Ca\textsuperscript{2+} dysregulation in Ryr1\textsuperscript{I4895T/wt} mice causes congenital myopathy with progressive formation of minicores, cores, and nemaline rods

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Contributed by David H. MacLennan, October 22, 2009 (sent for review October 15, 2009)

Ryr1\textsuperscript{I4895T/wt} (IT/+) mice express a knockin mutation corresponding to the human I4898T EC-uncoupling mutation in the type 1 ryanodine receptor/Ca\textsuperscript{2+} release channel (RyR1), which causes a severe form of central core disease (CCD). IT/+ mice exhibit a slowly progressive congenital myopathy, with neonatal respiratory stress, skeletal muscle weakness, impaired mobility, dorsal kyphosis, and hind limb paralysis. Lesions observed in myofibers from diseased mice undergo age-dependent transformation from minicores to cores and nemaline rods. Early ultrastructural abnormalities include sarcomeric misalignment, Z-line streaming, focal of cross-striations, and myofibrillar splitting and intermingling that may arise from defective myofibrillolysis. However, manifestation of the disease phenotype is highly variable on a Sv129 genomic background. Quantitative RT-PCR shows an equimolar ratio of WT and mutant Ryr1 transcripts within IT+ myofibers and total Ryr1 protein expression levels are normal. We propose a unifying theory in which the cause of core formation lies in functional heterogeneity among RyR1 tetramers. Random combinations of normal and either leaky or EC-uncoupled RyR subunits would lead to spatial differences in Ca\textsuperscript{2+} transients; the resulting heterogeneity of contraction among myofibrils would lead to focal, irreversible tearing and shearing, which would, over time, enlarge to form minicores, cores, and nemaline rods. The IT/+ mouse line is proposed to be a valid model of Ryr1-related congenital myopathy, offering high potential for elucidation of the pathogenesis of skeletal muscle disorders arising from impaired EC coupling.

calcium | central core disease | minicore disease | nemaline rod myopathy | ryanodine receptor

Congenital myopathies are a heterogeneous group of inherited skeletal muscle weakness disorders caused by mutations in structural, contractile, or regulatory muscle proteins (1, 2). Early clinical symptoms include neonatal hypotonia, generalized muscle weakness, respiratory distress, and skeletal deformities such as dislocated hips, pes cavus, and kyphoscoliosis. The penetrance of the disease is highly variable, but its course is typically slow or nonprogressive. Central core disease (CCD) and minicore disease (MmD), often referred to as core myopathies, and nemaline myopathies (NM) are the most common congenital myopathies. Their differential diagnosis is based on histopathological analyses of skeletal muscle biopsies that reveal cores, minicores, and nemaline rods (1–3). However, some cases of myopathy present with mixed histology so that cores are seen in combination with minicores or with nemaline rods (4–8). The etiology and pathogenesis of lesion formation is unclear and their presence does not always correlate with the severity of the clinical phenotype (5, 9, 10).

Many congenital myopathies have been linked to mutations in RYR1, encoding the type 1 ryanodine receptor/Ca\textsuperscript{2+} release channel (RyR1), a key protein in excitation-contraction (EC) coupling in skeletal muscle. RyR1 is a homotetramer of 565 kDa subunits, each containing 5,038 aa residues. In response to sarcolemmal depolarization, RyR1 channels are activated to release Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR), triggering muscle contraction. Mutations in RYR1 have been associated with malignant hyperthermia (MH) [Online Mendelian Inheritance in Man (OMIM) database no. 145600] (11), CCD (OMIM no. 117000) (11), MmD with external ophthalmoplegia (OMIM no. 255320) (12), congenital myopathy with cores and rods (6, 7), central nuclear myopathy (13), and neuromuscular disease with uniform type 1 fibers (14). For all of these disorders, Ca\textsuperscript{2+} dysregulation is a likely primary cause and a common etiology is possible.

Insights into the etiology of RyR1-related myopathies have come from in vitro functional studies of mutant RyR1 expressed transiently in heterologous and homologous cell culture systems (15–16). CCD-associated RyR1 mutations impair Ca\textsuperscript{2+} release channel function by rendering the channel either hypersensitive to stimuli (leaky) or unresponsive to activating ligands and depolarization of the sarcolemma (EC uncoupled). It is not yet clear how functionally different leaky and EC-uncoupled RyR1 mutant channels can induce similar skeletal muscle lesions and skeletal muscle weakness.

Answers to question of the etiology and pathogenesis of RyR1-related disorders are being sought through in vivo assessment of pathological changes produced in knockin (KI) mouse lines carrying RyR1 mutations. These include two mouse lines with leaky MH/CCD mutations R163C (17) and Y522S (18). We generated a KI mouse line (19) carrying the EC-uncoupling RyR1 mutation, I4895T, which corresponds to one of the most common CCD mutations in human RYR1, I4898T, assessed in vivo (11). In humans, occurrence of the heterozygous mutation is often associated with a severe clinical phenotype, but penetrance is variable (20). Ile-4898 is located in a highly conserved GGGIG\textsuperscript{4899} motif, proposed to form the selectivity filter of the Ca\textsuperscript{2+} release channel (21). In vitro functional studies have shown that all substitutions at position 4,898 reduce or disrupt Ca\textsuperscript{2+} release channel conductance (15, 16, 21). In particular, coexpression of WT and I4898T mutant RyR1 cDNAs to mimic the heterozygous disease state reduced global Ca\textsuperscript{2+} release by 60% (16). We reported (19) that homozygous Ryr1\textsuperscript{I4895T/I4895T} (IT/IT) mice are born paralyzed in all skeletal muscles, are unable to...
breathe, and die perinatally. We also showed that the IT/IT mutation disrupts Ca\(^{2+}\) release channel function without altering the integrity of the RyR1 protein complex or of the supramolecular structure of the Ca\(^{2+}\) release unit (CRU).

Here, we report our analyses of heterozygous RyR1\(^{I4898T/wt}\) (IT+/+) mice. We demonstrate that these mice exhibit a slowly progressive congenital myopathy with cores, minicores, and rods. We conclude that the IT/+ mouse line is a genetically and phenotypically valid model of an RyR1-related congenital myopathy. A unifying theory is presented to explain how similar pathogenic phenotypes can arise from functionally different RyR1 mutations.

**Results**

**IT/+ Mice Exhibit a Variable Myopathic Phenotype.** IT/+ mice were born at the predicted Mendelian frequency, without apparent skeletal deformities, and survived with no neonatal lethality. Nevertheless, neonatal signs of myopathy were observed. Specifically, IT/+ mice (\(n = 24\)) were flaccid and cyanotic during the first minutes after delivery (Fig. S1A), responded poorly to stimuli, and started breathing regularly 15–20 min after birth, compared to 5–7 min for WT littermates (\(n = 26\)). Progression of the disease in IT/+ mice was variable, ranging from mild to severe. Impairment of mobility was first noted as weakness of the hindlimb paralysis, whereas 4 males and 2 females (20%) appeared asymptomatic and had a normal lifespan of \(\approx 2\) years. With age, IT/+ mice developed dorsal kyphosis in the cervicothoracic region, likely because of overuse of the forelimbs for locomotion. At all ages, the size of IT/+ mice was comparable to that of controls (Fig. S1A and B; Fig. S1 B–E). At \(\approx 12\) months, 2 males and 2 females (14%) suffered complete hind limb paralysis, whereas 4 males and 2 females (20%) appeared asymptomatic and had a normal lifespan of \(\approx 2\) years. Gross pathological examination did not reveal muscle wasting, but showed very low fat deposits, which might account for the lower body weights of IT/+ mice.

**Histopathology of IT/+ Skeletal Muscles.** Structural abnormalities characteristic of congenital myopathy were sought for in hindlimb muscles of 6-weeks- to 24-month-old IT/+ mice. Histological and EM analyses were carried out on animals that displayed a disease phenotype, which was recognizable by 8 months (see above). Increased fiber size variability, increased endomysial spacing, and mild fibrosis were observed in all muscle groups at \(\approx 6–8\) months, but were most prominent in soleus (Fig. S2A and B). For this reason, we focused our attention on soleus muscle, which in 6-month-old IT/+ mice displayed a high range of fiber diameters, averaging 31 ± 20 \(\mu m\) (\(n = 143\)), compared to 24 ± 6 \(\mu m\) (\(n = 128\)) in WT animals. Fiber type distribution, assessed by NADH-staining, did not differ between WT and IT/+ mice in all age groups (Fig. S2 C and D and Table S1). Type I fiber hypotrophy/atrophy, characteristic of core myopathies (3), could be detected in individual fibers as early as 6 weeks, whereas type 2 fibers in young IT/+ mice appeared enlarged. In 18-month-old IT/+ mice, both fiber types showed reduced diameters (Table S1), suggestive of generalized fiber atrophy. The extent of central nucleation, indicative of skeletal muscle regeneration, did not differ between WT and IT/+ mice of all age groups. This observation is consistent with findings from core myopathy patients, who rarely exhibit central nucleation or active skeletal muscle regeneration (22). A single case of RYR1-related central nuclear myopathy (13) is apparently an exception.

Discrete foci of oxidative enzyme depletion, consistent with minicores, were observed in NADH-TR-stained longitudinal sections of IT/+ soleus fibers at \(\approx 12\) months of age (Fig. 1 C–E). The foci, 1–2 per 100 \(\mu m\) of myofiber length, were detected in \(\approx 20\%\) of both type 1 and type 2 fibers. They were predominantly eccentric (Fig. 1C), small and rounded, with an average diameter of 10–20 \(\mu m\). As in MmdD (8, 23), the main axis of each minicore (Fig. 1D) was in transverse orientation to the main axis of the fiber. Larger areas of oxidative enzyme depletion, consistent with cores, were observed in type 1 fibers of 20-month-old IT/+ mice (Fig. 1 F and G and Fig. S3 A–C). These cores often occupied a more central position and extended longitudinally for more than 50 \(\mu m\). Outside of the minicore/core areas, the oxidative staining of the IT/+ myofibers was uneven (Fig. 1 C–G and Fig. S3 A–C). The cross-banding pattern, reflecting the normal, highly-organized structure of the mitochondrial/sarcotubular network, was frequently distorted or absent and was
substituted by prominent longitudinal streaks of oxidative enzyme activity (Fig. 1 C, F, and G and Fig. S3 A–C). Whereas depletion of oxidative enzyme activity represents loss of mitochondria, increased intensity of oxidative staining peripheral to the core reflects abnormal mitochondrial clustering. The progressive formation of the patchy staining patterns outlining minicores and cores suggests an age-dependent expansion of core lesions in IT/+ myofibers.

Examination of toluidine blue-stained muscle sections from 6-month-old IT/+ mice (Fig. 2 A) revealed structural abnormalities consistent with minicore/core lesions. Minicore/core lesions were identified as well-demarcated areas of spatial disruption of sarcomeric and myofibrillar arrangement present within an individual myofiber. There was no apparent disruption of cell membrane integrity. They occurred with a frequency not greater than 1–2 per fiber, involved 5–40 consecutive sarcomeres, and were observed in ~14% of fibers. A linkage between these lesions and the minicores observed in NADH-TR staining (Fig. 1 C–F) was established by similarities in frequency and location, which were predominantly peripheral and extended across adjacent sarcomeres in a transverse direction. The lesions often had a perinuclear location and were in close proximity to blood vessels (Fig. 2 A and Inset), as described for minicores in core myopathy patients (4). The lesions showed age-dependent expansion (Table S2), so that by 18 months, they were present in ~65% of fibers and extended over larger areas. Such lesions were rarely found in WT control myofibers (Table S2). Overall, the resemblance between the lesions in IT/+ mice and minicore and core lesions in core myopathy patients (4, 8, 22) was striking.

An unexpected finding in the muscles of aged 18-month-old IT/+ mice was the presence of rod-like inclusions, which were clustered in areas apparently devoid of cross-striations and streamed along the length of the myofibers (Fig. 2 B). These inclusions were specific to IT/+ muscle samples and were found in ~15–20% of soleus fibers in two out of five aged IT/+ mice.

Electron Microscopy. Skeletal muscle fiber type was deduced from ultrastructural features specific for fast (type 2) and slow (type 1) fibers: Z-line thickness, relative mitochondrial abundance, and M-line appearance (24). Ultrastructural abnormalities were detected in both type 1 and type 2 myofibers of 6-week-old IT/+ mice (Fig. 3 A and B). Fig. 3 A shows z lines streaming and focal loss of a Z-disk (arrowheads). Note that intermyofibrillar spaces are reduced and mitochondria and SR are absent from this area (Upper Left diagonal), which is sharply delineated from a contiguous, normally structured region (Lower Right diagonal). Mitochondria in the unaffected area (asterisks) have a normal disposition and appearance. C shows that a type 2 fiber shows a well-demarcated lesion with gradual loss of sarcomeric organization from a structured (Upper Right) to an unstructured area (Center). Double-headed arrows show the variability of sarcomeric lengths in the core area. The arrowheads show how loss of sarcomeric register is transmitted to adjacent regions. D shows the central part of an unstructured core in a type 1 fiber. Z-lines (arrows) are wavy and disintegrating, intermyofibrillar space is reduced, and mitochondria and SR are absent. [Scale bars, 5 μm (A), 2 μm (B and C), and 1 μm (D).]

myofibrillar organization in a type 2 soleus myofiber. The myofibrils varied greatly in thickness and numerous sites of splitting and thinning were observed, suggesting possible defects in myofibrillogenesis in IT/+ mice. The focal abnormalities included sarcomeric shortening, insertion of an additional sarcomere, loss of sarcomeric register, and myofibrillar disorganization with focal loss of cross-striation. Myofibrillar intermingling and the loss of intermyofibrillar space within compacted regions was a frequent feature. We propose that these compacted areas represent the initial stages of core formation.
Transitions from structured to unstructured areas within the core (S2). In both type 1 and type 2 fibers, we observed abrupt shortening is highly irregular; some are overstretched, while the core area. The sarcomeres are out of register and their fiber. There is an insertion of two additional sarcomeres within five sarcomeres in a few adjacent myofibrils in a type 2 soleus myofibers of 6-week-old, IT/H11001.

mitochondria. The structure of the region contrasts with that of Z-line material within the area of compacted myofibrils. In B (3, 9, 10), Fig. 3 shows extensive Z-line streaming and focal loss of Z-line material within the area of compacted myofibrils. In this area, there is loss of intermyofibrillar space, of SR, and of mitochondria. The structure of the region contrasts with that of an adjacent, normally organized area in the same myofiber, with regularly positioned mitochondria and well-defined intermyofibrillar spacing.

The ultrastructural abnormalities that were first detected in myofibers of 6-week-old, IT/+ mice were more frequent and more pronounced in 6-month-old mice (Fig. 3 C and D and Table S2). In both type 1 and type 2 fibers, we observed abrupt transitions from structured to unstructured areas within the core lesions. Fig. 3C illustrates a core lesion involving approximately five sarcomeres in a few adjacent myofibrils in a type 2 soleus fiber. There is an insertion of two additional sarcomeres within the core area. The sarcomeres are out of register and their shortening is highly irregular; some are overstretched, while others are shortened maximally by about twofold, with sarcomere lengths of 0.76 μm vs. 1.7 μm in the unaffected area. Fig. 3D shows the central part of a structured core area in a type 1 soleus fiber. Although the sarcomeric pattern was relatively well preserved, the myofibrils were compacted and misaligned, intermyofibrillar spaces were absent, and Z-lines were wavy and disintegrated into small segments so that individual myofibrils could no longer be discerned. The mitochondria were absent from most of the area.

EM imaging confirmed the presence of nemaline rods in 18- to 20-month-old IT/+ mice. Fig. 4A shows extended areas of myofibrillar misalignment involving the entire width of the type 1 myofiber. In EM images, the inclusions observed in toluidine blue-stained sections (Fig. 2B) streamed throughout the length of the myofiber and aggregated in unstructured cores (Fig. 4A and B). The rods emanated from disintegrating Z-lines (Fig. 4B) and ranged in length from 0.2 to 2 μm; their lattice-like inner structure had a periodicity of ~13 nm by 17 nm (Fig. 4C). The remnants of thin filaments extended linearly from both ends of the rods. Structural features of the rods in IT/+ myofibers were strikingly similar to those described in NM and CCD with rods (3–6).

Ryr1 mRNA Levels and Protein Expression. The relative abundance of WT and mutant Ryr1 transcripts was determined in soleus muscles of 2-month-old male WT (n = 3) and IT/+ (n = 6) mice using allele-specific primers (ASP) and real-time RT-PCR. The WT and mutant Ryr1 transcripts differed by the two nucleotide bases that were introduced into the Ryr1 gene during mutagenesis (19). To achieve the highest level of specificity, the ASPs were designed to anneal to the mutated region, to include both nucleotide substitutions, and to terminate with a mismatch (SI Materials and Methods). In all IT/+ muscles sampled, the kinetics of PCR product accumulation from the mRNA pool were similar for both ASPs (Fig. 5A), yielding an average expression ratio of 1:1 between the allelic transcripts. Individual IT/+ samples showed small stochastic fluctuations in the levels of both WT and mutant transcripts.

Western blotting (Fig. 5B) revealed no differences in the expression of major contractile or Ca²⁺ regulatory proteins between 4-month-old IT/+ mouse muscles and age-matched controls. This result was expected because only 7% of all fibers were affected in young mice (Table S2).

Whole Muscle Contractility. Measurements of the characteristics of isometric contraction in isolated, intact, lumbrical (fast-twitch) and soleus (slow-twitch) muscles in 2-month-old IT/+ males (n = 6) and age- and sex-matched WT mice (n = 5) at 28 °C (Fig. S4A–D and Table S3) showed that force during a single twitch and submaximal tetanic contractions (i.e., <30 Hz) was 28–34% lower (P < 0.05) in IT/+ mice, compared to WT. A significant decrease (∼37%) in peak twitch force and maximal twitch rate of contraction (+df/dt) in mutated muscles was observed. No change was observed in −df/dt. There was also a trend for maximal tetanic force (P = 0.09) and +df/dt (P = 0.12) to be reduced across all stimulation frequencies in IT/+ mice. These data suggest that contractile function is impaired in both fast- and slow-twitch IT/+ muscle.
Discussion

Here we show that the 14895T mutation causes a congenital myopathy with minicores, cores, and rods in KI RyR1<sup>H11001</sup> mice. The manifestation and progression of myopathy in the mouse model recapitulates many features commonly observed in human congenital myopathies (1–3). Neonatal IT+/+ mice exhibit hypotonia and respiratory distress, but recover; the course of the disease is then slowly progressive. IT+/+ mice show impaired contractility at 2 months in both fast fiber-rich (lumbrical) and slow fiber-rich (soleus) muscles (Fig. S4). After 8 months, many mice exhibit varying degrees of impaired locomotion and, in 14% of all cases (n = 30), a complete paresis of the hindquarters (Fig. 1B and Fig. S1 B–E). Dorsal kyphosis, characteristic of congenital myopathies in humans (1–3), develops in 80% of aged IT+/+ mice. Variable penetrance is a common clinical feature of CCD/H11001 myopathies. Specifically, increased fiber size variability and intermingling, suggest sporadic, local abnormalities in myofibrillar organization, their focal thinning, misalignment, and denervation (33). Denervation and strenuous exercise are known to produce core-like lesions (33–35), which are strikingly similar to those observed in core myopathies. It is reasonable to suggest that the underlying cause of the ultrastructural abnormalities observed in these conditions is reduced RyR1-mediated Ca<sup>2+</sup> release, with EC uncoupling as a possible common denominator.

As with human RyR1-related congenital myopathy, the disease phenotype in IT+/+ mice is highly variable (Fig. 1A and B and Fig. S1 B–E), even though genetic variability is minimized on the stable Sv129 genomic background (19). Fluctuations in total RyR1 protein content and in levels of expression of WT and mutant alleles are also minimal, because total RyR1 protein levels are normal in heterozygous IT+/+ muscles (Fig. 5B) and real-time RT-PCR shows equimolar levels of mutant and WT RyR1 transcripts (Fig. S4).

A recent study of the single channel properties of mutant RyR1 (36) has shown that functional variability is an intrinsic feature of Ca<sup>2+</sup> release channels expressed in the heterozygous state. It was then proposed that the random combination of WT and mutant subunits in an RyR1 tetramer might contribute to phenotypic variability in RyR1-related disorders. The RyR1 population in heterozygotes would consist of six variants of RyR1 tetramers: homotetrameric WT channels of normal activity, homotetrameric mutant channels of compromised or null activity, and hetertetrameric channels of intermediate activity arising from four possible tetrameric arrangements of WT:mutant subunits (3:1; 1:3; 2:2, side by side; and 2:2, diagonally opposed). Our previous data showed that the homozygous 14895T mutation disrupted RyR1-mediated Ca<sup>2+</sup> release without altering the structural integrity of the RyR1 protein or of the supramolecular CRU complexes (19). Assuming that the formation of RyR1 tetrameric complexes in heterozygous IT+/+ mouse muscle occurs via random combination of WT and mutant subunits present in equimolar quantities, ~1/16 of all RyR1 tetramers would be fully active WT homotetramers, and ~1/16 could be functionally inactive IT/IT homotetramers; and 14/16 would exhibit an intermediate level of functional competence. At higher levels of assembly, the CRU would be formed from random combinations of functionally diverse RyR1 tetramers with normal dihydropyridine receptor (DHPR) complexes. This would not only increase the potential for inhomogeneity of Ca<sup>2+</sup> release within a CRU, but might also lead to disparity between sarcomeric domains controlled by functionally different CRUs.

We propose that heterogeneity of Ca<sup>2+</sup> release channel function, created at the levels of RyR1 tetramer and CRU assembly, results in a long-term spatial heterogeneity of Ca<sup>2+</sup> release that leads to a corresponding spatial heterogeneity of contractile force and, eventually, to impaired muscle activity. Stoichiometry of channel function is difficult to measure because of the intrinsic characteristics of ion gradients and channel gating. However, Feske et al. (37) have demonstrated that measurable changes in Ca<sup>2+</sup> channel density can be correlated directly with measurable changes in Ca<sup>2+</sup> channel function if conditions are created in which functional channels are limiting to ion flux. If we assume that RyR1 Ca<sup>2+</sup> release channel function in IT+/+ mice is limiting, then small fluctuations in the activity of a CRU could have significant effects on local Ca<sup>2+</sup> release and subsequent contractility of the sarcomeres governed by each CRU.

Our EM studies capture the apparent consequence of the heterogeneity of contraction that exists within IT+/+ myofibers (Fig. 3A–C). The discordance of contraction between adjacent myofibers is seen as disruption of sarcomeric register. Such discordance would create physical stresses within myofibers that

EC uncoupling and/or reduced RyR1-mediated Ca<sup>2+</sup> release are thought to play a key role in skeletal muscle disuse and wasting (30), in exercise-induced muscle damage (31), muscle aging (32), and denervation (33). Denervation and strenuous exercise are known to produce core-like lesions (33–35), which are strikingly similar to those observed in core myopathies. It is reasonable to suggest that the underlying cause of the ultrastructural abnormalities observed in these conditions is reduced RyR1-mediated Ca<sup>2+</sup> release, with EC uncoupling as a possible common denominator.
would lead to tearing and shearing between myofibrils and even to disruptions of the sarcotubular and mitochondrial systems that would, over time, cause physical and functional damage to Ca\(^{2+}\) regulatory, energetic, and contractile systems. We propose that, as these processes progress, small foci of damage would coalesce and manifest as minicores, cores, and rods that would compromise muscle function and force. Most importantly, the heterogeneity of Ca\(^{2+}\) release and contractile force that would occur, regardless of whether the heterozygous RyR1 mutation enhanced Ca\(^{2+}\) leak or caused EC uncoupling, could represent a common mechanism for core formation arising from functionally different RyR1 mutations. Thus we propose a unifying theory for the pathogenic mechanism leading to the formation of structural abnormalities in RyR1-related muscle disorders.

In conclusion, our results indicate that the I/T+ mouse line represents a unique and phenotypically valid model of RyR1-related congenital myopathy with minicores, cores, and rods. Aminable to systematic sampling, the model offers high potential for further unraveling of the abnormal molecular mechanisms that underlie the pathogenesis of core myopathies and of skeletal muscle disorders arising from EC uncoupling. Ultimately, it will be valuable for testing the efficacy of therapeutic strategies designed to combat progression of the disease phenotype.

**Materials and Methods**

**Animal Handling.** Experimental protocols for animal research were approved by the Institutional Animal Care and Use Committees at all universities involved in this study. The generation and genotyping of RyR1+/H11001 mice was described previously (19). For maintenance, I/T+ mice, generated on an Sv129 background, were crossed with WT 129S2/SvPasCrl mice (Charles River).

**Histology.** Mice were euthanized by cervical dislocation. Muscles were dissected within 10–15 min postmortem. Multiple transverse and longitudinal sections of vastus lateralis, gastrocnemius, EDL, tibialis anterior, and soleus muscles from I/T+ and WT littersmates were examined for abnormalities. Specimens of buffered formalin- or glutaraldehyde-fixed tissue were embedded in paraffin, sectioned in transverse orientation (3–5 mm), and stained with hematoxylin and eosin (3). For NADH-TR reactivity, fresh muscle specimens were frozen in isopentane cooled in liquid nitrogen, sectioned in an IEC cryostat, and stained (3). To discriminate minicore/core lesions from possible “edge” or “dissection contracture” artifacts only lesions located within central areas of sections and restricted to one myofiber were analyzed.

**Electron Microscopy.** Muscle samples were fixed immediately, without pinning, in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded ethanol series, followed by propylene oxide, and embedded in a Quetol-Spur resin mixture. Semithin longitudinal and transverse sections (0.6 μm) were stained with toluidine blue on a hot plate for 30 seconds. Thin sections (0.1 μm) were cut on an RMC600 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed in an FEI CM100 TEM. Cross-sectional fiber diameters were determined by standard methods (3).

**ACKNOWLEDGMENTS.** We thank Drs. Susan Brown, Imperial College, London, UK, and Nicole Monnier, University Hospital, Grenoble, France, for expert advice and stimulating discussions. This work was supported by Canadian Institutes of Health Research Grants MT 3339 and MOP 49493 to D.H.M., National Heart, Lung, and Blood Institute, and National Institutes of Health grants to J.G.S. and C.E.S., and a Howard Hughes Medical Institute Grant to C.E.S.