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Functional protection of dystrophic mouse (*mdx*) muscles after adenovirus-mediated transfer of a dystrophin minigene

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ABSTRACT **Fast skeletal muscles of *mdx* (X chromosome-linked muscular dystrophy) mice were injected after birth with a recombinant adenovirus containing a minidystrophin gene, a 6.3-kbp cDNA coding for the N- and C-terminal ends of dystrophin. Adult muscles were challenged by forced lengthening during tetanic contractions. Stretch-induced mechanical and histological damages were much reduced in injected muscles, in direct proportion of the number of fibers expressing minidystrophin. Damaged fibers were preferentially found among minidystrophin-negative regions. Minidystrophin confers an important functional and structural protection of limb muscles against high mechanical stress, even after a partial somatic gene transfer.**

Duchenne muscular dystrophy is caused by mutations within the dystrophin gene coding for a large subsarcolemma cytoskeletal protein (1). Depending on the nature of the mutation, dystrophin is either absent or present at reduced levels or in truncated forms. The phenotypes vary from the most severe Duchenne muscular dystrophy disorders to the mild forms of Becker muscular dystrophy (2). High-incidence (1/3500 boys), *de novo* mutations, heavy invalidation, premature death, and absence of treatment are strong incentives for devising gene therapy for Duchenne muscular dystrophy. We have reported the insertion into an adenovirus (Ad) vector (Ad-RSV; RSV is Rous sarcoma virus) of a shorter cDNA copy (6.3 kbp) of dystrophin (3), cloned from a 65-year-old Becker muscular dystrophy patient (4). The protein, called minidystrophin (mDys), contains a large deletion within the central rod domain of dystrophin, leaving intact the N- and C-terminal ends that interact with cytoskeletal actin and with a group of membranous glycoproteins, respectively (5, 6), sites thought essential for the function of dystrophin (7–10). In our previous work (3, 11), the recombinant Ad (Ad-RSVmDys) was injected once into muscles of newborn *mdx* mice, a dystrophin-deficient mutant (12). We showed a high efficiency of gene transfer, a correct subsarcolemma localization of mDys, its long-term expression, and the absence of histological signs of degenerative processes. Recently, two reports (13, 14) appeared showing that the expression of mDys in transgenic *mdx* mice prevented the development of dystrophic symptoms and led to normal isometric force in the diaphragm (14). The present work examines the recovery of the ability to sustain forced lengthening during contraction, an important aspect of muscle mechanics in the body (see *Results and Discussion*).

Twitch and tetanic force are normal in limb muscles of the adult *mdx* mice (15). However, when *mdx* fast skeletal muscles are submitted to forced lengthening during tetanic contractions, they produce much less force (16, 17). The force drop is irreversible and positively correlates with the number of damaged fibers (16, 17). Local disruption of the sarcolemma is the primary damage, as dystrophin-lacking fibers show a reduced mechanical resistance (18, 19). Stretch-induced damage is also observed in normal muscles, but on a much more reduced scale (16).

We report that mDys can considerably decrease the damage produced by large mechanical stress, in a whole fast muscle of *mdx* mice, even if gene transfer did not concern all fibers of the muscles.

MATERIALS AND METHODS

Muscle Preparation and Infection. The construction of the recombinant Ads Ad-RSVmDys and Ad-RSVβgal (where βgal is β-galactosidase) has been described (3, 20, 21). Ad-RSVmDys suspensions (2.5×10^9 plaque-forming units in 30 μl of saline) were injected with a 30-gauge needle into the center of the gastrocnemius of 12-day-old *mdx* mice, on one side ($n = 14$); the other side was injected with 30 μl of saline ($n = 7$) or with the Ad-RSVβgal ($n = 7$). When mice were 4 months old muscles were isolated under general anesthesia, which preserved circulation. The lateral part of the muscle was dissected out. The order of dissection of the Ad-RSVmDys limb vs. the other one was alternated from animal to animal.

Isolated muscles were submitted to repetitive stimulations (125 Hz) for 300 msec, at 5-min intervals. During the first 160 msec, tension was developed isometrically and then a forced lengthening (1 mm; i.e., +7%, at 11.1 mm/sec) was imposed. After relaxation the muscle was returned to the resting length.

Histology. At the end of the experiments, the muscles were soaked for 3 h in an oxygenated Krebs solution containing 1% orange procion (OP) to label damaged fibers and then frozen in isopentane/liquid N_2. For each muscle, three cryosections, 0.5 mm apart, were studied. All counts were made on the three sections, and the percentages of mDys$^+$, βgal$^+$, and OP$^+$ fibers (see below) were calculated over the total number of fibers (8–9000 fibers per muscle). (*i*) mDys was revealed with a polyclonal rabbit antibody recognizing both dystrophin and mDys (22). This primary antibody was visualized by using the Dakopatts rabbit PAP procedure (Glostrup, Denmark) coupled to horseradish peroxidase. mDys$^+$ fibers showed a dark brown precipitate outlining the fibers. (*ii*) βgal activity was revealed with 5-bromo-4-chloro-3-indolyl β-D-galactoside as substrate. OP$^+$ fibers were seen by their orange fluorescence excited with blue light. Counts of OP$^+$ fibers were made on the same sections as counts of mDys$^+$ fibers. Due to the dissection, several OP$^+$ fibers were present in the first layer of fibers, at the periphery, even in normal unstretched muscles; they were never included into the counts. Three other cryosections per muscle were also used for counting OP$^+$ fibers, and fibers with central nuclei or peripheral nuclei (hematoxylin/eosin staining).

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Abbreviations: mDys, minidystrophin; Ad, adenovirus; RSV, Rous sarcoma virus; βgal, β-galactosidase; OP, orange procion.

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Statistical Analysis. Student *t* tests were performed on the paired differences between results from each *mdx*/Ad-RSVmDys muscle pair (see Tables 1 and 2).

RESULTS AND DISCUSSION

Tetanic Contractions with Forced Lengthening. The mechanical response is illustrated at the Fig. 1. The force drop was quantified as $(F_1 - F_7)/F_1$, where F_1 is the isometric force developed just before lengthening in the first tetanus and F_7 is that of the seventh one (F_1 was not significantly different in *mdx* and Ad-RSVmDys muscle: 748 ± 64 vs. 812 ± 57 mN; mean ± SEM; $n = 7$). Table 1 gives the force drop observed for the seven pairs of Ad-RSVmDys and *mdx* gastrocnemius. In *mdx* muscles, the force drop averaged 63%. In the Ad-RSVmDys muscles, the force drop was reduced in all cases. The reduction was very variable, with an average difference of 20%, which is highly significant ($P < 0.001$). In muscles from normal age-matched mice, the force drop was only 20 ± 1.7% (mean ± SEM; $n = 7$; no animal or left–right difference, data not shown). Thus the Ad-RSVmDys muscles displayed the capacity to withstand forced lengthening halfway between that of *mdx* and normal muscles. Another series of seven young *mdx* mouse muscles were injected on one side with Ad-RSVmDys and on the other side with Ad-RSVβgal, as a control virus expressing a foreign protein. They were sacrificed at 60 days. The average force drop was 55% for Ad-RSVβgal-injected muscles, a value similar to that observed on *mdx* muscle (63%, Table 1), but the force drop in Ad-RSVmDys muscles was reduced in seven of seven cases and was on average 37% (individual data not shown). The highly significant ($P < 0.001$) difference, 18%, was similar to that obtained in the experiments shown in Table 1 (20%). Thus viral injection and infection have no protective effect *per se.*

Force Drop and mDys Expression. Gene transfer did not affect 100% of the fibers (3) thus Ad-RSVmDys muscles contained variable mixtures of mDys$^+$ and *mdx* fibers. mDys expression will be quantified hereafter as the percentage of mDys$^+$ fibers (this approach does not quantify the amount of mDys in individual mDys$^+$ fibers.) We have estimated (11) that mDys$^+$ fibers had a mDys content of ≈50% of the normal dystrophin content. Fig. 2*A* shows the inverse relationship between the size of the force drop and the percentage of mDys$^+$ fibers. The variations in the number of mDys$^+$ fibers explain the variations in force drop among the various Ad-RSVmDys muscles (Table 1). This was the case for each animal where the difference in force drop between each pair of *mdx* and Ad-RSVmDys muscles is plotted vs. mDys expression: the higher the latter, the larger the difference in force drop (Fig. 2*B*). Fig. 2*B* shows, by extrapolation, that if mDys expression was <10%, no difference could be detected with this test. Sections of Ad-RSVβgal-injected muscles were similarly examined for βgal expression. The percentage of βgal$^+$ fibers and the corresponding force drop are totally unrelated parameters (Fig. 2*C*).

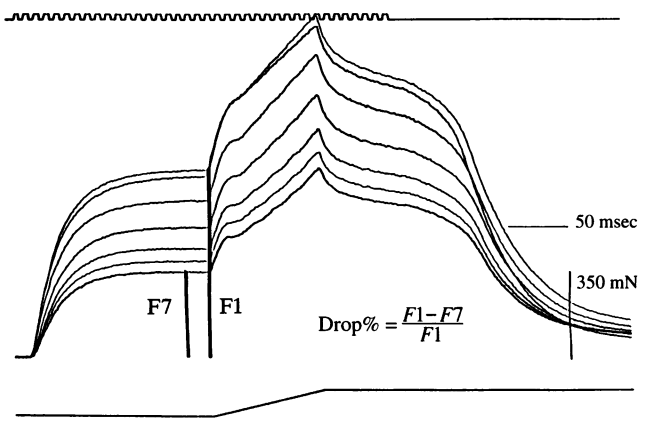

FIG. 1. Superimposed traces of tension produced by a gastrocnemius muscle during seven tetanic contractions with forced lengthening (top trace, duration of stimulation; middle traces, force records; bottom trace, length change).

Table 1. Force drop in paired muscles after seven tetani with imposed lengthenings

Animal	Force drop, % *mdx*	Force drop, % Ad-RSVmDys	Difference (*mdx* − Ad-RSVmDys)
A	82.5	74.2	8.3
B	64.4	58.5	6.0
C	59.6	44.3	15.3
D	54.2	29.2	25.0
E	67.1	43.2	23.9
F	54.3	33.2	21.1
G	62.4	25.1	37.3
Mean	63.5 ± 3.7	44.0 ± 6.6	19.5 ± 4.1*

Animals A–G are the same as in Fig. 2 *A* and *B*. Data are the mean ± SEM and the mean of the paired differences (±SEM; *, $P < 0.001$).

The Force Drop and the Number of Damaged Fibers. As in previous works (16, 17), damaged fibers were detected by the penetration of an extracellular dye, OP. The force drops and the percentages of OP$^+$ fibers are linearly correlated (Fig. 2*D*), and the same relationship holds for *mdx*, Ad-RSVmDys, and normal muscles. The slope of the relation is smaller than unity, as reported (16, 17). Most likely, penetration of OP was punctual and cross-sections might have missed it, by lack of complete longitudinal diffusion of the dye. Force drop and OP$^+$ fibers were observed even in normal muscles (Fig. 2*D*). This implies that among the OP$^+$ fibers counted in Ad-RSVmDys muscles, some were *mdx* fibers and some mDys$^+$ fibers. Do OP$^+$ fibers occur with the same frequency among all fibers, irrespective of the expression of mDys?

Stretch-Damaged Fibers and mDys Expression. From the previous counts, we selected the fibers that were simultaneously OP$^+$ and mDys$^+$ and those that were OP$^+$ and mDys$^-$ (i.e., remained *mdx*). Both counts were divided by the total number of either mDys$^+$ fibers or mDys$^-$ fibers, respectively. Table 2 shows that in all cases the occurrence of OP$^+$ damaged fibers was lower among the mDys$^+$ fibers; on average it was 3 times lower, a highly significant difference ($P < 0.001$). This difference is illustrated in Fig. 3. However, as seen in Fig. 3*D*, damaged OP$^+$ fibers are sometimes swollen and, sometimes, we experienced difficulties in unambiguously detecting the presence of mDys. To evaluate the fraction of OP$^+$ and mDys$^+$ fibers not detected, we made a similar analysis on stretched muscles from the normal mice, where 100% of the fibers are expected to be Dys$^+$. It turned out that only 67% of the OP$^+$ fibers can be detected as Dys$^+$ fibers. We probably underestimated mDys$^+$ fiber occurrence among OP$^+$ fibers in Ad-RSVmDys muscles, so that the numerator of the (OP$^+$ + mDys$^+$)/mDys$^+$ ratios should be multiplied by 1.49. This would change the mean ratio from 3.9 to 5.3 ($\times 10^{-2}$). Even then, the difference remains highly significant ($P < 0.001$). Nuclei of fibers expressing mDys have a peripheral location as in normal fibers (11), while in *mdx* fibers they remained central. We studied the distribution of the OP$^+$ fibers among the fibers with central nuclei and with peripheral nuclei. As seen in Table 2, the occurrence of damaged fibers is about 3 times lower in fibers with peripheral nuclei. However, the above analysis overlooks that mDys$^+$ fibers occurred in clusters, rather clearly defined from regions of *mdx* fibers (Fig. 3). OP$^+$ fibers were preferentially found in the *mdx* regions. This was quantified by measuring the density of OP$^+$ fibers in clusters of mDys$^+$ fibers and in *mdx* regions. Table 2 shows that

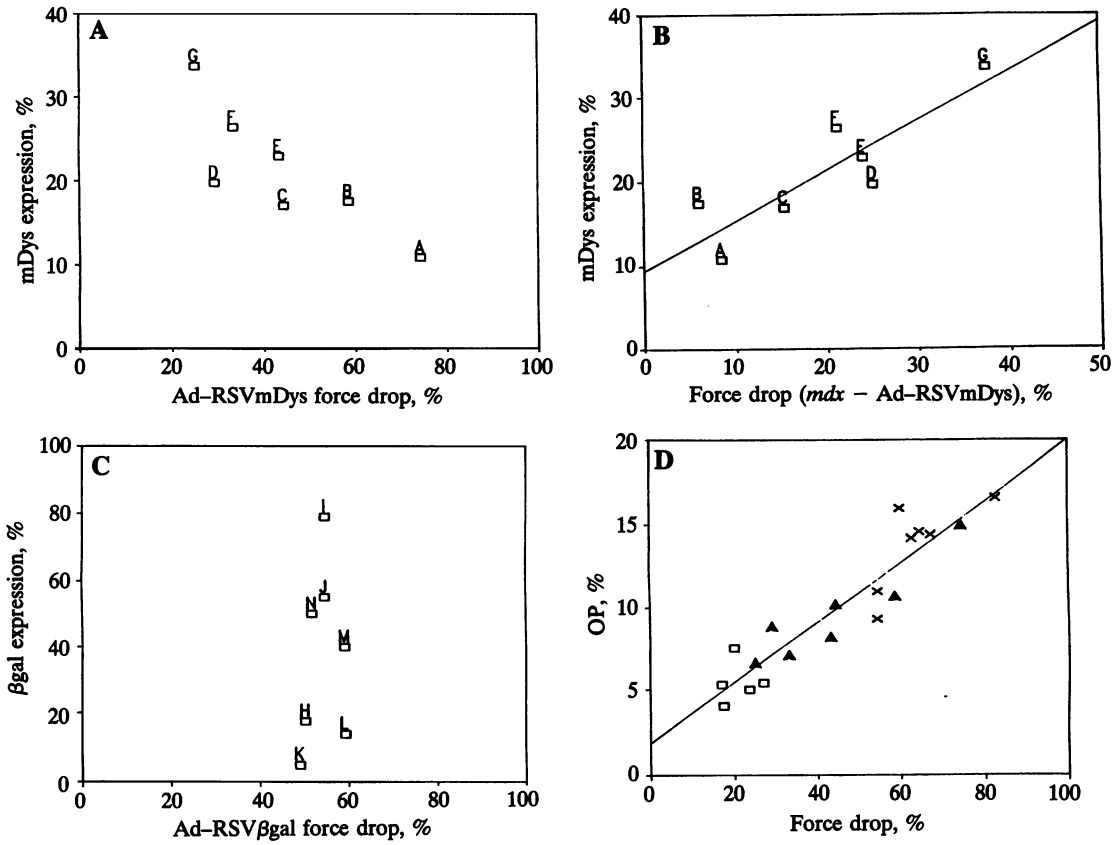

FIG. 2. (*A*) Force drop in Ad-RSVmDys muscles and the mDys expression. A–G, different animals of Table 1. (*B*) The force drop is expressed as the difference between paired *mdx* and Ad-RSVmDys muscles; A–G refer to the same animals as in *A*. (*C*) Force drop of Ad-RSVβgal-injected muscles and the expression of βgal; H–N refer to different animals. (*D*) Percentage of damaged fibers in muscles after the lengthening test, identified by OP labeling. Crosses, *mdx* muscles; triangles, Ad-RSVmDys muscles; squares, normal muscles (C57BL/10 mice).

the density of the OP^+ fibers was, on average, about 3 times lower in the $mDys^+$ clusters than in the *mdx* regions, and the difference, though variable, was found in seven of seven cases.

Contractions with forced lengthening, called "eccentric contractions," are not laboratory oddities. They occur during normal muscle function to produce "braking" movements, e.g., as during walking (23), but they are not restricted to the function of limb muscles: the diaphragm is subject to eccentric contraction during expiration (24). As dystrophin-deficient muscles are particularly sensitive to the high stress generated during these contractions (16, 17), it was thus very critical to see whether $mDys^+$ fibers not only recover a normal force (14) but also can sustain eccentric contractions.

This work demonstrates that mDys can considerably increase the resistance to damage produced by elevated mechanical stress in a whole muscle. Ad-mediated gene transfer, however, was partial and variable. Our experiments were thus performed on "mixed" muscles composed of *mdx* and of $mDys^+$ fibers, and the properties of each type of fiber could not be studied separately. Detection of $mDys^+$ and of OP^+ fibers brought our analysis to the fiber-to-fiber level. On average, fiber damage was about 3 times less frequent among $mDys^+$

Table 2. Distribution of the OP^+ fibers in Ad-RSVmDys muscles submitted to the imposed lengthening test

Animal	No. OP^+ and $mDys^+$ fibers: No. $mDys^+$ total ($\times 10^2$)	No. OP^+ and $mDys^-$ fibers: No. $mDys^-$ total ($\times 10^2$)	No. OP^+ and Np fibers: No. Np total ($\times 10^2$)	No. OP^+ and N_c fibers: No. Nc total ($\times 10^2$)	No. fibers per mm^2: $mDys^+$ region	No. fibers per mm^2: *mdx* region
A	4.4	17.3	4.9	25.1	23.1	83.2
B	7.8	12.1	7.4	23.8	14.0	57.1
C	5.1	11.4	7.1	17.9	31.0	66.3
D	3.9	10.1	6.9	15.3	17.8	56.8
E	1.5	10.3	7.9	12.4	10.3	46.5
F	2.2	8.8	5.4	12.9	16.0	40.4
G	3.1	8.3	4.3	10.1	18.4	53.0
Mean	3.9 ± 0.79	11.2 ± 1.2	6.3 ± 0.53	16.8 ± 2.2	18.7 ± 2.5	57.6 ± 5.3
MPD	7.2 ± 1.09*		10.5 ± 2.16*		39.0 ± 4.1*	

Relative occurrence of OP^+ fibers among the $mDys^+$ fibers and the *mdx* ($mDys^-$) fibers and the relative occurrence of OP^+ fibers among fibers with central nuclei (Nc) and with peripheral nuclei (Np) are shown. The density of damaged OP^+ fibers in regions of $mDys^+$ fibers and of *mdx* fibers, expressed as the number of fibers per mm^2 of each region area (measured by planimetry on low-magnification photographs), is also shown. Criteria: a given OP^+ fiber was considered to belong to a $mDys^+$ or *mdx* region if it was surrounded by, at least, five fibers of the same type, $mDys^+$ or *mdx*. Any OP^+ fiber located at the limits of two regions was counted as 0.5 for either region; these fibers contributed little to the counts. Data are the means ± SEM and the mean of the paired differences (MPD) (±SEM), which are highly significant (*, $P < 0.001$).

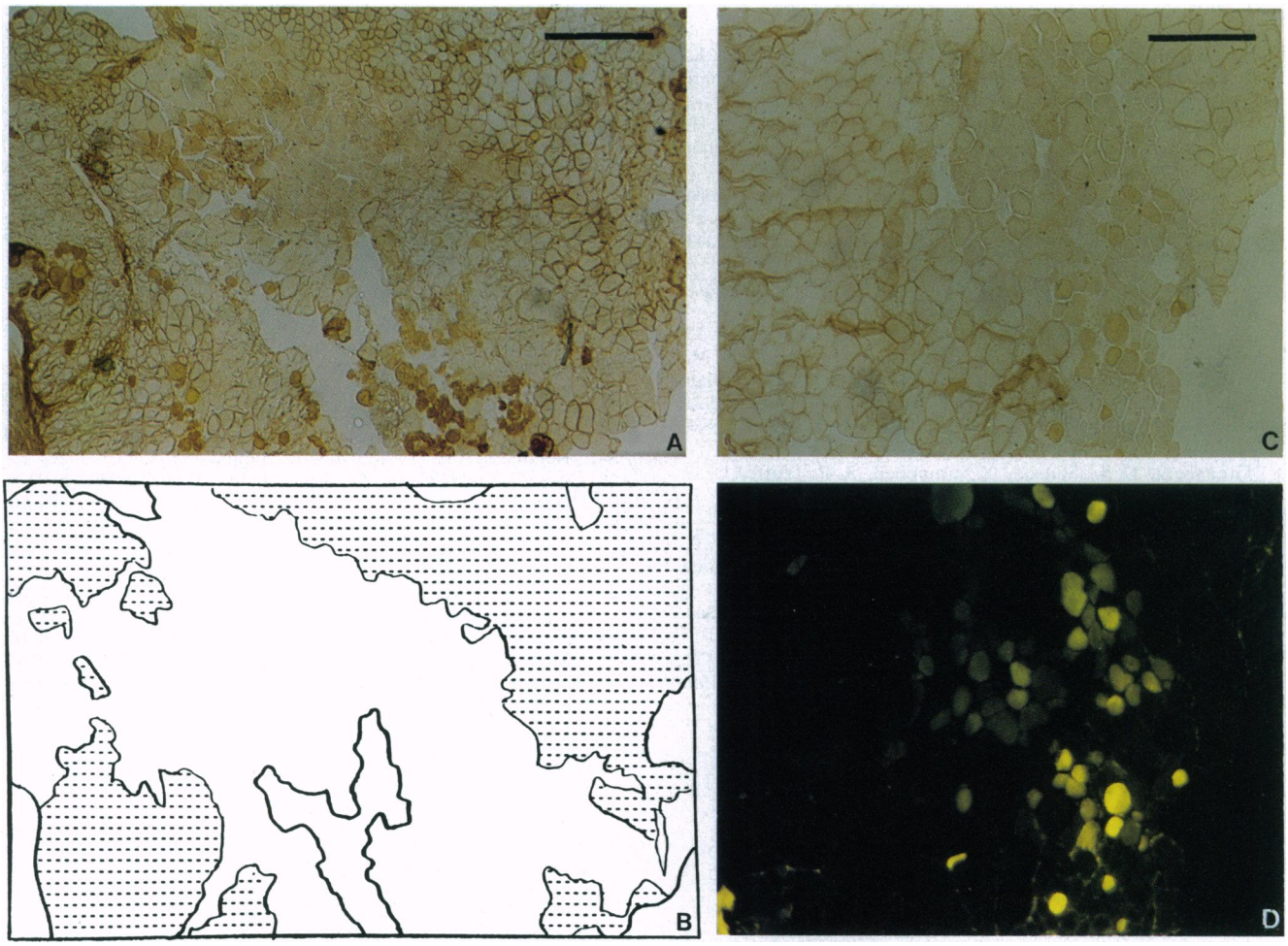

FIG. 3. (*A* and *B*) Low-magnification photograph (*A*) and a contour drawing (*B*) of a part of a transverse cross section of an Ad-RSVmDys gastrocnemius muscle, showing the area of mDys$^+$ (hatched) and *mdx* (blank) fibers. (*C* and *D*) Typical example showing the preferential occurrence of damaged OP$^+$ fibers in the area of *mdx* fibers. (*C*) Light microscopy, an mDys$^+$ area is shown on the left side of the photograph. (*D*) The corresponding epifluorescence image, showing the localization of OP$^+$ fibers, outside the mDys$^+$ area. (Bars: *A* and *B*, 500 μm; *C* and *D*, 200 μm.)

fibers (Table 2), and there is also a 3/1 ratio in the force drop difference between *mdx* and normal muscles (Table 1). This suggests that mDys$^+$ fibers could resist breaking as efficiently as normal ones (a definite proof would require experiments on a single fiber). Indeed, normal histological phenotype and the presence of the dystrophin-associated proteins in the sarcolemma parallel mDys expression (3, 13, 25, 26).

Furthermore, our results indicate the proportion of mDys$^+$ fibers needed to provide a significant benefit. On the one hand, a minimal threshold of around 10% of mDys$^+$ fibers seems necessary (Fig. 2*B*); on the other hand, extrapolation of the results of Fig. 2*A* suggests that mechanical resistance would be as good as normal muscle (i.e., a 20% force drop, Table 1) if about 40% of its fibers were mDys$^+$. Neither a transfer in all fibers nor an even distribution of the mDys$^+$ fibers seems required to confer apparent full protection of the whole muscle. This may be a consequence of the parallel arrangements of the fibers: the stronger mDys$^+$ fibers could bear most of the stress and protect the weaker *mdx* ones. This protective effect could be quantified: data of Table 1 and Fig. 2*D* show that, after lengthening, the average percentages of OP$^+$ fibers in *mdx* (0% mDys$^+$) on the one hand and in normal (similar to 100% mDys$^+$) muscles on the other hand were 13 and 4.5%, respectively. If we assume that, between these two extremes, there is an inverse linear relationship between the percentages of mDys$^+$ and of OP$^+$ fibers, a muscle containing, e.g., 33% mDys$^+$ fibers (as in mouse G, Fig. 2*D*) would be expected to contain 10.3% of OP$^+$ fibers. Instead, it contained only 6.6%. Interestingly, patient surveys suggest that either expression of 30–40% of normal dystrophin level in all fibers or 50% of evenly distributed normal nuclei protect muscles from severe damage (27, 28). This expression level previously found in injected muscles by Western blots (11) and the extrapolation to the mDys$^+$ fiber percentage (40%) necessary for a complete protective effect are both in the range of that found in patients where severe muscle damage are absent (27, 28). The Ad-mediated transfer seems the most efficient way to attain a high percentage of mDys$^+$ fibers (11) by direct somatic transfer to newborn animal. The expression of mDys so obtained enables *mdx* fibers to recover a mechanical resistance comparable to normal fibers, when challenged by the most stringent mechanical test. Functionally, mDys appears to be a satisfactory surrogate for dystrophin in restoring membrane strength.

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