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Expression of Deletion-Containing Dystrophins in *mdx* Muscle: Implications for Gene Therapy and Dystrophin Function

JEFFERY D. FRITZ, ISTVAN DANKO, STEVEN L. ROBERDS, KEVIN P. CAMPBELL, JEFFREY S. LATENDRESSE, AND JON A. WOLFF

Departments of Pediatrics and Medical Genetics, Waisman Center, University of Wisconsin-Madison, Madison, Wisconsin 53705 [J.D.F., I.D., J.S.L., J.A.W.] and Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242 [S.L.R., K.P.C.]

ABSTRACT

The expression of full-length dystrophin and various dystrophin deletion mutants was monitored in *mdx* mouse muscle after intramuscular injection of dystrophin-encoding plasmid DNAs. Recombinant dystrophin proteins, including those lacking either the amino terminus, carboxyl terminus, or most of the central rod domain, showed localization to the plasma membrane. This suggests that there are multiple attachment sites for dystrophin to the plasma membrane. Only those constructs containing the carboxyl terminus were able to stabilize dystrophin-associated proteins (DAP) at the membrane, consistent with other studies that suggest that this domain is critical to DAP binding. Co-localization with DAP was not necessary for membrane localization of the various dystrophin molecules. However, stabilization and co-localization of the DAP did seem to be a prerequisite for expression and/or stabilization of mutant dystrophins beyond 1 wk and these same criteria seemed important for mitigating the histopathological consequences of dystrophin deficiency. (***Pediatr Res* 37: 693–700, 1995**)

Abbreviations

DMD, Duchenne muscular dystrophy
PCR, polymerase chain reaction
pRSVDy, plasmid DNA encoding full length dystrophin
pRSVDy-B, plasmid DNA encoding Becker-like dystrophin
pSV40L, plasmid DNA encoding firefly luciferase
pRSVDy-SV40L, plasmid DNA encoding both full-length dystrophin plus luciferase
pRSVDy-B-SV40L, plasmid DNA encoding both Becker-like dystrophin plus luciferase
pRSVDy-A, plasmid DNA encoding amino-terminal domain-deleted dystrophin
pRSVDy-C, plasmid DNA encoding carboxyl-terminal domain-deleted dystrophin
pRSVDy-D, plasmid DNA encoding amino- and carboxyl-terminal domain-deleted dystrophin

Aberrations in dystrophin expression are responsible for DMD and Becker muscular dystrophy (1). It also appears that deficiencies of dystrophin-associated proteins may play an important role in DMD and other severe childhood myopathies (2). A promising cure for DMD is placement of the normal dystrophin cDNA into affected tissue. Previous transgenic studies in *mdx* mice—an animal model of DMD (3)—suggest that expression of recombinant dystrophin restores normal muscle morphology and function (4–6). Restoration of dystrophin expression in *mdx* muscle has also been observed after the injection of retroviral vectors (7), naked plasmid DNA (8), or adenoviral vectors (9). Dystrophin expression has been shown to improve myofiber survival in *mdx* muscle after dystrophin gene transfer either by injection of naked plasmid DNA (10) or adenoviral vectors (11).

The main issues concerning DMD gene therapy approaches are: *1*) transfer and expression of dystrophin in sufficient numbers of muscle fibers and *2*) prevention of disease progression by recombinant dystrophin expression. The transgenic *mdx* mouse studies imply that recombinant dystrophin expression can correct dystrophic muscle to the extent that it has normal muscle morphology and function (4–6). However, the results obtained with embryonic gene transfer may not extend to somatic gene transfer which would involve gene transfer at later development times. Somatic gene transfer using adenoviral vectors has resulted in stable dystrophin expression only when introduced into neonatal mice (12); a treatment situation difficult to mimic in all DMD patients. Also, limitations on the

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Correspondence and reprint requests: Jon A. Wolff, M.D., University of Wisconsin-Madison, Waisman Center, Room 355, 1500 Highland Avenue, Madison, WI 53705.
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size of the gene to be transferred is currently a problem with adenoviral vectors and has resulted in their use to transfer only a Becker-like dystrophin to dystrophic muscle (9,11). Using adenoviral vectors, it is at present not possible to compare the efficacy between Becker-like and full-length dystrophin expression in dystrophic muscle. Postnatal gene transfer into muscle by intramuscular injection of plasmid DNA has been used to transfer and express genes encoding both the Becker-like and full-length dystrophins in dystrophic muscle (8,10). Unfortunately, it remains difficult to assess the therapeutic value of this treatment on the muscle as a whole since less than 1% of the myofibers express dystrophin after intramuscular injection. But this approach does provide sufficient dystrophin expression to assess its therapeutic value at the myofiber level (10).

To further characterize the functional relationships of the various domains of dystrophin, and the importance of these domains in relationship to DMD gene therapy, we examined the expression of various deletion-containing dystrophins in *mdx* muscle after the intramuscular injection of plasmid DNA. The effect of dystrophin expression on dystrophin-associated protein expression, myofiber survivability, and percentage of centrally located nuclei were used to assess the functionality of the deleted dystrophin domains with regard to dystrophin function or therapeutic value toward DMD gene therapy. Previous studies have shown that the stability of expression of dystrophin in combination with a reporter gene can be used as an indirect indication of myofiber survival (10,11).

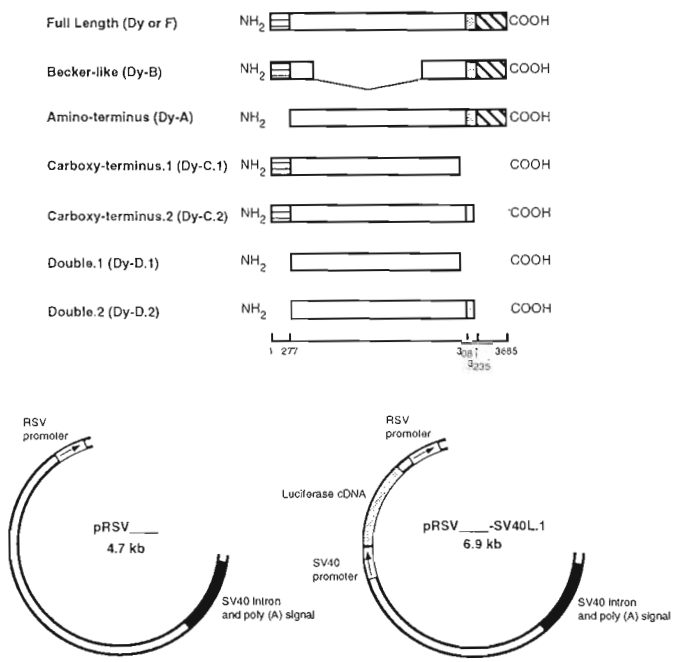

Figure 1. Diagrammatic representation of the dystrophin molecules encoded by the various expression plasmids injected into *mdx* muscle. The full-length dystrophin sequence and the borders of its varying domains was based on previously published sequence (36). The approximate amino acid boundaries corresponding to the amino-terminal (actin-binding, *horizontal shading*), spectrin-like repeats (*white*), cysteine-rich (*stippled shading*), and carboxyl-terminal (*diagonal shading*) domains are noted below the diagram of the double.2 molecule. The cDNA encoding each of these dystrophin molecules was ligated into either the pRSV or the pRSV-SV40L.1 vectors previously described (10).

METHODS

Mouse strains. The C57BL/6, Balb/c, and ICR strains were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The mdx^{4cv} and mdx^{5cv} strains (gift of V. Chapman) having indistinguishable phenotypes (13,14) were used interchangeably and are simply referred to as *mdx* mice (10) unless otherwise indicated. These specific strains were used because they have substantially lower number of revertants than the original *mdx* strain (14).

Plasmid construction, preparation, and injection. Plasmid DNAs pRSVDy, pRSVDy-B, pSV40L.1, pSV40L.2, and pRSVDy-SV40L.1 or pRSVDy-B-SV40L.1, were identical to those previously described (10). The pRSVDy-B construct encodes a dystrophin molecule lacking amino acids 664–2366 (Fig. 1, Becker-like). The pUC19 plasmid was obtained commercially (Life Technologies, Inc., Bethesda, MD).

An expression construct, pRSVDy-A, encoding a dystrophin lacking amino acids 1–277 (Fig. 1, amino terminus) was prepared using PCR primers Dys-*Not*I (5′-GGGCCCGCGGCCGCAATGATCACGGTCAGTCTAGCA-3′) and Dys-*Aat*II (5′-CCCGGGCTGACGTCCAGTCTTTATC-3′) to amplify the dystrophin cDNA region encoding amino acids 277–1526 from pRSVDy. The Dys-*Not*I primer has an ATG site located within it so that encoded dystrophin will start with a methionine and then continue with residue 277. This ensures that this mutant has identical 5′ sequences as the other mutant constructs not containing 5′ deletions. The resulting 3745-bp product was ligated into pCRII (Invitrogen) and the resulting plasmid digested with NotI and AatII. This fragment was ligated into pRSVDy (previously digested with *Not*I and *Aat*II) to yield pRSVDy-A.

Two carboxyl-terminal dystrophin mutant constructs, pRSVDy-C.1 and pRSVDy-C.2, were also prepared. Construct pRSVDy-C.1 was prepared by digesting pRSVDy with *Xho*I and *Sal*I, and then ligating the ends together. The pRSVDy-C.1 construct encodes a dystrophin molecule lacking amino acids 2975–3685 (Fig. 1, carboxyl terminus.1). Construct pRSVDy-C.2 was prepared by first amplifying a DNA fragment encoding dystrophin amino acids 2829–3195 by PCR using pRSVDy as template and Dys-*Ngo*MI (5′-CCCGGGGCCGGCAGGCACCTATTGG-3′) and Dys-*Sal*I (5′-GGGCCCGTCGACC TACTAGACACGGATCCTCCCTGTTCG-3′) primers. This PCR fragment was ligated into pCRII and the resulting plasmid digested with *Ngo*MI and *Sal*I. The digested fragment was then cloned into pRSVDy previously digested with *Ngo*MI and *Sal*I to yield pRSVDy-C.2. The pRSVDy-C.2 construct encodes a dystrophin molecule lacking amino acids 3196–3685 (Fig. 1, carboxyl terminus.2).

Constructs encoding dystrophin lacking both the amino- and carboxyl-terminal regions were prepared by combining the schemes described above. Construct pRSVDy-D.1 encodes a dystrophin molecule lacking both the amino- and carboxyl-terminal domains described for both pRSVDy-A and pRSVDy-C.1 (Fig. 1, double.1). It was prepared by digesting pRSVDy-A with *Xho*I and *Sal*I and ligating the resulting ends together.

Construct pRSVDy-D.2 encodes a dystrophin molecule lacking the regions defined by both pRSVDy-A and pRSVDy-C.2 (Fig. 1, double.2). It was constructed by inserting the *Ngo*MI/*Sal*I fragment of pRSVDy-C.2 into pRSVDy-A.

The cDNA sequences encoding these various dystrophin molecules were placed in expression vectors which either lacked or contained a region capable of encoding luciferase (Fig. 1). Vectors containing both luciferase and dystrophin cDNA have the SV40L.1 notation added to the plasmid (*i.e.* the plasmid encoding both the full-length dystrophin and luciferase was termed pRSVDy-SV40L.1). These vectors serve as indicators of myofiber survival since luciferase expression persists in *mdx* muscle only after transfer and expression of either full-length or Becker-like dystrophin (10).

All plasmids were purified by alkaline lysis and two CsCl gradients as previously described (15). Plasmid DNA injections into mouse quadriceps were done in 100 μL of normal saline (10). Specific amounts of plasmid DNA injected per quadriceps are given in the figure legends.

Immunohistochemistry, luciferase, and β-galactosidase assays. Dystrophin immunohistochemistry was performed using antibodies which recognized either the rod (16) (generously provided by L. Kunkel), amino-terminal or carboxyl-terminal domains of dystrophin (17) in combination with biotin-conjugated sheep anti-rabbit secondary antibody (Amersham Corp., Arlington Heights, IL) and streptavidin-Texas Red (Life Technologies). The number of dystrophin-positive myofibers per muscle was determined as previously described (8,10). Antibodies specific for α-dystroglycan (156-kD dystrophin-associated glycoprotein) (18), β-dystroglycan (43-kD dystrophin-associated glycoprotein) (18,19), and adhalin (50-kD dystrophin-associated glycoprotein) (20) were also previously described.

The proportion of dystrophin-positive fibers containing centrally located nuclei was determined as previously described (8). Briefly, *mdx* muscle sections were processed for dystrophin staining (as previously described) and co-stained with propidium iodide (Sigma, St. Louis, MO) to visualize both dystrophin-positive fibers and nuclei (21).

Determinations of luciferase and β-galactosidase expression were done using previously described methods (22,23).

The *t* test was used for statistical analysis.

RESULTS

Full-length and Becker-like dystrophins. Immunohistochemical staining of normal skeletal muscle with appropriate antibodies reveals dystrophin and dystrophin-associated proteins as a continuous staining along the plasmalemma of every muscle fiber. Except for rare revertant fibers, this staining can not be seen in *mdx* muscle.

The number of dystrophin-positive myofibers was determined at 1 wk and 2 mo after the intramuscular injection of 400 μg of pRSVDy and pRSVDy-B into *mdx* mouse muscle. Control muscles were injected with 400 μg of the plasmid pUC19 under similar conditions. The number of dystrophin-positive myofibers was corrected for revertant fibers by subtracting the number of dystrophin-positive fibers observed in muscles injected with the control plasmid pUC19 which averaged 4 ± 1 and 17 ± 0.5 (mean ± SE) at 1 wk and 2 mo after injection, respectively.

Expression of the full-length and Becker-like dystrophin persisted for at least 2 mo after intramuscular injection of the corresponding expression plasmid (Fig. 2). Dystrophin was visualized by immunohistochemical staining with dystrophin antibodies specific for either the amino-terminal, rod or carboxyl-terminal domain (Fig. 3*a–c*). Full-length dystrophin localized to the sarcolemmal membrane (Fig. 3*a–c*) and the localization of both the full-length and Becker-like dystrophin was similar (data not shown). The number of dystrophin-positive fibers increased slightly from 1 wk to 2 mo after plasmid DNA injection for samples expressing either the full-length or Becker-like dystrophins (Fig. 2). Concomitant with full-length dystrophin expression was the co-localization of the dystrophin-associated proteins adhalin (Fig. 3*d*), α-dystroglycan and β-dystroglycan (Fig. 4*b* and *d*). Similar observations were made in *mdx* muscle expressing Becker-like dystrophin (data not shown).

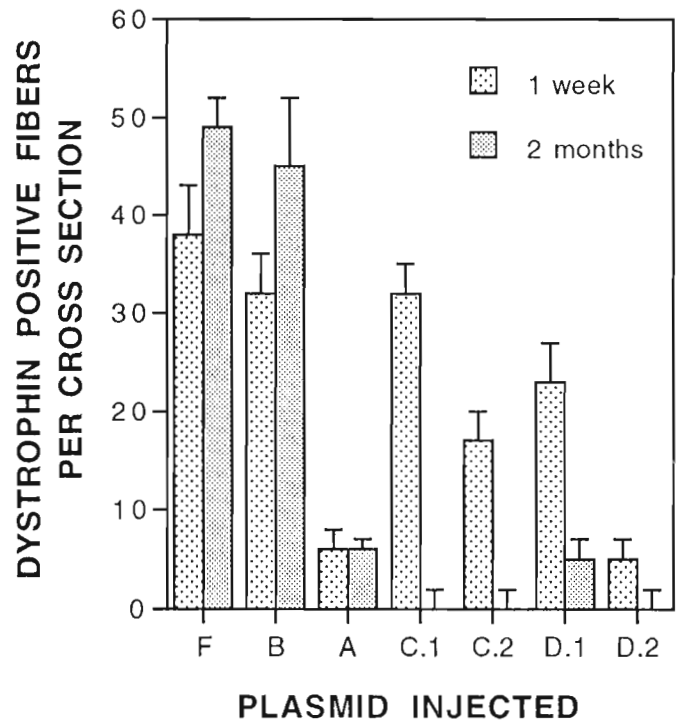

Figure 2. Duration of expression for the various dystrophin molecules in *mdx* muscle. Mice, 4–6 wks of age, were given intramuscular injections of 400 μg of full-length dystrophin expression plasmid pRSVDy (*F*), 400 μg of Becker-like expression plasmid pRSVDy-B (*B*), 400 μg of amino-terminally deleted dystrophin expression plasmid pRSVDy-A (*A*), 400 μg of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.1 (*C.1*), 400 μg of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.2 (*C.2*), 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.1 (*D.1*), or 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.2 (*D.2*). Mice were assayed for dystrophin expression at 1 wk and 2 mo after plasmid injection. At least six different quadriceps muscles (from at least six mice) were examined for each treatment group. Values represent the mean number of dystrophin-positive fibers observed per muscle at the site of plasmid DNA injection, corrected for the number of revertant fibers. *Bars* indicate SE.

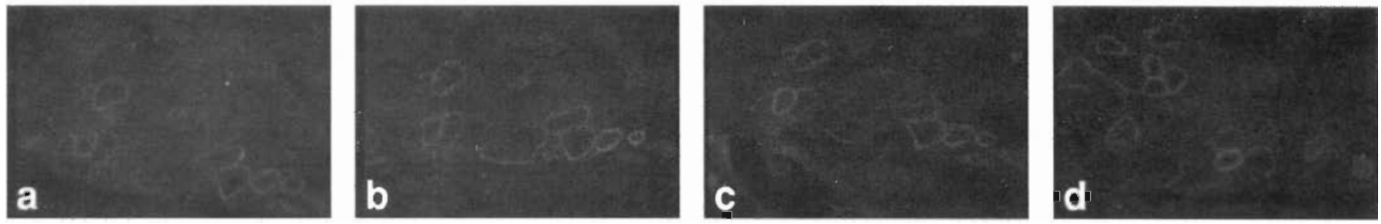

Figure 3. Immunohistochemical detection of dystrophin and adhalin in *mdx* muscle 1 wk after injection of full-length dystrophin expression plasmid pRSVDy. Serial sections stained with antibodies specific for the amino-terminal (*a*), spectrin-like repeat (*b*), and carboxyl-terminal (*c*) domains of dystrophin and for the dystrophin-associated protein adhalin (*d*). Magnification, ×62.5.

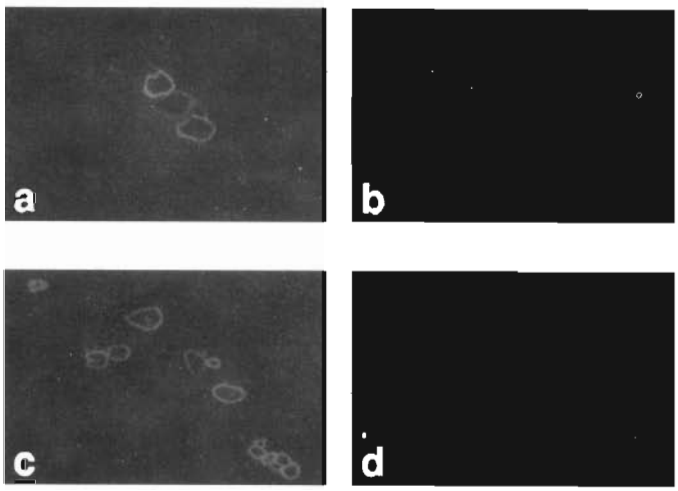

Figure 4. Immunohistochemical colocalization of dystrophin with dystrophin-associated proteins α- and β-dystroglycan in *mdx* muscle 1 wk after injection of full-length dystrophin expression plasmid pRSVDy. Two pairs of serial sections (*a*, *b*, and *c*, *d*) are shown. Sections (*a*) and (*c*) are stained with antibody specific for the spectrin-like repeat (rod), domain of dystrophin. Sections (*b*) and (*d*) were stained with antibodies specific for α- or β-dystroglycan, respectively. Magnifications are ×62.5 for (*a*, *b*) and ×40 for (*b*, *d*).

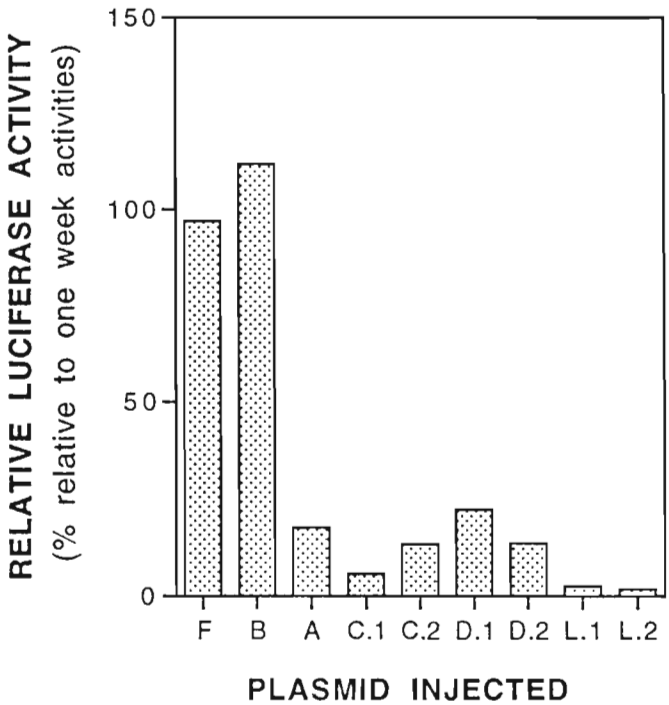

Figure 5. Stability of luciferase expression in *mdx* mouse muscle. Mice, 4–6 wk of age, were given intramuscular injections of 50 μg of pRSVDy-SV40L.1 (*F*), 50 μg of pRSVDy-B-SV40L.1 (*B*), 50 μg of pRSVDy-A-SV40L.1 (*A*), 50 μg of pRSVDy-C.1-SV40L.1 (*C.1*), 50 μg of pRSVDy-C.2-SV40L.1 (*C.2*), 50 μg of pRSVDy-D.1-SV40L.1 (*D.1*), 50 μg of pRSVDy-D.2-SV40L.1 (*D.2*), 10 μg of pSV40L.1 (*L.1*), or 10 μg of pSV40L.2 (*L.2*). Mice were assayed for luciferase expression at 1 wk and 2 mo after plasmid injection. At least six different quadriceps muscles (from at least six mice) were examined for each treatment group. Values represent the relative luciferase activity remaining at 2 mo compared with levels observed at 1 wk for each sample.

The levels of luciferase activity were determined at 1 wk and 2 mo after injection of the plasmid containing both the dystrophin and luciferase genes. Luciferase expression persisted at similar levels between 1 wk and 2 mo after plasmid DNA injection in *mdx* muscle expressing either full-length or Becker-like dystrophin (Fig. 5). Another indication of improved *mdx* myofiber health was the significantly decreased number of centrally located nuclei observed in *mdx* muscle expressing either full-length or Becker-like dystrophin 2 mo after plasmid DNA injection (Fig. 6).

Amino terminus-deleted dystrophin. The number of myofibers expressing a dystrophin which lacked its actin-binding domain was very low at both 1 wk and 2 mo in *mdx* muscle after intramuscular injection of the respective plasmid (Fig. 2). Using a panel of antibodies specific for different domains of dystrophin, revertant fibers were distinguished from those expressing the amino-terminal domain-deleted dystrophin. Those fibers expressing the amino-terminal domain-deleted dystrophin were visualized with dystrophin antibodies specific for the carboxyl-terminal domain (Fig. 7*b*), but were not visualized with the amino-terminal domain specific antibody (Fig. 7*a*). This dystrophin molecule localized to the sarcolemmal membrane and co-localized with the dystrophin-associated protein α-dystroglycan (Fig. 7*c*). Co-localization with adhalin was also observed during the expression of the amino-terminal domain-deleted dystrophin (data not shown). Luciferase expression in *mdx* muscle expressing the amino-terminal domain-deleted dystrophin did not persist to the same degree as that observed in *mdx* muscle expressing either the full-length or Becker-like dystrophin (Fig. 5). The percentage of dystrophin-positive fibers containing centrally located nuclei was greater in *mdx* muscle expressing the amino-terminal domain-deleted dystrophin number than *mdx* muscle expressing either the full-length or Becker-like dystrophin. But, this percentage was less than that observed in *mdx* muscle injected with control plasmid, pUC19 (Fig. 6).

Carboxyl terminus-deleted dystrophins. Expression of dystrophins containing carboxyl-terminal domain deletions was observed at 1 wk after intramuscular injection of the corresponding plasmid DNA but did not persist at 2 mo after

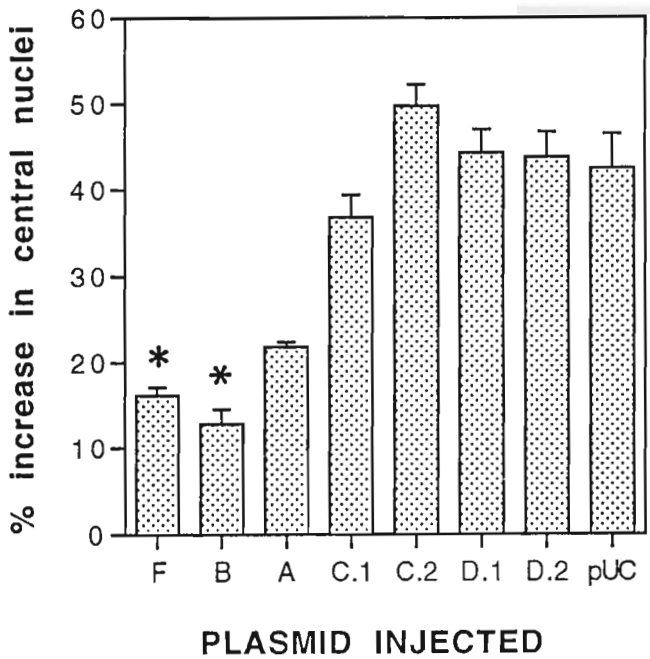

Figure 6. Percentage of dystrophin-positive fibers containing centrally located nuclei 2 mo after intramuscular injection of dystrophin-encoding plasmid DNA into *mdx* muscle. Mice, 4–6 wk of age, were given intramuscular injections of 400 μg of full-length dystrophin expression plasmid pRSVDy (*F*), 400 μg of Becker-like expression plasmid pRSVDy-B (*B*), 400 μg of amino-terminally deleted dystrophin expression plasmid pRSVDy-A (*A*), 400 μg of carboxy-terminally deleted dystrophin expression plasmid pRSVDy-C.1 (*C.1*), 400 μg of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.2 (*C.2*), 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.1 (*D.1*), 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.2 (*D.2*), or 400 μg of pUC19 (*pUC*). At least 250 dystrophin-positive fibers observed from at least four different muscles (from at least four mice) were examined for each sample. (*Statistically significant at $p < 0.05$.)

injection (Figs. 2 and 8). Using a panel of antibodies specific for either the amino-terminal, rod or carboxyl-terminal domains of dystrophin, it was possible to distinguish revertant fibers from those expressing the carboxyl-terminally deleted dystrophins. Fibers expressing the carboxyl-terminal domain-deleted dystrophin were visualized with the anti-dystrophin antibodies specific for the amino-terminal (Fig. 8*a*) and rod (Fig. 8*b*) domains, but not with the antibodies specific for the carboxyl-terminal domain (Fig. 8*c*). These truncated dystrophins localized to the sarcolemmal membrane but α-dystroglycan sarcolemmal expression was not restored by expression of this construct (Fig. 8*d*). Sarcolemmal expression of the other dystrophin-associated proteins studied was also not restored (data not shown). Fewer myofibers expressed dystrophin lacking a portion of the cysteine-rich domain and its carboxyl-terminal domain (Fig. 1, C.1) compared with myofibers expressing either the full-length, Becker-like, or dystrophin with the entire cysteine-rich domain and its carboxyl-terminal domain deleted (Fig. 1, C.2) at 1 wk after plasmid DNA injection (Fig. 2). Luciferase expression did not persist to the same degree in *mdx* muscle expressing the carboxyl-terminal domain-deleted dystrophin compared with that expressing either the full-length, Becker-like, or actin-binding domain-deleted dystrophins (Fig. 5). Compared to *mdx* muscle injected with pUC19, muscle injected with the carboxyl-terminally deleted dystrophin constructs had little difference in the number of dystrophin-positive fibers containing centrally located nuclei (Fig. 6).

Amino and carboxyl-terminus-deleted dystrophins. Expression of dystrophins lacking both the amino-terminal domain and the carboxyl-terminal domain was observed only at 1 wk after plasmid DNA injection into *mdx* muscle (Figs. 2 and 9). At this time, these double-deleted dystrophins localized to the sarcolemma and could be differentiated from revertant fibers on the basis of their reactivity with only the antibody specific for the rod domain of dystrophin (Fig. 9*b*). No dystrophin-associated proteins were observed in *mdx* muscle expressing these molecules (Fig. 9*d* and data not shown). Also, *mdx* muscle expressing the double-deleted dystrophins did not show persistent luciferase expression (Fig. 5) or decrease in the number of centrally located nuclei observed 2 mo after plasmid DNA injection (Fig. 6).

DISCUSSION

All the dystrophin constructs studied were expressed at 1 wk after plasmid DNA injection in *mdx* muscle to different extents (Figs. 2–4 and 7–9). The low level of revertant fibers in the particular strain of *mdx* mice used in this study (13) and the observation that revertant fibers do not show cytoplasmic

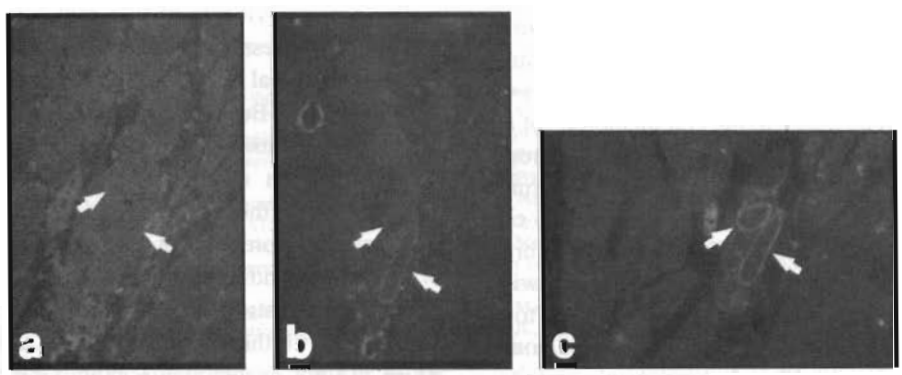

Figure 7. Immunohistochemical detection of dystrophin and α-dystroglycan in *mdx* muscle 1 wk after injection of amino-terminally deleted dystrophin expression plasmid pRSVDy-A. Serial sections stained with antibodies specific for the amino-terminal (*a*), or carboxyl-terminal (*b*) domains of dystrophin and antibody specific for α-dystroglycan (*c*). *Arrows* indicate the presumably transfected fibers expressing amino-terminally deleted dystrophin molecules. Magnification was ×40 except for (*a*) which was ×62.5.

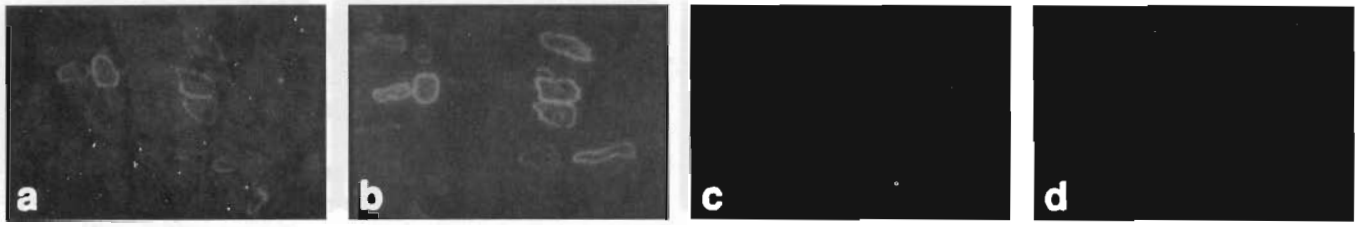

Figure 8. Immunohistochemical detection of dystrophin and α-dystroglycan in *mdx* muscle 1 wk after injection of of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.1. Serial sections stained with antibodies specific for the amino-terminal (*a*), spectrin-like repeat (*b*), and carboxyl-terminal (*c*) domains of dystrophin, and for for α-dystroglycan (*d*). Magnification, ×62.5.

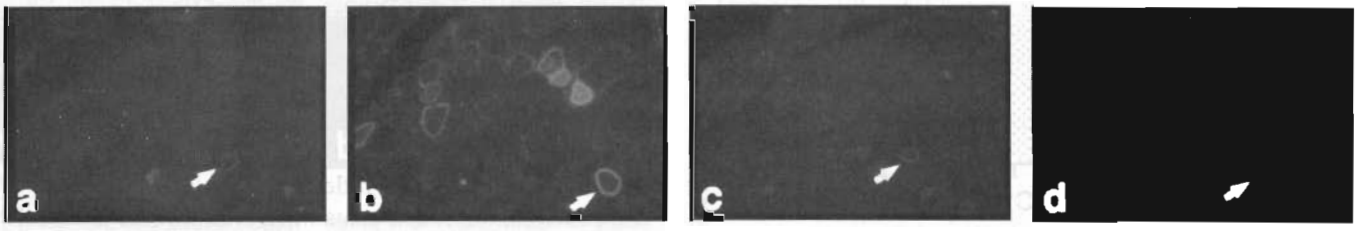

Figure 9. Immunohistochemical detection of dystrophin and adhalin in *mdx* muscle 1 wk after injection of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.1. Serial sections stained with antibodies specific for the amino-terminal (*a*), spectrin-like repeat (*b*), and carboxyl-terminal (*c*) domains of dystrophin and for adhalin (*d*). *Arrows* indicate a presumed revertant fiber. Magnification, ×40.

dystrophin staining (8) suggested that full-length and Becker-like dystrophin expression resulted from the cDNA within the injected plasmid DNA. Using a panel of antibodies specific for either the amino-terminal, rod, or carboxyl-terminal domains of dystrophin, it was possible to distinguish revertant fibers from those expressing either the amino- or carboxyl-terminally deleted dystrophins (Figs. 6–9). The differentiation of myofibers expressing the deletion-containing dystrophins from revertants further suggested that dystrophin expression and gene transfer did occur in *mdx* muscle after intramuscular plasmid DNA injection.

As previously reported (8–11), both the full-length and Becker-like dystrophins were localized at the sarcolemmal membrane (Fig. 3–4). These results were consistent with the sarcolemmal localization of dystrophin in Becker dystrophy patients that express a dystrophin lacking regions of the rod domain (34,35). After injection of the dystrophin genes lacking the amino or carboxyl terminus, the respective dystrophin proteins also were localized to the sarcolemmal membrane (Fig. 7–8). These results were consistent with observations in DMD patients in which dystrophins lacking either the amino- or carboxy terminus were localized to the sarcolemmal membrane (24–33). Similarly, independent localization of dystrophin amino and carboxyl terminus to the cell membrane of *mdx* muscle cells has recently been reported (45). Both our results and the human studies suggest that neither the amino-terminal nor the carboxyl-terminal domains of dystrophin are completely necessary for trafficking dystrophin to the membrane. These results suggest that there may be multiple regions within the dystrophin molecule responsible for its trafficking to the membrane. It is not unlikely that the portion of the rod domain not deleted from the Becker-like dystrophin also contains sarcolemmal localization cues for dystrophin and may suggest the mechanism by which the double deleted molecules localized to the membrane.

Another important indication of dystrophin function after plasmid injection is its ability to restore expression of the dystrophin-associated proteins. Expression of either the full-length or Becker-like dystrophins resulted in restoration and co-localization with the dystrophin-associated proteins in individual myofibers. This is in agreement with the expression of dystrophin-associated proteins in transgenic mice expressing full-length dystrophin (4–6). Although low numbers of dystrophin-positive myofibers were observed after the injection of plasmids encoding the amino terminus-deleted dystrophin (Fig. 2), co-localization of dystrophin-associated proteins and amino terminus-deleted dystrophin were observed (Fig. 7). However, no dystrophin-associated proteins co-localized with the fibers expressing the carboxyl-terminally deleted dystrophins (Fig. 8). This suggests that the carboxyl-terminal domain of dystrophin was essential for restoration and co-localization with the dystrophin-associated proteins. These results are in agreement with the ability of the carboxyl-terminal domain of dystrophin to complex with dystrophin-associated proteins (37–41).

We have previously shown that stability of dystrophin or luciferase expression can be used as indirect indication of myofiber survival in *mdx* mouse muscle (10). Expression of the full-length and Becker-like dystrophin persisted for at least 2 mo after intramuscular plasmid DNA injection (Fig. 2). Also in agreement with our previous study (10), co-expression of luciferase with the full-length and Becker-like dystrophin enabled stable expression of luciferase. These results also agree with similar findings using adenovirus-mediated transfer of Becker-like dystrophin (9,11). In contrast, co-expression of luciferase with the amino or carboxy terminus-deleted dystrophins did not enable stable expression of luciferase (Fig. 5). These results are consistent with the Duchenne phenotype observed in patients expressing dystrophins lacking either the amino or carboxyl terminus (24–33).

It was of interest that expression of the amino terminus-deleted dystrophin was not able to improve myofiber survivability or decrease the number of centrally located nuclei even though it enabled expression of the dystrophin-associated proteins (Figs. 2 and 5–7). This suggests that expression of the dystrophin-associated proteins and their interaction with the carboxyl-terminal domain of dystrophin was not sufficient to correct for the dystrophic phenotype in *mdx* muscle expressing the amino-terminal domain-deleted dystrophin. However, only those dystrophin constructs that restored dystrophin-associated protein expression resulted in improved myofiber survivability and a decrease in central nuclei. Most likely, myofiber survival requires a network of protein interactions encompassing the amino terminus of dystrophin and cytoplasmic elements such as actin (42), the carboxyl terminus of dystrophin and the dystrophin-associated proteins (37–41), and the dystrophin-associated proteins and extracellular elements such as laminin (18,43,44).

In summary, of the dystrophin molecules studied, only the full-length or Becker-like dystrophins exhibited long-term expression, co-localization with dystrophin-associated proteins, and persistent luciferase expression after plasmid DNA injection into *mdx* muscle (Figs. 2–5 and 7–9). This suggests that those *mdx* myofibers expressing either of these dystrophins were less susceptible to myofiber degeneration. Another indication of improved myofiber health was the decreased number of centrally located nuclei observed in *mdx* muscle expressing either full-length or Becker-like dystrophin (Fig. 6). Restoration of normal muscle morphology and function has been previously observed in transgenic *mdx* mice expressing dystrophin (4–6). This study suggests that after intramuscular injection of plasmid DNA, *mdx* myofibers expressing either full-length or Becker-like dystrophin are corrected for dystrophin deficiency and other secondary effects associated with dystrophin deficiency. Thus postnatal gene transfer may be beneficial to DMD patients provided that sufficient numbers of the myofibers express either the full-length or Becker-like DMD gene.

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