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CONTRACTILE PROPERTIES OF SKINNED MUSCLE FIBRES FROM YOUNG AND ADULT NORMAL AND DYSTROPHIC (*mdx*) MICE

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SUMMARY

1. Single muscle fibres were enzymatically isolated from the soleus and extensor digitorum longus (EDL) muscles of genetically dystrophic *mdx* and normal (C57BL/10) mice aged 3–6 or 17–23 weeks.

2. Fibres of both muscles were chemically skinned with the non-ionic detergent Triton X-100 (2% v/v). Ca^{2+}- and Sr^{2+}-activated contractile responses were recorded and comparisons were made between several contractile parameters of various fibre types of normal and dystrophic mice of similar age.

3. There were no significant differences in the following contractile parameters of skinned fibres of normal and *mdx* mice of the same age: sensitivity to activating Ca^{2+} (pCa_{50}) or Sr^{2+} (pSr_{50}) and differential sensitivity to the activating ions ($pCa_{50} - pSr_{50}$). However the maximum isometric tension (P_o) and the frequency of myofibrillar force oscillations in EDL fast-twitch fibres of young *mdx* mice were significantly lower than those of soleus fast-twitch fibres of the same animals, or fast-twitch fibres (EDL or soleus) of normal mice.

4. Age-related differences were apparent in some contractile parameters of both normal and *mdx* mice. In particular the steepness of force–pCa and force–pSr curves increased with age in normal mice, yet decreased with age in fibres of *mdx* mice.

5. A fluorescent probe, ethidium bromide, which interchelates with DNA, was used with laser-scanning confocal microscopy to determine the distribution of myonuclei in fibres. Fibres isolated from either muscle type of normal animals displayed a characteristic peripheral spiral of myonuclei. Fibres from muscles of *mdx* mice displayed three major patterns of nuclear distribution; the normal peripheral spiral, long central strands of nuclei, and a mixture of these two patterns.

6. The contractile characteristics of *mdx* fibres were not markedly influenced by the nuclear distribution pattern in that there were no discernible differences in the major contractile parameters (the Hill coefficients n_{Ca} and n_{Sr}, which are associated with the steepness of the Ca^{2+} and Sr^{2+} activation curves, pCa_{50}, pSr_{50}, pCa_{50}-pSr_{50}) of skinned fibres possessing peripheral or central nuclei. However, except for n_{Sr}, these values were all lower in individual fibres which displayed similar proportions of central and peripheral nuclei. The presence of mixed nucleation and absence of

fibres with embryonic contractile characteristics in *mdx* mice suggest that the dystrophin-negative fibres can repair locally occurring muscle damage.

INTRODUCTION

The description of an X chromosome-linked murine mutant (Bulfield, Siller, Wight & Moore, 1984), C57BL/10 *mdx*, has provided an animal model of muscular dystrophy which is allelic to the most common of human dystrophies, Duchenne muscular dystrophy (DMD). Both conditions are similar in that the product of the DMD (*mdx*) gene, dystrophin, is missing from all muscle tissues (Hoffman, Brown & Kunkel, 1987). A structural role for the protein is supported by its localization to the inner plasmalemmal surface through immuno-histochemistry, and by evidence of structural homology with structural proteins such as spectrin and α-actinin (for review see Beam, 1988). However, the absence of dystrophin is not equally damaging to muscle tissue in the DMD and *mdx* conditions. Individuals with DMD suffer from severe and progressive muscle deterioration leading to eventual death. In contrast, apart from a peak period of muscle fibre necrosis and regeneration between the ages of 2 to 6 weeks and maintained, elevated serum kinase levels (Glesby, Rosenmann, Nylen & Wrogemann, 1988), *mdx* mice display no major symptoms of dystrophy (Dangain & Vrbova, 1984) and may have a normal life expectancy (Carnwath & Shotton, 1987). The difference is puzzling and underlies the need to determine the physiological role for dystrophin in muscle.

We have previously reported (Fink, Stephenson & Williams, 1990), that muscle fibres isolated from individuals afflicted with Duchenne muscular dystrophy (DMD) and from the autosomal mutant form of murine dystrophy 129ReJ *dy/dy*, display a number of contractile characteristics that are different from those of fibres from control animals. Although DMD and *dy/dy* dystrophies share a number of etiological features, muscle fibres of the *dy/dy* murine model do not lack dystrophin (Love, Morris, Ellis, Fairbrother, Marsden, Bloomfield, Edwards, Slater, Parry & Davies, 1991). Here we have investigated contractile properties of individual skinned skeletal muscle fibres from both *mdx* and control (C57BL/10 ScSn) mice to see if any features of the contractile responses were modified by this X-linked dystrophy. Although this type of investigation may not directly test the physiological role played by dystrophin in intact muscle it will uncover possible secondary effects of dystrophin absence that may be mediated through fibre degeneration/regeneration cycles and fibre repair.

We have also taken advantage of the depth discrimination (optical sectioning ability) of confocal microscopes to unequivocally distinguish in each individual isolated fibre between peripherally and centrally located myonuclei stained with the fluorophore ethidium bromide. Since it is thought that the presence of centro-nucleation is indicative of a regenerated muscle fibre (Harris & Johnson, 1978), we were able to relate measured contractile properties with the regenerative status of each individual muscle fibre under investigation.

Preliminary findings of this investigation have previously been communicated (Williams, Head & Stephenson, 1989; Head, Lynch, Stephenson & Williams, 1991).

METHODS

Muscle preparations

Single muscle fibres were obtained from either the soleus (mixed fast- and slow-twitch) or the extensor digitorum longus (EDL; fast-twitch) muscles from C57BL/10 ScSn mice, and from the X chromosome-linked muscular dystrophy mutant C57BL/10 *mdx*. All animals were painlessly killed by rapid cervical dislocation. Control and dystrophic animals were chosen from two age groups: (i) young, 3–6 weeks (encompassing the period of peak muscle fibre necrosis), and (ii) adult, 17–23 weeks (post-necrotic period). Single muscle fibres were isolated from intact murine muscles with an enzymatic technique previously described (Head, Stephenson & Williams, 1990). Briefly, ablated muscles were incubated in Tyrode solution (mM: 2·5 Ca^{2+}, 10·0 glucose, 135 NaCl, 4 KCl, 1 $MgCl_2$ 0·33 NaH_2PO_4, 10 HEPES; pH 7·3) containing 0·2% collagenase (Sigma Type IV) for 60 min at 30–35 °C. After this time the muscle bundle was removed from the enzyme mixture and rinsed (twice) and resuspended in a high Na^{2+}-containing relaxing solution containing 50 mM EGTA (Head *et al.* 1990). Gentle swirling of the solution liberated a large number of individual muscle fibres (70–90% of total) which were maintained in the same relaxing solution at 5 °C until required for experiments.

Skinned fibre preparations

Single fibres enzymatically liberated from normal and dystrophic muscles were attached to a force recording apparatus: they were clamped at one end in a pair of stainless-steel forceps and tied at the other end with braided silk (Deknatel size 9·0) to the pin of an AE801 strain-gauge transducer. The sarcolemmal barrier and internal membrane systems were removed by incubation of fibres with 2% Triton X-100 in a K^+-based relaxing solution (Type A, see later) for 10 min (see Head *et al.* 1990).

Average sarcomere length of all fibres was determined from the diffraction maxima produced by a He–Ne laser used to illuminate the mounted preparations. The length of all fibres was adjusted after mounting to the force recording apparatus so that sarcomere length for all normal and dystrophic fibres was between 2·6 and 2·9 μm with an average value (mean ± S.E.M.) of 2·67 ± 0·01 μm ($n = 171$).

Solutions

The experimental solutions (A, B, H and K) were similar to those previously employed in the laboratory in all other studies of the function of skinned mammalian muscle fibres (Fink, Stephenson & Williams, 1990; Head *et al.* 1990; Lynch, Stephenson & Williams, 1991), and contained (mM): 117 K^+, 36 Na^+, 1 Mg^{2+}, 60 HEPES, 8 adenosine triphosphate (ATP), 10 creatine phosphate; pH 7·10. The solutions differed from each other as follows. (i) Solution A: 50 mM $EGTA^{2-}$ (ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'* tetraacetate ions), (ii) solution B: 49–49·5 mM Ca-$EGTA^{2-}$ with excess $EGTA^{2-}$ (0·5–1·0 mM), (iii) solution H: 50 mM HDTA (hexamethylenediamine-*N,N,N',N'*-tetraacetate ions) or (iv) solution K: 40 mM Sr$EGTA^{2-}$ with 10 mM excess $EGTA^{2-}$. Solutions of differing pCa ($pCa = -\log_{10}[Ca^{2+}]$ with $6{\cdot}40 < pCa < 5{\cdot}0$) or Sr^{2+} ($pSr = -\log_{10}[Sr^{2+}]$ with $6{\cdot}50 < pSr < 4{\cdot}0$) concentrations were obtained by mixing (in various proportions) stock solutions B/A or K/A, respectively. Solutions of $pCa < 5{\cdot}0$ and $pSr < 4{\cdot}0$ were prepared separately.

Further details concerning fibre preparation, fibre attachment and solutions are outlined elsewhere (Fink *et al.* 1990; Head *et al.* 1990). All experiments were performed at room temperature (22–25 °C).

Data analysis

Assessment of the effects of Ca^{2+} and Sr^{2+} activation was achieved by construction of force–pCa and force–pSr curves for the contractile responses of each individual skinned fibre as previously described in detail (Fink *et al.* 1990; Wilson & Stephenson, 1990; Lynch *et al.* 1991). The fibre type designation could be determined from the experimental relations of each fibre by calculating the following parameters: pCa_{50} and pSr_{50} values (reflecting relative sensitivity to each activating ion); the difference $pCa_{50} - pSr_{50}$ which reflects the differential sensitivity to the two

activating ions; and the Hill coefficients (n) which reflect the steepness of the curve described by the theoretical relation $Pr = K[X^{2+}]^n/(1+K[X^{2+}]^n)$ which best fits the data points, where Pr is the relative force, $[X^{2+}]$ the concentration of divalent cations (Ca^{2+} or Sr^{2+}), and K is a constant. The presence and frequency of force oscillations of myofibrilar origin was also used as a diagnostic feature for fibre type classification.

The procedure for classifying fibres into respective types, on the basis of their contractile characteristics has been described in detail previously (Wilson & Stephenson 1990; Lynch *et al.* 1991). Individual fibres were only allocated to one of the two major fibre-type groups, fast-twitch or slow-twitch, if their force–pCa and force–pSr curves were judged to be of similar steepness as determined from an index of similarity, $I_s = (n_{Ca} - n_{Sr})/(n_{Ca} + n_{Sr}) < 0{\cdot}1$. Fast-twitch fibres were characterized by steep ($n > 2{\cdot}6$) force–pCa(pSr) relations and large ($> 1{\cdot}0$ log unit) $pCa_{50} - pSr_{50}$ values. Further sub-classification of fast-twitch fibres is also possible if a fibre exhibits high-frequency (> 1 Hz) force oscillations of myofibrilar origin, although in this study we have not attempted to further subdivide this fibre pool into the various fast-twitch fibre types. Slow-twitch fibres can be distinguished by shallow force–pCa(pSr) curves ($n < 2{\cdot}6$) and $pCa_{50} - pSr_{50}$ values usually less than 0·5. Low-frequency ($< 0{\cdot}4$ Hz) force oscillations are often exhibited.

Absolute maximum force responses (N cm^{-2}) were determined from the initial force responses in solutions of maximal Ca^{2+} and Sr^{2+} concentration. Absolute values were standardized for cross-sectional area which was determined after immersion of each fibre in aqueous solution, and corrected for the fibre swelling which occurs following skinning of each fibre (average swelling of cross-sectional area of each fibre was by a factor of 1·69). The cross-sectional area of some fibres was also determined during analysis for nuclear location (see later) under an inverted microscope containing an eyepiece calibrated graticule.

Statistics

All results are presented as the mean ± S.E.M. Student's t test was used to compare differences in the single fibre contractile parameters between the normal and dystrophic groups in both age groups (young and adult). Differences between the contractile properties of all groups were considered statistically different if $P < 0{\cdot}05$.

Localization of fibre nuclei

After analysis of the contractile and regulatory properties of individual skinned preparations, some fibres were removed from the force recording apparatus and incubated for 10 min in a K^+-based relaxing solution (Type A) containing ethidium bromide (5 μM; Molecular Probes, OR, USA). This fluorescent compound associates preferentially with cell DNA and RNA and allowed cell nuclei to be imaged with an Olympus IMT-2 inverted microscope coupled to a laser-scanning confocal microscope (Lasersharp MRC-500, BioRad). The fluorescence of ethidium bromide was excited with the 488 nm band of an argon ion laser and emission was collected, after passing through a long-pass filter (cut-on 515 nm), by a photomultiplier tube under manual black-level and gain control. The apparent thickness of the optical sections of muscle fibres was controlled by adjusting a pin-hole aperture in the emission path. A z-section thickness for optical sections of between 5 and 10 μm could be used to unequivocally distinguish between centrally placed and peripheral nuclei. The distribution of myonuclei within an individual fibre was described as (i) peripheral (arranged in peripherally located helices), (ii) central (arranged in long central strands) and (iii) mixed (combination of both previous patterns in the same fibre segment). Further details of the imaging system have been described elsewhere (Williams, Cody, Gehring, Parish & Harris, 1990; Williams, 1990).

RESULTS

Morphology of single muscle fibres

An enzymatic technique employing collagenase (0·2%, Sigma Type IV), previously described (Head *et al.* 1990) for dissociation of fibres from muscles of the dystrophic autosomal mutant 129/ReJ (*dy/dy*), can liberate high yields (70%) of single intact muscle fibres from skeletal muscles of control and *mdx* mice. Single fibres from the soleus and EDL muscles of control animals in both age groups were identical to those

described in other mammalian systems; elongated and unbranched and showed no evidence of cell necrosis or damage. The nuclei of fibres from both muscle types were invariably peripheral in location, and generally distributed in spirals (helices) around the fibre (see Fig. 6).

In the young group of *mdx* mice (3–6 weeks), apart from the classical normal muscle fibre seen in control animals, a number of other fibre morphologies were apparent. Some fibres showed necrotic regions adjacent to sections of fibre which displayed normal fibre morphology (cross-striations and peripheral nuclei; see Fig. 1*A*). Small myotube-like attachments were present in fibres which had long strands of centrally placed nuclei (Fig. 1*B*). Some individual fibres displayed great variation in diameter, and in the fibre shown in Fig. 1*C* a newly regenerated region with distinct central nuclei bordered a clearly supercontracted region (Fig. 1*D*). Other fibres showed focal-point lesions where membrane integrity was completely compromised (Fig. 1*E*). Regenerating myotubes with complex morphology were also evident (Fig. 1*F*). Mechanical damage to one branch of this syncitium induced contraction in the entire complex establishing that this structure did not represent three closely associated but individual fibres. All these morphologies were apparent in both EDL and soleus muscles but seemed to be more prevalent in EDL muscle fibres in the juvenile age group.

In the adult *mdx* group (17–23 weeks) many more abnormalities of fibre structure were apparent as we have described elsewhere (Williams *et al.* 1989; Head, Williams & Stephenson, 1992). In this study the majority of fibres which were selected for mounting to the force recording apparatus exhibited normal, elongated fibre morphology with little evidence of fibre branching, as judged by their appearance under a dissecting microscope (magnification × 40).

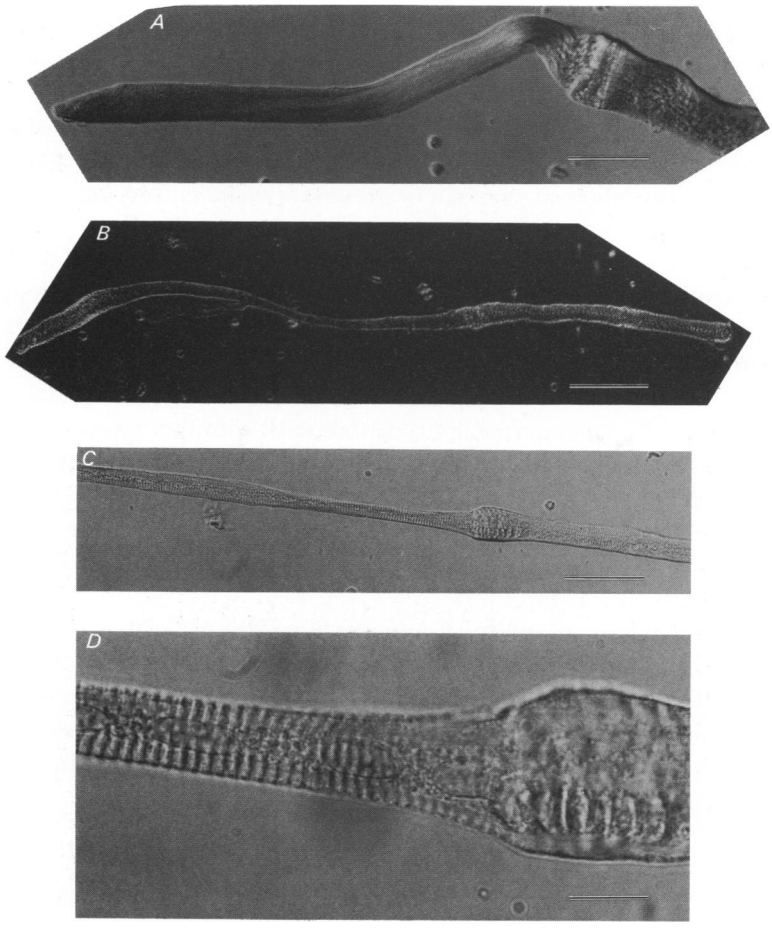
Physiological properties of skinned fibres

A number of contractile parameters from individual skinned muscle fibres of normal and dystrophic animals in both age groups were investigated. The results for normal and dystrophic animals are summarized in Tables 1 and 2, respectively.

Muscle fibres from normal animals

As we have shown previously fast- and slow-twitch murine muscle fibres from normal animals can be readily distinguished by using characteristics of their force–pCa and force–pSr relations. We have presented in a number of previous publications examples of the force–pCa and force–pSr relations for these murine fibre types, and as such these curves are not repeated here (Fink, Stephenson & Williams, 1986; Head *et al.* 1990).

The soleus muscle of normal (C57BL/10) animals possessed both fast- and slow-twitch fibres. In the young group (3–6 weeks of age) 45% (nine of twenty fibres) of the total sampled fibre pool comprised fast-twitch fibres, and this proportion was slightly higher in the adult (17–23 week) group (52%; eleven of twenty-one fibres). Fast-twitch fibres of the soleus differed markedly from slow-twitch fibres mainly with respect to differential sensitivity to Ca^{2+} and Sr^{2+} ($pCa_{50} - pSr_{50}$), and the frequency of myofibrillar force oscillations. Fast-twitch soleus fibres were 16 times more sensitive to Ca^{2+} than Sr^{2+}, and some exhibited 'high' frequency (> 2 Hz) force



oscillations. There was less discrimination between sensitivity to Ca^{2+} and Sr^{2+} (2-fold) in slow-twitch fibres, and all of these fibres also displayed lower frequency (0·3 Hz) oscillations of myofibrillar origin.

Age-dependent differences were not apparent when comparing pCa_{50}, pSr_{50} or $pCa_{50} - pSr_{50}$ values for either fibre type of the soleus muscle (Table 1). However, the steepness of force–pCa and force–pSr curves for soleus fibres was generally greater with age as evidenced by Hill coefficients n_{Ca} and n_{Sr} which were significantly higher in the adult than young group. A similar age-dependent increase in steepness of the force–pSr curve was exhibited by soleus slow-twitch fibres but was not evident in the force–pCa curve of the same fibres.

All individual fibres (twenty-six total) sampled from the EDL muscle of normal mice of both age groups were classified as fast-twitch fibres. There were no obvious differences in contractile characteristics (pCa_{50}, pSr_{50}, $pCa_{50} - pSr_{50}$) of EDL fibres between the two age groups (see Table 1). However, these fibres displayed a few contractile characteristics which were significantly different from those of the fast-twitch pool of the soleus muscle. The EDL fibre pool possessed steeper force –pCa and

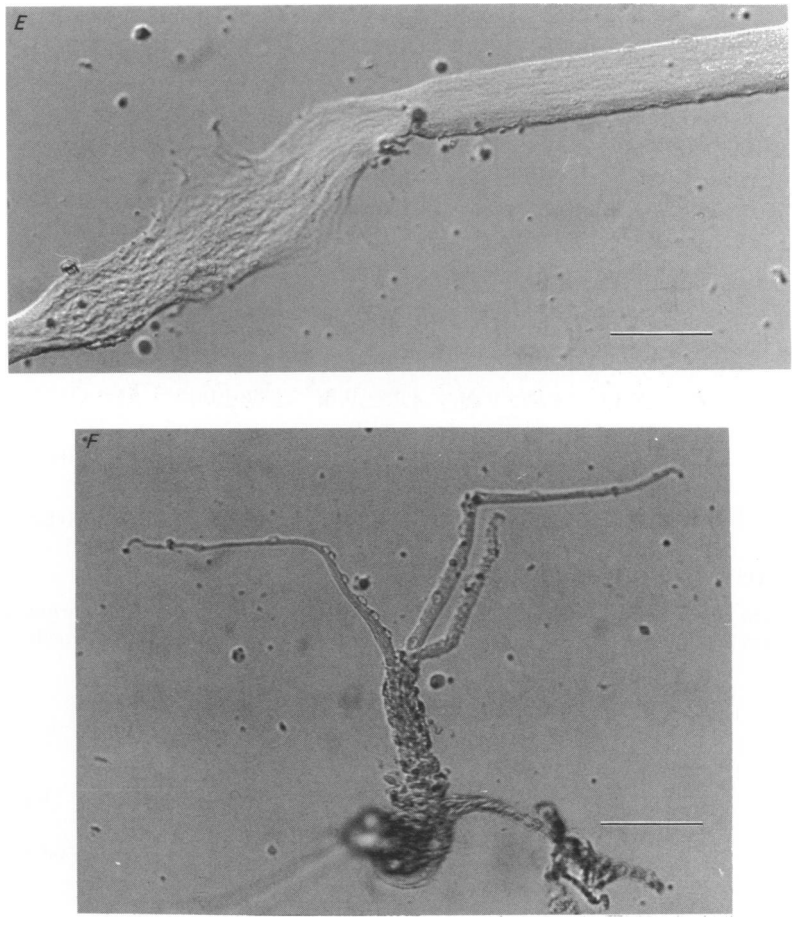


Fig. 1. Examples of some of the morphologies found in enzymatically isolated muscle fibres of *mdx* mice (aged 4–5 weeks). *A*, soleus fibre showing normal morphology (cross-striations and peripheral nuclei) adjacent to a necrotic fibre region. *B*, regenerated EDL fibre with central nuclei and myotubular attachments. *C*, soleus fibre exhibiting large variations in diameter and necrotic region, which under higher magnification (*D*) shows strings of central nuclei. *E*, EDL fibre showing localized area where membrane integrity was compromised. *F*, complex regenerating fibre from a soleus muscle. Scale bars: *A*, 75 μm; *B*, 120 μm; *C*, 75 μm; *D*, 15 μm; *E*, 90 μm; *F*, 120 μm.

force–pSr curves than those of the soleus fast-twitch fibre pool. These EDL fibres also generated higher maximal force responses (P_o) in both young and adult animals (see Fig. 3). However, it must be remembered that this EDL fibre pool contains a mixture of fast-twitch oxidative (FOG) and glycolytic (FG) fibres whereas the soleus fast-twitch pool represents FOG fibres exclusively (Anderson, Bressler & Ovalle, 1988).

Muscle fibres from dystrophic mdx animals

Some interesting differences were detected in the contractile parameters of *mdx* EDL and soleus muscle fibres in comparison to those of fibres from the same muscles of normal animals. The EDL muscle fibres from the young age group exhibited

TABLE 1. Characteristics of normal muscle fibres

	Fibre type					
	Soleus				EDL	
	Fast twitch		Slow twitch		Fast twitch	
	Young (9)	Adult (11)	Young (11)	Adult (8)	Young (10)	Adult (16)
pCa_{50}	5·87 ± 0·03 (9)	5·77 ± 0·04 (11)	6·07 ± 0·03 (11)	6·04 ± 0·03 (8)	5·91 ± 0·02 (10)	5·75 ± 0·04 (16)
n_{Ca}	3·48 ± 0·02 (9)	4·37 ± 0·46* (11)	3·10 ± 0·21 (11)	2·80 ± 0·20 (8)	5·20 ± 0·30 (10)	4·74 ± 0·15 (16)
pSr_{50}	4·67 ± 0·06 (8)	4·51 ± 0·07 (10)	5·78 ± 0·03 (10)	5·66 ± 0·03 (8)	4·69 ± 0·01 (10)	4·55 ± 0·03 (14)
n_{Sr}	3·20 ± 0·14 (8)	4·11 ± 0·31* (10)	2·93 ± 0·18 (10)	3·39 ± 0·17* (8)	5·05 ± 0·24 (10)	4·30 ± 0·26 (14)
$pCa_{50} - pSr_{50}$	1·19 ± 0·03 (8)	1·23 ± 0·05 (10)	0·30 ± 0·02 (10)	0·38 ± 0·02 (8)	1·22 ± 0·03 (10)	1·23 ± 0·02 (14)
Frequency of myofibrillar oscillations (Hz)	2·0 ± 0·0 (2)	2·07 ± 0·66 (3)	0·32 ± 0·05 (4)	0·31 ± 0·02 (8)	2·43 ± 0·22 (4)	—

All values are expressed as the mean ± S.E.M. with the number of fibres in parentheses.
* Fibres from adult animals were significantly different to those of the same type from the young animals.

TABLE 2. Characteristics of *mdx* muscle fibres

	Fibre type					
	Soleus				EDL	
	Fast twitch		Slow twitch		Fast twitch	
	Young (15)	Adult (23)	Young (6)	Adult (14)	Young (29)	Adult (16)
pCa_{50}	5·94 ± 0·02 (18)	5·86 ± 0·03 (23)	6·08 ± 0·02 (6)	6·11 ± 0·03 (14)	5·84 ± 0·03 (29)	5·81 ± 0·03 (16)
n_{Ca}	4·72 ± 0·24 (18)	4·23 ± 0·29 (23)	3·48 ± 0·56 (6)	3·22 ± 0·25 (14)	4·80 ± 0·22 (29)	4·30 ± 0·34 (16)
pSr_{50}	4·76 ± 0·02 (18)	4·59 ± 0·03 (23)	5·75 ± 0·06 (6)	5·77 ± 0·03 (14)	4·68 ± 0·03 (29)	4·57 ± 0·03 (16)
n_{Sr}	4·70 ± 0·22 (18)	4·04 ± 0·24* (23)	4·11 ± 0·73 (6)	3·72 ± 0·39 (14)	4·75 ± 0·23 (29)	3·97 ± 0·26* (16)
$pCa_{50} - pSr_{50}$	1·19 ± 0·02 (18)	1·27 ± 0·02 (23)	0·32 ± 0·04 (6)	0·34 ± 0·02 (14)	1·17 ± 0·02 (29)	1·24 ± 0·02 (16)
Frequency of myofibrillar oscillations (Hz)	2·1 ± 0·10 (2)	1·92 ± 0·34 (7)	0·29 ± 0·11 (4)	0·20 ± 0·04 (13)	0·55 ± 0·5 (7)	1·73 ± 0·28 (4)

All values are expressed as the mean ± S.E.M. with the number of fibres in parentheses.
* Fibres from adult animals were significantly different to those of the same type from the young animals.

myofibrillar force oscillations of a frequency not seen in any other fibre group or age group in this study as is evident in the force traces shown in (Fig. 2). This frequency (0·55 ± 0·5 Hz, $n = 7$) was significantly lower ($P < 0·05$) than that seen in the soleus

fast-twitch group ($2{\cdot}1 \pm 0{\cdot}1$ Hz, $n = 2$) from similarly aged *mdx* mice, or in the soleus fast-twitch group ($2{\cdot}0 \pm 0{\cdot}0$ Hz, $n = 2$) and EDL fast-twitch fibre groups ($2{\cdot}43 \pm 0{\cdot}22$ Hz, $n = 4$) from normal mice of similar age.

The maximum force generating capacity (P_0) of some muscle fibre groups from *mdx* mice was not significantly different from that of the respective fibre group in normal

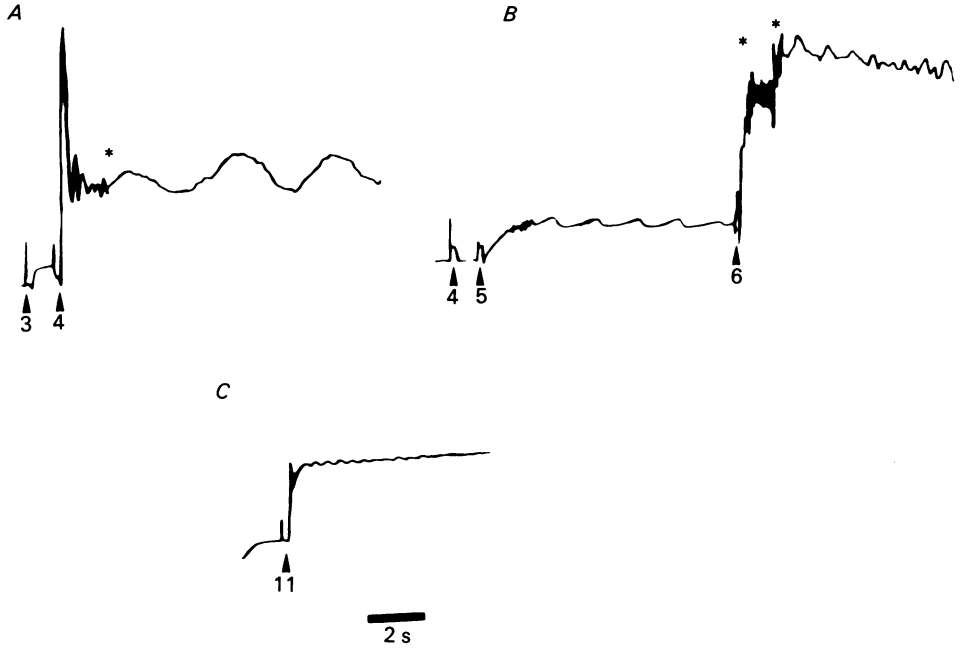


Fig. 2. Force traces showing the presence of force oscillations in submaximally activated muscle fibres of young (3–6 week) *mdx* mice. *A*, centrally nucleated soleus fibre with low-frequency (0·21 Hz), high-amplitude oscillations. Average force, 11·6% P_0; sarcomere length, 2·72 μm; diameter, 46 μm. *B*, EDL fibre showing irregular oscillations (frequency range 0·5–2·0 Hz). Average force, 5 and 29% P_0; sarcomere length, 2·75 μm; diameter, 90 μm. *C*, high-frequency (2·5 Hz), low-amplitude oscillations in a soleus fibre. Average force, 18% P_0; sarcomere length, 2·60 μm; diameter, 62 μm. Numbered arrows indicate the pCa (3–6) or pSr (11) of the activating solutions (3, 6·69; 4, 6·42; 5, 6·10; 6, 5·87; 11, 5·68). The chart speed was 15 times lower prior to the point indicated by the asterisk in panel *A* and between the asterisks in panel *B*.

animals (Fig. 3) although maximum force levels tended to be lower in the adult *mdx* groups. However, force levels were statistically significantly lower in the *mdx* soleus fast- and slow-twitch fibre types in comparison to fibres of the same groups of adult normal mice, a decrease which is similar to that reported in other dystrophic muscle types (Fink *et al.* 1986, 1990).

There was a marked tendency for the steepness of force–pCa and force–pSr curves of fast-twitch fibres to be lower in fibre groups of adult animals. This trend was apparent in both the EDL fast-twitch fibres and the fast-twitch fibres of the soleus, but was only statistically significantly different for the steepness values for force–pSr curves. This decreased steepness of force–pSr curves was accompanied by significant decreases in the sensitivity to activating cations with lower pCa_{50} and pSr_{50} values

evident in fibres of older animals (see Table 1). These changes were evident in both fast-twitch fibre pools (soleus and EDL), but not in the slow-twitch fibre pool of the soleus.

A total of eight fibres sampled from the soleus muscle of *mdx* mice (both young and adult) exhibited contractile characteristics which represented a mixture of those

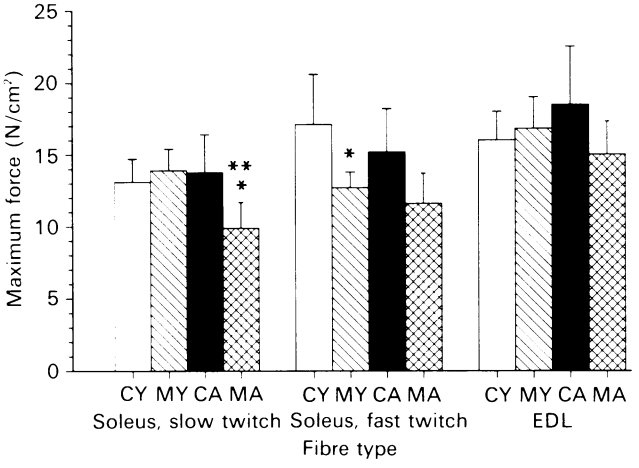


Fig. 3. Average maximum isometric force induced by Ca^{2+} for fibres of all fibre-type groups. Abbreviations denote: CY, control young; MY, *mdx* young; CA, control adult; and MA, *mdx* adult animals. Significant differences ($P < 0{\cdot}05$) existed between control and *mdx* groups of same age (*) and between *mdx* of different age (**) for some soleus fibre type populations. Vertical bars indicate the S.E.M. value. Fibre numbers for each group are contained in Tables 1 and 2.

shown by the fast- and slow-twitch fibre types and were therefore not assigned to the major fibre pools, fast-twitch and slow-twitch, and their contractile characteristics were not listed in Table 2. Instead these fibres were treated as 'mixed' fibres. Of these, four were also analysed for myonuclear distribution, and their contractile characteristics are discussed later. Of the four remaining mixed fibres three possessed force–pCa curves which were much steeper than the respective force–pSr curves ($n_{Ca} > n_{Sr}$). It was also evident in these fibres that the differential cation sensitivity was intermediate to that of the major fibre type groups ($0{\cdot}55 < pCa_{50} - pSr_{50} < 0{\cdot}95$). The remaining mixed fibre exhibited the flat force–pCa and force–pSr curves (n_{Ca}, $n_{Sr} = 2{\cdot}0$) characteristic of slow-twitch fibres, but with a large differential cation sensitivity ($pCa_{50} - pSr_{50} = 1{\cdot}00$) usually found in fast-twitch fibres. All fibres sampled from the muscles of normal animals could be classified into the well defined fast-twitch and slow-twitch pools of fibres.

Distribution of myonuclei in single fibres

The enzyme treatment used to isolate the muscle fibres was successful in removing the large number of satellite cells located between the basal lamina and fibre sarcolemma that may have potentially been stained by this technique. The observed pattern of nuclei, therefore, exclusively reflects the location of myonuclei.

All individual fibres from normal EDL and soleus muscles that were stained with the DNA/RNA selective fluorophore ethidium bromide displayed the normal, peripheral, helical pattern of nuclei as shown in Fig. 4*A*. In contrast, the majority of individual fibres sampled from both soleus and EDL *mdx* muscles showed evidence

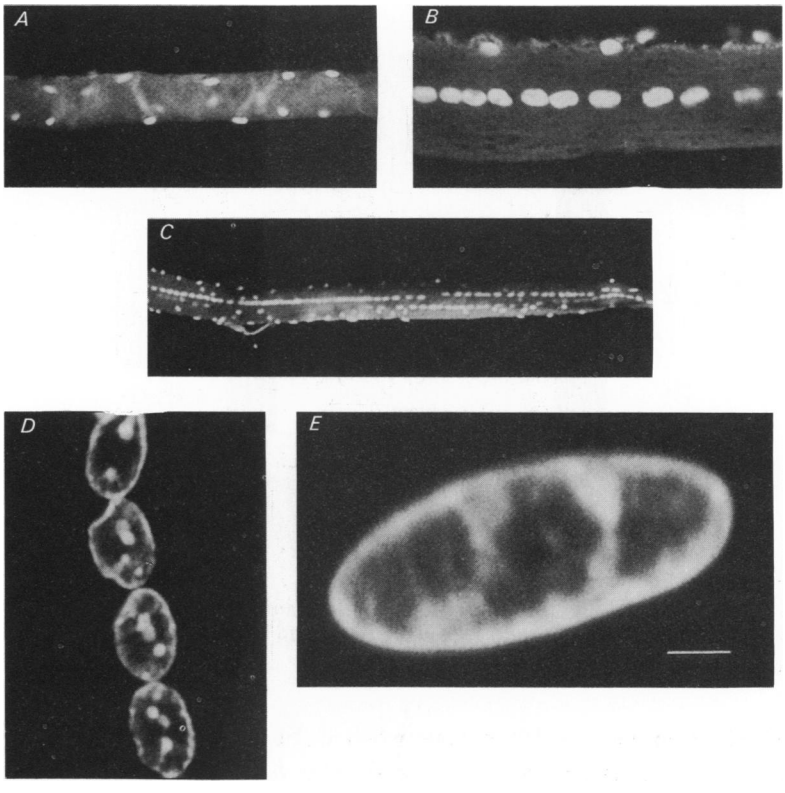


Fig. 4. Location of myonuclei of individual fibres fluorescently labelled with ethidium bromide and imaged with laser-scanning confocal microscopy. Soleus fibres from a normal mouse (*A*) with typical peripheral spiral of nuclei, and from an *mdx* mouse displaying (*B*) predominantly central nuclei of (*C*) a mixture of peripheral and central nuclei. Higher magnification images of a section of a centrally located strand of nuclei and a single central nucleus (*D*) of an *mdx* muscle fibre show that heterochromatin is located mainly peripherally which is characteristic of a cell which has entered a synthetic (G1) phase of activity. The scale bar in panel *E* relates to all panels and denotes: *A*, 45 μm; *B*, 30 μm, *C*, 70 μm; *D*, 8 μm and *E*, 2 μm.

of either a predominantly centrally located string(s) of myonuclei (Fig. 4*B*) or, even more interestingly, a combination of central nuclei and the peripheral pattern of nuclei (Fig. 4*C*). High magnification images of fluorescently labelled centrally located strings (Fig. 4*D*) or an individual central nucleus (Fig. 4*E*) indicate that the majority of heterochromatin is located around the edge of each nucleus (euchromatic) and this pattern is usually found in the nuclei of cells that are quiescent, but which have been moved from the G0 (resting) phase to the G1 (synthetic) phase of the cell cycle. The transition is usually initiated by cell trauma such as membrane damage,

Ca^{2+} influx and nerve injury (Grounds, 1991), and in this case probably indicates the nuclei are actively synthesizing RNA sequences coding for proteins required in fibre regeneration (Anderson, Ovalle & Bressler, 1987).

The *mdx* fibres which still retained the normal peripheral arrangement of myonuclei were generally in the young age group suggesting that, as previously

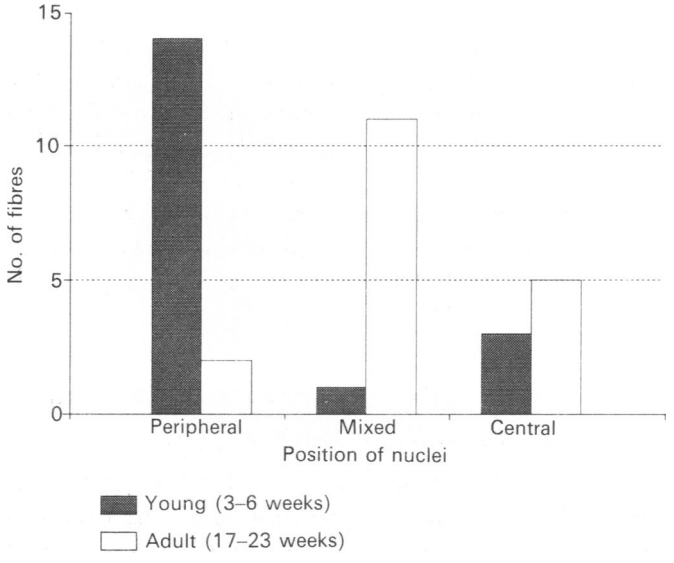


Fig. 5. Histogram showing the difference in the location of nuclei identified by ethidium bromide staining between individual muscle fibres from *mdx* animals of young (3–6 weeks) and adult (17–23 weeks) groups.

documented (Bulfield *et al.* 1984; Carnwath & Shotton, 1987), the appearance of central nuclei is closely linked to age of the fibre and progression of disease symptoms. This is more directly illustrated in Fig. 5 which shows the relationship between the age of the animal from which each muscle fibre was isolated and the estimated location of myonuclei in the fibre. It is evident that although most fibres in the young age group were peripherally nucleated nearly all fibres from both *mdx* muscle types showed some evidence of centronucleation (mixed plus central) in the adult age group (17–23 weeks). It is also apparent that few of these fibres are completely centrally nucleated, but instead exhibit variable degrees of mixing of the two nuclear distribution patterns. This observation supports the idea of a gradual or progressive process, rather than a dramatic or catastrophic process as would be exhibited in fibre degeneration/regeneration. All fibres from the normal animals were peripherally nucleated in all age groups.

The contractile characteristics of *mdx* muscle fibres which displayed a predominantly central distribution of myonuclei (see Fig. 5) were generally indistinguishable from those of fibres which displayed mostly peripheral nuclei. For example, in adult soleus fast-twitch fibres, where we were able to accurately describe nuclear location in a total of eleven fibres, pCa_{50} ($5{\cdot}88 \pm 0{\cdot}08$), pSr_{50} ($4{\cdot}63 \pm 0{\cdot}09$) and $pCa_{50} - pSr_{50}$ ($1{\cdot}33 \pm 0{\cdot}06$) were seen to be identical in both of these fibre groups

($n = 4$ in both groups). However, the steepness of force–pCa and force–pSr curves was markedly lower in the centrally nucleated fibres (n_{Ca} $3{\cdot}78 \pm 0{\cdot}38$; n_{Sr} $2{\cdot}98 \pm 0{\cdot}20$, $n = 4$) than in fibres that had peripheral nuclei (n_{Ca} $4{\cdot}73 \pm 0{\cdot}4$; n_{Sr} $4{\cdot}28 \pm 0{\cdot}32$; $n = 4$). If we assume that centronucleated fibres are regenerated, and therefore less mature than peripherally nucleated fibres of adult animals, then this steepening trend is similar to that which we described earlier as occurring with the aging of fibres.

The contractile parameters pCa_{50}, pSr_{50} and n_{Ca} for adult soleus fast-twitch fibres with similar numbers of central and peripheral nuclei ($n = 3$, mixed, see Fig. 5) were significantly lower than those of either centrally or peripherally nucleated fibres ($5{\cdot}68 \pm 0{\cdot}04$, $4{\cdot}42 \pm 0{\cdot}04$, and $2{\cdot}80 \pm 0{\cdot}18$, respectively), and indistinguishable from those of peripherally nucleated fibres for n_{Sr} ($4{\cdot}37 \pm 0{\cdot}78$) and $pCa_{50} - pSr_{50}$ ($1{\cdot}26 \pm 0{\cdot}05$). These fibres were classified in the previously mentioned group of 'mixed' fibres because they exhibited a large difference between n_{Ca} and n_{Sr} and as such did not satisfy the criterion that the index of similarity (I_s) for Ca^{2+} and Sr^{2+} activation is smaller than 0·1 (see Methods).

DISCUSSION

Contractile characteristics of single fibres

Several of the major contractile properties of individual skinned fibres isolated from skeletal muscles of the *mdx* mouse are virtually indistinguishable from those of fibres of normal mice. Apart from the observation of a unique frequency of myofibrillar force oscillations in EDL muscle fibres of young *mdx* mice there were very few differences detected in the other analytical parameters (n_{Ca}, n_{Sr}, pCa_{50}, pSr_{50}, $pCa_{50} - pSr_{50}$), derived from force–pCa and force–pSr relations for fast- or slow-twitch fibres of soleus and EDL muscles. In addition, maximum force levels of the *mdx* muscle fibres were generally not significantly different from those of normal animals, although *mdx* soleus fast- and slow-twitch fibres exhibited marked and significant reductions in force generating capacity with respect to the same fibres of the young groups.

In contrast, we have previously shown (Fink *et al.* 1986) that the individual muscle fibres from a strain of a mice with an autosomal dystrophy (129ReJ *dy/dy*) in which dystrophin levels are normal, had several characteristics distinct from those of the normal population of control fibres. For example both the fast-twitch and slow-twitch fibres from these dystrophic mice were less sensitive to activating [Ca^{2+}] and produced significantly lower maximum tension than their normal counterparts. In addition, a recent study of contractile properties of single skinned fibres isolated from biopsied muscle samples of human Duchenne muscular dystrophy sufferers also showed a number of contractile properties which differed from those of normal fibres (Fink *et al.* 1990). Most interestingly there were very few type IIB fibres in the DMD muscle sample. Instead, a significant number of fibres with embryonic characteristics were identified supporting the notion of a large turnover (degeneration/regeneration) of fibres in the DMD dystrophic muscle. Maximum force levels were also depressed compared to normal muscle fibres and it was only part of the slow-twitch DMD fibre group which produced near-maximum force levels. These observations, coupled with

the similarity in contractile and activation properties of muscle fibres from normal and *mdx* dystrophic animals would suggest that the absence of the sub-sarcolemmal protein dystrophin has little influence on the expression and function of contractile and regulatory proteins in *mdx* muscle. However, we cannot overlook the possibility that during fibre degeneration and regeneration other contractile isoforms (such as embryonic forms) are transiently expressed before being replaced with adult isoforms.

The marked differences found between fibres of dystrophic 129ReJ mice, human DMD sufferers and their respective controls are therefore more likely linked to the more severe clinical symptoms exhibited in these dystrophies, than to the lack of membrane-associated dystrophin. In addition, the results of the present study may have important implications for the focus of treatment strategies designed to reverse the deleterious symptoms of muscular dystrophy. If the contractile properties of dystrophic muscle fibres are not compromised by the lack of dystrophin as we have shown, but rather non-specifically by the degeneration/regeneration processes, then procedures aimed at halting or reversing these deleterious cycles may represent sufficient treatment strategy. For example, the implantation of normal myoblasts into dystrophic muscle results in mosaic fibres possessing normal and dystrophic nuclei (Law, Goodwin & Wang, 1988; Karpati, Pouliot, Carpenter & Holland, 1989; Partridge, Morgan, Coulton, Hoffman & Kunkel, 1989; Huard, Labrecque, Dansareau, Robitaille & Tremblay, 1991), which can express dystrophin, and potentially reduce any susceptibility of the muscle fibre membrane to suffer stress-induced injury. If such treatments are carried out before significant deterioration in muscle function is detected then it is apparent from the results presented here, and in our related studies of muscle function in other dystrophies (Fink *et al.* 1986, 1990; Head *et al.* 1990) that muscle function will be preserved at near-normal levels.

The contractile characteristics of individual *mdx* fibres were not significantly dependent on the location of myonuclei, whether peripheral, central or a mixed pattern of nuclei. However, the steepness of the force–pCa curve (n_{Ca}) was directly dependent on the degree of peripheral nucleation of individual *mdx* muscle fibres, with peripherally nucleated fibres exhibiting steeper force–pCa curves than fibres exhibiting complete centronucleation. If we assume that fibres exhibiting a predominantly central distribution of nuclei represent either regenerated fibres, or fibres in which there has been a large turnover of structural, myofibrillar and regulatory proteins in repair processes (and in both cases the fibres could be considered juvenile) then the mechanism underlying the observation of an increased steepness in peripherally nucleated (mature) fibres may be similar to that which we report here to accompany the normal aging of muscle fibres.

Fibre type distributions

There was little evidence of significant fibre type differences between normal and dystrophic muscles in either age group as determined by contractile characteristics of single fibres. Anderson *et al.* (1988) also reported no significant differences between the histochemically determined fibre type proportions of soleus muscles of *mdx* and control mice at either of the ages studied (3–4 weeks and 32 weeks). This observation would support our earlier contention that there may be low turnover of some muscle fibres in *mdx* dystrophy. In some other histochemical studies of the fibre type

distributions in *mdx* muscles the soleus was found to have a larger proportion of slow-twitch (type I) fibres than found in the normal control muscles. Although Marshall, Williams & Goldspink (1989), reported that the proportion of slow-twitch (type I) fibres in soleus muscles diminished between 3 and 52 weeks of age (from 73 to 65%) while in the same time period in normal muscles the proportion increased (from 22 to 30% while diminishing to 14% at 12 weeks) the changes reported were small. In contrast, Carnwath & Shotton (1987) reported 58 ± 4% slow-twitch (type I) fibres at 26 weeks in *mdx* (27 ± 4% in normal mice), of which 5% were thought to represent original (non-regenerated) fibres.

Position and distribution of fibre nuclei

The coupling of confocal microscopy and highly fluorescent compounds that interchelate with intracellular DNA/RNA allowed for precise localization of myonuclei in isolated skeletal muscle fibres. The presence of centronucleation in muscle fibres especially as displayed by the long, unbroken strands of nuclei evidenced in Figs 1 and 4 has implications for the estimation of maximum force levels for these fibres. Absolute force levels are standardized to fibre cross-sectional area calculated from the diameter, measured optically with the fibre immersed in solution. In the presence of central nuclei a significant portion (20 to 50 μm^2) of the total cross-sectional area is devoid of myofibrils (e.g. see Fig. 1*D*) and does not contribute to force production. In *mdx* fibres maximum force production may therefore be underestimated with this error being largest in small calibre (diameter 20–25 μm) muscle fibres (error 3 to 16%) and insignificant in large fibres (> 50 μm; < 2% error).

It has generally been suggested that the central position of a myonucleus in a muscle fibre can be used as indication that the fibre had been regenerated or replaced (Harris & Johnson, 1978). In these circumstances the centronucleation would arise from the activation and fusion of myoblast precursor (satellite) cells within the intact basement of membrane of a necrotic dystrophic muscle fibre (for example see Fig. 1*B* and *F*). These resultant muscle fibres, the product of secondary myogenesis, retain centronucleation as there is little evidence that the nuclei can migrate to the fibre periphery (Carnwath & Shotton, 1987). Secondary myogenesis, or fibre replacement, would seem to have little scope therefore for producing muscle fibres which exhibit the variety of myonuclear mixing illustrated by the *mdx* fibres used in this study. The nuclear patterns exhibited by these fibres would exclusively be those of the myonuclei rather than satellite cells or other mononuclear cells. These cell types were removed by enzymatic digestion of extracellular connective tissue and basement membranes with collagenase, and detergent treatment of individual mounted fibres with Triton X-100 to remove the sarcolemma.

It is most likely that the fibres exhibiting a mixed population of central and peripheral nuclei represent fibres which have been focally damaged (for example, see Fig. 1*A* and *C*), potentially stress-induced injuries complicated by the lack of membrane-associated dystrophin (Head *et al.* 1992). These damage sites do not lead to total necrosis of the parent fibre as contraction clots will form to seal the damage site (Shafiq, 1970). Instead the fibre may be repaired through activation and infiltration by satellite cells which eventually fuse to span the damaged region producing a new fibre area with centrally located nuclei. Peripheral myonuclei in the

area surrounding the damage site will also migrate centrally and invade the contraction clot region. Gradually fibres which are continually damaged may acquire a large proportion of central nuclei and it is likely that the variability of mixing reflects the number of damage/repair cycles that fibres have gone through. Fibres from older *mdx* animals will have higher probability of exhibiting a larger proportion of central nuclei. All nuclei were peripheral in location in normal fibres of both age groups.

The similarity of contractile characteristics of single skinned fibres from normal and *mdx* mice, the presence of mixed nucleation in some *mdx* fibres, and the absence in *mdx* mice of fibres with embryonic contractile characteristics as commonly seen in DMD muscle allow us to conclude that the dystrophin-negative fibres can survive repeated bouts of focal damage through repeated repair cycles and that fibres with central nucleation are not necessarily regenerating muscle fibres.

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