

# Normal Myogenic Cells from Newborn Mice Restore Normal Histology to Degenerating Muscles of the mdx Mouse

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**Abstract.** Dystrophin deficiency in skeletal muscle of the x-linked dystrophic (mdx) mouse can be partially remedied by implantation of normal muscle precursor cells (mpc) (Partridge, T. A., J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel. 1989. *Nature (Lond.)*. 337:176-179). However, it is difficult to determine whether this biochemical "rescue" results in any improvement in the structure or function of the treated muscle, because the vigorous regeneration of mdx muscle more than compensates for the degeneration (Coulton, G. R., N. A. Curtin, J. E. Morgan, and T. A. Partridge. 1988. *Neuropathol. Appl. Neurobiol.* 14:299-314). By using x-ray irradiation to prevent mpc proliferation, it is possible to study loss of mdx muscle fibers without the complicating effect of simultaneous fiber regeneration. Thus, improvements in fiber survival resulting from any potential therapy can be detected easily (Wakeford, S., D. J. Watt, and

T. A. Partridge. 1990. *Muscle & Nerve.*)

Here, we have implanted normal mpc, obtained from newborn mice, into such preirradiated mdx muscles, finding that it is far more extensively permeated and replaced by implanted mpc than is nonirradiated mdx muscle; this is evident both from analysis of glucose-6-phosphate isomerase isoenzyme markers and from immunoblots and immunostaining of dystrophin in the treated muscles. Incorporation of normal mpc markedly reduces the loss of muscle fibers and the deterioration of muscle structure which otherwise occurs in irradiated mdx muscles. Surprisingly, the regenerated fibers are largely peripherally nucleated, whereas regenerated mouse skeletal muscle fibers are normally centrally nucleated. We attribute this regeneration of apparently normal muscle to the tendency of newborn mouse mpc to recapitulate their neonatal ontogeny, even when grafted into 3-wk-old degenerating muscle.

**I**MPLANTATION of myogenic cells into skeletal muscles of animals suffering from recessively inherited myopathies has been conducted largely with a view to developing methods of therapy in man (Law et al., 1988; Morgan et al., 1988; Partridge et al., 1989; Karpati et al., 1989; Watt et al., 1982, 1984). For the demonstration of "biochemical rescue" by implantation of muscle precursor cells (mpc)<sup>1</sup> from normal mice, the dystrophin-deficient mdx mouse (Bulfield et al., 1984; Hoffman et al., 1987) has proven most useful; the extent of replacement of the missing gene product being verified quantitatively on immunoblots and histologically by immunostaining (Partridge et al., 1989). Up to 30% of fibers in treated muscles were converted from dystrophin negative to dystrophin positive. Normal human myoblasts can also fuse with mdx muscle in vivo and synthesize dystrophin in a small percentage of fibers (Karpati et al., 1989).

Despite its genetic and biochemical homology to Duchenne

muscular dystrophy (DMD) (Hoffman et al., 1987), the mdx mouse has limitations as a model of this disease. As in DMD subjects, the mdx mouse suffers widespread degeneration of its skeletal muscle fibers. This is most striking between 20 and 100 d but persists, less conspicuously, throughout its life (Carnwath and Shotton, 1987; Coulton et al., 1988a). However, the counterbalance of regenerating new fibers results in an animal never severely weakened and eventually growing larger and stronger than the wild-type (Anderson et al., 1987; Coulton et al., 1988b).

To convert the mdx mouse into a model where we can evaluate any potential therapy in terms of improved muscle fiber survival, we have inhibited the regeneration of its muscle by applying local high doses of x rays (Wakeford et al., 1990). The resulting failure to replace degenerating fibers leads to atrophy and to histological disorganization as the few surviving fibers lose their normal close-packed fascicular arrangement and become progressively separated by a loose cellular connective tissue. Here, we show that the incorporation of wild-type neonatal mpc into such preirradiated mdx muscle counteracts this massive fiber loss and leads to the retention of an almost normal histological appearance.

1. *Abbreviations used in this paper:* DMD, Duchenne muscular dystrophy; EDL, extensor digitorum longus; GPI, glucose phosphate isomerase; MPC, muscle precursor cells; TA, tibialis anterior; X irradiation, x-ray irradiation.

## Materials and Methods

### Strains of Mouse

As hosts, we used mdx nude mice, which were derived as described previously (Partridge et al., 1989). These were of Gpi-1s<sup>a</sup> allotype. As mpc donors, we used newborn normal mice of C57Bl/10ScSn or C57Bl/6J strains, or C57Bl/10 mdx mice, all of Gpi-1s<sup>b</sup> allotype.

### X-ray Irradiation

16-d-old mdx nu/nu mice were anesthetized (Watt et al., 1982) and their right legs were subjected to 18 Grays of x-ray irradiation (X irradiation), the rest of the body being protected by a 3-mm-thick lead shield (Wakeford et al., 1990).

### Cell Implantation

Muscle cells for implantation were prepared by enzymatic disaggregation of neonatal mouse muscle (Watt et al., 1982), and pellets containing  $5 \times 10^5$  cells were prepared. This is a mixture of myogenic and nonmyogenic cells (Morgan, 1988). Between 3 and 5 d after irradiation, host mice were anesthetized and an incision was made in the skin overlying the tibialis anterior (TA) muscle of the right leg. Cell pellets were taken up into the tip of a 5- $\mu$ l Hamilton syringe which was inserted at a shallow angle along the length of the muscle and withdrawn as the contents were expelled. The TA and the extensor digitorum longus (EDL) muscles were each injected with 3–5  $\mu$ l of culture medium containing  $5 \times 10^5$  cells or, as a control, of culture medium alone, in a single injection site. The skin was then closed with fine sutures.

Wild-type C57Bl/10 muscle cells were injected into five mice 3 d after X irradiation and wild-type C57Bl/6J muscle cells were injected into four mice 4 d after X irradiation. Medium alone was injected into four mice 3 d after X irradiation and mdx muscle cells were injected into three mice 5 d after X irradiation. This age, 19–21 d, was chosen such that the mdx muscle should be about to undergo massive necrosis (Coulton et al., 1988a).

### Analysis of Muscles

The TA, EDL, and peroneus muscles were removed from the irradiated and contralateral (control) legs at 7, 10, 14, 21, 29, 49, and 67 d after the implantation of cells or medium. The muscles of the right leg and of seven of the left legs were bisected transversely. The proximal half was mounted in Tissue Tec OTC compound and frozen in isopentane cooled to  $-165^\circ\text{C}$  in liquid nitrogen. The distal half was fixed in formalin and processed for wax histology. For the remaining left legs, only wax histology of the distal halves of the muscles was examined.

Three 5- $\mu$ m-cryostat sections, spaced 100  $\mu$ m apart, were cut from each of the muscles into which mpc had been implanted. The glucose-6-phosphate isomerase (EC 53.1.9; GPI) isoenzymes from one section, at each level, were separated by IEF and visualized (Morgan et al., 1987). The remaining sections were stained with haematoxylin and eosin for histological examination. The relative proportions of donor and heterodimer GPI were estimated by reflectance-scanning photographs of the separated GPI isoenzymes on a Shimadzu dual wavelength TLC scanner. Absolute quantitation is made unreliable by multiple banding of each GPI isozyme in IEF gels and by the problem of ensuring photography in the linear phase of development of the staining reaction. However, the scans do give reliable ordinal data on the relative activities of the isomers present in a given section. This in turn is a good sample of average host/donor composition of the muscle as a whole (Peterson and Pena, 1984). 8- $\mu$ m-thick cryostat sections cut from each of the muscles from the injected legs and from the contralateral legs of five mice, were thawed onto slides coated with gelatin (0.5%) and chrome alum (0.2%) for immunofluorescent visualization of dystrophin (Partridge et al., 1989). This gives a good indication of host/donor composition of the muscle fibers local to the section (Karpati et al., 1989; Partridge et al., 1989). The serial cryosection was stained with haematoxylin and eosin for histological examination.

After GPI isoenzyme analysis and dystrophin immunostaining, the remaining portions of TA muscles removed 29–67 d after injection of wild-type mpc, mdx mpc, or medium alone were solubilized and total muscle protein was subjected to dystrophin immunoblotting (Hoffman et al., 1988). The immunoblot, after use for dystrophin detection, was reprocessed with mAb D2 (Kaprielian and Fambrough, 1987) for detection of a fast twitch

isoform of the Ca<sup>2+</sup> Mg<sup>2+</sup> ATPase, as a control for the muscle protein content of each lane.

Sections cut from three levels, 100  $\mu$ m apart, of the paraffin-embedded distal portions of the injected and contralateral muscles were stained with haematoxylin and eosin for routine histological analysis. The percentage of centrally nucleated fibers was counted on a section from the mid-zone of each muscle. The percentage of dystrophin-positive fibers was counted on the immunocytochemically stained section from each muscle.

Detailed results from the analysis of TA, EDL, and peroneus muscles injected with wild-type mpc and mdx mpc are shown in Fig. 1.

## Results

### X-irradiated Muscles Injected with Culture Medium

TA, EDL, and peroneus muscles of four mice were examined at 29, 49 (two mice), and 67 d after injection of medium. Nonirradiated muscles of the left legs of these animals were of typical mdx appearance, with many close-packed muscle fibers (a large proportion of which were centrally nucleated) and areas of necrosis and regeneration (Fig. 2, *a* and *b*).

Muscles of the right legs, which had been irradiated at 16 d and injected with culture medium at 19 d, all showed the histopathological features of irradiated mdx muscles described previously (Wakeford et al., 1990), with marked atrophy involving loss of muscle fibers (Fig. 3 *a*). Necrotic fibers were seen, but there was no evidence of regeneration. The few surviving muscle fibers, only small proportions of which (0.5–3.6%) were centrally nucleated, were of large average diameter and were separated from one another by a loose cellular connective tissue (Fig. 3 *b*). As expected, these muscles contained only host type GPI (Fig. 4).

In one peroneus, examined at 49 d after injection of medium, a group of three dystrophin-positive fibers was found; another, examined at 67 d after injection of medium, contained one dystrophin-positive fiber. These sporadic, dystrophin-positive fibers occur in mdx muscles, singly, or in clusters of up to four contiguous profiles, and are thought to reflect somatic cell mutation (Hoffman et al., 1990). These “revertant” positive fibers are found with a frequency in our mdx colony of much less than one profile per section of EDL, TA, or peroneus muscle and are not augmented by high doses of x rays, so they do not interfere significantly with identification of positive fibers derived from injected normal mpc.

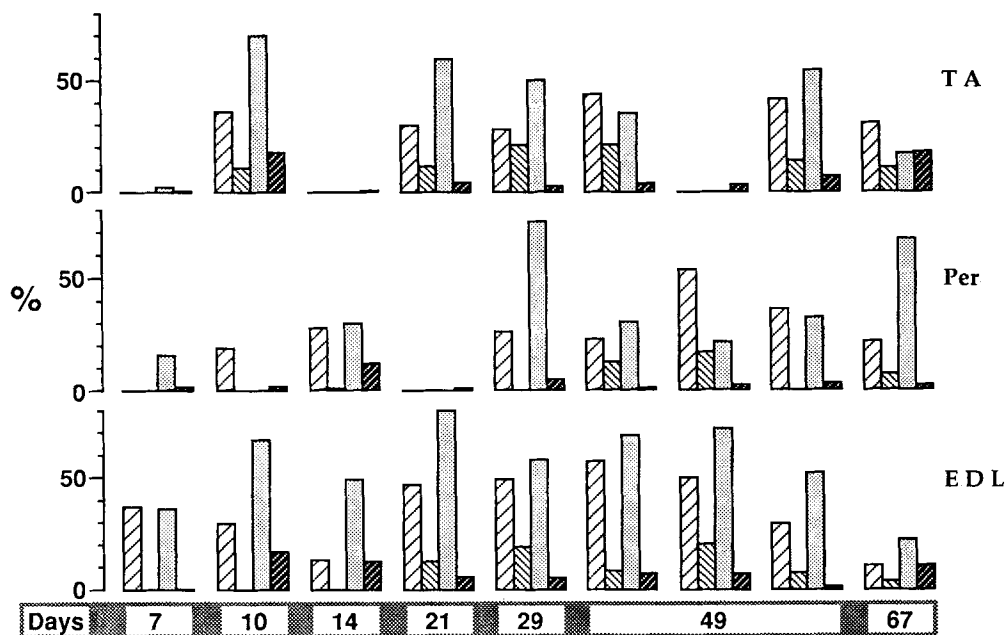
On immunoblots of irradiated TA muscles that had been injected with medium alone, no dystrophin was detected (Fig. 5).

### X-irradiated Muscles Injected with Wild-Type mpc

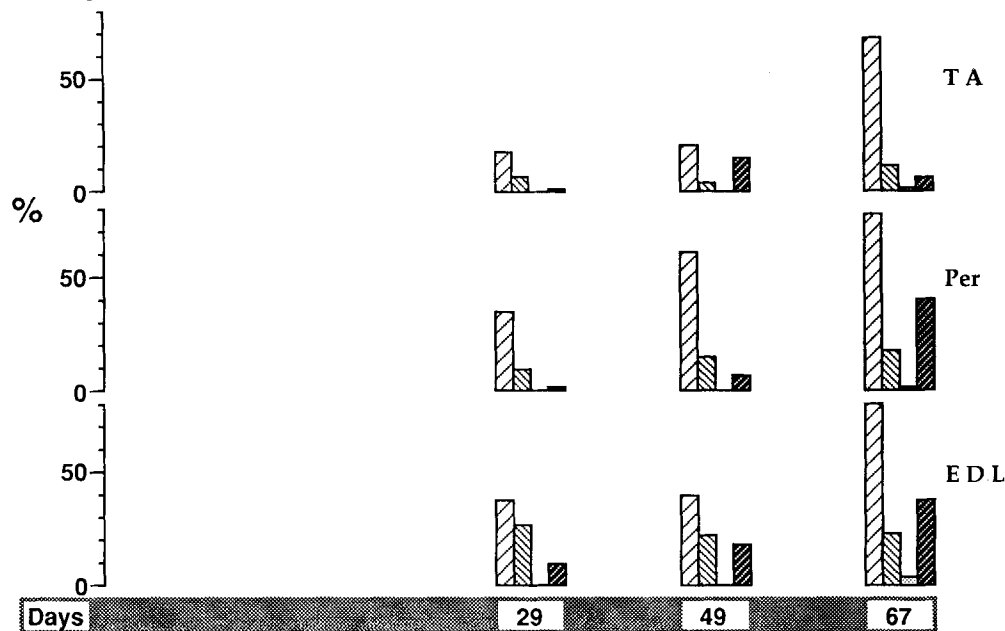
Detailed results of the GPI isoenzyme analysis, dystrophin immunostaining, and degree of central nucleation of each individual muscle are shown in Fig. 1 *a*.

At 7 d after injection, host and donor, but no heterodimer GPI, were detected in the TA muscle, host alone being found in the EDL and peroneus muscles. In the immunostained section of the TA (Fig. 6), the site of injection could be identified as a linear track of small, disoriented dystrophin-positive myotubes, surrounded by a solid zone of correctly oriented dystrophin-positive muscle fibers extending some 200  $\mu$ m on either side of the injection track. Comparison

**a. grafted with normal muscle cells**



**b. grafted with mdx muscle cells**



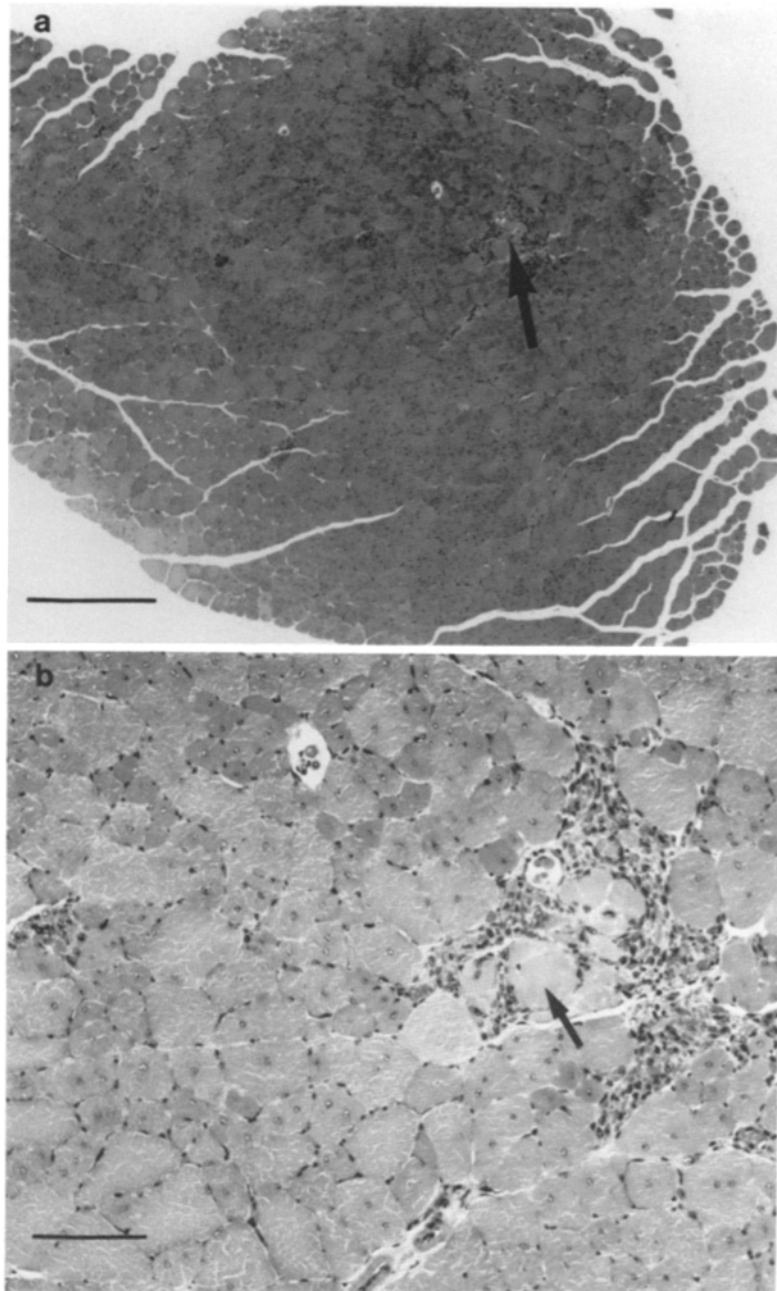
*Figure 1.* Summary of results on irradiated mdx muscles injected with mpc. Numbers along the bottom show the time elapsing between injection of mpc and examination of the muscle. At each time three muscles were examined: TA, peronei, and EDL. For each muscle, four results are indicated: (▨) percent GPI isomer present in the donor form; (▩) percent GPI present in heterodimer form; (□) percent muscle fibers that stain positive for dystrophin; and (■) percent muscle fibers containing centrally placed nuclei. (a) Muscles injected with normal mpc; (b) muscles injected with mdx mpc.

with the serial cryostat section stained with haematoxylin and eosin, shows that this dystrophin-positive zone comprised both peripherally and centrally nucleated muscle fibers, the latter predominating close to the injection site. Wax sections from the distal half of the injected muscle again contained mainly peripherally nucleated muscle fibers, with a few centrally nucleated fibers located close to the injection site (Fig. 7).

Considering these early stages as a whole, of the nine muscles examined 7-14 d after injection of wild-type mpc, seven contained dystrophin-positive muscle fibers. Six of these

contained donor, but only one contained a significant amount of heterodimer GPI, suggesting that the implanted myogenic cells had mainly formed new muscle fibers of purely donor origin. Even by 10 d, in two muscles, these dystrophin-positive fibers, many of which were very small, comprised >60% of the total muscle fibers. As in the TA examined 7 d after mpc injection, the dystrophin-positive fibers were predominantly located in one block surrounding a small site of disturbed structure, the presumed injection site.

In two muscles examined at 7 d after injection, small num-



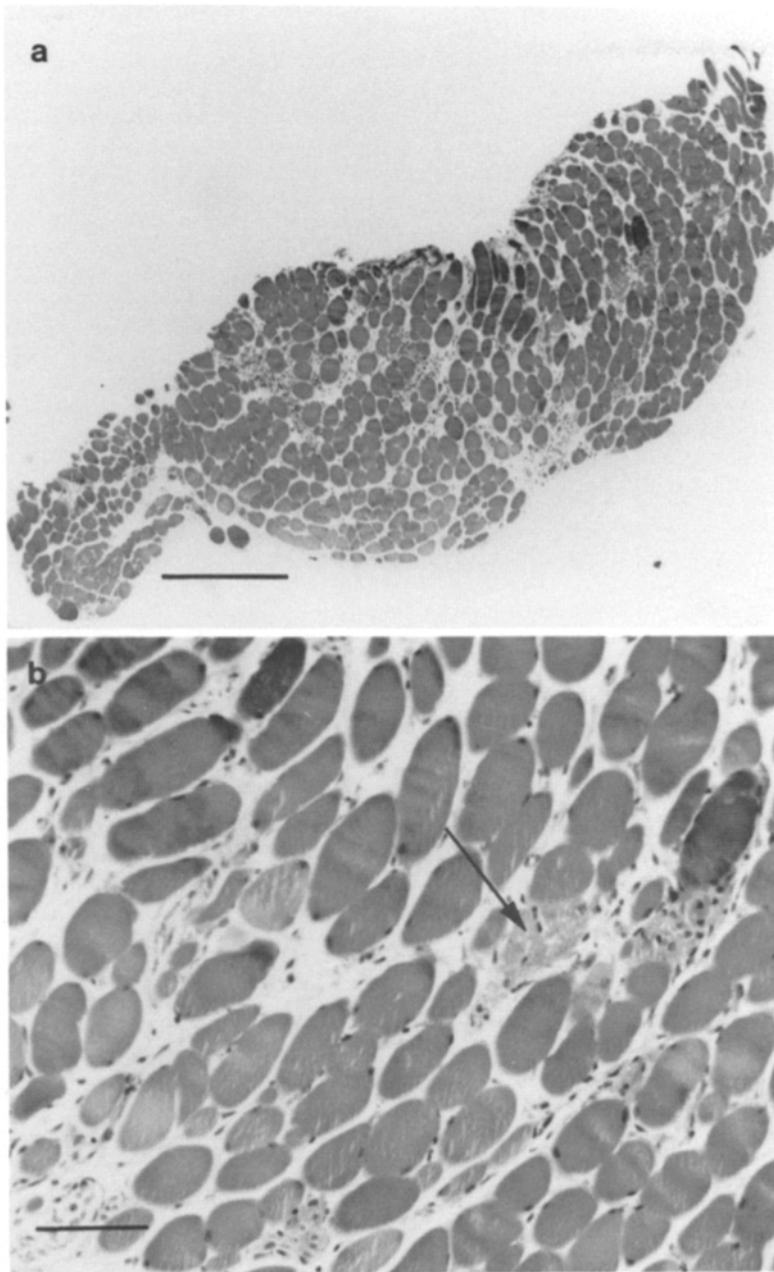
**Figure 2.** Left TA muscle of a 68-d-old mdx nu/nu mouse. This is the nonirradiated contralateral control of the muscle shown in Figs. 8 and 9. (a) Low power, showing closely packed muscle fibers with some areas of necrosis, inflammation, and regeneration (*arrow*). Bar, 500  $\mu$ m. (b) High power, showing a typical mdx myonecrotic lesion, with a small group of necrotic fibers (*arrow*) and regenerating fibers. The majority of the fibers (67% in the muscle as a whole) contained centrally placed nuclei. Paraffin section stained with hematoxylin and eosin. Bar, 100  $\mu$ m.

bers of dystrophin-positive fibers but no donor or heterodimer GPI were found. Conversely, no dystrophin-positive fibers were found at 10 d in one peroneus muscle which contained some donor GPI. We can only explain these findings by the fact that the sections used for the enzyme runs were made from a slightly different part of the muscle to that immunostained for dystrophin. This, in agreement with the lack of heterodimer GPI, suggests that the dystrophin-positive fibers were very short and were not in cytoplasmic continuity with host muscle fibers at this stage.

There was a sudden change in the pattern of regeneration between 14 and 21 d after injection, in that many mosaic fibers were formed. Analysis of GPI isoenzyme in irradiated muscles examined from 21 to 67 d after implantation of

wild-type mpc showed that where donor cells were present, a variable proportion had become incorporated into the host muscle fibers, for both donor and heterodimer GPI were found in 14 out of 18 muscles examined. In many of these muscles the donor contribution, in terms of GPI subunits (i.e., donor enzyme plus half the heterodimer), was 50% or more (Fig. 1).

All of the muscles which contained donor, with or without heterodimer, GPI also contained appreciable proportions, >70% in some instances, of dystrophin-positive fibers. In general, high proportions of donor and heterodimer GPI were associated with high proportions of dystrophin-positive fibers, but discrepancies were noted in individual muscles. These may reflect differences in the distribution of the two



**Figure 3.** TA muscle of an mdx nu/nu mouse. The leg of this mouse had been X-irradiated 52 d previously, and 3 d later culture medium only had been injected. (a) The magnification as Fig. 2 a, showing the massive atrophy of the muscle, with overall loss of fibers and loss of fascicular structure. Bar, 500  $\mu\text{m}$ . (b) High power. Most of the surviving fibers are peripherally nucleated, rounded, and separated from each other by loose connective tissue. There is some necrosis (arrow) but no regeneration. Paraffin section stained with hematoxylin and eosin. Bar, 100  $\mu\text{m}$ .

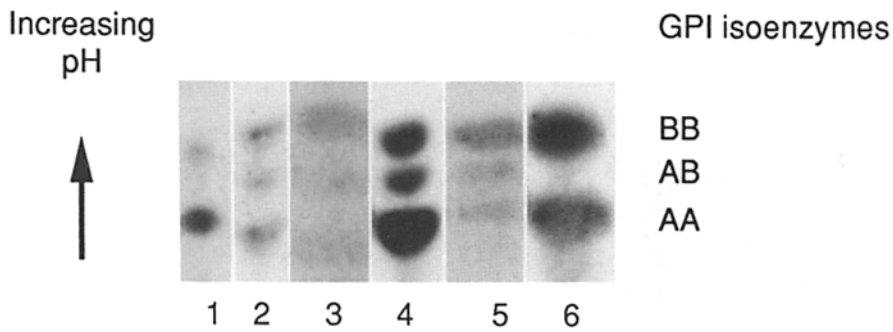
markers within the muscle. In genetically mosaic muscle fibers, dystrophin is often discontinuously distributed (Karpapati et al., 1989; Partridge et al., 1989; Watkins et al., 1989) as is seen in parts of Fig. 8, indicating a degree of restriction to territories around dystrophin-competent myonuclei, whereas GPI isozyme subunits equilibrate freely along the length of the fibers (Frair and Peterson, 1983) and give an averaged estimate of muscle genotype.

In muscles containing a high proportion of dystrophin-positive muscle fibers, the remaining negative areas, i.e., host mdx fibers that had survived since birth, were usually disposed in large compact blocks (Fig. 8). Within some of these blocks the occasional dystrophin-positive fiber could be seen (rather more than would be expected on the basis of somatic "reversion"), indicating that injected mpc had proba-

bly penetrated these regions. Transverse mid muscle sections of muscles which contained high proportions of dystrophin-positive fibers were much larger than irradiated muscles of equivalent age that had been injected with medium alone (compare Figs. 3 and 9).

Between 21 and 49 d after injection, all muscles had a low proportion (1.1–7.2%) of centrally nucleated fibers. Apart from occasional small foci of necrosis and regeneration, the fibers were closely packed, arranged in fascicles, and were virtually indistinguishable from wild-type mouse muscle (Fig. 9, a and b). The majority of centrally nucleated fibers seen in the hematoxylin- and eosin-stained frozen sections which could be identified with certainty on the dystrophin-stained sections were dystrophin positive.

The three muscles from the mouse examined 67 d after in-



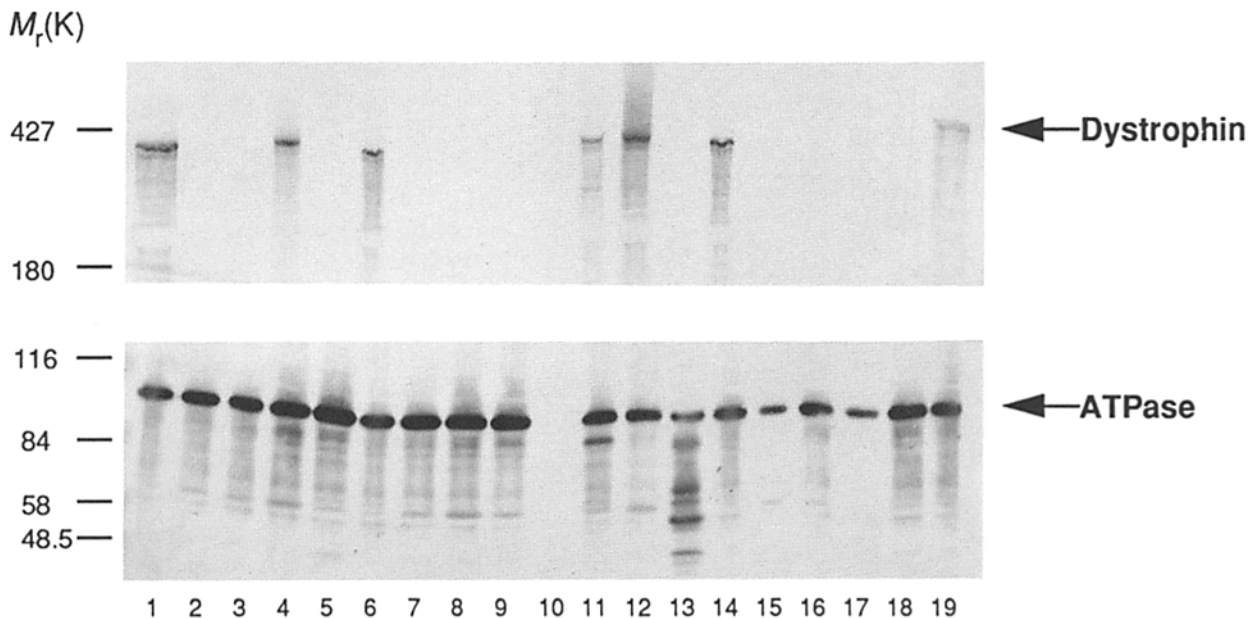
**Figure 4.** GPI isoenzymes, separated by IEF, from cryostat sections of the following. (Lane 1) Irradiated mdx nu/nu TA muscle, into which newborn C57Bl/6J mpc had been implanted 10 d earlier. This muscle contained host and donor GPI isoenzymes only and contained dystrophin-positive fibers. (Lane 2) Irradiated mdx nu/nu TA muscle, into which newborn C57Bl/10 mpc had been implanted 29 d earlier. This muscle contained host, donor, and heterodimer GPI isoenzymes and contained dystrophin-

positive fibers. (Lane 3) Irradiated mdx nu/nu TA muscle, into which newborn C57Bl/10 mpc had been implanted 49 d earlier. This muscle contained host, donor, and heterodimer GPI isoenzymes and contained dystrophin-positive fibers. (Lane 4) Irradiated mdx nu/nu TA muscle, into which newborn mdx mpc had been implanted 29 d earlier. This muscle contained host, donor, and heterodimer GPI isoenzymes and contained no dystrophin-positive fibers. (Lane 5) Irradiated mdx nu/nu TA muscle, into which newborn mdx mpc had been implanted 67 d earlier. This muscle contained host, donor, and heterodimer GPI isoenzymes and contained 3.2% dystrophin-positive fibers. (Lane 6) Control: a mixture of C57Bl/6J and 129/ReJ skeletal muscles, to show that the AA and BB GPI isoenzymes do not reassociate to form the heterodimeric AB GPI isoenzyme on IEF.

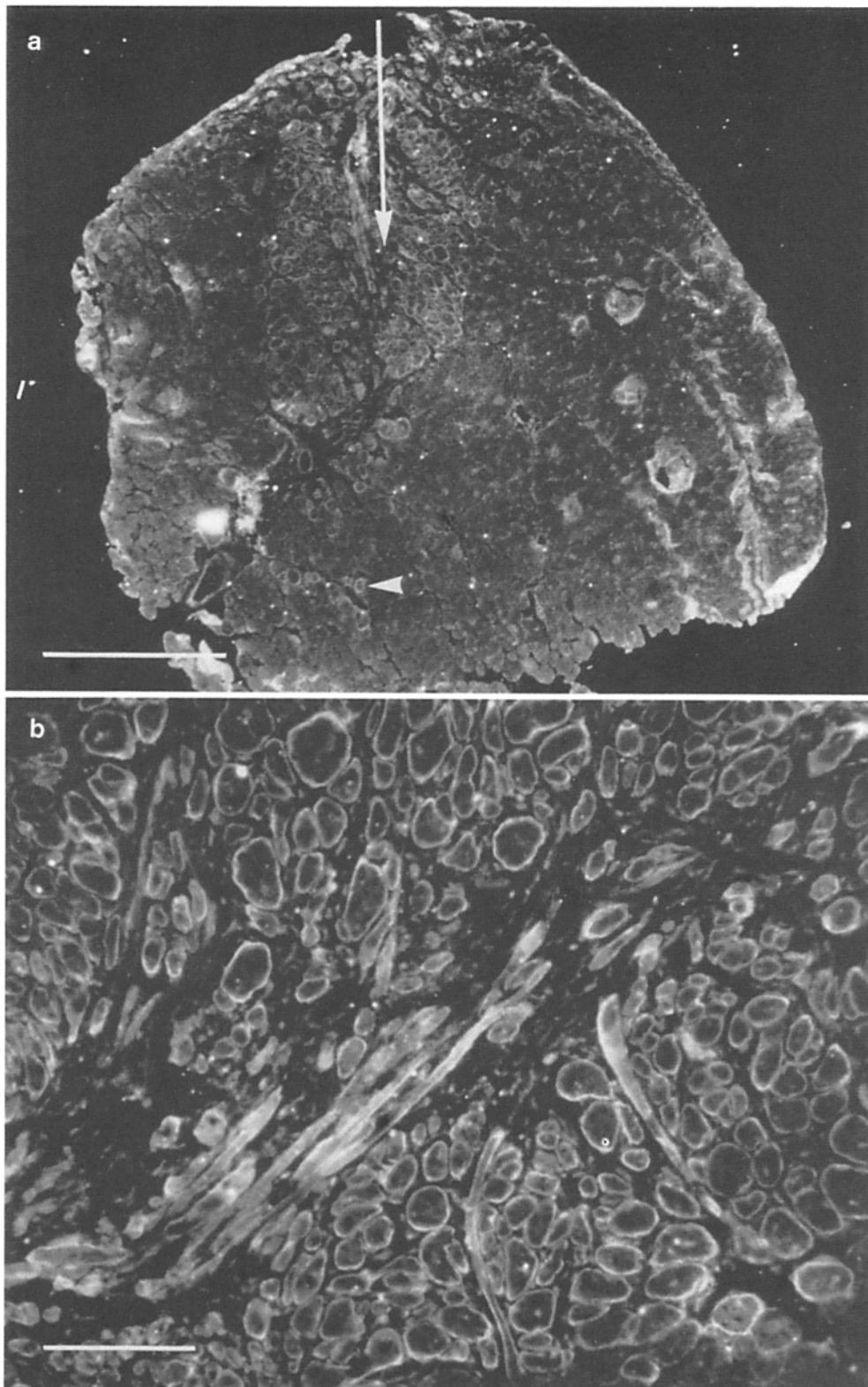
jection were particularly informative. Two of them, the EDL and TA, contained low proportions of dystrophin-positive fibers, but high proportions of centrally nucleated muscle fibers (17.7 and 10.7%, respectively). The peroneus, by contrast, contained a high proportion of dystrophin-positive

fibers (67%) and a very low proportion (2%) of centrally nucleated muscle fibers.

Immunoblots of dystrophin from irradiated mdx TA muscles examined 29–67 d after implantation of wild-type mpc, revealed almost normal levels of this protein in all but one



**Figure 5.** Immunoblot detection of dystrophin in TA muscles. The gel was first processed for detection of dystrophin with antibody to the 60-kD dystrophin peptide. Subsequently it was reprocessed with a mAb to the fast muscle isoform of  $\text{Ca}^{2+}$   $\text{Mg}^{2+}$ -ATPase, as a control for muscle protein loading. (Lanes 1, 6, 14, and 19) Normal C57Bl/10 control TA muscle. (Lane 2) Irradiated mdx nu/nu TA muscle, into which medium only had been implanted 32 d earlier. (Lane 3) Irradiated mdx nu/nu TA muscle, into which newborn mdx mpc had been implanted 32 d earlier. (Lane 4) Irradiated mdx nu/nu TA muscle, into which newborn C57Bl/10 mpc had been implanted 32 d earlier. (Lanes 5, 13, and 18) mdx nu/nu TA muscle from an uninjected contralateral leg. (Lanes 7 and 8) Irradiated mdx nu/nu TA muscle, into which medium only had been implanted 52 d earlier. (Lane 9) Irradiated mdx nu/nu TA muscle, into which newborn mdx mpc had been implanted 52 d earlier. (Lanes 10–12) Irradiated mdx nu/nu TA muscle, into which newborn C57Bl/10 mpc had been implanted 52 d earlier. (No muscle protein was present in the lane 10 sample.) (Lane 15) Irradiated mdx nu/nu TA muscle, into which medium only had been implanted 67 d earlier. (Lane 16) Irradiated mdx nu/nu TA muscle, into which newborn mdx mpc had been implanted 67 d previously. (Lane 17) Irradiated mdx nu/nu TA muscle, into which newborn C57Bl/10 mpc had been implanted 67 d previously. *Note:* Only the TA muscles which had been injected after irradiation with normal mpc contained dystrophin. In most cases, this was present at close to normal levels.

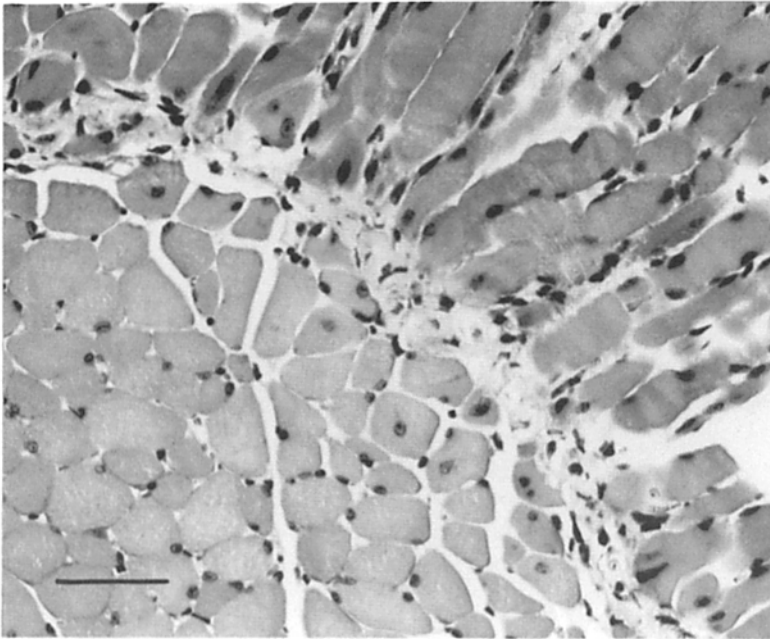


**Figure 6.** TA muscle of an mdx nu/nu mouse. This had been X irradiated at 16 d of age, and 4 d later, injected with  $5 \times 10^5$  C57Bl/6J mpc. The muscle was removed 7 d after implantation, and immunostained for dystrophin. The injection site is clearly visible (*arrow*) as a linear track of small disoriented myotubes surrounded by a zone of dystrophin-positive muscle fibers. The dystrophin-positive fibers seen found at a distance of several hundred micrometers from the injection site (*arrowhead*) are more numerous than would be expected to occur by "reversion." Cryostat section, (*a*) Low power. Bar, 500  $\mu\text{m}$ . (*b*) High power. Bar, 100  $\mu\text{m}$ .

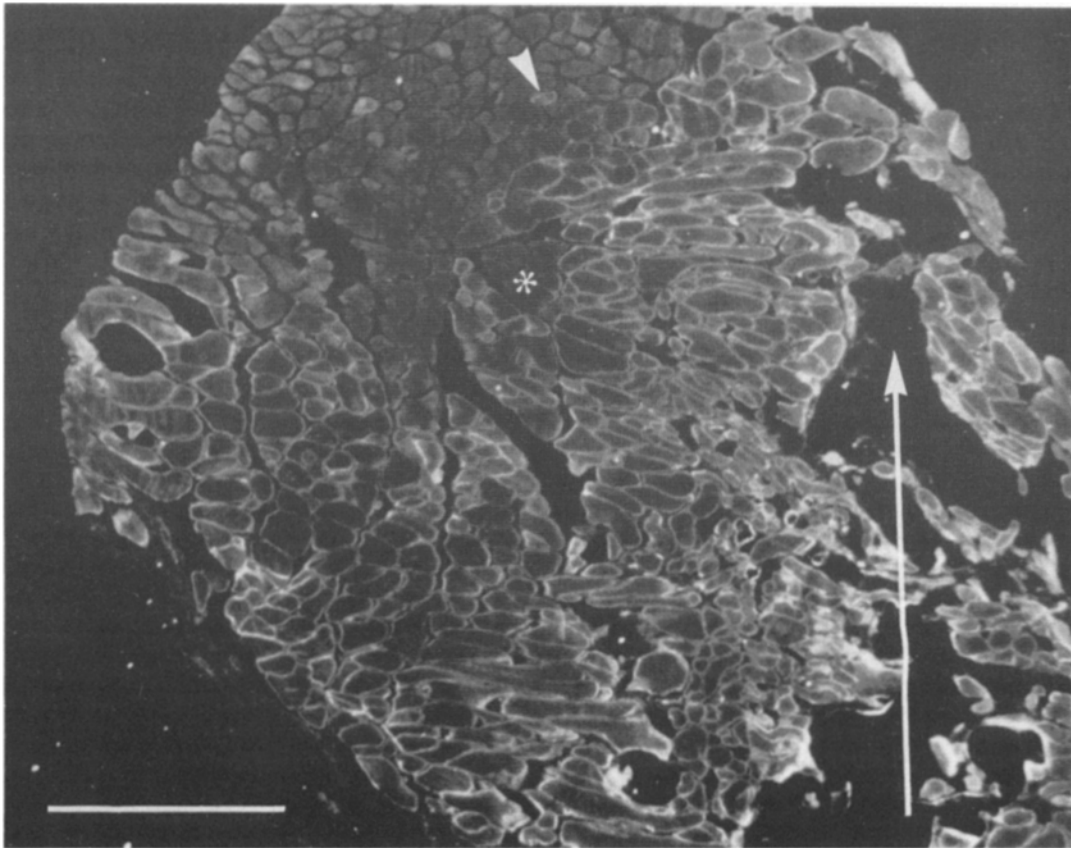
(Fig. 5, lane 17). The exception, examined 67 d after implantation, contained no detectable dystrophin on the immunoblot, few dystrophin-positive fibers, and only traces of donor and heterodimer GPI (Fig. 1), indicating either that insufficient mpc had been retained, or that they had not thrived in the muscle.

#### ***X-irradiated Muscles Injected with mdx mpc***

Muscles were examined from one mouse at 29, 49, and 69 d after injection of mdx mpc. The results are summarized in Fig. 1 *b*. All nine of the muscles examined from the injected right legs contained host, donor, and heterodimer GPI.

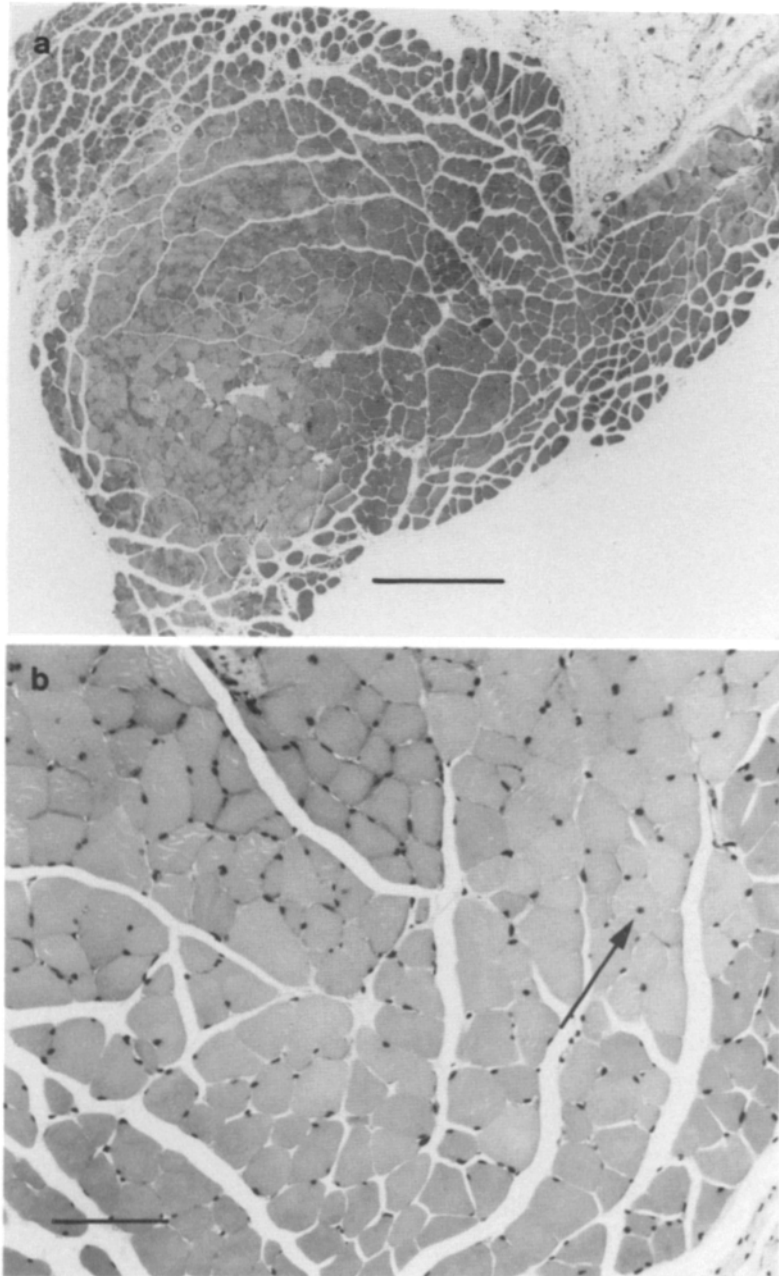


**Figure 7.** Wax section cut from distal half of the muscle shown in Fig. 6. This section contains part of the site of the injection of normal mpc. A few fibers close to this site are centrally nucleated but the majority are peripherally nucleated. Stained with hematoxylin and eosin. Bar, 100  $\mu\text{m}$ .



**Figure 8.** Low power dystrophin immunostain of a TA muscle of an mdx nu/nu mouse. This muscle had been X irradiated 52 d previously and injected 3 d later with  $5 \times 10^5$  C57Bl/10 mpc. This shows the majority of the muscle, containing a large block of dystrophin-positive muscle fibers adjacent to a block of dystrophin-negative fibers. The blank area (*arrow*) is occupied by fibrous and fatty tissue and is presumed to be the injection site. Within the predominantly dystrophin-negative area are several isolated dystrophin-positive fibers (*arrowhead*), more than we would expect to occur by reversion, suggesting that normal mpc have penetrated this region, but that the majority of mdx fibers in this region have not yet degenerated. At the interface, some fibers (\*) show dystrophin staining on the side facing their dystrophin-positive neighbors and no staining on the side facing their negative neighbors. This pattern of mosaicism suggests that the section is passing obliquely through an interface between a dystrophin-positive and dystrophin-negative segment of these fibers. Cryostat section. Bar, 500  $\mu\text{m}$ .





**Figure 9.** Hematoxylin and eosin stained wax section of the muscle shown in Fig. 8. (a) Low power magnification, showing that the muscle contains closely packed muscle fibers, with areas of necrosis and regeneration. Bar, 500  $\mu\text{m}$ . (b) High power magnification of area close to the injection site. Apart from one or two centrally nucleated fibers (arrow), this muscle is histologically normal. Bar, 100  $\mu\text{m}$ .

There was a clear increase in contribution of donor GPI subunits with time in these muscles, implying a progressive displacement of the host component of these muscles by the implanted mdx mpc.

At 29 d after injection, all three muscles contained low proportions of centrally nucleated muscle fibers and, in this respect, were entirely comparable with muscles of equivalent age injected with wild-type mpc. At 49 and 67 d however, there was a tendency to a higher level of central nucleation, reaching 31 and 34% of all fibers in two of the muscles examined at 67 d after injection. Muscle bulk and muscle fiber number appeared even greater in mdx-injected muscles than in muscles of equivalent age injected with wild-type mpc. This preservation of muscle size and fiber number was particularly evident in the TA muscles 67 d after injection with mdx mpc (Fig. 10, a and b). However, these older mus-

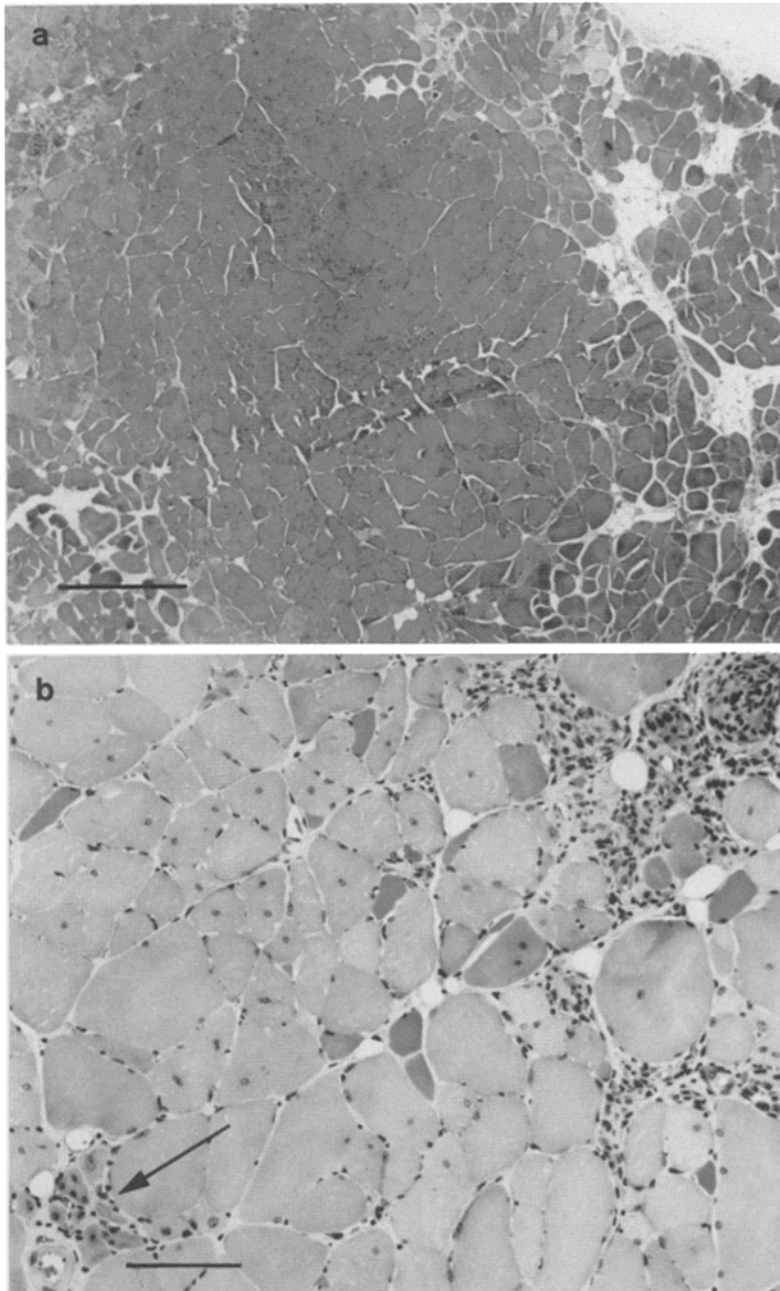
cles exhibited all of the features of nontreated mdx muscles; i.e., abnormally shaped fibers, central nucleation, wide variation in fiber size, fiber necrosis, and regeneration.

In muscles of the oldest mouse, examined 67 d after injection with mdx mpc, unusually large numbers of revertant dystrophin-positive fibers were seen (Fig. 1 b); seven in the TA, three in the EDL, and two in the peroneus.

No dystrophin was detected on immunoblots of the TA muscles into which mdx mpc had been injected (Fig. 5).

### Discussion

How to reconcile the mild clinical phenotype seen in the mdx mouse with the unremitting decline in muscle function which characterizes DMD has been a subject of much speculation. Two histological correlates of the loss of muscle func-



**Figure 10.** TA muscle of an mdx nu/nu mouse. This muscle had been X irradiated 71 d earlier and injected 4 d later with  $5 \times 10^5$  mdx mpc. (a) Low power, showing that there is good preservation of muscle bulk. Bar, 500  $\mu$ m. (b) High power, showing features typical of mdx muscle; i.e., central nucleation (31%), a wide variation in fiber size, necrosis, and myotubes (arrow). Paraffin section stained with hematoxylin and eosin. Bar, 100  $\mu$ m.

tion in DMD patients are (a) lack of effective muscle fiber regeneration and (b) massive fibrosis and fatty replacement of muscle. These contrast with the modest increase in fibrous tissue (Marshall et al., 1989) and spectacular regeneration seen in the mdx mouse. This inverse relationship between myogenesis and fibrosis has led to proposals that these two phenomena may be linked. For instance, basic fibroblast growth factor, tending to stimulate mpc proliferation and to inhibit fibroblast proliferation, may provide a causal link, interspecies differences in the balance of these two effects being held to account for the difference between DMD and mdx (DiMario et al., 1989). Equally, it seems likely that deposition of dense fibrous and fatty tissue interspersed between the muscle fibers in DMD might directly depress the effective-

ness of myogenesis by constraining cell movement and by diminishing tissue perfusion.

Other evidence suggests an intrinsic loss of proliferative capacity by myogenic cells in early DMD muscles (Blau et al., 1983), probably in consequence of enforced cycling of these cells in diseased muscle. In the present experiments, the endogenous regenerative powers of the resident myogenic cells in mdx muscle was abolished by radiation (Wakeford et al., 1990), to provide an experimentally convenient simulation of the myofiber loss which occurs in dystrophin-deficient DMD muscle. We cannot exclude the possibility that this high dose of radiation has additional effects on the muscle which may alter the ability of implanted mpc to colonize it. However, this same radiation dose, ap-

plied to normal mouse muscle at the same age, produces no detectable effect other than the predicted decrease in growth (Wakeford et al., 1990).

By combined use of GPI markers, dystrophin immunostaining, and standard histological sections, we have monitored the effects of implanting myogenic cells into irradiated mdx muscle. The extent to which wild-type mpc colonize preirradiated mdx muscles is a marked improvement over our previous studies where, in nonirradiated regenerating mdx muscles, we found, at best, 30–40% dystrophin-positive fibers and 30–40% of normal dystrophin levels on immunoblots (Partridge et al., 1989). In the present study, preirradiated mdx muscles were far more extensively permeated and replaced by implanted normal mpc; in many, dystrophin-positive fibers comprised 60–80% of the total and nearly normal levels of dystrophin were seen in the immunoblots. More encouraging still, this high level of biochemical rescue was accompanied by retention or acquisition by these muscles of a nearly normal histological phenotype. Thus, rather perversely, the lack of myogenic potential of endogenous myogenic cells in muscles of DMD patients may actually enhance the efficacy of myoblast transfer therapy in this disease.

This colonization of preirradiated mdx muscles by normal mpc appears to proceed in two stages. Up to 14 d after injection of the mpc, donor isoenzyme was found in several muscles, usually in the absence of heterodimer, implying that the numerous dystrophin-positive fibers present in these muscles were of purely donor origin. By contrast, from 21 d onward, heterodimer GPI was usually present in all muscles where donor GPI was found; in many cases the total donor contribution equaling or exceeding that of the host. Thus, there is a rapid transition from muscle fibers of purely donor origin to the formation of mosaic muscle fibers, with no evidence of further change after 21–29 d. It seems unlikely that these mosaic fibers could have formed by co-regeneration of host and donor mpc, since the radiation dose used reduces muscle fiber regeneration from endogenous mpc to the limits of detectability. Equally, normal muscle fiber growth, involving incorporation of donor mpc into host muscle fibers, can account for few if any mosaic fibers, for the major phase of incorporation of myoblasts into growing muscle fibers during growth should already have occurred by 35–40 d of age (Cardasis and Cooper, 1975), when we first find significant numbers of mosaic fibers. Moreover, previous attempts to achieve incorporation of injected myoblasts during muscle fiber growth, even in younger mice, have produced only low levels of mosaicism (Karpati et al., 1989; Watt et al., 1984; Morgan et al., 1988; Partridge et al., 1989). Rather, the myogenesis undertaken by the injected cells is probably associated with the massive myonecrosis which occurs at this time in mdx muscle, irradiated or otherwise (Wakeford et al., 1990), and which appears to be a most effective stimulus to mosaic fiber formation by injected myoblasts (Watt et al., 1982; Morgan et al., 1988; Partridge et al., 1989). In the absence of endogenous mdx mpc, mosaic fibers could be formed by fusion of donor mpc into the surviving segments of partly necrotic host muscle fibers or by fusion of the pure donor-derived myofibers formed during the first 14 d with segments of host muscle fiber. In effect, therefore, the injected cells appear to repair degenerating host muscle fibers,

a mechanism which has been presumed by others (Law et al., 1990) but not critically tested.

Analysis of dystrophin content by immunoblotting and by immunofluorescence confirms the picture of a progressive widespread replacement of necrotic host muscle fibers by injected wild-type myogenic cells. Even by 7 d, dystrophin-positive fibers of purely donor origin are seen over a large proportion of the muscle, up to 200  $\mu\text{m}$  from the presumed injection site, implying a very rapid lateral migration of these cells through the connective tissue components of the muscle. We do not attribute this to dispersal by passive carriage in fluid under pressure from the injection, for, in other experiments, small particles of tattoo dye incorporated in the cell suspension remain closely associated with the well-defined areas of tissue disruption we identify as injection sites. These findings complement previous demonstrations of a rapid longitudinal movement of myogenic cells within regenerating muscle (Schultz et al., 1985).

Peronei were not deliberately injected with mpc, but most showed evidence of heavy infiltration by the implanted cells, suggesting that these cells had migrated in from injected neighbors. This accords with previous evidence of movement of mpc between muscles, as well as within them (Watt et al., 1987; Morgan et al., 1987) and gives encouragement to the cause of myoblast implantation therapy. However, it is likely that mpc suspension had leaked out of the injected muscles and thus directly contacted the peroneus, but even then, they would have had to penetrate the peroneal epimysium. We can think of no irrefutable test of the more trivial explanation that we had injected mpc into the peronei by mistake, but hope that we would not make this error sufficiently often to account for the consistency of our results.

The sporadic, brightly dystrophin-positive fibers, found in irradiated mdx muscles which had been injected with either growth medium only, or with mdx mpc, are also of interest. Similar fibers found in untreated mdx muscle appear to contain essentially full-length dystrophin, probably a consequence of somatic mutations which reconstitute transcription of a functional message from the gene (Hoffman et al., 1990). The finding of a much higher than normal number of such fibers in muscle of the oldest mouse injected with mdx mpc suggests that the proliferation of the injected mpc may lead to increased incidence of such somatic mutations. Whatever the basis, the occurrence of these fibers emphasizes the need for caution when analyzing the efficacy of myoblast transfer, particularly in human trials, where dystrophin may be the only marker of survival and incorporation of donor mpc into the host muscle fibers (Law et al., 1990).

Two incidental findings are of special interest in that they raise questions about the mechanisms of degeneration and regeneration in the mdx myopathy. First, dystrophin-negative fibers are found in large distinct blocks within the treated muscles. It is not clear why these surviving, peripherally nucleated fiber profiles should be so compactly grouped in irradiated muscles that had been injected with mpc, while the equivalent population of surviving mdx fiber profiles is diffusely distributed in muscle that had been irradiated but not injected with mpc. Perhaps the large block of dystrophin-positive fibers formed adjacent to the injection site acts to protect this minor population of fibers furthest from the in-

jection site from such physiological stress as may predispose them to degeneration and replacement by injected mpc. Alternatively, the surviving dystrophin-negative fiber profiles may be continuous with dystrophin-positive segments out of the plane of section. Certainly, dystrophin sometimes shows a segmental distribution (Karpati et al., 1989; Partridge et al., 1989), and some fibers in Fig. 8 appear to have been obliquely sectioned through a transition between dystrophin-positive and dystrophin-negative segments. The implication, that dystrophin may exert a protective effect on remote parts of the syncytial muscle fiber, runs counter to suggestions of local mechanical function for this molecule (Karpati et al., 1989).

The second point of interest, is the rapid acquisition of a normal peripherally nucleated appearance by muscle derived from injected mpc. This is clearest where wild-type mpc had been injected; here GPI, and dystrophin immunoblot and immunostaining all indicate that around half of the muscle was derived from the injected mpc, but only a minority of fiber profiles are centrally nucleated. More directly, at 7 and 10 d after injection of wild-type mpc, most of the dystrophin-positive fibers, which GPI results tell us are derived entirely from donor mpc, were already peripherally nucleated. At 29–49 d after injection of wild-type mpc, centrally nucleated fibers never comprised >7%. By 69 d, the most highly dystrophin-positive muscle contained only 2% centrally nucleated fibers whereas the two muscles with low incidences of dystrophin-positive fibers contained 10 and 17% centrally nucleated fibers.

It is difficult to reconcile this data with the long-term retention of centrally positioned myonuclei seen in regenerated fibers in mouse muscle after grafting (Partridge and Sloper, 1977; Grounds et al., 1980; Morgan et al., 1989) or myonecrosis in the mdx mouse (Bulfield et al., 1984; Bridges, 1986; Carnwath and Shotton, 1987; Coulton et al., 1988 a). Together with an earlier observation of rapid peripheral nucleation in regenerates of minced neonatal muscle (Grounds et al., 1980), it suggests the simple explanation that the developmental age of the myogenic cells determines whether muscle fibers become centrally or peripherally nucleated; neonatal mpc form fibers in which the myonuclei move rapidly to the periphery, older (3-wk) mpc form fibers where the myonuclei remain central. A similar perinatal "maturation" of mouse myoblasts has been noted previously in vitro (Cossu et al., 1983).

The retention of peripheral nucleation up to 67 d by muscles with a high percentage of dystrophin-positive fibers, suggests that fusion of normal mpc into mdx fibers and consequent synthesis of dystrophin may protect these fibers from subsequent degeneration. Two muscles, examined 67 d after injection, which contained a low proportion of dystrophin-positive fibers also contained higher than usual proportions of centrally nucleated fibers, apparently in dystrophin-positive areas. These muscles may have degenerated late, and thus regenerated from older normal mpc that had lost their neonatal properties.

Where mdx mpc were injected, dystrophin cannot be used as a marker, but the rapid proportional increase in donor and heterodimer GPI indicates that, here too, a significant proportion of the muscle fibers are of wholly donor or mosaic origin and have therefore been formed by regeneration. Again, central nucleation was initially low at 29 d, rising to 15%

at 49 d and, in two of three muscles, to >30% by 69 d. The gradual increase in central nucleation of muscle fibers presumably reflects continuing myonecrosis of fiber segments after implantation of mdx mpc. Interestingly, this rise occurs 7–10 wk after injection (10–13 wk of age), whereas it might be expected to run on continuously from the time of injection. The reasons for this are currently being examined.

The continuing degeneration/regeneration in irradiated muscle injected with mdx mpc is also manifested as a continuous rise in donor and heterodimer GPI content over the 67 d of observation. It contrasts with the rapid stabilization, by ~21 d, of both GPI isoenzyme pattern and dystrophin content in mdx muscles injected with normal mpc. This relative stability is the best evidence to date, although still circumstantial, for a functional benefit arising from dystrophin expression in dystrophic muscles treated by myoblast transfer. Together with the remarkable histological normalization of irradiated mdx muscle by injection of wild-type muscle precursor cells, it gives cause for optimism with regard to the use of myogenic cells for treatment of inherited myopathies. It suggests that the paucity of effective host mpc in DMD (Blau et al., 1983) may be advantageous in encouraging the dispersion of injected mpc and it demonstrates that there is no need for effective host regeneration in order to form mosaic fibers. In fact, in the mdx mouse, this lack of competition from host myogenic cells leads to a massive en bloc conversion of muscle fibers to dystrophin positivity, showing that the injected wild-type mpc readily penetrate the potential structural barriers within skeletal muscle, the perimysial connective tissues ensheathing fascicles, the endomyssial tissue within the fascicles, and the basement membranes surrounding individual muscle fibers. Further investigation of this model should reveal the limits of dispersion of myogenic cells within degenerating skeletal muscle and whether myogenic cells obtained from different developmental stages of muscle differ in the efficiency with which they can restore or maintain normal muscle histology and function.

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