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Defective muscle basement membrane and lack of M-laminin in the dystrophic *dy/dy* mouse

(autosomal recessive muscular dystrophy/dystrophin-related protein/genetic disease)

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ABSTRACT **M-laminin is a major member of the laminin family of basement membrane proteins. It is prominently expressed in striated muscle and peripheral nerve. M-laminin is deficient in patients with the autosomal recessive Fukuyama congenital muscular dystrophy but is normal in patients with the sex-linked Duchenne and Becker muscular dystrophies. We have examined M-laminin expression in mice with autosomal recessive muscular dystrophy caused by the mutation *dy*. The heavy chain of M-laminin was undetectable in skeletal muscle, heart muscle, and peripheral nerve by immunofluorescence and immunoblotting in homozygous dystrophic *dy/dy* mice but was normal in heterozygous and wild-type nondystrophic mice. Immunofluorescence confirmed the presence of other major basement membrane proteins in the dystrophic mice. Very low levels of M-laminin heavy chain mRNA were detected by Northern blotting of muscle and heart tissue from *dy/dy* mice, suggesting that M-laminin heavy-chain mRNA may be produced at very low levels or is unstable. Information about the chromosomal localization of the M heavy-chain in human and mouse suggests that a mutation in the M-chain gene causes the muscular dystrophy in *dy/dy* mice. The *dy* mouse may provide a model for autosomal muscular dystrophies in humans and facilitate studies of functions of M-laminin.**

The laminins are a family of basement-membrane proteins. Each laminin has three homologous subunits: a 400-kDa heavy chain and two distinct 200-kDa light chains (1–5). Different laminins exhibit distinct patterns of expression in a tissue and in a developmentally specific manner. A-laminin (laminin A-B_1-B_2, heavy chain A, light chains B_1 and B_2) is the first to be expressed in development and was the first to be isolated and characterized from parietal yolk sac cells. M-laminin (laminin M-B_1-B_2), with a 400-kDa M chain in place of the A chain, is the major laminin in basement membranes of striated muscle, peripheral nerve, and placental trophoblast (6–8). In general, M-laminin seems to appear later than A-laminin in development. For example, A-laminin is present in early embryonic muscle in rodents, whereas M-laminin appears in the more mature muscle (6). In the normal adult, basement membranes of muscle fibers contain M-laminin exclusively; the A-laminin is restricted to the neuromuscular junctions and the blood vessels (9). Other laminins include S-laminins (10), in which the B_1 chain has been replaced by the homologous S chain, and kalinin and K-laminin, which contain the A-chain homologue K (11). Laminins with the S chain are found in neuromuscular and myotendinous junctions and in blood vessels, whereas laminins with the K chain are found in the epithelial basement membrane of the skin.

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Due to the relatively large size of the laminin subunits, one may expect the genes for these chains to be large and perhaps relatively frequent targets for mutations. Mutations in a subunit of kalinin are the cause of lethal junctional epidermolysis bullosa (12). Mutations in the A, B_1, or B_2 chains have not yet been described and may be lethal. We recently showed that patients with the autosomal recessive muscular dystrophy Fukuyama congenital muscular dystrophy (FCMD) (13) have reduced M-laminin in muscle and peripheral nerve, as determined by immunofluorescence (14). The FCMD mutation was recently linked to chromosome 9 (15), whereas the human M-chain gene is located on chromosome 6 (16). The reduction of M-laminin in the FCMD patients might therefore be an indirect consequence of a mutation in a different gene.

Several spontaneous mutations in the mouse affect muscle function. The *mdx* mouse has a mutation in the dystrophin gene and lacks dystrophin protein (17). The *mdx* mouse is thus the murine equivalent of the Duchenne/Becker muscular dystrophy patient (18), although it has a much milder phenotype than patients with dystrophin defects. Another mutation in the mouse causing muscular dystrophy is the autosomal recessive *dy* (19). Homozygous *dy/dy* mice have a more severe phenotype than the *mdx* mouse. Aside from having a progressive muscular disease, these mice are smaller than the littermates and die between 2 and 6 mo of unknown cause. Dystrophin and the dystrophin-related protein (DRP) are normal in the *dy/dy* mice (20, 21).

We show here that *dy/dy* mice lack M-chain protein and have very low levels of M-chain mRNA. The lack of M chain apparently leads to a defect in the basement membrane as visualized by transmission electron microscopy. The lack of M chain in the homozygous *dy/dy* mouse is therefore the likely cause of the dystrophic phenotype. The *dy* gene has been localized close to the gene for the DRP on mouse chromosome 10 (22). The location of the DRP gene in human (chromosome 6q24) is close to the gene for the M chain (6q22–23; 16). Because of the extensive homologies between these segments of human chromosome 6 and mouse chromosome 10, it appeared likely that the M-chain gene is at or close to the *dy* locus in mouse. In fact, this has been confirmed very recently (Y. Yamada, personal communication).

MATERIALS AND METHODS

Animals. Homozygous (*dy/dy*) and control (+/?) mice of strains 129/ReJ-*dy* and 129 B6F1/J-*dy* were purchased from The Jackson Laboratories and used at different ages as indicated.

Abbreviations: DRP, dystrophin-related protein; FCMD, Fukuyama congenital muscular dystrophy; FITC, fluorescein isothiocyanate.

Antibodies. Three M-chain-specific polyclonal antibodies were used: (*i*) affinity-purified rabbit antibodies to the 60-kDa fragment from the C terminus of the human M chain (composing repeats 4–5 in the so-called G domain, the domain of laminin heavy chains that lacks a counterpart in the light chains; ref. 6), (*ii*) antiserum to a synthetic peptide derived from a sequence in the second repeat in the G domain (7), and (*iii*) antiserum to the 300-kDa N-terminal segment of mouse M chain (23). Polyspecific antisera to rat and human laminins (7, 24), reacting with several laminin subunits were also used as was a rabbit antiserum to mouse type IV collagen (25). Monoclonal rat antibodies to perlecan and entactin were supplied by A. Ljubimov (Cedar–Sinai Medical Center) (26). Antiserum to mouse fibronectin was as described (27).

Immunofluorescence. Mouse skeletal muscle and heart were collected, embedded in OCT, and frozen immediately in 2-methylbutane at −70°C. Frozen tissue sections (10–20 μm) were cut on a cryostat and fixed in ice-cold acetone for 10 min. Sections were incubated with primary antibodies at 1:50 to 1:200 dilution for 2 hr at 37°C. After washing with phosphate-buffered saline three times, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or anti-mouse IgG at 37°C for 1 hr. The sections were mounted with 20% (vol/vol) glycerol and examined under a Zeiss fluorescence microscope.

Northern Blotting. Total RNA was isolated from skeletal and heart muscle of mice of different ages using acid guanidinium thiocyanate–phenol–chloroform extraction. RNA was separated on a 0.9% agarose/formaldehyde gel and nicked using a UV crosslinker (Stratagene). The RNA bands were transferred to Hybond-N (Amersham) and fixed at 80°C for 2 hr. Hybridization was performed by using a ^{32}P-labeled 3-kb cDNA probe corresponding to the 3′ end of the human M-chain cDNA (10^7 cpm/ml; specific activity 10^9 cpm/μg; ref. 7) at 42°C overnight in a buffer containing 50% (vol/vol) formamide, 5× standard saline citrate (SSC), 5× Denhardt's solution, herring sperm DNA at 200 μg/ml, and 7% dextran sulfate. After hybridization, the nylon filter was washed twice at a final stringency of 0.5× SSC/0.1% SDS at 50°C for 20 min. The filter was exposed to Kodak XAR film at −70°C for 24 hr with intensifying screen.

PCR. mRNA was reverse-transcribed and amplified using described primers (28).

Southern Blotting. Genomic DNA was isolated from the liver of 4-week-old mice (+/? and *dy/dy*). Ten micrograms of genomic DNA was cut with 100 units each of different restriction enzymes, including *Hin*dIII, *Bam*HI, *Eco*RI, *Pst* I, and *Xba* I at 37°C overnight. DNA was separated on a 0.7% agarose gel, nicked, and transfered to Hybond-N. Hybridization was performed by using a ^{32}P-labeled 458-bp rat M-chain cDNA probe equivalent to residues 501–969 in the human M-chain cDNA (10^7 cpm/ml; specific activity 6×10^8 cpm/μg; ref. 7) at 42°C overnight in a buffer containing 50% formamide, 5× SSC, 5× Denhardt's solution, and herring sperm DNA at 100 μg/ml. After hybridization, the nylon filter was washed at a final stringency of 0.1× SSC/0.1% SDS at 50°C for 50 min. The filter was exposed to Kodak XAR film at −70°C for 17 hr with intensifying screen.

SDS/PAGE and Immunoblot Analysis. Laminins were extracted from tissues as described (23). Briefly, frozen tissue samples were thawed and immediately homogenized [0.1 g (wet weight) of tissue per ml] in extraction buffer without EDTA (0.15 M NaCl/0.05 M Tris·HCl, pH 7.4/2 mM phenylmethylsulfonyl fluoride/aprotinin at 2 μg/ml at 4°C using a Polytron homogenizer. The homogenate was centrifuged at 16,000 × *g* for 15 min, and the supernatant was discarded. The tissue residue was resuspended in 2 vol of extraction buffer/10 mM EDTA and incubated for 1 hr with frequent mixing before centrifugation as before. The protein content of the EDTA extracts was estimated by using the bicinchoninic acid assay (Pierce). Aliquots of the EDTA extracts were fractionated by SDS/PAGE using 4–12% gradient gels (NOVEX, San Diego) with reducing conditions and high-molecular-weight markers (GIBCO). Proteins were electrophoretically transferred to Immobilon poly(vinylidene difluoride) (Millipore) and processed for immunoblotting (6) using affinity-purified antibodies at 3 μg/ml or antisera at 1:500 dilution. Immunoreactive protein bands were visualized by using the enhanced chemiluminescence method (Amersham).

Transmission Electron Microscopy. Specimens of stretched and unstretched leg muscles from 8-week-old normal and dystrophic mice were fixed in 70% Karnovsky's fixative, rinsed in 0.1 M cacodylate buffer (pH 7.2), postfixed in 1% OsO_4 in cacodylate buffer, and treated with 1% tannic acid (pH 7.0) to enhance the basement membrane (29). After a rinse in 1% Na_2SO_4/0.1 M cacodylate buffer, the tissue specimens were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 201 electron microscope.

RESULTS

Skeletal muscle and peripheral nerve from the homozygous *dy/dy* mouse contained no laminin M chain as analyzed by indirect immunofluorescence with affinity-purified M-chain-specific antibodies (Fig. 1*a*). Heterozygous or wild-type littermates (+/?) of the same sex contained normal M-laminin as did mice of other strains. The presence and localization of other basement membrane components in *dy/dy* mice appeared normal, as evidenced by staining with polyspecific antisera against laminins (Fig. 1*a*), with antiserum against type IV collagen (Fig. 1*b*), and with monoclonal antibodies against the heparan sulfate proteoglycan perlecan (Fig. 1*b*) and entactin (data not shown). Examination of tissue sections from other organs normally containing M-laminin, including heart (Fig. 1*c*) and testis (data not shown), showed that the *dy/dy* mice lacked M chain in these tissues also.

Immunoblotting was used to analyze laminins in the muscle of *dy/dy* mice. The M chain in M-laminin is processed into two major fragments of 300 kDa and 80 kDa in normal tissue (7). The antipeptide and the anti-300 kDa antisera detected the 300-kDa N-terminal fragment of the M chain (Fig. 2*a*), and the affinity-purified antibodies to the C-terminal segment of the M chain detected the 80-kDa fragment (Fig. 2*b*) in muscle from normal or heterozygous mice. None of the three M-chain-specific antisera detected any M chain in the *dy/dy* mouse (Fig. 2 *a* and *b*). Polyspecific antiserum to human laminin from placenta, which reacts with several of the laminin heavy (A and M) and light chains (B_1, B_2, and S) detected protein bands in the nondystrophic mice with apparent molecular masses of 400, 300, and 200 kDa (Fig. 2*c*). These were the appropriate molecular weights for the A chain, the N-terminal segment of the M chain, and for B_1/B_2/S chains, respectively. These antisera detected 400-kDa and 200-kDa bands in dystrophic mice but did not detect a 300-kDa band, again suggesting that the M chain is absent in these mice but that A and B chains are present (Fig. 2*c*). Antisera to fibronectin detected fibronectin in approximately equal amounts in the samples from normal and dystrophic mice (data not shown).

Northern blotting of RNA from skeletal muscle and heart of normal and dystrophic mice showed that the ≈10-kb message for the M chain was significantly reduced in the skeletal muscle and barely detectable in heart of the dystrophic mice (Fig. 3). RNA samples from a total of six dystrophic mice and six control mice between 4 and 6 weeks of age were tested by this technique. The M-chain message was reduced in the dystrophic mice to between 6 and 48% compared with the controls. Some of the variations observed in the reduction

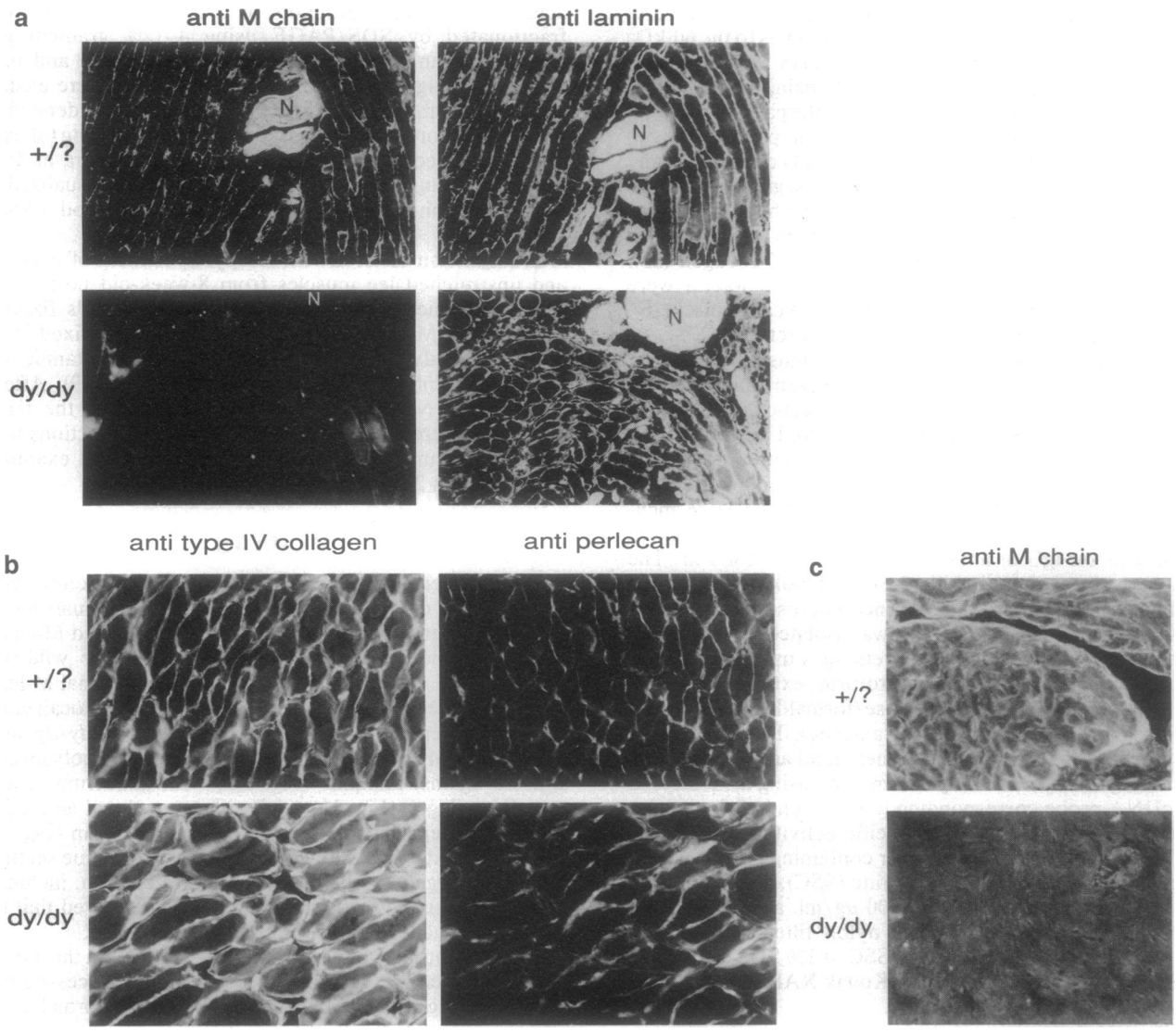

FIG. 1. (*a*) Adjacent sections of skeletal leg muscle from 5-week-old dystrophic (*dy/dy*) and normal (+/?) mice were stained with affinity-purified anti-M chain (20 μg/ml) or with polyspecific anti-laminin, 1:200. The dystrophic mouse showed a complete lack of reactivity with the M-chain-specific antibodies in the basement membranes of muscle fibers and peripheral nerve (N), whereas an immunoreactivity comparable to that seen in heterozygote or wild-type mice was obtained with the antiserum reacting with several laminin subunits. (*b*) Sections of skeletal muscle stained with antiserum to type IV collagen and monoclonal antibodies to perlecan (undiluted hybridoma culture medium) showed stainings of similar intensities in normal and dystrophic mice. (*c*) Sections of heart muscle stained with affinity-purified anti-M-chain antibody. No immunoreactivity was seen in the heart of a dystrophic homozygous mouse.

may be due to the inability to distinguish heterozygous and wild-type mice among the controls. Reverse transcription-PCR was used to confirm the identity of the message in the *dy/dy* mice. With the primers used, a band of the expected size (≈500 bp) was obtained from both *dy/dy* and control mice (data not shown).

Southern blotting of genomic fragments generated by several restriction enzymes did not detect any differences between homozygous and heterozygous or wild-type mice, suggesting that there were no major deletions in the *dy/dy* mice, at least not in the 3′ portion of the M-chain gene that the probe detected (data not shown).

Immunofluorescence and immunoblotting results showed that the dystrophic mice were missing the M chain of M-laminin but had a normal complement of other basement membrane components. To study the muscle basement membrane in more detail at high resolution we examined the muscle tissue by transmission electron microscopy. Significant abnormalities were detected in the basement membrane in both stretched and unstretched *dy/dy* muscle that were not seen in normal mice in particular if the tissue specimens were treated with tannic acid (Fig. 4). The basement membrane in the dystrophic mice was variable, often thin, and sometimes absent.

DISCUSSION

The phenotype of the dystrophic mouse *dy* is due to a spontaneous mutation that is inherited in an autosomal recessive manner. Dystrophin and the DRP are both normal in *dy/dy* mice (20). We show here that the homozygous *dy/dy* mouse lacks M-chain protein and has very low amounts of M-chain mRNA. It is possible that the *dy* mutation is a mutation in the M-chain gene and that the *dy/dy* mouse phenotype is the result of this mutation. This is evidence that loss of an extracellular matrix protein may directly cause a muscular dystrophy.

Electron microscopy showed that the muscular basement membrane was fragmented or absent in the dystrophic mice, whereas it was continuous in normal mice. Because immuno-

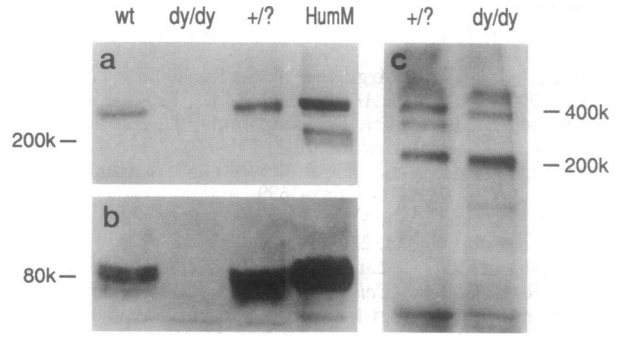

FIG. 2. Extracts of skeletal muscle from dystrophic and normal mice were fractionated by SDS/PAGE, and protein bands were transferred to Immobilon and stained with different antibodies. (*a*) Antiserum against the 300-kDa N-terminal segment of the M chain. (*b*) Affinity-purified antibodies to the C-terminal segment of the M chain. (*c*) Polyspecific antiserum to human placental laminin. The dystrophic mice lack both the 300-kDa N-terminal segment (*a* and *c*) and the 80-kDa C-terminal segment (*b*). The dystrophic mice contain laminin heavy (400 kDa) and light (200 kDa) chains in apparently normal amounts (*c*). wt, wild type, HumM, human M-laminin isolated from placenta (7).

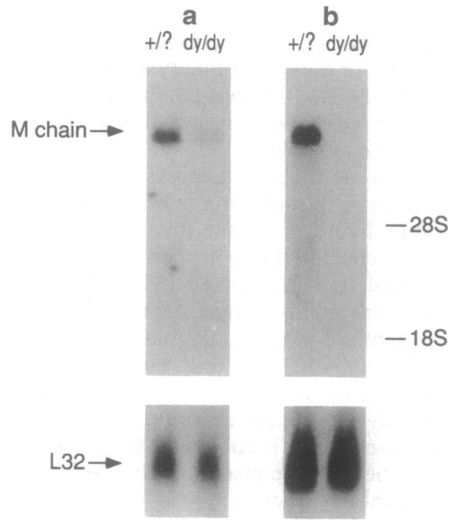

FIG. 3. Northern blotting of 30 μg of RNA from leg muscles of 4-week-old normal and dystrophic mice (*a*) and from hearts of 5-week-old normal and dystrophic mice (*b*). The level of the ≈10-kb M-chain message was significantly reduced in the muscle tissues from dystrophic mice. Levels of message for ribosomal protein L32 were used as an internal RNA-loading control.

fluorescence showed that other basement-membrane components—including type IV collagen, entactin, and perlecan—were present, the lack of M chain appears to cause the abnormal basement-membrane structure seen at the electron microscopy level. Immunofluorescence and immunoblotting suggested that the muscle did contain another laminin heavy chain, probably the A chain. Increased expression of A-laminin has been shown also in the muscle of FCMD patients (14). The expression of A-laminin is probably due to the regenerative response of the muscle to work-induced injury. On the other hand, the presence of basement-membrane components does not necessarily result in a basement membrane as defined by electron microscopy. In the neonatal rat liver, M-laminin is present in the perisinusoidal space, but a basement membrane is not seen (30). The thinness of the basement membrane in the *dy/dy* mouse muscle may result from basement-membrane proteins being produced in abnormal proportions or to the basement membrane being degraded.

The defective basement membrane and the dystrophic phenotype of the *dy* mouse suggests that M-laminin has specific properties not shared with A-laminin in muscle. For example, M-laminin may transmit a specific signal to the muscle cell, which is required for mature muscle function. It

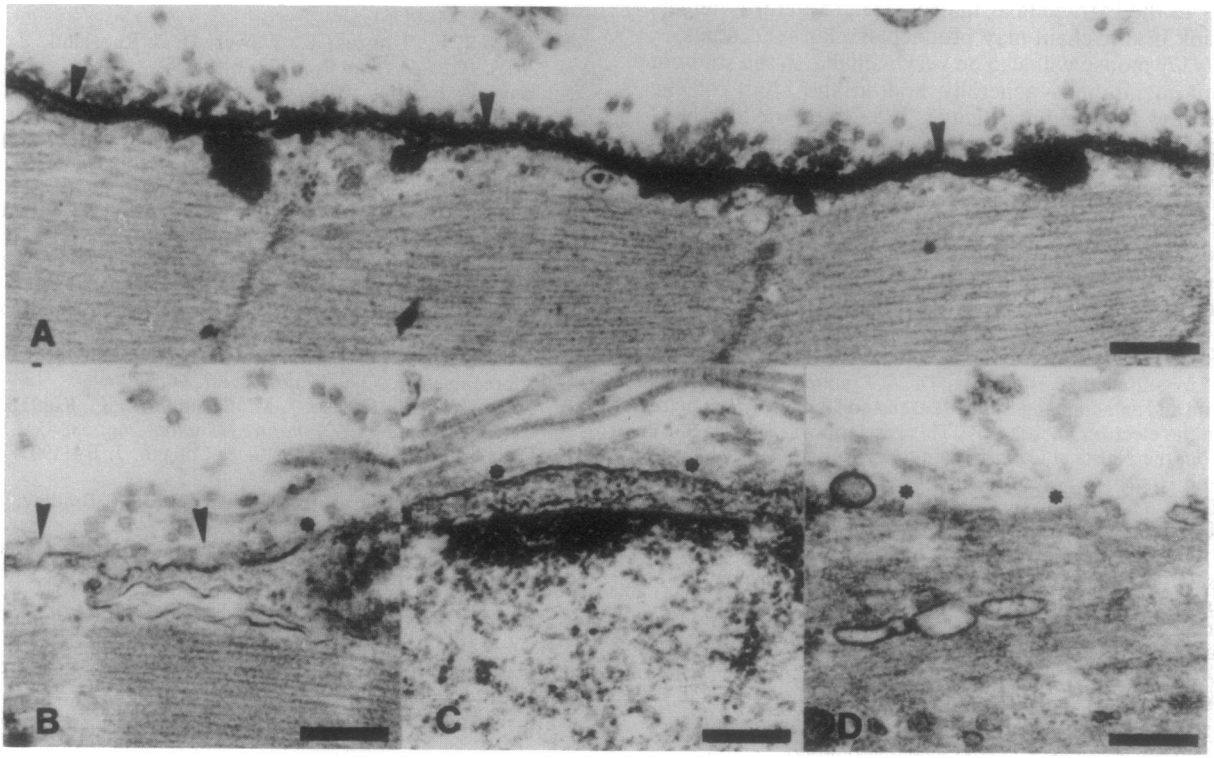

FIG. 4. Transmission electron microscopy of skeletal muscle from 8-week-old normal (*A*) and dystrophic (*B–D*) mice. Arrowheads point at basement membrane, and asterisks indicate where the basement membrane is missing. [Bars = 0.53 (*A*), 0.29 μm (*B*), 0.37 μm (*C*), 0.30 μm (*D*).]

is interesting to compare the observation made here that A-laminin does not appear to compensate for the lack of M-laminin in mature muscle in the dystrophic mice with the observation that the DRP cannot compensate for the lack of dystrophin in the *mdx* mouse or in patients with Duchenne/Becker muscular dystrophy (31).

M-laminin is predominantly expressed in striated muscle and peripheral nerve, but also in the brain, in the developing and regenerating liver, and in the thymus and testis (16, 30, 32). It has been reported that peripheral nerves in the *dy/dy* mice are incompletely myelinated (33). This may thus be caused by the lack of M-laminin. In normal mice, the expression of M-laminin in the testis increases during puberty (H.X. and E.E., unpublished work), suggesting that M-laminin has a role in testis maturation and sexual function. The fact that the *dy/dy* mice do not reproduce (19) may not be due only to muscle weakness, it could also be due to incomplete maturation of the testis caused by the lack of M chain, resulting in sexual dysfunction.

The *dy* gene has been localized to the distal part of mouse chromosome 10, close to the gene for the DRP (22). Because in humans, the genes for the M chain and the DRP are closely linked on the homologous chromosome 6, we deduce that the M-chain gene in mouse is localized close to the DRP gene. We propose that the M-chain gene is included in the *dy* locus in the mouse. Preliminary gene analysis using Southern blotting with M-chain probes showed that the M-chain gene is not deleted in the dystrophic mice. However, a deletion in the 5′ end of the gene or a small change would not be detected by this analysis.

Muscular dystrophies, for which the molecular basis is known, have until now involved defects in the cytoskeleton (dystrophin) in Duchenne and Becker muscular dystrophy and in the dystrophin-associated membrane complex in severe childhood autosomal recessive muscular dystrophy (SCARMD; ref. 34). It is interesting that loss of M-laminin could cause muscular dystrophy because laminins have been implicated in linking dystrophin via the associated membrane complex to the extracellular matrix (35). Therefore, defects in any link in this chain may produce similar syndromes.

The *dy/dy* mouse will also be very valuable in the study of basement membranes generally and in the study of the function of M-laminin in particular. We expect the lack of M chain to result in abnormalities not only in muscle but also in other tissues that normally express M-laminin. These include the central and peripheral nervous systems, kidney mesangium, thymus, testis, and various exocrine glands (16, 32, 36). Detailed studies of the development of these tissues and differentiation of cells within them may shed more light on the function of M-laminin.

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