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Genetics

X chromosome-linked muscular dystrophy (*mdx*) in the mouse

(animal model)

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ABSTRACT **An X chromosome-linked mouse mutant (gene symbol, *mdx*) has been found that has elevated plasma levels of muscle creatine kinase and pyruvate kinase and exhibits histological lesions characteristic of muscular dystrophy. The mutants show mild clinical symptoms and are viable and fertile. Linkage analysis with four X chromosome loci indicates that *mdx* maps in the *Hq Bpa* region of the mouse X chromosome. This gives a gene order of *mdx–Tfm–Pgk-1–Ags*, the same as for the equivalent genes on the human X chromosome.**

There are two major forms of X chromosome-linked muscular dystrophy in man: Duchenne (McKusick number, 31020) and Becker (McKusick number, 31010) (1–3); recent linkage analyses with restriction fragment-length polymorphisms suggests that they could be allelic (4, 5). For neither of these syndromes is the primary lesion or a homologous animal model known. Several mouse mutants exhibit myopathies (6, 7); the most investigated of these are the autosomal *dy* and dy^{2J} mutants (6). There is, however, controversy over whether the *dy* mutants are myogenic or neurogenic in origin (8–10) and, therefore, their suitability as animal models of Duchenne/Becker muscular dystrophy (11).

Therefore, it is of considerable interest that a spontaneous mutation (*mdx*) arose in our inbred C57BL/10 colony of mice that is X chromosome-linked and produces viable homozygous animals that have high serum levels of muscle enzymes and exhibit histological lesions similar to human muscular dystrophy.

MATERIALS AND METHODS

Animals. C57BL/10ScSn inbred mice were originally obtained from M. Festing (MRC Laboratory Animals Centre, Carshalton, Surrey, U.K.) and further inbred for five generations prior to the spontaneous appearance of the X chromosome-linked muscular dystrophy (*mdx*) mutation. Segregation analysis was performed on animals within this stock. The position of *mdx* on the X chromosome was located by crossing to four mutants: Mottled-blotchy (Mo^{blo}), Tabby (*Ta*), sparse-fur (*spf*) and Hypophosphataemia (*Hyp*) (all the gift of M. Lyon, MRC Radiobiology Unit, Harwell, Oxon, U.K.).

Enzyme Assays. Pyruvate kinase (PK; EC 2.7.1.40) activity was determined by a semiautomated enzyme assay (12) and expressed as μmol/min per ml of whole blood ± SEM. In some experiments the blood was fractionated into plasma and erythrocytes after withdrawal by heart puncture into heparinized tubes. The blood was centrifuged at 1,000 × g_{avg} for 15 min, and the plasma was withdrawn; the leukocyte and interface layers were discarded, and the erythrocytes were resuspended twice in 0.15 M NaCl and recentrifuged and then were lysed in 0.05 M Tris, 7.4/1 mM EDTA/1 mM dithiothreitol/0.1% Triton X-100. Cellulose acetate electrophoresis and staining of creatine kinase (EC 2.7.3.2) was performed according to the protocol provided by Helena Laboratories (Beaumont, TX).

Histology. All mice were examined clinically before being killed, and as many muscles as possible were surveyed. Ether-killed mice were skinned, eviscerated, fixed in 10% Formal/saline, halved in the median plane, decalcified, dismembered, and subdivided so that about 16 blocks resulted from each mouse. These were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. This made it possible for a large number of skeletal muscles and other tissues to be examined histologically. Some selected sections were stained with picro-Mallory trichrome, and some frozen sections of Formalin-fixed muscles were stained with Sudan IV for the demonstration of fat; in some muscles, nerve endings were demonstrated by the cholinesterase method of Coupland and Holmes (13). For electron microscopy, small pieces of the gastrocnemius and soleus muscles were fixed in 5% glutaraldehyde in 0.075 M cacodylate buffer, washed in buffer, refixed in 1% osmium tetroxide in cacodylate buffer, and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate.

RESULTS

Discovery and Inheritance of the Mutant. In a screening program designed to discover enzyme activity mutants of glycolysis in the mouse (14, 15), some animals spontaneously appeared in our C57BL/10 reference inbred line that had at least 3-fold higher blood levels of PK activity. Because we had already discovered mutations at the structural gene loci for both the muscle-type (16) and the liver-erythrocyte-type (17) PK isoenzymes and several putative regulatory gene mutations (unpublished results), we considered this new variant worthy of detailed analysis (18).

Crosses within our C57BL/10 strain showed that the variant high-PK-activity phenotype segregated as a recessive X chromosome-linked mutation (Table 1); homozygotes have slightly reduced viability and fertility. The biochemical analysis immediately produced complications: enzyme levels were not affected in liver (or any other tissue), and the variant enzyme had an altered electrophoretic mobility, the K_m for phospho*enol*pyruvate, resistance to tryptic digest, and interaction with fructose diphosphate. All these characteristics indicated a tissue-specific mutant affecting post-translational modification of the enzyme (18), which would be inconsistent with its recessive X-linked inheritance and the inactive-X-chromosome hypothesis (19).

This apparent paradox was resolved when it was realized that these biochemical characteristics of the variant enzyme were identical to those of the muscle-type isoenzyme coded for by the *Pk-3* locus (16). Fractionation of whole blood from mutant animals showed that the extra enzyme activity solely resided in the plasma fraction, washed erythrocytes having similar activity to control animals (Table 2). In man the spontaneously occurring X chromosome-linked syn-

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Abbreviation: PK, pyruvate kinase.

Table 1. Segregation of the high-PK phenotype

Parents	PK activity in offspring* Females	Males
Both normal (C57BL/10)	7.84 ± 0.47 (6)	
Both mutant (C57BL/10)	22.2 ± 3.0 (14)	
Mutant ♀ × DBA/2 ♂	8.84 ± 0.13 (7)	25.8 ± 0.46 (5)
DBA/2 ♀ × mutant ♂	8.30 ± 0.11 (7)	8.22 ± 0.14 (5)
C57BL/10 ♀ × mutant ♂	8.23 ± 0.21 (22)	8.18 ± 0.20 (23)

*Enzyme activity is expressed as μmol/min per ml of whole blood (mean ± SEM); the number of animals is shown in parentheses.

drome Duchenne muscular dystrophy (31020) (3) also produces high levels of PK (20, 21) in the serum and other cytosolic muscle-specific isoenzymes, especially creatine kinase (22). An analysis of plasma isoenzymes in mutant and normal C57BL/10 mice showed extremely high levels of the muscle-type (but not the heart- or brain-type) isoenzyme in the mutant (Fig. 1). We therefore decided to perform a histological analysis of mutant and normal mice.

Histological Characterization of the Myopathy. A cursory examination of the gastrocnemius and soleus muscles from one male (*mdx*/Y) and one female (*mdx*/*mdx*) adult mutant mouse uncovered myopathic lesions similar in many respects to those of muscular dystrophy. Therefore, a more detailed survey was undertaken on one male and one female C57BL/10 mutant (*mdx*/*mdx*, *mdx*/Y) and on control (+/+, +/Y) mice of ages 4 days and 3, 5, and 9 weeks and on one pair (*mdx*/Y, +/Y) of 12-month-old male mice. Heterozygote females (*mdx*/+) were not examined in detail, but histologically and biochemically they looked like control animals (+/+, +/Y). Only the 12-month-old mutant mouse showed any evidence of clinical disease in the form of muscular tremors and mild incoordination. No histological lesions were observed in any of the muscles examined in the 4-day-old mutant mice (either *mdx*/*mdx* or *mdx*/Y), but by the age of 3 weeks, changes had occurred in many muscles of the limbs (though not of the head, neck, and trunk) of both male and female mice. There was excessive atrophy with loss of normal muscle fibers. In some areas—the musculus quadriceps femoris for instance—there was a variation in fiber size, degeneration of some fibers, and marked concentration of densely-stained sarcolemmal nuclei with phagocytic cells in place of lost fibers (Fig. 2*a*). A few otherwise normal fibers showed centralized nuclei. Striations were retained in most fibers, even the atrophic ones. At 5 weeks the changes were very similar, but a few muscle fibers were completely necrotic with focal reaction of multinucleated giant cells.

In *adult mice* (9 weeks old), the characteristic change was centralization of the nuclei within the otherwise normal muscle fibers (Fig. 2*b*), almost all of the fibers being affected in most of the skeletal muscles examined, including those of the head, neck, and trunk. There was considerable variation in fiber size due to enlargement of some and atrophy of others (Fig. 2*c*). Degeneration and necrosis of individual fibers was not uncommon, and it was accompanied by proliferation of the sarcolemmal nuclei, phagocytic cells, and endomysial fibroblasts. There were areas of somewhat basophilic fibers with prominent vesicular nuclei; these were interpreted as regenerating muscle tissue. Long chains of nuclei were frequent in muscle fibers that showed no other deviation from the normal, but this was encountered only in this group of older animals (Fig. 2*d*). Although mild fibrosis and some evidence of phagocytosis of necrotic muscle tissue had developed in these mice, there was no replacement of lost muscle by fat cells.

Table 2. PK activity in fractions of whole blood from normal and mutant mice

Fractions	PK activity* Normal	Mutant
Plasma	0.276 ± 0.027	16.0 ± 0.048
Erythrocyte	6.94 ± 0.083	7.49 ± 0.084

*Enzyme activity is expressed as μmol/min per ml of whole blood (mean ± SEM of four samples).

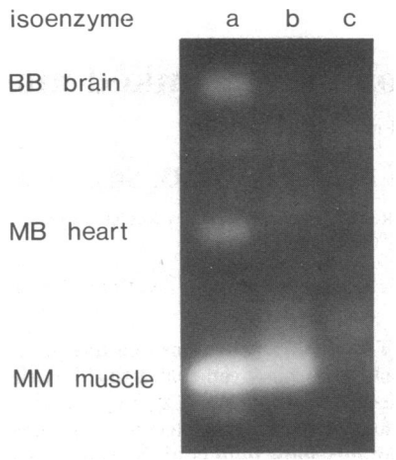

FIG. 1. Electrophoresis of serum creatine kinase in mutant (lane b) and normal (lane c) animals. Electrophoresis was performed according to the Helena Laboratories (Beaumont, Texas) creatine kinase procedure, with standards in lane a.

In the one 12-month-old mouse that had shown clinical locomotor symptoms, the muscle lesions were essentially similar. The intermysial reaction was more pronounced, and phagocytosis of necrotic muscle tissue was evident. Although there were foci of fat cell accumulation in some areas of muscle tissue, it is not clear whether this signified replacement of lost fibers, but it also was found in the midst of normal muscle tissue.

By the methods used, abnormalities were not detected in the brain or spinal cord. Cholinesterase reactions of normal intensity and distribution were observed at nerve endings. In addition to the major changes seen by light microscopy, ultrastructural examination revealed that dilation of the sarcoplasmic reticulum was an early change in affected myofibrils (Fig. 3*a*). Electron-dense bodies developed in the mitochondria (Fig. 3*b*), the latter subsequently swelling and degenerating. There was disruption of the plasmalemma and basal lamina. Ultimately the normal myofibrillar architecture of bands and lines disappeared, and myofilaments disintegrated and became misaligned. There was phagocytosis of the myofibril by macrophages and polymorphonuclear leucocytes (Fig. 3*c*). Granular material and moderately increased amounts of collagen were present in the widened intermyofibrillar spaces. Remnants of sarcoplasmic reticulum and some mitochondria were often preserved despite degeneration of the myofibril.

Therefore, the mutant has an acquired, progressive, degenerative myopathy that is X chromosome-linked and probably myogenic in origin, although detailed examination of the nervous system will be necessary before neural involvement can be discounted. Thus, there are reasonable grounds for accepting the condition as, according to the definition of Adams (1), a muscular dystrophy. We named it *X-linked muscular dystrophy*, gene symbol: *mdx*.

Linkage Analyses. From the segregation data of crosses between mutant and normal animals (Table 1) X chromosome-linked inheritance of *mdx* was established. Further linkage analyses were performed between *mdx* and four genes spanning the complete mouse X chromosome: *spf*

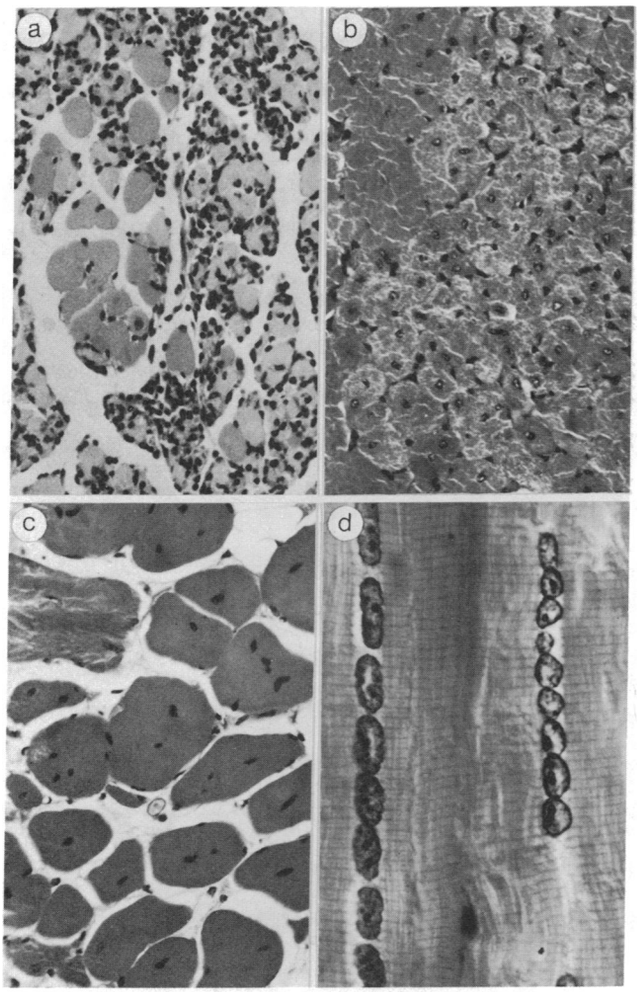

FIG. 2. (*a*) Dystrophic mouse, 3 weeks old. Muscle fibers in cross section showing variation in size, proliferation of sarcolemmal nuclei, and infiltration by phagocytes and connective tissue cells. (Hematoxylin and eosin; ×80.) (*b*) Dystrophic mouse, 9 weeks old. The muscle fibers in cross section show no obvious abnormality apart from the centrally placed nuclei. (Hematoxylin and eosin; ×80.) (*c*) Adult dystrophic mouse. Swollen muscle fibers showing one or more centrally placed nuclei (Hematoxylin and eosin; ×80.) (*d*) Adult dystrophic mouse. Longitudinally cut muscle fibers with normal striations, showing long chains of nuclei. (Hematoxylin and eosin; ×260.)

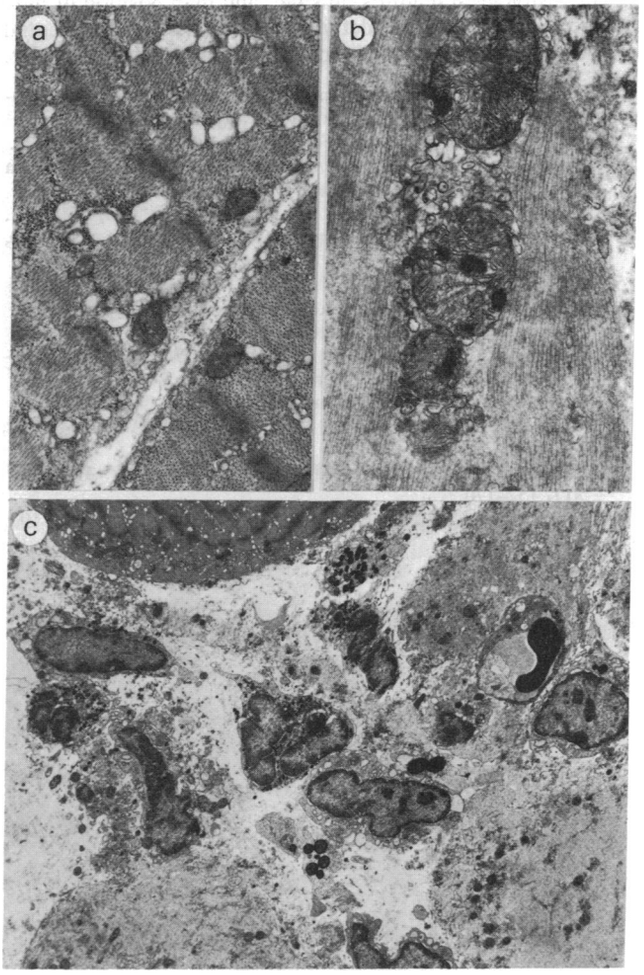

FIG. 3. Gastrocnemius muscle of dystrophic mouse. (*a*) The sarcoplasmic reticulum of the upper myofibril is dilated, whereas that of the lower myofibril is of normal dimensions. (×12,800.) (*b*) Electron-dense bodies in mitochondria. (×18,100.) (*c*) Macrophages and polymorphs associated with degenerated myofibrils. Myofibrils (*Lower Right*) show loss of Z lines and disintegration of myofilaments, plasmalemma, and basal lamina, although some mitochondria and remnants of sarcoplasmic reticulum are present. (× 2,700.)

(sparse-fur), *Ta* (Tabby), Mo^{blo} (mottled-blotchy), and *Hyp* (hypophosphataemia). The results of the linkage analyses give the following numbers in each phenotypic class in a total of 125 animals: *spf/mdx* (males only scored), 10 +/*mdx*, 7 *spf*/+, 1 *spf/mdx*, and 3 +/+; *Ta/mdx* (both sexes scored), 10 +/*mdx*, 9 *Ta*/+, 0 *Ta/mdx*, and 2 +/+; Mo^{blo}/*mdx* (both sexes scored), 13 +/*mdx*, 11 *Mo*/+, 1 *Mo/mdx*, and 4 +/+; and *Hyp/mdx* (both sexes scored), 23 +/*mdx*, 10 *Hyp*/+, 10 *Hyp/mdx*, and 10 +/+. These data give the distance (centimorgans): *spf/mdx*, 19 ± 8.6; *Ta/mdx*, 9.5 ± 6.4; *Mo/mdx*, 17 ± 6.7; and *Hyp/mdx*, 38 ± 6.7. This suggests that the gene order is centromere-*spf-mdx-Ta-Mo-Hyp*, localizing *mdx* in the *Hq-Bpa* segment of the mouse X chromosome.

DISCUSSION

Several animal models that exhibit muscular degeneration have been found (6, 7, 11). The most investigated are the *dy* and dy^{2J} mutants (6, 8) in the mouse over which there is controversy concerning whether they are myogenic or not (8, 9, 10) and their suitability as animal models for human muscular dystrophy. There are distinct similarities between the *mdx* mutant and the *dy* mutant (6). However, there are also differences in that *dy* mutants show progressive hind limb paralysis at 3–4 weeks of age and fragmentation of atrophic fibers into spindle cells with fat replacement, whereas the *mdx* mutant is viable.

Morphological differences between the *dy* mutant and Duchenne dystrophy were apparent to early investigators (23) and somewhat similar discrepancies were found between *mdx* mutants and human dystrophy. Whether these variations indicate a different etiology or a species response is unknown. Although the *mdx* mutant has a belated onset of mild clinical symptoms compared with both Duchenne and Becker muscular dystrophy, they have several histopathological features in common: all have elevated plasma PK and creatine kinase levels, and there is preliminary evidence that they are all myogenic in origin.

More importantly *mdx* in mice and Duchenne/Becker in humans [which may be allelic (4, 5)] are all X chromosome-linked. The positioning of *mdx* in the *Hq Bpa* region of the mouse X chromosome gives the gene order: *mdx–Tfm–Pgk-1–Ags* for the mouse X chromosome, similar to the order of Duchenne/Becker muscular dystrophy with the homologous genes on the human X chromosome (24).

The histological features, linkage, and map position of *mdx* make it worthy of more detailed examination as a potential animal model of X chromosome-linked muscular dystrophy.

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