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In vivo muscle gene transfer of full-length dystrophin with an adenoviral vector that lacks all viral genes

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Duchenne muscular dystrophy (DMD) is an important target for gene transfer because of the disease's high frequency and devastating course. To date, adenoviral vector-mediated gene transfer for DMD has been unavailable because (1) adenoviral vectors were unable to accommodate the full-length dystrophin cDNA (14 kb); and (2) adenoviral vectors induced inflammatory reactions in the gene transfer recipient. We addressed both problems with a novel adenoviral vector that contains no viral genes and encodes 28.2 kb of foreign DNA including both the full-length dystrophin cDNA with the muscle creatine kinase promoter for transcriptional control and a lacZ marker gene. This report presents the in vivo expression of dystrophin and β-galactosidase from this vector in skeletal muscle of the mdx mouse, a mutant mouse that lacks dystrophin. Somatic delivery of the vector by intramuscular injection in 6-day-old mice resulted in the expression of full-length, recombinant dystrophin at the muscle membrane. Dystrophin-associated proteins were restored in muscle fibers expressing recombinant dystrophin. Mdx muscle injected with our vector showed a decrease in the proportion of fibers with nuclei located centrally; centrally placed nuclei in muscle fibers are characteristic of cycles of degeneration and regeneration suffered by dystrophin-deficient muscle tissue. These results are strong evidence that adenoviral vector-mediated full-length dystrophin delivery provides substantial somatic function.

Keywords: *Duchenne muscular dystrophy; dystrophin; gene transfer; adenoviral vector*

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of skeletal muscle caused by a defect in the dystrophin gene. The dystrophin gene is very large, comprising 2.4 megabases of genomic DNA encoding a 14 kb cDNA. Some mutations of the dystrophin gene result in the absence of the protein dystrophin at the muscle membrane.[1–3] The apparent consequence of this loss of dystrophin is instability of the membrane resulting in muscle fiber degeneration. Progressive cycles of muscle fiber degeneration and regeneration ultimately give way to failure of the regenerative mechanism, replacement of muscle tissue with fibrous and fatty connective tissue, and inevitable early death usually by age 25 years due to cardiopulmonary failure.[4] The goal of gene transfer for this disease would be to rescue muscle tissue by providing the dystrophin cDNA to a sufficient number of fibers before the failure of the regenerative process. Dystrophin protein generated from the cDNA would link the contractile apparatus with the extracellular matrix through its interaction with restored dystrophin-associated proteins.

Adenoviral vectors have significant potential utility for muscle gene transfer because of their relative safety, high titer and efficient transduction of postmitotic cells.[5] Two of the problems with adenoviral vectors, however, are their limited insert capacity and their tendency to trigger a cellular immune reaction. First-generation adenoviral vectors with deletions of the E1 and E3 regions of the viral genome have an insert capacity of only 8 kb,[6] too small to accommodate the entire 14 kb dystrophin cDNA and the promoter needed to control transcription. Therefore, truncated dystrophin cDNAs have been obtained either by cloning them from mildly affected Becker muscular dystrophy (BMD) patients[7] or by introducing in-frame deletions in the central region (rod domain) of the full-length recombinant dystrophin cDNA.[8] These shortened genes have shown efficacy in transgenic mice.[9,10] Several studies have demonstrated that first-generation adenoviral vectors encoding truncated dystrophin cDNAs can be used to deliver truncated dystrophin protein to skeletal muscle in the *mdx* mouse model by intramuscular injection.[8,11–13] However, treating a biochemical deficiency with a milder allele of the same disease is not ideal. Furthermore, the first-generation adenoviral vectors produce late viral proteins that induce a T cell-mediated immune response.[14]

Using novel technology, we recently generated a new adenoviral vector specifically to address the issues of limited insert capacity and the production of immunogenic viral proteins. The principal features of this large capacity vector are that: (1) it can package up to 30 kb of foreign DNA; and (2) it has no viral genes.[15] The first application of this large capacity adenoviral vector system,

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AdDYSβgal, was designed for gene transfer for DMD and contains a two-part expression cassette: (1) the full-length murine dystrophin cDNA under control of the murine muscle creatine kinase (MCK) promoter including the first intron; and (2) the *E. coli lacZ* gene under control of the cytomegalovirus (CMV) promoter. The vector is propagated in the 293 cell line with necessary viral functions provided by the cell line and a helper virus. Purification of the vector is accomplished by cesium chloride banding that, by current technology, leaves a helper virus level of 1% or less remaining in the purified vector stock. The helper virus is a first-generation adenoviral vector with an additional deletion of the packaging signal in order to favor packaging of the vector, which has a wild-type packaging signal, over the helper. We showed that this recombinant virus efficiently infected cultured primary muscle cells, and the foreign DNA independently expressed both dystrophin and β-galactosidase *in vitro*.[15] Here we report the expression from AdDYSβgal in dystrophin-deficient *mdx* mice *in vivo*, and demonstrate the first successful somatic delivery of full-length dystrophin at high levels to skeletal muscle.

Results

Expression and localization of dystrophin, β-galactosidase and dystrophin-associated proteins in vector-injected mdx muscle

Full-length dystrophin and β-galactosidase were both expressed in muscle fibers after an intramuscular injection of the AdDYSβgal vector into 6-day-old *mdx* mice (Figure 1A and C). Most fibers expressing dystrophin also express β-galactosidase, as would be expected. In a few fibers, dystrophin is detected, but not β-galactosidase. This discrepancy most likely occurs because the immunohistochemical detection of dystrophin, which is localized to the muscle membrane, is more sensitive than the histochemical detection of β-galactosidase, which is diffused throughout the cytoplasm. Very rarely, a muscle fiber expresses β-galactosidase, and not dystrophin. The simplest explanation for this phenomenon would be that dystrophin expression along the length of an infected muscle fiber occurs regionally in the vicinity of the nuclei containing the vector DNA. Dystrophin and β-galactosidase expression were not observed in the opposite hind limb that was sham-injected with diluent buffer except for rare revertant fibers normally seen in *mdx* muscle (Figure 1D and Figure 2B, D, F).[16]

We examined dystrophin and β-galactosidase expression from the vector at three time-points after intramuscular injection of AdDYSβgal in 6-day-old mice. At 2 weeks after injection of 2×10^7 vector particles in the right gastrocnemius muscle, membrane-localized dystrophin was detected by immunohistochemistry in regions of the vector-injected muscle, but not in the sham-injected left gastrocnemius (Figure 2A and B). At 4 weeks after injection of 2×10^7 vector particles, large areas of muscle expressed dystrophin (Figure 2C), and high levels of full-length dystrophin were detected by Western blot analysis (approximately 50% of normal levels corresponding to approximately 50% of fibers expressing dystrophin in cryosections) (Figure 3). The dystrophin expressed from the vector showed no evidence of degradation or shortened forms. At this same time-point after injection of 1×10^8 vector particles, the region of muscle expressing dystrophin was even more extensive (data not shown). At 6 weeks after injection of 2×10^7 vector particles, dystrophin continued to be expressed, but the proportion of fibers expressing dystrophin had decreased (Figure 2E). At all time-points, the expression of β-galactosidase from the vector paralleled the expression of dystrophin (data not shown).

The distribution of dystrophin expressed from AdDYSβgal and dystrophin-associated proteins (DAPs) restored to treated muscle was analyzed *in vivo* by immunohistochemistry. Full-length dystrophin expressed from the vector in *mdx* muscle was correctly localized to the sarcolemma (Figure 1C and Figure 2A, C, E). The DAPs, α-sarcoglycan, γ-sarcoglycan (not shown), β-dystroglycan and syntrophin, were expressed at the sarcolemma of those fibers expressing dystrophin in the vector-injected limb and were absent from the sham-injected limb (Figure 4).

Therapeutic effect of vector administration on mdx histopathology

In cross-sections of normal skeletal muscle, cell nuclei are found at the periphery of muscle fibers. In contrast, centrally placed nuclei are characteristic of fibers that have undergone degeneration and subsequent regeneration. Therefore, the ongoing muscle fiber degeneration and regeneration in *mdx* mouse muscle results in an increasing proportion of fibers with centrally placed nuclei as the mouse ages. In our studies, *mdx* mice receiving either 2×10^7 or 1×10^8 vector particles in a single intramuscular injection at day 6 of life and killed 4 weeks after injection demonstrated large areas of muscle with a marked decrease in the proportion of fibers with centrally placed nuclei and a decrease in the variation in fiber size (Figure 1B). Entire cross-sections of vector-injected and sham-injected gastrocnemius muscles were analyzed for the percentage of fibers harboring centrally placed nuclei (Table 1). Four weeks after a 2×10^7 vector particle injection, the percentage of muscle fibers with centrally placed nuclei was 10.8 ± 5.5 (mean ± standard deviation for the entire cross-section) for the vector-injected gastrocnemius muscle in contrast to 40.1 ± 7.4 for the sham-injected opposite gastrocnemius muscle. We also calculated the percentage of fibers with centrally placed nuclei in selected regions of the cross-section of vector-injected muscle in which most fibers expressed dystrophin. The analysis limited to these regions showed that $7.4 \pm 6.0\%$ of fibers had centrally placed nuclei. We observed similar results in mice injected with a larger amount of AdDYSβgal (Table 1). Four weeks after a 1×10^8 vector particle injection, the percentage of fibers with centrally placed nuclei, analyzed for the entire cross-section of muscle, was 17.4 ± 4.8 for the vector-injected gastrocnemius muscle in contrast to 51.5 ± 0.3 for the sham-injected opposite gastrocnemius muscle. The analysis limited to regions of vector-injected muscle with most fibers expressing dystrophin showed that $7.3 \pm 1.8\%$ of fibers had centrally placed nuclei. In contrast, 4 weeks after an intramuscular injection of only 2×10^6 vector particles no morphological difference was observed between vector-injected and sham-injected muscles (Table 1). This observation was consistent with the finding that very few fibers expressed dystrophin 4 weeks after a 2×10^6 vector particle injection (data not shown). Although mice collected at 6 weeks

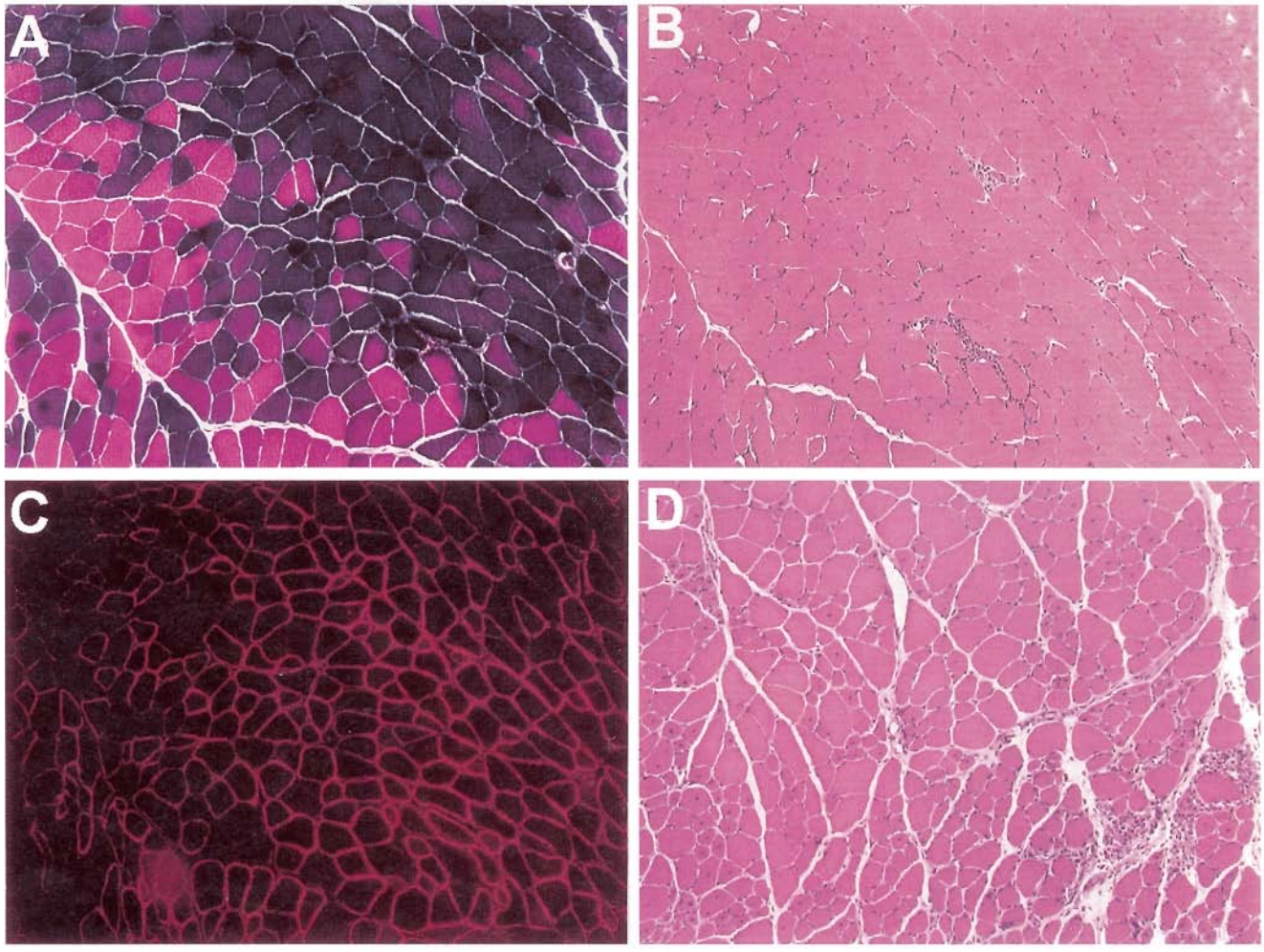

Figure 1 *Expression of dystrophin and β-galactosidase and histologic improvement after intramuscular injection of mdx mice with AdDYSβgal. Six-day-old mice were injected with 2×10^7 AdDYSβgal particles (serial sections in A, B and C) in the right gastrocnemius muscle or sham-injected with diluent buffer (D) in the left gastrocnemius muscle and analyzed for dystrophin (C) and for β-galactosidase (A and D) 4 weeks later. (A) β-Galactosidase expression in mdx muscle, delivered by AdDYSβgal. Counterstain is hematoxylin and eosin. (B) A serial section stained with hematoxylin and eosin to show muscle morphology. The improvement in histopathology in regions expressing dystrophin from AdDYSβgal contrasted with the sham-injected muscle shown in D. (C) A serial section of the same muscle demonstrated dystrophin localized to the sarcolemma. (D) No β-galactosidase expression was observed in sham-injected control muscle. Counterstain is hematoxylin and eosin. Magnification ×120.*

after a 2×10^7 vector particle injection had a lower proportion of fibers expressing dystrophin (Figure 2E), the areas expressing dystrophin showed a more normal histology than the sham-injected opposite limb (data not shown).

Discussion

We have achieved the first demonstration of efficient gene delivery of full-length dystrophin to *mdx* skeletal muscle fibers *in vivo* using a novel adenoviral vector, AdDYSβgal. This accomplishment had not been possible with first- or second-generation adenoviral vectors because their maximum capacity for foreign DNA was approximately 8 kb,[6] too short for the 14 kb dystrophin cDNA. Our vector accommodated the entire dystrophin cDNA and the 6.5 kb muscle-specific promoter, MCK.

After an intramuscular injection of AdDYSβgal into neonatal *mdx* mice, high levels of expression of both dystrophin and β-galactosidase from the vector were achieved. Other investigators have shown that an intramuscular injection of a first-generation adenoviral vector may provide long-term gene expression when the injection is performed during the first few days of life.[12,17] The longevity of recombinant gene expression may be more transient if the vector injections are performed in adult mice, as has been shown for first-generation adenoviral vectors.[14] For the large capacity adenoviral vectors lacking all viral genes, it will be important to test the longevity of recombinant gene expression after injections into adult mouse muscle.

Dystrophin expressed from the AdDYSβgal vector localized properly to the sarcolemma where it was evenly distributed around the muscle membrane in cryosections of *mdx* mouse muscle, a mutant mouse that lacks dystrophin. Recombinant dystrophin was expressed as the full-length molecule at up to 50% of the levels of normal muscle; these levels corresponded to approximately 50% of the fibers expressing dystrophin. Western blot analysis showed no evidence of dystrophin degradation or of

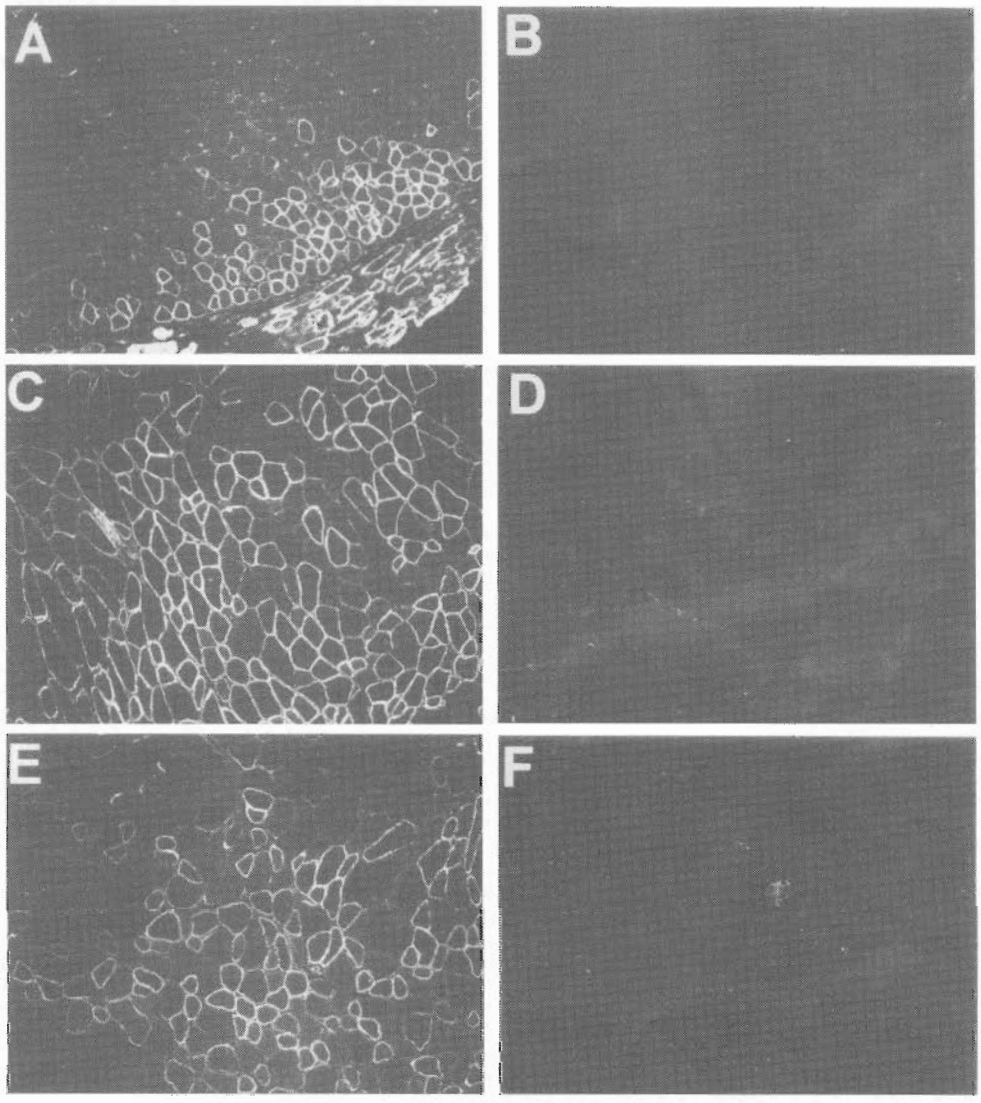

Figure 2 *Time course of dystrophin expression in mdx muscles following injection of 2×10^7 particles of AdDYSβgal. Dystrophin expression in the vector-injected right gastrocnemius muscles 2 (A), 4 (C), and 6 (E) weeks following vector injection. Absence of dystrophin expression in the sham-injected left gastrocnemius muscles 2 (B), 4 (D), and 6 (F) weeks following injection. Magnification × 120.*

truncated dystrophin molecules. Finally, in those fibers expressing dystrophin, dystrophin-associated proteins were restored and localized to the muscle membrane; this finding is characteristic of normal, healthy muscle and absent in *mdx* mouse muscle. All of these results point toward the ability of the recombinant full-length dystrophin, delivered by an adenoviral vector, to function normally.

We have shown that the MCK promoter in our vector retains its natural requirements in order to initiate transcription both *in vitro* and *in vivo*. These requirements include a tissue specificity for muscle tissue and a cell differentiation specificity for myotubes in culture or muscle fibers *in vivo*. Dystrophin from the vector is not expressed in myoblasts in culture, but is expressed after differentiation of myoblasts into myotubes.[15] Our *in vivo* studies show that dystrophin is not expressed in liver of mice receiving AdDYSβgal by an intravenous injection despite efficient transduction of hepatocytes, evidenced by expression of β-galactosidase from the vector (PRC and SK, unpublished observations). The ultimate goal of gene transfer for patients with DMD would be to deliver the vector by an intravascular route to achieve widespread distribution to muscle. Therefore, the use of muscle-specific promoters may be critical in the future so that systemically delivered vectors only express dystrophin in muscle. However, even with simple local delivery by intramuscular injection of either first-generation adenoviral vectors or AdDYSβgal, β-galactosidase expression from the vector was detected in the liver[18] (PRC, unpublished observations) and in the spinal cord[19] (PRC, unpublished observations).

Dystrophin is an essential structural component of the skeletal muscle membrane. Therefore, the observation of a significant beneficial structural effect is a reliable indication of functional recovery due to the provision of dystrophin to the muscle membrane. In *mdx* mouse muscle, as in human muscle afflicted with DMD, one characteristic of progressive cycles of degeneration and regeneration is an increase in the proportion of fibers with cen-

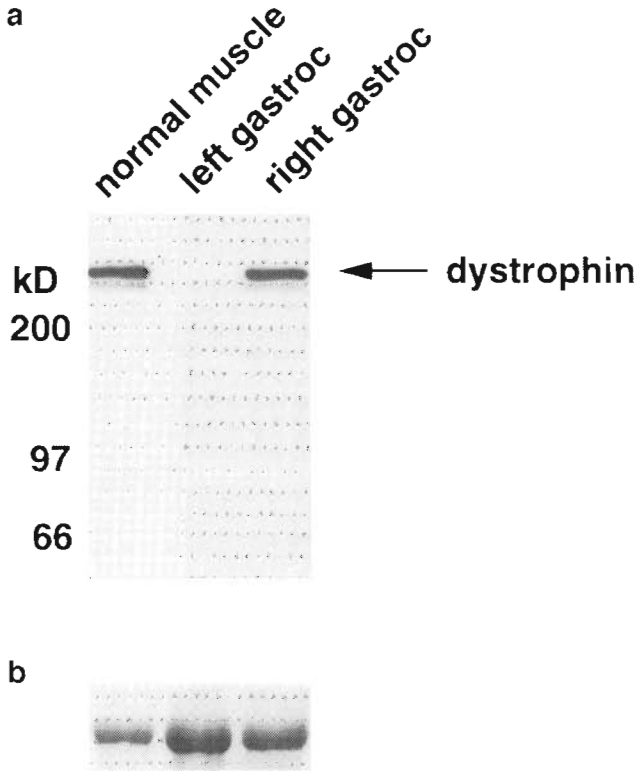

Figure 3 *Western blot analysis for dystrophin 4 weeks after injection with 2×10^7 particles of AdDYSβgal. (a) Western blot of vector-injected right gastrocnemius and sham-injected left gastrocnemius. Normal muscle demonstrates full-length dystrophin expression. (b) Coomasie Blue-stained gel after transfer to immunoblot (a).*

trally located nuclei; in healthy muscle, the nuclei are found near the cell membrane. We observed a significant improvement in muscle histopathology in *mdx* muscle treated with the AdDYSβgal vector by somatic gene transfer. In animals that received 2×10^7 or 1×10^8 vector particles, high levels of dystrophin were expressed in large regions of the injected muscle 4 weeks after injection. These levels were associated with a marked decrease in the percentage of fibers with centrally placed nuclei when compared with the sham-injected control muscle from the opposite limb. Intramuscular injection of a viral vector results in regions of the muscle cross-section that show expression of the gene delivered by the vector separated by regions of the muscle that do not show expression of the recombinant gene. Analysis of the entire cross-section of muscle for the proportion of fibers with centrally placed nuclei showed a significant difference between vector-injected and sham-injected muscle. We also calculated the proportion of fibers with centrally placed nuclei in selected regions of muscle where the majority of fibers expressed dystrophin from the vector. In these regions, the beneficial effect was even more marked. This analysis provides an indication of the potential of dystrophin to prevent dystrophic changes in muscle if a high level of transduction were uniformly achieved by somatic gene delivery. Clearly, in a clinical setting, a systemic means of widespread, uniform vector delivery would be preferable to intramuscular injection.

We observed a decrease in dystrophin expression by 6 weeks following vector administration. There are several possible reasons to explain this observation. Most likely, a T cell-mediated immune response against β-galactosidase could have resulted in the loss of transgene-positive fibers over time. T cell-mediated and B cell antibody-mediated immune responses against β-galactosidase expressed from an intramuscular injection of a first-generation *lacZ*-encoding adenoviral vector have been described recently.[20] Other factors may also have contributed to the loss of transduced fibers, but were probably of lesser significance. The expression of late viral proteins has been thought to be the central problem with the use of first-generation adenoviral vectors for somatic gene transfer. A cell-mediated immune response directed against late viral proteins was shown to have resulted in the elimination of fibers infected with a first-generation adenoviral vector.[14] In our vector, all viral genes are eliminated and, therefore, viral proteins are not produced. However, the low level of helper virus, a modified first-generation adenoviral vector, in the AdDYSβgal preparation could have contributed to a cell-mediated elimination of muscle fibers. As a third possible explanation for the loss of transgene-positive fibers over time, the episomal DNA might have been unstable in the cell nuclei. However, first-generation vectors were maintained over long periods in immunocompromised mice[14,21] or in mice that were administered very high levels of vector within the first few days of life potentially inducing immune tolerance.[12,17] Furthermore, naked DNA was maintained and continued to express transgenes in skeletal muscle for long periods.[22] Finally, the expression of full-length dystrophin itself could have induced a T cell-mediated immune response. The *mdx* mouse has a premature chain terminating mutation in the dystrophin gene that codes for a truncated protein which is therefore unstable. It is possible that the *mdx* mouse may not be immunologically tolerant to all of the epitopes of the full-length dystrophin protein. Recent reports have demonstrated host immune responses to recombinant transgene-encoded proteins delivered by first-generation adenoviral vectors.[23,24] These host immune responses have been shown to limit transgene expression, particularly when the human homologue of the recombinant protein is expressed in the mouse. Further studies will be required to detect any anti-dystrophin immune response induced by recombinant dystrophin in a dystrophin-deficient host when the protein is delivered by an adenoviral vector that lacks all viral genes. Experiments are underway to determine the relative contributions of all of the above-mentioned possible mechanisms that could result in the loss of recombinant gene expression from the vector over time.

In conclusion, we have shown efficient delivery of the full-length dystrophin cDNA to skeletal muscle of the *mdx* mouse *in vivo* by a new adenoviral vector. This is the first demonstration of providing full-length dystrophin to skeletal muscle by somatic gene transfer. We have demonstrated that delivery of recombinant dystrophin using this adenoviral vector resulted in the restoration of skeletal muscle membrane stability *in vivo*. Future studies will be directed toward the study of the response of muscle tissue to the vector and the proteins expressed by the vector, improvements in vector design, and development of systemic methods of adenoviral vector delivery to skeletal muscle.

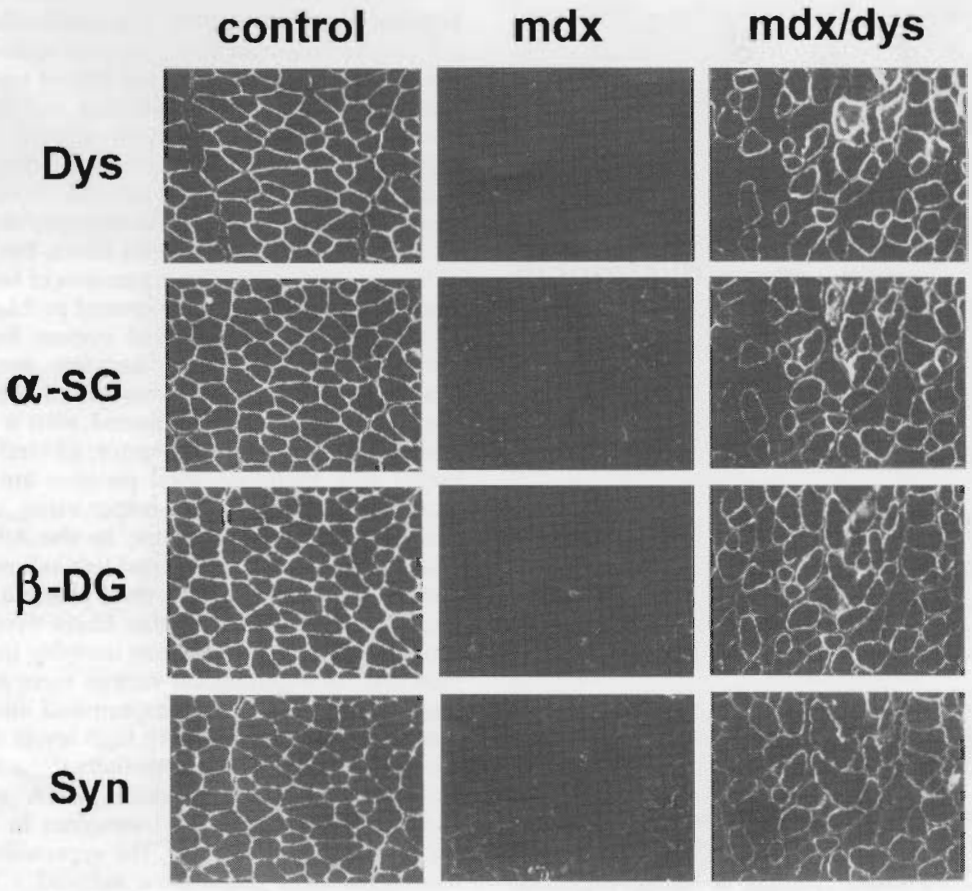

Figure 4 *Restoration of dystrophin-associated proteins 4 weeks following intramuscular injection of 2×10^7 particles of AdDYSβgal in mdx mice. Expression of dystrophin (Dys), α-sarcoglycan (α-SG), β-dystroglycan (β-DG) and syntrophin (Syn) was demonstrated immunohistochemically in normal mouse muscle (control), sham-injected mdx muscle (mdx) and AdDYSβgal-injected mdx muscle (mdx/dys). Magnification ×160.*

Materials and methods

Preparation of AdDYSβgal

The vector was developed as previously reported.[15] After propagation in 293 cells, AdDYSβgal was purified on three successive equilibrium cesium chloride gradients. The purified band was dialyzed against two changes of PBS^{2+} with 5% glycerol. Vector titer was determined by infecting 293 cells to limiting dilution and staining 18 h later for β-galactosidase. The contamination of the pur-

Table 1 Total fiber counts in treated (right) and control (left) muscles

Mouse	*No. viral particles injected*	*Part of section sampled*[a]	*Left*			*Right*		
			No. fibers with central nuclei	*Total no. of fibers*	*Percentage of fibers with central nuclei*	*No. fibers with central nuclei*	*Total no. of fibers*	*Percentage of fibers with central nuclei*
105#4	2×10^6	Entire	1516	4874	31.1	1547	4095	37.8
105#7	2×10^6	Entire	2926	7438	39.3	2464	7406	33.3
110#3	2×10^7	Entire	1956	4318	45.3	841	5706	14.7
		Selected				326	2800	11.6
110#4	2×10^7	Entire	923	2649	34.8	298	4321	6.9
		Selected				53	1689	3.1
112#4	1×10^8	Entire	2704	5276	51.3	1397	6709	20.8
		Selected				107	1262	8.5
112#5	1×10^8	Entire	2243	4336	51.7	813	5799	14.0
		Selected				122	2021	6.0

[a]Counts were done on the entire cross-section of muscle. Selected regions represent a sub-section in which the majority of fibers expressed dystrophin.

ified vector preparation with helper virus, as determined by plaque assay and by Southern blot analysis of isolated DNA, was 1% or less.

In vivo transduction of mdx mouse muscle with AdDYSβgal

Six-day-old *mdx* mice were each given a 5–10-μl intramuscular injection of purified AdDYSβgal in the posterior right hind limb. The same volume of PBS^{2+} with 5% glycerol was injected in the posterior left hind limb. Duplicate mice were used for each dose and collection time. Experimental mice injected with 2×10^6 particles were killed at 2 and 4 weeks after injection. Mice injected with 2×10^7 particles were killed at 2, 4 and 6 weeks after injection. Mice injected with 1×10^8 particles were killed at 4 weeks after injection. The left and right gastrocnemius muscles were snap-frozen in liquid nitrogen-cooled isopentane.

Detection of β-galactosidase, dystrophin and DAPs in cryosections of skeletal muscle

Muscle blocks were cryosectioned at 5–10 μm. Histochemical staining for β-galactosidase was performed as previously described.[25] Muscle sections were incubated with X-gal staining solution at 37°C for 12–16 h and subsequently counterstained with hematoxylin and eosin. Dystrophin was detected using the affinity purified anti-dystrophin antibody d10[16,26,27] as the primary antibody, biotinylated donkey anti-sheep antibody as the secondary antibody, and Texas-Red-labeled streptavidin for detection. α-Sarcoglycan, β-dystroglycan and syntrophin were detected using a rabbit polyclonal antibody against the α-sarcoglycan (adhalin) C-terminal peptide,[28] an affinity-purified sheep polyclonal antibody against dystroglycan fusion protein,[29] and an affinity-purified sheep polyclonal antibody against syntrophin fusion protein[30] for the primary antibody, respectively. After incubation with biotinylated secondary antibodies, sections were labeled with FITC-conjugated streptavidin.

To obtain percentages of fibers harboring centrally placed nuclei, serial sections were stained for β-galactosidase and immunostained for dystrophin to identify regions of β-galactosidase and dystrophin-expressing fibers and with hematoxylin and eosin to identify the position of nuclei within muscle fibers. A representative section from the mid-portion of each muscle was analyzed. Counts of total fiber number and fibers with centrally placed nuclei were made from hematoxylin and eosin-stained sections.

Protein extraction and Western blot analysis for dystrophin expression

Muscle homogenates were prepared by homogenizing several sections from the same tissue block used for immunohistochemical analysis in 50 mM Tris pH 7.4, 5 mM EDTA, 5 mM EGTA and 0.2% SDS. Homogenates were incubated on ice for 30 min and then centrifuged at 16 000 *g* at 4°C. Supernatant protein concentrations were assayed using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). SDS-PAGE and Western blot analysis were performed, as previously described.[8]

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