Heterokaryon Myotubes with Normal Mouse and Duchenne Nuclei Exhibit Sarcolemmal Dystrophin Staining and Efficient Intracellular Free Calcium Control

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Duchenne and *mdx* muscle tissues lack dystrophin where it normally interacts with glycoproteins in the sarcolemma. Intracellular free calcium ($[Ca^{2+}]_i$) is elevated in Duchenne and *mdx* myotubes and is correlated with abnormally active calcium-specific leak channels in dystrophic myotubes. We fused Duchenne human and normal mouse myoblasts and identified heterokaryon myotubes by Hoechst 33342 staining to measure the degree to which dystrophin introduced by normal nuclei could incorporate throughout the myotube at the sarcolemma and restore normal calcium homeostasis. Dystrophin expression in myotubes was determined by immunofluorescence and confocal laser scanning microscopy. Dystrophin was expressed at the sarcolemma in normal mouse and heterokaryon myotubes, but not in Duchenne myotubes. In heterokaryons, extensive dystrophin localization occurred at the sarcolemma even where only Duchenne nuclei were present, indicating that dystrophin does not exhibit nuclear domains. Heterokaryon, normal mouse and Duchenne myotube $[Ca^{2+}]_i$ was measured using fura-2 and fluorescence ratio imaging. Heterokaryon and normal mouse myotubes were found to maintain similar levels of $[Ca^{2+}]_i$. In contrast, Duchenne myotubes had significantly higher $[Ca^{2+}]_i$ ($p < 0.001$). Furthermore, the ability of heterokaryons to maintain normal $[Ca^{2+}]_i$ did not depend on greater numbers of normal nuclei than Duchenne being present in the myotube. These results support the view that dystrophin expression in heterokaryons allows for efficient control of $[Ca^{2+}]_i$.

INTRODUCTION

Several theories attempt to explain muscle necrosis in Duchenne muscular dystrophy in view of an abnormal expression of dystrophin (see review, Rojas and Hoffman, 1991). In one case, a lack of dystrophin in muscle tissues is thought to weaken the sarcolemma, producing repeated cycles of segmental plasma membrane tearing and repair that eventually destroy it. In this view, dystrophin maintains the mechanical-structural stability of the muscle architecture. In the second case, an absence of dystrophin in muscle fibers causes membrane glycoproteins normally associated with dystrophin to disperse and/or degrade, resulting in increased extracellular calcium permeability, leading to calcium-dependent protease activation and abnormal proteolysis (Turner *et al.*, 1991, 1993). In this view, dystrophin is

believed to have a role regulating integral membrane proteins (Ohlendieck and Campbell, 1991). In both instances, an absence of dystrophin is responsible for muscle degeneration although its exact physiological role in muscle tissues remains unknown.

Dystrophin, the missing gene product in Duchenne muscular dystrophy (Hoffman *et al.*, 1987), is normally expressed on the cytoplasmic side of the muscle fiber sarcolemma (Bonilla *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988; Carpenter *et al.*, 1990; Byers *et al.*, 1991) and is also located at the plasma membrane in myotubes formed shortly after myoblast fusion (Miranda *et al.*, 1988; Klamut *et al.*, 1989; Huard *et al.*, 1991). Dystrophin is a 125-nm rod-like protein (427 kDa, MW) with four domains and shares sequence homology with several cytoskeletal proteins (Koenig *et al.*, 1988). It is proposed to exist as a homodimer where anti-parallel dys-

trophin monomers interact by their N-terminal domain (α -actinin sequences) with cortical actin filaments and by their C-terminal domain (unique dystrophin sequences) with sarcolemmal glycoproteins (Ervasti and Campbell, 1991). In addition to being expressed at the sarcolemma in muscle fibers, dystrophin is also found at low levels in the triadic junctions (Knudson *et al.*, 1988; Bornemann and Schmalbruch, 1991) and at higher levels at the neuromuscular (Huard *et al.*, 1992) and myotendinous junctions (Samitt and Bonilla, 1990; Tidball and Law, 1991).

The idea that dystrophin serves a primarily mechanical role preventing stress at the membrane and decreasing the probability of tears is appealing in its simplicity; however, the permeability to sodium and levels of intracellular sodium were found to be the same in dystrophic and normal muscle, ruling out nonspecific membrane leaks (Turner *et al.*, 1991). On the other hand, total calcium (Bertorini *et al.*, 1982; Dunn and Radda, 1991) and intracellular free calcium ($[Ca^{2+}]_i$) concentrations (Mongini *et al.*, 1988; Sanchez *et al.*, 1988; Turner *et al.*, 1988; Bakker *et al.*, 1993) were found to be significantly elevated in muscle tissues lacking dystrophin. $[Ca^{2+}]_i$ is twofold higher in isolated *mdx* flexor digitorum brevis (*fdb*) fibers (Turner *et al.*, 1988), and 25% higher in *mdx* and Duchenne myotubes in culture compared with normal myotubes when incubated in standard 1.8 mM extracellular calcium ($[Ca^{2+}]_o$) saline solution. In addition, significant differences in $[Ca^{2+}]_i$ between normal and dystrophic myotubes were seen after a 30-min challenge with 18 mM $[Ca^{2+}]_o$ (Fong *et al.*, 1990). Patch clamp analysis has identified leak channels selective for calcium with a greater probability of opening (P_o) in dystrophic myotubes. These abnormally active calcium leak channels could account for the elevated resting $[Ca^{2+}]_i$ levels in Duchenne and *mdx* muscle cells (Fong *et al.*, 1990; Turner *et al.*, 1991). Similarly, *mdx* cerebellar granule neurons possessed a calcium-specific leak channel that was more active than normal neurons (Haws and Lansman, 1991). Furthermore, *mdx* neurons had significantly higher resting levels of $[Ca^{2+}]_i$ and exhibited poor calcium control during brief periods of calcium stress (Hopf and Steinhardt, 1992).

In support of a role for calcium in muscle fiber damage, *mdx* fibers with stably elevated $[Ca^{2+}]_i$ levels exhibited an enhanced rate of muscle protein degradation (Turner *et al.*, 1988; MacLennan *et al.*, 1991). Therefore, a problem in $[Ca^{2+}]_i$ homeostasis occurs in dystrophin-deficient muscle and neural tissues, which may be linked to the pathophysiology of the disease. This link between the presence of dystrophin and a measurable index of normal cell physiology presents an opportunity to test whether the reintroduction of dystrophin expression can re-establish normal cell function, an important prerequisite for the success of myoblast fusion therapy (Partridge, 1991).

Myoblast fusion between co-cultured Duchenne human and normal mouse myoblasts has allowed us to

reintroduce dystrophin expression to determine if dystrophin localizes correctly throughout the fused cells at the sarcolemma and restores $[Ca^{2+}]_i$ control. Using the mouse-human heterokaryon enabled us to distinguish nuclei type to determine nuclear ratios and its effect on $[Ca^{2+}]_i$ control and dystrophin expression with reference to distance from normal nuclei.

MATERIALS AND METHODS

Muscle Cell Cultures

Satellite cell myoblasts were isolated from normal mouse (C57B1/10ScSn) hindlimb muscle according to the procedure described by Dimario and Strohman (1988). Human primary myoblast cultures, purified of fibroblasts, originated from a 5-mo-old male donor with Duchenne muscular dystrophy and were provided to us by Richard G. Ham (University of Colorado, Boulder, CO). Normal human myoblasts were obtained from Terry D. Heiman-Patterson (Thomas Jefferson University, Philadelphia, PA). Human and mouse myoblasts were proliferated for 3–5 d in Ham's F-10 medium containing 20% fetal bovine serum (Hyclone Logan, UT), and 2% chick embryo extract on collagen-coated 60-mm petri dishes (Falcon Lincoln Park, NJ). When myoblasts reached 80% confluency, differentiation was initiated by removing cells with trypsin (0.025%)/EDTA (0.01%), replating cells at a density of 10^5 cells/ml, and culturing cells in Dulbecco's modified eagle medium with 2.5% horse serum (Hyclone). For heterokaryon cultures, Duchenne myoblasts were first plated at a cell density of 10^5 cells/ml and incubated overnight in differentiation medium. After cell attachment, normal mouse myoblasts were added at cell densities of 1.2×10^4 cells/ml. All muscle cell cultures were incubated in a water-saturated, 37°C incubator, under an atmosphere of 5% CO_2 /95% air, and were maintained by regular medium changes every 3 d. Myotubes were formed after 2–4 d in cell-fusion medium. All culture media contained penicillin-G and streptomycin sulfate (Sigma, St. Louis, MO) at their usual working concentrations.

Tissue culture plastic will not adequately transmit light in the near ultraviolet wavelength range needed to excite fura-2. Therefore, myoblasts had to be cultured in 35-mm plastic petri dishes modified by drilling a 20-mm diameter hole through the center and permanently attaching a glass coverslip to the bottom with 100% silicone rubber aquarium sealant (Dow Corning, Midland, MI). For dystrophin immunofluorescence staining, myoblasts were cultured in four-chamber glass slide flasks (Lab-Tek, Naperville, IL). All petri dishes and glass slides were treated with 0.01% calf skin collagen, type I (Sigma), in Puck's saline and sterilized with ultraviolet light.

Fura-2 Intracellular Calcium Measurements

Intracellular free calcium measurements using fura-2 and fluorescence ratio imaging were made in elongated myotubes, similar in size and shape, and at the same age in cell culture from 6 to 13 d of differentiation. Myotubes were loaded with 10 μ M fura-2/AM for 1 hour, in the dark, at room temperature, in Puck's saline solution with 8% dialyzed fetal bovine serum (Gemini Bioproducts, Calabasas, CA). To facilitate probe uptake into the myotube, fura-2/AM (Molecular Probes, Eugene, OR) at a 10-mM stock concentration in dimethyl sulfoxide was mixed 1:1 with 25% (wt/vol) Pluronic F-127 (Molecular Probes). Fura-2/AM is cell-permeant and readily enters the cell interior where nonspecific cytoplasmic esterases hydrolyze off the acetoxymethyl ester groups releasing fura-2, the cell impermeant form. After fura-2 loading, myotube cultures were washed three times with rodent saline solution to remove unincorporated fura-2. The rodent saline solution consisted of the following (in mM): 138 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1.06 $MgCl_2$, 5.6 glucose, and 12.4 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), $pH_o = 7.2$. The $CaCl_2$ concentration was raised to 18 mM to stress myotubes with 10-fold higher $[Ca^{2+}]_o$.

Fura-2 ratio images were obtained using a Nikon Fluorite $\times 40$ objective (Garden City, NY; NA 1.30 oil) on a Zeiss IM-35 inverted

microscope (Thornwood, NY) converted for microfluorimetry. An alternating filter wheel contained interference filters (for excitation mode ratioing) set to deliver light at 385 and 345 nm with half-band widths of 10 nm. A 395-nm dichroic mirror in the light path directed excitation and emission light to their appropriate destinations. Fura-2 fluorescence emissions occurring after passage through a 450-nm-long pass filter was quantitated using an air-cooled, Dage 66 silicon-intensified target camera (Dage-MTI, Michigan City, IN). The signal was digitized with a Gould 5000 image processor under the control of a PDP 11/73 microcomputer (Digital Equipment, Marlboro, MA). Myotube ratio images were obtained approximately every 3.5 s and were permanently stored on a Panasonic (Secaucus, NJ) optical laser disk during the experiment. Myotube $[Ca^{2+}]_i$ concentrations were determined from laser disk stored images using the formula described by Grynkiewicz *et al.* (1985): $[Ca^{2+}]_i = K_d (R - \nu R_{min}) / (\nu R_{max} - R)$, where R_{max} is the fura-2 ratio in saturating calcium solution, R_{min} is the fura-2 ratio in zero calcium solution (ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid [EGTA]-buffered), R is the fura-2 ratio from the myotube, ν is 0.71 the correction factor for fura-2 fluorescence in the myotube (Fong *et al.*, 1990), and K_d is the fura-2 effective dissociation constant at 37°C.

Heterokaryons were identified following $[Ca^{2+}]_i$ measurements by Hoechst 33342 staining. A stock concentration of Hoechst 33342 (1 mg/ml) was diluted 1/1000 with rodent saline solution and incubated with myotubes for 10 min at 23°C. Mouse nuclei have punctate chromatin staining and human nuclei appeared smooth in fluorescence when excited at 385 nm (Blau *et al.*, 1983; Huard *et al.*, 1991).

The myotube $[Ca^{2+}]_i$ values are nanomolar and are reported as the mean $\pm$ SEM; n is the sample number. Statistical significance was determined by the Student's *t* test and $[Ca^{2+}]_i$ differences where $p < 0.05$ were considered significant.

Immunocytochemical Detection of Dystrophin

The dystrophin polyclonal antibody was made against a chemically synthesized decapeptide representing the predicted sequences from the last 10 amino acids of the carboxy-terminal (C-terminal) portion of human dystrophin (Ervasti *et al.*, 1990). The dystrophin antiserum was a gift from Dr. Kevin P. Campbell (University of Iowa, Iowa City, IA). The secondary antibody was a F(ab)2' donkey anti-rabbit immunoglobulin conjugated to tetramethyl rhodamine isothiocyanate (TRITC), and normal donkey serum was obtained from Jackson ImmunoResearch Laboratories (Avondale, PA).

Muscle cell cultures undergoing early stages of differentiation were probed for dystrophin expression by indirect immunofluorescence. Myotubes were washed one time with phosphate-buffered saline (PBS) and fixed with acetone:methanol (1:1) for 3 min at room temperature. Myotubes were then blocked with 5% normal donkey serum in PBS for 1 h. The donkey serum was removed, and dystrophin antibody was diluted 1:1000 with PBS, 2.5% donkey serum, and 0.05% tween-20 before being added to appropriate myotube chambers. The control myotube samples contained normal donkey serum in place of dystrophin antibody, and all myotube samples were incubated overnight (21°C). Afterwards, myotubes were exposed for 1 h to secondary antibody diluted 1:250 with PBS, 1% donkey serum, and 0.05% tween-20. All incubations occurred at 21°C, in a dark, humidified chamber, and after each treatment, myotube chambers were washed three times, over 15 min, with PBS and 0.05% tween-20. Dystrophin labeled myotubes were mounted in a glycerol:PBS (1:1) medium containing 5% *n*-propyl gallate (Sigma) as an antioxidant. A glass coverslip was mounted over the myotubes, and the edges were sealed with nail polish. Hoechst 33342 was used as a last step in the dystrophin immunostaining procedure to stain myotube nuclei.

Confocal myotube images were obtained with a laser scanning confocal microscope system (BioRad MRC 600, Cambridge, MA) equipped with a Nikon Fluor $\times 40$ (1.30 NA) oil-immersion objective. The green line (514 nm) of the argon ion laser was used for fluorescence excitation, and the resulting fluorescence emission occurring at ≥ 550 nm was collected. Each myotube image was scanned 10 times, averaged, and analyzed directly by the MRC 600 software to give the pixel intensity profile of dystrophin myotube staining.

RESULTS

Dystrophin Expression

We induced cell fusion between normal mouse and Duchenne myoblasts to reintroduce normal nuclei, and therefore normal dystrophin, into dystrophic myotubes. To produce heterokaryons with equal or greater numbers of Duchenne nuclei, it was necessary to initiate Duchenne cultures at a density that was eight-times greater than mouse. Therefore, we cocultured Duchenne (10^5 cells/ml) and normal mouse (1.2×10^4 cells/ml) myoblasts and differentiated them by placing them in reduced serum growth medium. Myotubes were observed 2–4 d after incubation in differentiation medium, but were not used until 1 wk later to allow for a brief period of development.

Confocal laser scanning microscopy and immunofluorescence was used to probe 1.5–2 wk-old normal mouse and Duchenne myotubes, and heterokaryons for their ability to express dystrophin and to determine its location in the cell (Figures 1 and 2). Normal mouse myotubes and heterokaryons exhibited strong fluorescence labeling of dystrophin at the sarcolemma in confocal images. In some instances, dystrophin occurred as patches of strong accumulation at the sarcolemma while other areas were reduced for dystrophin labeling. In contrasts, Duchenne myotubes did not show any evidence of dystrophin labeling at the sarcolemma. We also observed no sarcolemmal dystrophin staining in cultures of *mdx* myotubes or normal mouse fibroblasts. These results support the specificity of antibody labeling of dystrophin in normal mouse and heterokaryon myotubes.

To better determine dystrophin labeling at the myotube sarcolemma, confocal images were traversed several times by a line that was perpendicular to the length of the myotube and fluorescence intensities of the secondary antibody were measured along the line. In Figure 1, normal mouse myotubes generated sharp peaks at each point where the line crossed the sarcolemma indicating that dystrophin was localized there. Also, the interior of the myotube exhibited greatly reduced fluorescence compared with the sarcolemma. However, some internal myotube areas showed positive staining, possibly representing dystrophin before being transferred to its muscle surface location. In contrast, Duchenne myotubes exhibited a lack of dystrophin fluorescence staining and an absence of peaks for dystrophin at the sarcolemma. In Duchenne myotubes, the profile of dystrophin expression showed small increases as the line passed over the interior of the myotube. This profile most likely reflects nonspecific secondary antibody retention because serum-blocked controls also produced low intracellular fluorescence but no sarcolemmal staining.

Normal mouse myotubes were investigated further for dystrophin labeling in longitudinal sections using confocal laser scanning microscopy (Figure 3). At the

Figure 1. Immunofluorescence localization of dystrophin in normal mouse and Duchenne myotube cultures. (A and C) Confocal laser scanning microscope myotube images of rhodamine antidystrophin immunofluorescence. (B and D) Graphical representation of dystrophin fluorescence staining profile along a line traversing through the long axis of normal mouse and Duchenne myotubes. (A) Normal mouse myotube with intense dystrophin staining at the sarcolemma. (B) Profile of dystrophin staining in the normal mouse myotube illustrates the intensity of dystrophin labeling at the sarcolemma as shown by sharp peaks occurring each time the indicator line crosses over the plasma membrane. In contrast, dystrophin labeling of the interior of the myotube was low compared with the sarcolemma. (C) Duchenne myotube lacking dystrophin fluorescence staining at the sarcolemma. (D) The profile of dystrophin staining in the Duchenne myotube also showed no dystrophin labeling of the myotube sarcolemma. As in the normal myotube a low fluorescence signal was seen in the interior of the Duchenne myotube. Myotubes were cultured for 13 d before fixing with acetone:methanol (1:1) and adding antidystrophin antibody. Bar, 10 μ m.

apical region of the myotube, dystrophin exhibited a lattice network expression over the myotube sarcolemma. As the confocal plane was moved inward into the myotube, the presence of dystrophin labeling at the sarcolemma was clearly visible as shown by strong fluorescence at this area. Also, nuclei appeared as dark spots inside the myotube showing that the dystrophin antibody was not labeling membrane nonspecifically. At the bottom of the myotube, near the sarcolemma and glass substrate interface, dystrophin expression occurred in a repetitive banding pattern reflective of sarcomere formation. The differences in dystrophin expression at the apical and basal areas of the myotube sarcolemma may occur as a result of myotube matu-

ration. Alternatively, it may reflect an effect of the cell culture dish surface.

Heterokaryons were recently reported to exhibit a mode of gene expression for myosin heavy chains and a membrane protein that was suggestive of nuclear domains (Pavlath *et al.*, 1989). Therefore, we were interested in determining whether heterokaryons produced by the fusion of normal mouse and Duchenne myoblasts also expressed dystrophin in nuclear domains. Figure 4 shows an elongated heterokaryon at 10 d of differentiation with clearly visible nuclei under brightfield microscopy. The heterokaryon consisted of three normal mouse nuclei and one Duchenne nucleus (at arrow) based on Hoechst 33342 staining, which labels the

Figure 2. Immunofluorescence localization of dystrophin in a Duchenne human-normal mouse heterokaryon myotube. Confocal laser scanning microscope myotube images were analyzed for dystrophin antibody staining as described in Figure 1. (A) Heterokaryon with three normal mouse nuclei and one isolated Duchenne nucleus located at the bottom of the myotube (see arrowhead in Figure 4). Dystrophin staining occurred at the sarcolemma in areas of the myotube occupied by normal mouse nuclei and a Duchenne nucleus. (B) Dystrophin expression at the sarcolemma was similar over the areas occupied by normal mouse and Duchenne nuclei as shown by the height of the peaks as the indicator line passed over the sarcolemma. In contrast, there was no dystrophin labeling of the nuclear membranes. (C) Dystrophin staining at the sarcolemma could also be detected over a distance 300 μm distal to the Duchenne nucleus in an area where no other nucleus was present. The end region of the elongating myotube showed strong dystrophin labeling. (D) The staining of dystrophin was strong at various locations along the length of the myotube, and in areas where staining was low, sarcolemmal dystrophin localization was still observed. The end region of the myotube in (C) shows that peaks for dystrophin are two times greater than peaks seen in areas occupied by normal nuclei. Nuclear domains for dystrophin were not observed in heterokaryons. Bar, 15 μm .

mouse nucleus as punctate and the Duchenne nucleus as smooth in fluorescence (Blau *et al.*, 1983). Dystrophin labeling was detected in the area occupied by normal nuclei as shown by bright fluorescence and distinct peaks at the sarcolemma (Figure 2). In addition, the plasma membrane area around the isolated Duchenne nucleus was also stained for dystrophin. Furthermore, we found that dystrophin labeling at the sarcolemma extended down the length of the myotube $\sim 300 \mu\text{m}$ beyond the Duchenne nucleus where no other nucleus was present. The sarcolemmal dystrophin labeling at the area most distal to the Duchenne nucleus was almost twofold greater than labeling in the area of normal nu-

clei. This result shows that dystrophin expression in heterokaryons does not reflect the presence of nuclear domains and that normal sarcolemmal localization of dystrophin can occur over the entire hybrid myotube.

Calcium Regulation

Resting $[\text{Ca}^{2+}]_i$ levels were measured in normal and dystrophin-deficient muscle cells at myoblast and myotube stages. We found no significant difference in resting $[\text{Ca}^{2+}]_i$ levels in mouse myoblasts ($104 \pm 8 \text{ nM}$, $n = 31$, normal mouse vs. 109 ± 6 , $n = 44$, *mdx*, $p > 0.5$) or in human myoblasts ($133 \pm 9 \text{ nM}$, $n = 47$, normal human

Figure 3. Fluorescence confocal images of 16-d-old normal mouse myotubes labeled by rhodamine antidystrophin immunofluorescence. (A) Dystrophin appears as a lattice network at the apical surface area of the myotube. (B and C) As the focus plane is moved inward the presence of dystrophin at the sarcolemma is clearly visible as shown by bright fluorescence labeling. (C) In contrast, the interior is only weakly fluorescent and the nucleus appears as dark spots inside the myotube. In addition, near the sarcolemma and glass interface, dystrophin staining occurs as bands resembling sarcomeres (see arrowhead). (D) At the basal area of the myotube surface, dystrophin appears in a lattice network. The sarcolemmal location of dystrophin can also be seen in neighboring myotubes at various positions in the focus plane. Bar, 25 μm .

vs. 114 ± 9 , $n = 67$, Duchenne, $p > 0.2$). However, after 4–8 days of differentiation, significant differences arose in $[\text{Ca}^{2+}]_i$ in mouse myotubes (82 ± 5 nM, $n = 66$, normal mouse vs. 111 ± 7 , $n = 65$, *mdx*, $p < 0.001$) and in human myotubes (89 ± 8 nM, $n = 79$, normal human vs. 118 ± 8 , $n = 85$, Duchenne, $p < 0.02$).

We produced heterokaryons to determine if resting $[\text{Ca}^{2+}]_i$ levels return to normal after the sarcolemmal re-expression of dystrophin in dystrophic myotubes. We measured $[\text{Ca}^{2+}]_i$ in normal, dystrophic, and heterokaryon myotubes incubated in standard (1.8 mM $[\text{Ca}^{2+}]_o$) saline solution using fura-2 and fluorescence ratio imaging technology (Figure 5A). Normal mouse and heterokaryon myotubes between 6 and 13 days of differentiation were found to maintain similar resting levels of $[\text{Ca}^{2+}]_i$ (151 ± 5 nM, $n = 69$, normal mouse;

168 ± 6 nM, $n = 42$, heterokaryon). In contrast, Duchenne myotubes at similar periods in differentiation maintained significantly ($p < 0.001$) elevated resting levels of $[\text{Ca}^{2+}]_i$ (241 ± 10 nM, $n = 72$). In addition, when exposed to 18 mM $[\text{Ca}^{2+}]_o$ saline solution, normal mouse and heterokaryon myotubes continued to maintain significantly lower levels of $[\text{Ca}^{2+}]_i$ from Duchenne myotubes over a 30-min period. Because we were measuring calcium in two different species, the differences in $[\text{Ca}^{2+}]_i$ reported between normal mouse and Duchenne human myotubes may not accurately indicate that the dystrophic myotubes have an abnormally elevated level of $[\text{Ca}^{2+}]_i$. Therefore, in one experiment, we compared resting $[\text{Ca}^{2+}]_i$ levels in normal mouse, normal human, Duchenne, and heterokaryon myotubes. Our results show that normal mouse (123 ± 8 nM, n

Figure 4. Fluorescence and brightfield photomicrographs of a Duchenne human-normal mouse heterokaryon myotube shown in Figure 2. (A) A 10-d-old heterokaryon with three normal mouse nuclei that appear punctate in fluorescence and one Duchenne nucleus that appears smooth fluorescence (see arrowhead). (C) No other nuclei are present in the myotube process that extends $\sim 300 \mu\text{m}$ beyond the Duchenne nucleus. (B and D) Brightfield photomicrographs of the heterokaryon. Heterokaryons were labeled with $1 \mu\text{g/ml}$ Hoechst 33342 for 10 min to identify mouse and human nuclei. Photomicrographs were taken using a Nikon Fluorite $\times 40$ objective (NA 1.30 oil) on a Nikon Diaphot inverted microscope. Bar, $25 \mu\text{m}$.

$= 18$), normal human ($147 \pm 7 \text{ nM}$, $n = 11$), and heterokaryon ($141 \pm 9 \text{ nM}$, $n = 11$) myotubes all maintained lower resting levels of $[\text{Ca}^{2+}]_i$ than Duchenne ($201 \pm 19 \text{ nM}$, $n = 17$) myotubes. These results show that Duchenne myotubes are defective in calcium control as shown by elevated levels of $[\text{Ca}^{2+}]_i$ and this calcium difference is not due to species differences. Therefore, dystrophin expression in Duchenne muscle cells corrects $[\text{Ca}^{2+}]_i$ toward normal levels suggesting that the presence of dystrophin is normally essential to maintaining calcium homeostasis.

Because heterokaryons were variable in their composition of normal mouse and Duchenne nuclei, it was possible that a difference in $[\text{Ca}^{2+}]_i$ could be found if we compared heterokaryon populations that were primarily normal mouse or Duchenne in nuclear content. Figure 5B shows heterokaryon $[\text{Ca}^{2+}]_i$ levels in hybrid myotubes that contained equal or greater numbers of Duchenne or normal mouse nuclei. Heterokaryons with greater numbers of Duchenne nuclei (19% of the heterokaryon samples) had an $[\text{Ca}^{2+}]_i$ of $178 \pm 19 \text{ nM}$ (n

$= 7$); heterokaryons with an equal number of normal and Duchenne nuclei (43% of the heterokaryon samples) had an $[\text{Ca}^{2+}]_i$ of $169 \pm 6 \text{ nM}$ ($n = 16$); and heterokaryons with a greater number of normal nuclei than Duchenne (38% of the heterokaryon samples) had an $[\text{Ca}^{2+}]_i$ of $148 \pm 10 \text{ nM}$ ($n = 14$). Although a slightly higher $[\text{Ca}^{2+}]_i$ was found in heterokaryons with greater numbers of Duchenne nuclei than normal, no significant $[\text{Ca}^{2+}]_i$ differences occurred as a consequence of nuclear composition.

Resting $[\text{Ca}^{2+}]_i$ levels from normal and dystrophic myotubes incubated in saline solutions with 1.8 mM $[\text{Ca}^{2+}]_o$ show their efficiency at controlling calcium. When we compared individual myotube resting $[\text{Ca}^{2+}]_i$ levels, a gaussian distribution of calcium ranges was found for normal mouse myotubes and heterokaryons (Figure 6). In contrast, Duchenne myotubes had two peak calcium ranges. One peak occurred in the normal calcium ranges while the other peak was $\sim 160 \text{ nM}$ higher in the calcium range (36% of sample). Furthermore, in only one instance of normal mouse (1% of

Figure 5. Intracellular free calcium ($[\text{Ca}^{2+}]_i$) measurements estimated by fura-2 and fluorescence ratio imaging. (A) Normal mouse, Duchenne, and heterokaryon myotubes (6–13 d old) were incubated in a rodent saline solution with 1.8 mM $[\text{Ca}^{2+}]_o$ during $[\text{Ca}^{2+}]_i$ measurements. Normal mouse and heterokaryon myotubes maintained similar levels of resting $[\text{Ca}^{2+}]_i$; however, Duchenne myotube resting $[\text{Ca}^{2+}]_i$ was 30% higher. (B) Heterokaryons were grouped into one of three types and resting $[\text{Ca}^{2+}]_i$ levels were determined. Heterokaryons were grouped according to whether they contained more Duchenne, more normal mouse, or an equal number of Duchenne and normal mouse nuclei. Heterokaryons did not exhibit significant resting $[\text{Ca}^{2+}]_i$ differences from each other regardless of their nuclear content. The calculations used to determine $[\text{Ca}^{2+}]_i$ levels in myotubes are described in MATERIALS AND METHODS.

sample) and heterokaryon myotubes (2% of sample) did we find $[\text{Ca}^{2+}]_i$ levels in the range of Duchenne myotubes with higher resting calcium. Therefore, these findings suggest the possibility that Duchenne myotubes initially appear normal but gradually lose their ability to control $[\text{Ca}^{2+}]_i$ as they undergo further development.

DISCUSSION

These results showed that dystrophin was expressed throughout the myotube in all mouse-human heterokaryons as has been reported for heterokaryons between *mdx* mice and normal rat myoblasts (Huard *et al.*, 1991). Heterokaryons with normal mouse nuclei and Duchenne nuclei expressed dystrophin over the areas occupied by the Duchenne nuclei. In the most extreme example, the expression of dystrophin continued ~300 μm down the length of the myotube beyond the last nucleus, which was a Duchenne nucleus. Furthermore, our results show that dystrophin is at the sarcolemma, at its recognized functional position. Therefore, our re-

sults do not support nuclear domains for dystrophin expression in myotube heterokaryons. Nuclear domains have been reported in myotube heterokaryons for the expression of other proteins such as the myosin heavy chain and a surface membrane protein (Pavlati *et al.*, 1989). Our confocal serial images of dystrophin immunostaining in normal myotubes showed regional differences for dystrophin labeling over the apical and basal surfaces of the myotube. At the apical region, myotubes showed dystrophin in a diffuse, lattice network, over the sarcolemma. In contrast, distinct bands of dystrophin labeling occurred at the myotube-glass substrate interface. This area of the myotube may represent a more functional organization of myofibrils, and

Figure 6. The distribution of $[\text{Ca}^{2+}]_i$ levels in individual myotubes from cultures of normal mouse, Duchenne, and heterokaryons between 6 and 13 d of differentiation. Heterokaryon and normal mouse myotubes maintained a similar single peak distribution of resting $[\text{Ca}^{2+}]_i$. The calcium range where most myotubes were found was ~160 nM $[\text{Ca}^{2+}]_i$ for heterokaryons and ~150 nM $[\text{Ca}^{2+}]_i$ for normal mouse. In contrast, Duchenne myotubes exhibited two peak distributions of resting $[\text{Ca}^{2+}]_i$. One peak of $[\text{Ca}^{2+}]_i$ occurred at approximately normal levels at 180 nM $[\text{Ca}^{2+}]_i$, while the other peak was almost two times higher at 340 nM $[\text{Ca}^{2+}]_i$. Myotubes were incubated in rodent saline solution with 1.8 mM $[\text{Ca}^{2+}]_o$ and fura-2 $[\text{Ca}^{2+}]_i$ measurements occurred over a period of 20 min at 35°C. The calculations used to determine $[\text{Ca}^{2+}]_i$ levels in myotubes are described in MATERIALS AND METHODS.

the corresponding repetitive antibody staining pattern may indicate dystrophin localization to sites over the I- and M-band regions of the sarcomeres (Porter *et al.*, 1992).

We have used fura-2 responses to measure the levels of free intracellular calcium and to compare calcium regulation in normal, dystrophic, and heterokaryon myotubes. To avoid possible artifacts, it is necessary to calibrate fura-2 responses in cytoplasm in both myotube types by clamping intracellular free calcium to known levels with microinjected Ca^{2+} /EGTA-buffered solutions (Turner *et al.*, 1988). This procedure gives rise to a correction factor that was the same for normal and *mdx* mouse fibers. Also, permeabilized myotubes in solutions at physiological levels of free intracellular calcium gave similar correction factors (Fong *et al.*, 1990). Recently another group has confirmed our findings of elevated $[\text{Ca}^{2+}]_i$ levels in *mdx* myotubes using fura-2 calibrated by saponin permeabilization in known extracellular calcium-EGTA/bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA)-buffered solutions (Bakker *et al.*, 1993). Other free calcium indicators like quin-2 (Mongini *et al.*, 1988), indo-1 (Sarabia and Klip, 1989), and calcium-selective microelectrodes (Sanchez *et al.*, 1988) showed significantly higher levels of $[\text{Ca}^{2+}]_i$ in dystrophin-deficient muscle cells, although absolute levels can not be compared by these different methods. There are two other independent methods which indicate higher levels in *mdx* fibers and myotubes. The higher level of protein degradation can be shown to be entirely correlated with the fura-2-measured free intracellular calcium manipulated independently of fiber type (Turner *et al.*, 1988). The kinetics of calcium sequestration can also be directly correlated with the fura-2-measured levels in myotubes (Turner *et al.*, 1991). Therefore, the $[\text{Ca}^{2+}]_i$ differences between normal and dystrophic myotubes do not result from changes in fura-2 fluorescence due to differences in cytoplasmic factors or compartmentalization in the two myotube types.

We have shown that heterokaryons exhibit normal levels of $[\text{Ca}^{2+}]_i$ that did not depend on the presence of more normal than Duchenne nuclei in the myotube. Although it may be possible that heterokaryon $[\text{Ca}^{2+}]_i$ measurements simply reflect Duchenne myotubes with normal $[\text{Ca}^{2+}]_i$, we do not believe this is the case for the following reason. When the distribution of individual myotube $[\text{Ca}^{2+}]_i$ levels were analyzed, we found a single peak $[\text{Ca}^{2+}]_i$ level for heterokaryon myotubes which resembled the distribution of $[\text{Ca}^{2+}]_i$ observed in normal mouse myotubes. In contrast, two peaks for resting $[\text{Ca}^{2+}]_i$ were observed in Duchenne myotubes (Figure 6). In addition, only 2% of heterokaryon and 1% of normal mouse myotubes were found with elevated resting $[\text{Ca}^{2+}]_i$ levels compared with 36% of Duchenne myotubes. This strengthens the view that re-expression of dystrophin in heterokaryon myotubes restores $[\text{Ca}^{2+}]_i$ control. If heterokaryon myotubes were

unaffected by the presence of normal nuclei then a greater incidence of myotubes with elevated $[\text{Ca}^{2+}]_i$ levels should have been found in our large population of recorded heterokaryon myotubes. This did not occur. Therefore, resting $[\text{Ca}^{2+}]_i$ were at the lower normal levels in Duchenne human-normal mouse myotube heterokaryons, and this did not depend on the proportion of normal to dystrophic nuclei in the hybrid myotubes.

The defect in muscle $[\text{Ca}^{2+}]_i$ control in *mdx* and Duchenne is correlated with altered calcium leak channel activity (Turner *et al.*, 1988; 1991; Fong *et al.*, 1990), which results from calcium-dependent proteolysis (Turner *et al.*, 1993). In *mdx* myotubes, calcium leak channel Po is high and $[\text{Ca}^{2+}]_i$ is significantly elevated above normal. However, 50 μM leupeptin can prevent the increase in calcium leak channel activity and free calcium levels in the absence of dystrophin, which suggests that dystrophin may function to shield calcium leak channels from proteolysis (Turner *et al.*, 1993). We hypothesize that in dystrophic muscle the absence of dystrophin increases the amount of calcium-dependent proteolysis of leak channels increasing their Po and resting $[\text{Ca}^{2+}]_i$ and results in more proteolysis in a positive feedback system (Turner *et al.*, 1993). Different initial levels of myotube activity might lead to higher calcium values, which by means of this positive feedback system could give rise to a distinct population of Duchenne myotubes with elevated $[\text{Ca}^{2+}]_i$ levels.

We have shown that heterokaryon myotubes express dystrophin, incorporate it throughout the fused myotubes at the sarcolemma, and produce resting $[\text{Ca}^{2+}]_i$ levels that are significantly lower than dystrophin-deficient Duchenne myotubes. These results are promising for the suggested use of myoblasts for cell-fusion therapy in Duchenne muscular dystrophy (Karpati *et al.*, 1989; Morgan *et al.*, 1990; Partridge, 1991).

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