine dystrophin cDNA construct that is missing amino acids 45–273 (Fig. 1 a). This deletion is similar to exon 3–7 deletions found in many patients, except it retains the first 13 amino acids of exon 3 and extends to the middle of exon 8, thereby maintaining an open reading frame. The internally truncated protein joins amino acid 44 to the middle of hinge 1, eliminating virtually the entire  $\text{NH}_2$ -ter-



**Figure 1.** Generation of transgenic mice expressing dystrophin with an actin-binding domain deletion. (a) Diagram of the vector used to generate the transgenic mice (not to scale). The murine dystrophin cDNA containing a deletion 'Δ' between internal PstI and NsiI sites was regulated by the  $-3300$  to  $+7$  bp MCK promoter/enhancer, and the SV40 poly-adenylation site, either with or without the SV40 VP1 intron inserted in a NotI site. Indicated are cDNA sequences that encode the actin-binding domain I of dystrophin, four hinge regions, 24 spectrin-like repeats, and the cysteine-rich (CR) and alternatively spliced COOH-terminal (CT) domains. (b) Western blot analysis of total muscle proteins in control (C57), dystrophin (*mdx*), and transgenic (lines 1F and 2F) mice. Transgenic muscle from both lines expressed an  $\sim 400$ -kD protein. Expression of the transgene in line 1F was approximately the

same as the dystrophin levels in control mice in both quadriceps and diaphragm. Expression of the transgene in line 2F was  $\sim 2$ – $3$  times the level of dystrophin in the quadriceps muscle and approximately equal to the level of dystrophin in diaphragm muscle of control mice. Upper arrowhead indicates the position of the full-length 426-kD dystrophin; the lower arrowhead indicates the position of the 400-kD truncated dystrophin.

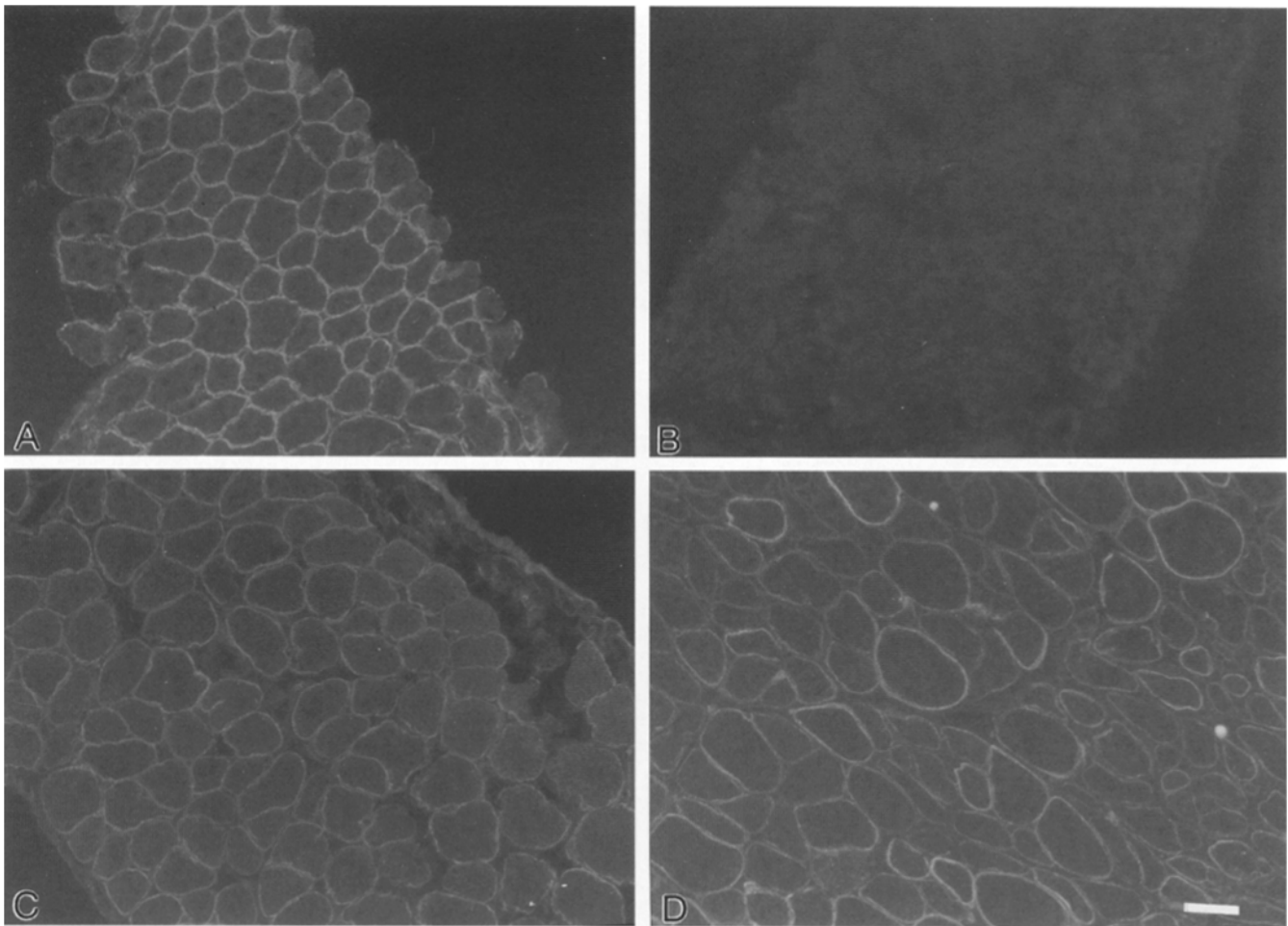


**Figure 2.** Immunofluorescence localization of dystrophin in quadriceps muscle. Dystrophin staining at the sarcolemma appears uniform in the quadriceps muscle of control (*A*) and transgenic lines  $\Delta$ abd-1F (*C*) and  $\Delta$ abd-2F (*D*). Note the absence of dystrophin labeling in *mdx* muscle (*B*). Bar is 50 microns.

minimal domain of dystrophin (Koenig and Kunkel, 1990). Mice positive for the transgene were bred onto an *mdx* genetic background to generate lines expressing the transgene-encoded protein in the absence of endogenous dystrophin. Transgenic mouse lines were established from 12  $F_0$  animals and analyzed by immunoblot to determine which were expressing normal or above normal levels of the internally truncated dystrophin (data not shown). In contrast to other transgenic dystrophin mice we have generated, a level of expression significantly greater than wild-type was not obtained in any of the 12 lines expressing this construct (Cox et al., 1993; Phelps et al., 1995). This lack of high level expression may be due to an inherent instability of a protein with this deletion, as has been suggested for human patients with similar deletions (Beggs et al., 1991). Based upon the Western blot analysis, two independent lines of transgenic mice ( $\Delta$ abd-1F and  $\Delta$ abd-2F) were chosen for detailed analysis. Expression of the transgene in line  $\Delta$ abd-1F produced  $\sim 80\%$  of control dystrophin levels in both quadriceps and diaphragm (Fig. 1 *b* and data not shown). Expression of the transgene in line  $\Delta$ abd-2F (which unlike the 1F transgene included a VPI intron) generated  $\sim 2\text{--}3$  times the control levels of dystrophin in

quadriceps muscles and similar to control levels of dystrophin in diaphragm (Fig. 1 *b* and data not shown). Dystrophin expression in three additional lines was moderate to low in quadriceps and diaphragm muscles so these latter mice were not extensively analyzed (see below).

We next examined both quadriceps and diaphragm muscles using antibodies that recognize the COOH terminus of dystrophin to determine if the internally truncated protein was localized correctly in the *mdx* muscles (Cox et al., 1994). As in control mice, the dystrophin was localized at the sarcolemma membrane (Figs. 2 and 3). In quadriceps of both transgenic lines, the signal was uniform and present in all fibers (Fig. 2, *C* and *D*). While line  $\Delta$ abd-1F also displayed uniform expression of dystrophin in the diaphragm muscles (Fig. 3 *C*), line  $\Delta$ abd-2F diaphragm muscle contained areas of fibers displaying uniform staining mixed with areas displaying highly variable levels of staining, including some regions with little or no staining. In many areas a fiber-to-fiber variability was also observed in which very large diameter fibers were intensely stained while nearby smaller diameter fibers displayed very low-level staining (Fig. 3 *D*). We have previously described a number of transgenic lines with mosaic patterns of expres-

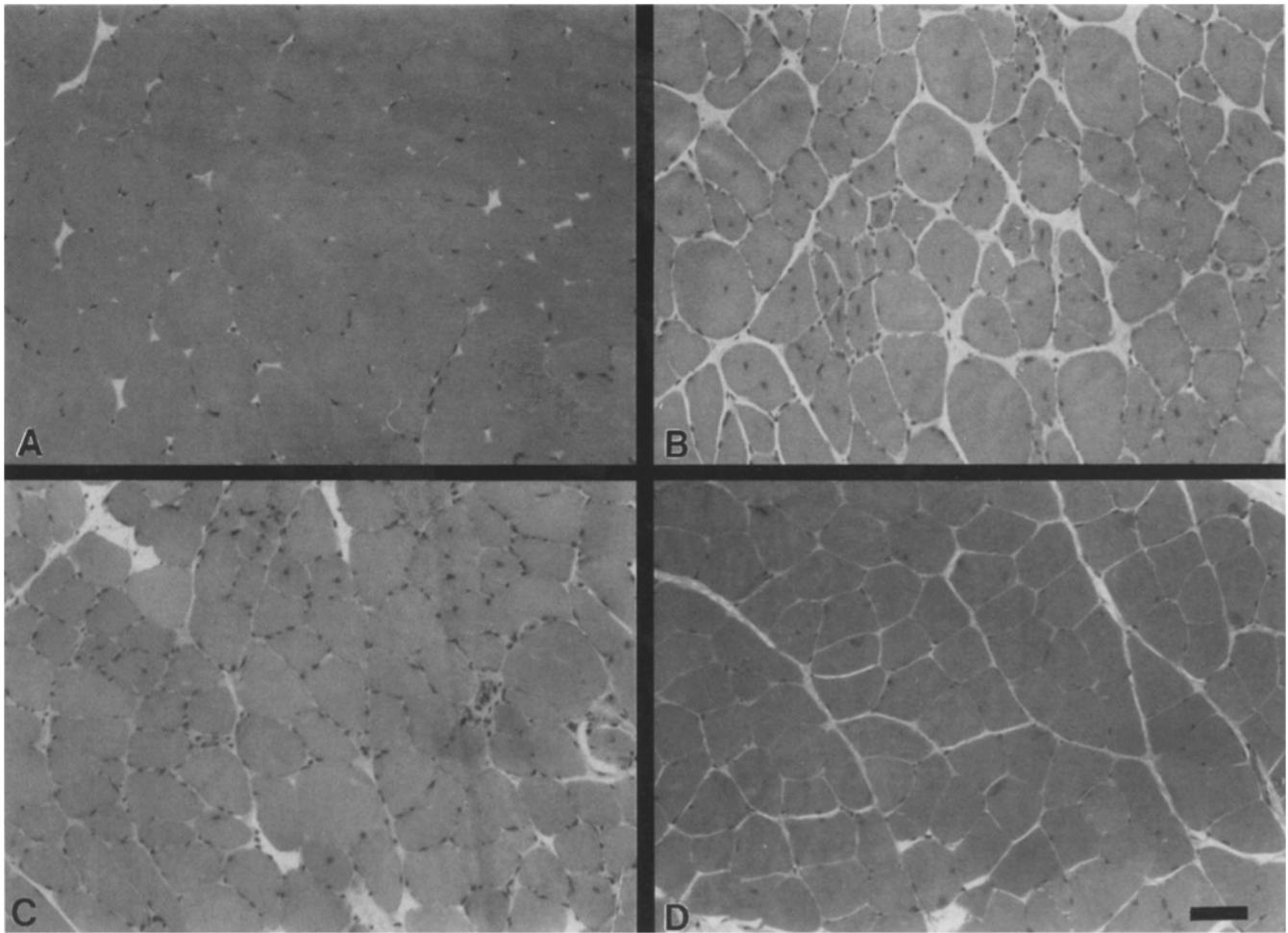


**Figure 3.** Immunofluorescence localization of dystrophin in diaphragm muscle. Dystrophin is localized uniformly at the sarcolemma in control muscle (A), but is not detected in *mdx* muscle (B). Muscle from transgenic line  $\Delta$ abd-1F (C) also displays a uniform staining pattern, but the intensity of the staining is slightly lower than in control muscle. Transgenic line  $\Delta$ abd-2F (D) displays a more variable staining pattern. In some regions, large diameter fibers are more intensely stained than nearby smaller diameter fibers (D). Other regions of the diaphragm from line  $\Delta$ abd-2F display areas with no staining interspersed with region of moderate staining. Bar is 50 microns.

sion, which results from a position effect variegation due to chromatin differences at the site of integration of each particular transgene array in a line of animals (Rafael et al., 1994; Phelps et al., 1995; Milot et al., 1996). Three other lines of mice displayed either very low expression of dystrophin in diaphragm or were more mosaic than line  $\Delta$ abd-2F (not shown). Highly mosaic dystrophin expression leads to a dystrophic phenotype, even with full-length dystrophin (Phelps et al., 1995), so we did not further analyze these latter lines of mice.

To determine the effect of the domain I deletion, diaphragm and quadriceps muscle sections were examined morphometrically. The absence of dystrophin in *mdx* mice leads to cycles of muscle fiber degeneration and regeneration, variable fiber diameters, and a gradual increase in fiber necrosis (Bulfield et al., 1984; Torres and Duchon, 1987; Stedman et al., 1991). These muscles also display variable amounts of infiltration by mononuclear cells, indicative of an inflammatory response, accompanied by a progressive replacement of the muscle with adipose and connective tissue (fibrosis). Histological analysis indicated that quadriceps muscle in the transgenic mice was much

less affected than in *mdx* mice (Fig. 4). Few necrotic fibers were observed and little fibrosis was evident, although a variation in fiber size was apparent. The percentage of muscle fibers with centrally located nuclei is indicative of previous cycles of degeneration/regeneration, and is inversely correlated with the overall ability of a dystrophin molecule to protect muscle from necrosis and regeneration (Phelps et al., 1995). The percentage of centrally nucleated fibers observed in quadriceps muscles of 3–4-month-old mice was  $\sim$ 8% for transgenic *mdx* line  $\Delta$ abd-1F and  $\sim$ 7% for line  $\Delta$ abd-2F compared with  $<$ 1% for age-matched C57BL/10 and  $\sim$ 80% for *mdx* (Table I). These data indicate that in quadriceps muscles, the internally truncated protein is able to prevent the appearance of most dystrophic symptoms. In *mdx* mice, the diaphragm muscles show more degeneration and necrosis of fibers (Stedman et al., 1991) and a greater loss of force and power (Cox et al., 1993) than limb muscles do and consequently more closely resemble the phenotype observed in DMD patients. In the  $\Delta$ abd-1F line morphological analysis of the diaphragm muscle revealed a relatively normal appearance, with few detectable necrotic fibers (Fig. 5 C).



**Figure 4.** Histological analysis of quadriceps muscle. Haematoxylin and eosin staining of control C57BL/10 (A) and transgenic *mdx* muscle of lines  $\Delta$ abd-1F (C) and  $\Delta$ abd-2F (D) reveal little or no fibrotic infiltration of the muscles compared with the dystrophic pathology observed in *mdx* mice (B). Few necrotic fibers were observed in control or transgenic mice, and little inflammation was apparent; however, a variation in fiber size in the transgenic muscle was noted as was an increased number of fibers with centrally located nuclei. Bar is 50 microns.

The percentage of diaphragm muscle fibers with centrally located nuclei averaged  $\sim$ 11% for line  $\Delta$ abd-1F, compared with  $<$ 0.2% for age-matched C57BL/10 control mice and  $\sim$ 55% for *mdx* mice (Table I). These results suggest that in diaphragm muscle, as in the quadriceps, the deleted protein is able to prevent most of the dystrophic symptoms normally present in *mdx* mice. In contrast, the diaphragm muscle of line  $\Delta$ abd-2F had a variable mor-

phology from one region of the muscle to another. This line of mice displayed a very mosaic pattern of dystrophin expression, and, as a result, some regions of muscle looked very healthy (Fig. 5 D) while others looked similar to *mdx* muscle (Fig. 5 E). Three other lines of mice with low or extremely mosaic dystrophin expression displayed a diaphragm muscle morphology similar to *mdx* mice (not shown).

**Table I.** Centrally Nucleated Fibers in Muscles of Mice

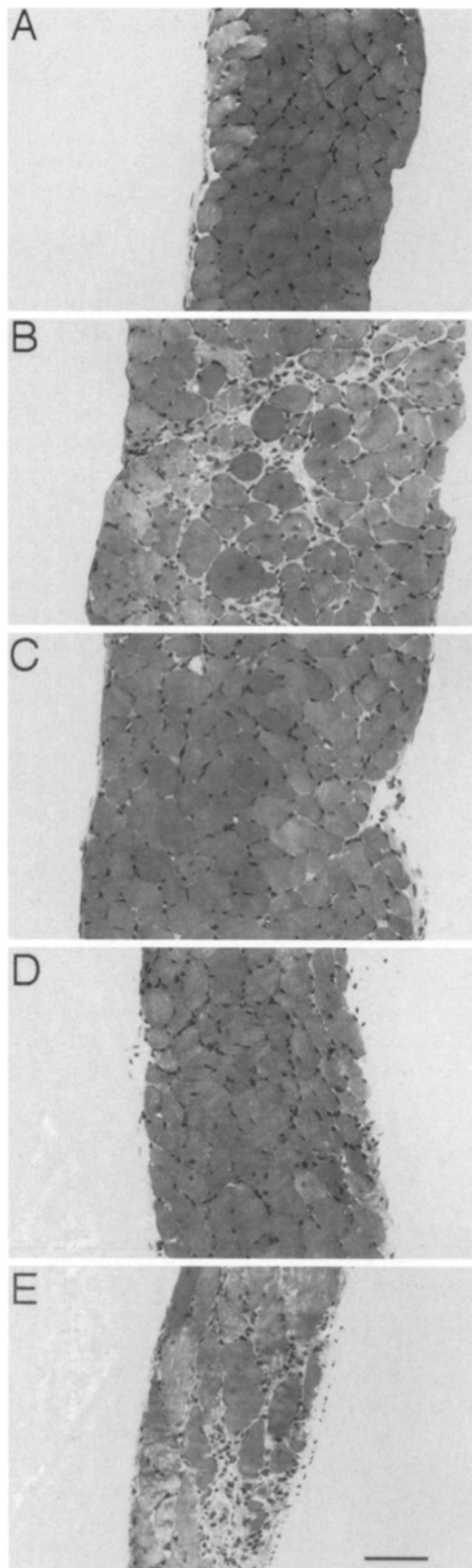
Line	Animal*	Percentage of centrally nucleated fibers	
		Diaphragm	Quadriceps
C57		0.2	0.7
1F	3647	10.8	5.3
1F	4436	11.8	13.6
2F	4045	ND	10.0
2F	4805	ND	4.5
<i>mdx</i>		54.8	88.7

\*All mice tested were 3–4 months of age. ND, not determined.

### Functional Analysis of the Truncated Dystrophin

The diaphragm muscles from *mdx* mice display a significant functional deficit, manifested by dramatic reductions in the generation of both force and power (Cox et al., 1993; Rafael et al., 1994; Phelps et al., 1995). To maximize potential differences between the transgenic and *mdx* mice, mechanical properties of muscles were measured in mice 6–7 months of age. By this age diaphragm muscles from *mdx* mice display a 57% reduction in specific force compared with age matched control mice (C57BL/10). In contrast, diaphragm muscles from transgenic  $\Delta$ abd-1F mice displayed only a 27% reduction in specific force (Table II). Similarly, the normalized power generated by diaphragm muscles of





**Figure 5.** Histological analysis of diaphragm muscle. Haematoxylin and eosin staining of diaphragm muscle strips from control C57BL/10 (A), *mdx* (B), transgenic line  $\Delta$ abd-1F (C), and two examples from transgenic line  $\Delta$ abd-2F (D and E). As in the quadriceps muscle (Fig. 4), the transgenic *mdx* diaphragm from line  $\Delta$ abd-1F displays little fibrosis, few necrotic fibers, and only a

**Table II.** Contractile Properties of Diaphragm Muscles of Mice

Line*	Specific force <sup>‡</sup> (kN/M <sup>2</sup> )	Normalized power <sup>‡</sup> (W/kg)
C57	250 ± 29	60 ± 18
$\Delta$ abd1F (5)	182 ± 15	42 ± 6
<i>mdx</i> (6)	108 ± 22	20 ± 6

\*The number of animals tested is given in parentheses following the name of the mouse line.

<sup>‡</sup>Data are expressed as the mean followed by the value for one standard error of the mean.

transgenic *mdx* mice was 30% less than control mice, compared with a 64% decrease for the *mdx* mice (Table II). These data demonstrate that the deleted protein is semi-functional, being able to prevent the appearance of much, but not all, of the pathological defects displayed by *mdx* mice.

Since the internally truncated dystrophin appeared to function close to normal in the absence of wild-type dystrophin, we determined if its expression would have any effect in the presence of full-length dystrophin. Such a dominant negative effect might be predicted for a protein that functions as a dimer, and would indicate whether the mutant protein could compete for binding sites or perhaps form unstable or nonfunctioning dimers able to perturb the normal function of dystrophin. To address this issue we performed morphological analysis of transgenic line  $\Delta$ abd-2F after crossing it onto a wild-type background. However, no differences between transgenic or wild-type mice in either the percentage of myofibers with centrally located nuclei or the morphological state of the quadriceps or diaphragm muscles were observed (data not shown).

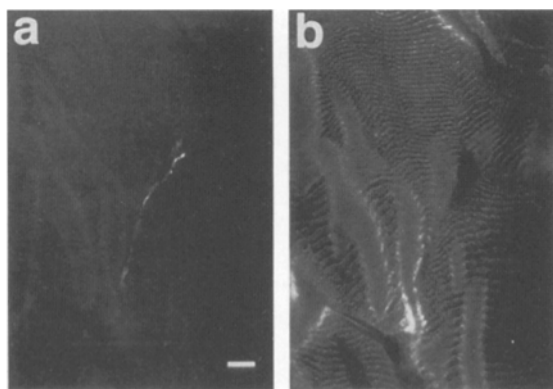
#### Sarcomeric Localization of the Truncated Dystrophin

A number of reports have localized dystrophin to the sarcolemma in a punctate pattern at both M and Z-line costameres (Straub et al., 1992; Porter et al., 1992; Masuda et al., 1992). In addition, Masuda et al. (1992) observed regular internal cross striations in longitudinal sections of skeletal muscle fibers using antibodies specific for dystrophin. Straub and colleagues (1992) observed similar internal staining with dystrophin antibodies, but attributed the signal to a cross reaction of their antibodies with  $\alpha$ -actinin. This localization may be due to interactions between the NH<sub>2</sub> terminus of dystrophin and elements of the cytoskeleton. To determine whether the actin-binding domain deletion in our transgenic mice would affect the myofibrillar localization of dystrophin, we examined longitudinally oriented quadriceps muscle sections using ultrathin cryosections, which due to their decreased focal plane, allow for a much sharper image with greater resolution than normal thin sections. Our antibodies against the COOH-terminal

slight increase in centrally nucleated fibers compared with control muscle. Transgenic line  $\Delta$ abd-2F, which is an independently derived line from line 1F, displayed variable expression by immunofluorescence. Similarly, a variable pathology was observed in the diaphragm from animals of this line. Some regions displayed a relatively normal diaphragm muscle morphology (D), while other regions displayed moderate to extensive dystrophy (E). Bar is 100 microns.

domain of dystrophin recognize a region lacking homology with  $\alpha$ -actinin, but to ensure that these antibodies do not cross-react with other proteins in *mdx* mice, *mdx* quadriceps muscle sections were tested by double immunofluorescence labeling with antibodies against dystrophin and  $\alpha$ -actinin. The results indicated that the dystrophin antisera did not cross-react with  $\alpha$ -actinin or other myofibrillar proteins (Fig. 6). Analysis of ultrathin sections in control mice revealed dystrophin staining at the sarcolemma in discrete patches at both M- and Z-lines (*arrowheads*), and internally in the myofiber in a striated pattern corresponding to the Z-line (*arrows*), and, weakly, at the M-line (Fig. 7 A). In the ultrathin sections of transgenic *mdx* skeletal muscles a similar pattern of staining at both the sarcolemma and at the internal regions of the myofiber was observed (Fig. 7 B). Hence, the localization of dystrophin does not appear disturbed by a deletion in the actin-binding domain. Confocal microscopic analysis of a single teased fiber from control quadriceps muscle double stained with antisera against the dystrophin COOH terminus and  $\alpha$ -actinin confirmed that the major intracellular dystrophin staining colocalized with  $\alpha$ -actinin at the Z-line (not shown). Similar staining is observed in control mice with antibodies we raised against the NH<sub>2</sub>-terminal domain of dystrophin (not shown). These results confirm earlier reports of sarcomeric dystrophin localization that used NH<sub>2</sub>-terminal antibodies, and show the same results are obtained with COOH-terminal antibodies that do not cross react with  $\alpha$ -actinin (Masuda et al., 1992; Porter et al., 1992).

Since the NH<sub>2</sub>-terminal domain of dystrophin binds actin, we determined if the deletion had any effect on the localization pattern of actin in skeletal muscle. In addition, the localization of dystrophin at costameres led to the hypothesis that dystrophin is associated with other components of the cytoskeleton. Therefore, the localization patterns of actin and of the costamere-associated protein  $\alpha$ -actinin were determined. Filamentous actin localization was similar in both control mice and in the transgenic *mdx* mice, as



**Figure 6.** Analysis of dystrophin antisera on *mdx* mouse muscle. Ultrathin cryosections from *mdx* mouse quadriceps were immunostained with (a) affinity-purified COOH-terminal dystrophin antisera or (b) antisera against  $\alpha$ -actinin. No cross reactivity with  $\alpha$ -actinin or other cytoskeletal proteins was observed either on these thin sections or by Western analysis (Cox et al., 1994). A positively staining region from a 'revertant' *mdx* myofiber in a is visible. Bar is 10 microns.

judged by phalloidin staining (Fig. 7, C and D). In both the control and transgenic *mdx* mice  $\alpha$ -actinin staining was observed in a striated pattern at the Z-lines, in a pattern similar to that observed for dystrophin (Fig. 7, E and I). Hence, at the light microscopy level, transgenic *mdx* skeletal muscle does not display any perturbation of the localization of dystrophin or these other two cytoskeletal proteins.

## Discussion

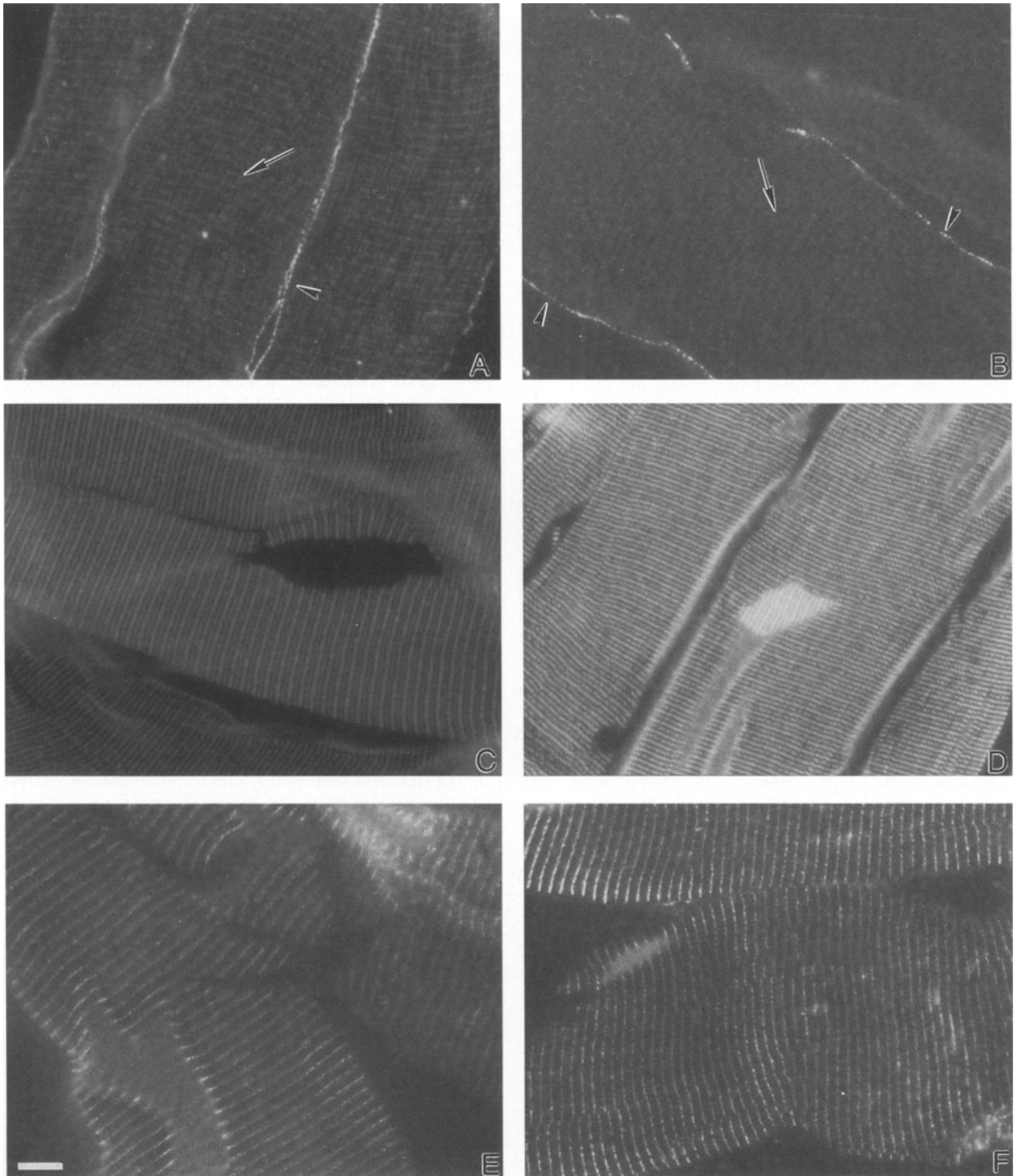
Correlation between the molecular nature of mutations at the DMD locus and clinical phenotypes has provided considerable insight into the importance of different functional domains of dystrophin. Several groups have observed that in-frame deletions in domain I result in a greater phenotypic variability with a clinical course that is more severe than expected for typical BMD patients (Malholtra et al., 1988; Baumbach et al., 1989; Koenig et al., 1989; Beggs et al., 1991; Comi et al., 1994; Muntoni et al., 1994). Beggs and colleagues (1991) observed that patients with domain I deletions typically had low levels of dystrophin, relatively early onset of symptoms, and severe progression of muscle degradation. Muntoni and colleagues (1994) reported that patients with in-frame deletions within domain I display a less intense pattern of immunohistochemical staining and a faster progression of skeletal muscle degeneration compared with patients affected by deletions in the central rod domain. These results suggest that domain I is important for the normal function of dystrophin. Alternatively, the very low levels of dystrophin observed in patients with domain I deletions could suggest that the more severe than expected phenotypes result primarily from decreased expression of a fully functional protein.

Mice that expressed the truncated protein at or above normal levels manifested relatively few of the pathological abnormalities found in *mdx* mouse quadriceps and diaphragm muscles. The diaphragms of these mice generated levels of force and power closer to those observed in control than in *mdx* mice (Table II). In addition, no obvious alterations were observed in the localization of dystrophin, actin, or  $\alpha$ -actinin between the transgenic and control mice. These results indicate that normal expression of a dystrophin protein with a deletion in domain I leads to what might be termed a "mild Becker" phenotype in *mdx* mice.

Interestingly, none of the twelve lines of transgenic mice we generated expressed the transgene at the high levels (5–50 times normal) we observed for other dystrophin constructs that used similar gene regulatory elements (Cox et al., 1993; Rafael et al., 1994; Phelps et al., 1995). Human patients with in-frame deletions in domain I also express very low levels of the truncated proteins. Consequently, this inability to obtain high level expression in mice may be due to an instability of the protein due to an improper folding or to weakened linkages with the cytoskeleton. Either condition might cause the protein to be more accessible to protease digestion.

Development of such a mild phenotype in mice after deletion of the vast majority of the dystrophin actin-binding domain is surprising, particularly since deletion of the cysteine-rich COOH-terminal DGC-binding site leads to the development of a severe dystrophy in humans and mice





**Figure 7.** Immunofluorescence analysis using ultrathin cryosections. Longitudinally oriented 0.5 micron cryosections of quadriceps muscle from control (A, C, and E) and transgenic *mdx* mice (B, D, and F) were stained with anti-dystrophin antibodies (A and B), phalloidin (C and D) or anti- $\alpha$ -actinin antibodies (E and F). Dystrophin staining was observed at the sarcolemma in discrete patches at both M- and Z-lines (arrowheads), and internally in the myofiber in a striated pattern corresponding to the I band (arrows), and, weakly, at the M-line. No differences were noted between the localization of dystrophin,  $\alpha$ -actinin, or actin between control and transgenic-*mdx* animals. Bar is 10 microns.

(Hoffman et al., 1991; Rafael et al., 1996). Current theories of dystrophin function suggest any mutation that leads to a disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix should be associated with a severe dystrophy (Campbell, 1995). Since our transgenic mice display a relatively mild phenotype, and since some patients with 5' gene deletions develop BMD rather than DMD, it seems likely that dystrophin can attach to the subsarcolemmal cytoskeleton in a more complex manner than has generally been assumed. Several mechanistic interpretations seem reasonable to explain these observations. Dystrophin may contain multiple actin-binding sites, such that deletion of one site would enable actin binding by a remaining site or sites. These multiple actin-binding sites could normally function simultaneously in a dystrophin protein. Alternatively, deletion of a primary actin-binding domain could lead to conformational changes that unmask cryptic binding sites not efficiently used in an intact protein. Another possibility is that dystrophin can bind to multiple components of the actin cytoskeleton, such that disruption of a link with actin might not seriously affect a direct connection with other proteins, such as talin, aciculin, or calmodulin.

Analysis of multiple patients with deletions in domain I has suggested that exon 3 and exon 5 are important for dystrophin function (Muntoni et al., 1994). We and others have shown that the actin-binding domain of dystrophin contains at least three sites of interaction between actin and dystrophin: ABS1 (amino acids 18-27, in exon 2), ABS2 (amino acids 131-148, in exon 6), and ABS3 (amino acids 91-117, in exon 5) (Levine et al., 1990, 1992; Fabrizio et al., 1993; Corrado et al., 1994; Jarrett and Foster, 1995). The deletion construct used in this study extends from the middle of exon 3 to the middle of exon 8 and thus joins amino acid 44 to the middle of hinge 1, removing ABS2 and ABS3 but retaining ABS1. It has been suggested that ABS2 and ABS3 are more critical to dystrophin function than is ABS1. Jarrett and Foster (1995) have shown that the binding of ABS 2 and 3 to actin is of a much greater affinity than the binding of ABS1 alone to actin. The majority of patients with in-frame domain I deletions that retain ABS1 display a severe BMD phenotype (Beggs et al., 1992; Comi et al., 1994; Muntoni et al., 1994). These studies and our results suggest an intact domain I is not essential for function and that the severe phenotype seen in these patients is due to the lower levels of dystrophin. We cannot rule out the possibility that the truncated protein in our transgenic mice is binding weakly to actin at ABS1 or at other unidentified sites.

Additional unidentified protein-binding sites could exist in the dystrophin NH<sub>2</sub> terminus, which might facilitate formation of a broad multi-component linkage with the subsarcolemmal cytoskeleton. The only detectable form of dystrophin in some BMD patients with an exon 3-7 deletion is generated by translation initiation in exon 8 (Winnard et al., 1995), which suggests an interaction with the cytoskeleton distal to exon 8. Other evidence indicates dystrophin can bind to additional cytoskeletal proteins, including talin (Senter et al., 1993), aciculin (Belkin and Burridge, 1995), and at the NH<sub>2</sub> terminus to calmodulin (Bonet-Kerrache et al., 1994; Jarrett and Foster, 1995; Winder and Kendrick-Jones, 1995). Hori and colleagues

(1995) have demonstrated that the NH<sub>2</sub>-terminal half of dystrophin is broadly protected from proteolysis *in vivo*, suggesting that a large region of the molecule interacts with cytoskeletal components. Additional observations, such as the demonstration that dystrophin is phosphorylated *in vitro* by a variety of protein kinases, suggest that the NH<sub>2</sub>-terminal domain could also be involved in regulating the association of dystrophin with other cytoskeletal elements (Madhavan and Jarrett, 1994; Senter et al., 1995). Thus, phenotypic effects caused by deletions in the NH<sub>2</sub>-terminal domain could also be caused by an improper regulation of dystrophin-cytoskeletal interactions rather than an inability to bind actin *per se*. Regardless of the precise mechanism by which NH<sub>2</sub>-terminal deletions lead to dystrophy, our data indicate that increased accumulation of the deleted protein can lead to an amelioration of symptoms. Attachment of dystrophin to the cytoskeleton via actin is likely weakened by such deletions, and having a larger pool of protein available for binding may help maintain greater overall strength in the cytoskeletal-extracellular matrix linkage.

Since many severely affected BMD patients accumulate less dystrophin than more mildly affected patients, it has been suggested that improvement of a patient's phenotype might be attained if the level of dystrophin accumulation could be boosted (Beggs et al., 1991). The degree of phenotypic improvement that could be attained would obviously vary with the type of mutation present in a patient and whether critical functional domains are removed from the expressed protein. The unknown stability of truncated dystrophin proteins resulting from various deletion and splicing mutations has complicated interpretation of the effect of those mutations on various dystrophin functional domains (Roberts et al., 1994; Amalfitano et al., 1996). Our study indicates that moderate functional deficits resulting from deletions in the NH<sub>2</sub>-terminal domain of dystrophin are exacerbated by low level expression of the relatively unstable, truncated protein. Hence, although an intact actin-binding domain may not be essential, it does contribute to an important function of dystrophin. The deleterious effect of loss of this function can be lessened significantly by overproduction of the truncated protein. This result is in marked contrast to deletions of the central rod domain, and to many in the COOH-terminal domain of dystrophin. We and others have demonstrated in transgenic mice that deletion of portions of the rod domain generate proteins that are almost fully functional even at levels only 20% of the control value (Phelps et al., 1995; Wells et al., 1995), whereas deletion of the cysteine-rich domain leads to a severe phenotype, even when control levels of the protein are expressed (Rafael et al., 1996). Continued efforts to develop detailed structure/function models for dystrophin using data from patients need to consider the effect of a particular deletion on the accumulation of the truncated dystrophin.

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*Note Added in Proof.* Recent data suggests that dystrophin may bind actin filaments via multiple sites throughout the NH<sub>2</sub>-terminal and rod domains, which could explain the mild phenotype in our transgenic animals (James Ervasti, personal communication).

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