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Research Article

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Merosin-deficient Congenital Muscular Dystrophy

Partial Genetic Correction in Two Mouse Models

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Abstract

Humans and mice with deficiency of the α2 subunit of the basement membrane protein laminin-2/merosin suffer from merosin-deficient congenital muscular dystrophy (MCMD). We have expressed a human laminin α2 chain transgene under the regulation of a muscle-specific creatine kinase promoter in mice with complete or partial deficiency of merosin. The transgene restores the synthesis and localization of merosin in skeletal muscle, and greatly improves muscle morphology and integrity and the health and longevity of the mice. However, the transgenic mice share with the nontransgenic dystrophic mice a progressive lameness of hind legs, suggestive of a nerve defect. These results indicate that the absence of merosin in tissues other than the muscle, such as nervous tissue, is a critical component of MCMD. Future gene therapies of human MCMD, and perhaps of other forms of muscular dystrophy, may require restoration of the defective gene product in multiple tissues. (*J. Clin. Invest.* 1998. 102:844–852.) Key words: muscular dystrophy • merosin • gene targeting • gene therapy • transgenic mice

Introduction

Merosin belongs to the laminin family of basement membrane proteins (1, 2). These are high molecular weight heterotrimeric proteins composed of three subunits, α, β, and γ. Merosin is the collective name for laminins with the α2 subunit (laminin-2: α2-β1-γ1 and laminin-4: α2-β2-γ1). Laminin α2 is prominently expressed in striated muscle as well as in a number of other tissues including peripheral nerve, the central nervous system, thymus, thyroid, intestine, and testis (3–10). The function and importance of laminin α2/merosin in these nonmuscle tissues are not yet known.

A form of congenital muscular dystrophy is caused by mutations in the *LAMA2* gene, causing deficiency of the α2 subunit of laminin-2/merosin. The disease is termed merosin-deficient congenital muscular dystrophy (MCMD)[1] (11–13). The levels of laminin α2 are reduced also in two congenital muscular dystrophies of unknown genotypes, the Fukuyama congenital muscular dystrophy (14), and the muscle-eye-brain disease (15).

MCMD is one of the most severe muscular dystrophies. Most patients lacking merosin are never able to walk. As this subgroup of CMD has only recently been recognized, comprehensive analysis of the disease is yet to be completed. Mouse models for MCMD are available. We have generated a null mutant mouse, dy^W, by homologous recombination in embryonic stem (ES) cells[2] (16). The experimental dy^W mouse and the spontaneous mutant *dy* mouse (17), which expresses very low levels of merosin, are both severely affected. Another spontaneous mutant mouse, dy^{2J}, has a less severe disease (18). The dy^{2J} mouse has a point mutation in the *lama2* gene that results in the expression of near normal amounts of a slightly truncated protein (12, 13). Recently, another experimental mouse, null mutant for *lama2*, was described (19). A characteristic of human MCMD and merosin-deficient muscular dystrophy in mice is that motor nerves are poorly myelinated (20–23), suggesting that merosin may be important in nervous tissue in addition to muscle.

We used the experimental mutant dy^W and the spontaneous mutant dy^{2J} mouse models of MCMD to determine whether correction of merosin deficiency in muscle would correct the clinical disease in the mice and, thus, potentially serve as a paradigm for gene therapy of MCMD in patients. Because many of the genes mutated in muscular dystrophies are considered relatively muscle specific, and as the myopathic phenotype is predominant in these diseases, muscle has been the focus for attempts toward the treatments. To this end, all clinical and experimental cell and gene therapy protocols for the treatment of Duchenne muscular dystrophy and *mdx* mice have been directed at restoring the expression of dystrophin in muscle only (24–26). Indeed, muscle-specific correction of dystrophin deficiency in *mdx* mice appeared to be successful in correcting the disease, despite the fact that dystrophin is normally expressed in multiple tissues. The results of these studies have been hailed as harbingers of future gene therapy in patients with Duchenne muscular dystrophy.

In the present study, we show that expression of a human *LAMA2* transgene in skeletal muscle of dy^W null mutant mice dramatically improves the muscle disease in these animals, as judged by histology of muscle, weight gain, and longevity of the animals. The expression of the transgene had less effect on the dy^{2J} mice, which have a less severe disease to begin with. Although the general condition of the transgenic mice was far better than that of the nontransgenic littermates, the trans-

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1. *Abbreviations used in this paper:* CK, creatine kinase; ES cells, embryonic stem cells; MCK, muscle-specific creatine kinase; MCMD, merosin deficient congenital muscular dystrophy.

genic dy^W and dy^{2J} mice develop the severe lameness and joint contractures of hindlegs that are characteristic of all strains of merosin-deficient mice and are likely caused by the persistent deficiency of merosin in peripheral nerves.

Methods

Mice. The generation of null mutant dy^W mice by homologous recombination in ES cells (16) will be described elsewhere. Briefly, a replacement vector was prepared from a genomic clone for *lama2,* in which a cassette containing the *lacZ* and *neo* genes was inserted 23 bp downstream of the ATG initiation site. The diphteria toxin A gene was inserted at one end of the genomic clone to select against random insertion events (27). The linearized vector was electroporated into CJ7 ES cells (28), and cells from selected clones of targeted ES cells were injected into C57BL/6J blastocysts. Resulting chimeric males were bred to BL/Swiss females, and heterozygous mice were bred to homozygosity for the dy^W gene. The dy^{2J} mice were originally from The Jackson Laboratory (Bar Harbor, ME). They were maintained on C57BL/6J background by breeding in The Burnham Institute animal facility. FVB/N and CD-1 mice were purchased from Taconic Farm (Germantown, NY) and Charles River (Boston, MA), respectively. All mice were fed standard laboratory rodent diet (Purina, St. Louis, MO) and water ad libitum. No special provisions were made for dystrophic mice other than that some food was presented to all mice on the cage floor for easy access.

Genotyping of dy^W and dy^{2J} mice. Genotyping of dy^W mice was performed by PCR. Mouse tail DNA (0.2 μg) was amplified by 35 cycles (1 min at 94°C, 1 min at 53°C, and 1 min at 72°C) on a thermal cycler. Sense and antisense primers of mouse *lama2* gene (5′-ACTGCCCTTTCTCACCCACCCTT-3′ and 5′-GTTGATGCGCTTGGGAC-3′) were used to detect a 247-bp fragment of the wild-type allele. Another antisense primer corresponding to the 5′ end of the *LacZ* gene (5′-CGACAGTATCGGCCTCAG-3′) was used to detect a 446-bp fragment of the mutated allele. Ligase chain reaction (29) was used to detect the G to A point mutation in the *Lama 2* gene of dy^{2J} mice. Six primers were designed: A: 5′-GGTATGGATTGCTGCTGTTCTGAT-3′; Bw: 5′-CCAGTGCCATAGAAAAACATATATAATACATAC-3′; Bm: 5′-CCAGTGCCATAGAAAAACATATATAATACATAT-3′; Cw: 5′-ATCAGAACAGCAGCAATCCATACCG-3′; Cm: 5′-ATCAGAACAGCAGCAATCCATACCA-3′; D: 5′-TATGTATTATATATGTTTTTCTATGGCACTGG-3′. Two sets of primers named wild-type set (primer A, Bw, Cw, and D) and mutant set (primer A, Bm, Cm, and D) were arranged for the normal and mutant genes. The wild-type primer set only amplifies the genomic DNA of wild-type and heterozygous dy^{2J} mice; the mutant primer set only amplifies the genomic DNA of heterozygous and homozygous dy^{2J}. Ligase chain reaction was carried out with pfu DNA ligase from Stratagene (La Jolla, CA) on a thermal cycler. The reaction products were separated on 3% agarose gel.

Preparation of transgene construct. Full length human laminin α2 cDNA (30) was inserted into the HindIII site of pCCLMCK-II (31), a plasmid that contains the mouse muscle creatine kinase (MCK) promoter driving the specific expression in striated muscle. This generates the plasmid pCCLMCK-hα2, in which the NotI site was converted into a SwaI site. A 15.2-kb DNA fragment named MCK-hα2 was released with SalI and SwaI and used for one cell embryo microinjection.

Production of human laminin α2 transgenic mice on wild-type background. The 15.2-kb DNA fragment, MCK-hα2, was microinjected into the male pronucleus of fertilized mouse eggs from FVB/N mice. Microinjected eggs were transplanted into the oviducts of pseudopregnant female CD-1 mice and carried to term. Mice were screened by PCR with tail genomic DNA, and transgene-positive founders were selected. To test for the expression of the human laminin α2 in transgenic founder mice, immunofluorescence staining was performed on tail muscle using the monoclonal antibody 5H2, which recognizes human but not mouse laminin α2 (32). Homozygous transgenic mice were generated from the breeding of heterozygotes.

Production of transgenic mice on dy^W and dy^{2J} backgrounds. Female homozygous transgenic mice were bred to male heterozygous dy^W mice, followed by sib breeding to generate mice heterozygous for the transgene and homozygous for dy^W. These mice will be referred to as transgenic dy^W (or dy^W[T]). Male homozygous transgenic mice were bred to female dy^{2J}/dy^{2J} mice, and male offspring were bred back to female dy^{2J}/dy^{2J} mice to produce mice heterozygous for the transgene and homozygous for dy^{2J}, hereafter referred to as transgenic dy^{2J} (or dy^{2J}[T]).

Identification of mice with transgene by PCR. PCR on mouse tail DNA was performed with two human laminin α2 cDNA primers (HM64791: 5′-CAAAGTATCTGTGTCTTCAGGA-3′, HM6946-5′-GAATGTAATCACACGTACAGC-3′), giving a 467-bp product from DNA of transgenic mice.

Determination of transgene copy number by Southern blot analysis. 10 μg of tail DNA from founder mice Nos. 4, 27, and 44 or 10 μg of wild-type mouse genomic DNA mixed with 30, 60, 120, 240, 480, 960, or 1920 pg of pCCLMCK-hα2 plasmid DNA were digested with HindIII at 37°C overnight. 30 pg of pCCLMCK-hα2 correspond to one copy of the transgene. Southern blot hybridization was done as described before (33) using a rat laminin α2 cDNA as a probe (See Fig. 1 *A*).

Protein detection by immunofluorescence and immunoblotting. Three anti-laminin α2 antibodies (5H2, Ab 804, and Ab 1301) were used (32). 5H2 is a mouse monoclonal antibody which binds to the COOH-terminal 80-kD segment of the human but not mouse laminin α2. Ab 804 is a preparation of affinity-purified rabbit polyclonal antibodies to the same 80-kD segment; this antibody preparation reacts with both human and mouse laminin α2. Ab 1301 is a rabbit antiserum raised against a synthetic peptide. This antiserum reacts with the NH_2-terminal 300-kD segment of both human and mouse laminin α2. Immunofluorescence and immunoblotting were performed as previously described (33).

Histology. Mice were anesthetized with Avertin (15 mg/ml, 0.017 ml/g) and perfused with 10 ml Z-fix (Sigma Chemical Co., St. Louis, MO) through the left ventricle. The muscle and attached bone from the leg were cut into pieces of 5-μm thickness and were post-fixed in Bouin's fixative for 3 d followed by decalcification. Tissues were then embedded in paraffin and sectioned at a thickness of 5 μm for hematoxylin–eosin staining.

Serum creatine kinase activity. Mice were anesthetized with Avertin, and ~ 100 μl of blood were collected from the orbital sinus. Blood samples were kept at room temperature for 10 min and on ice for 30 to 60 min and centrifuged at 8000 *g* at 4°C for 10 min. 20 μl of serum were used to measure creatine kinase (CK) activity with the CK10 kit from Sigma Chemical Co.

Statistical analysis. All data are expressed as mean±SEM. The *n* values indicate the number of data points. Two groups were compared with unpaired Student's *t* test. A value of $P \leq 0.05$ was regarded as significant.

Results

Generation of transgenic mice. We generated transgenic mice on wild-type and on two mutant mouse backgrounds, the null mutant dy^W with complete deficiency of laminin α2 and the dy^{2J} with partial deficiency. The dy^{2J} mice were included in these experiments because the nature of the gene and protein defects in dy^{2J} mice is well characterized (12, 13), but the molecular consequences of the protein defect are not understood. To distinguish between the transgene product and the endogenous product in these experiments, a feature particularly important in the analysis of the dy^{2J} mice, we used the human laminin α2 gene and took advantage of species-specific antibodies.

The human laminin α2 transgenic mice were first produced and characterized in FVB/N mice, which are homozygous for the wild-type *lama2* gene. 56 mice were born from microinjected fertilized eggs. 15 (27%) of the mice carried the transgene as detected by PCR on genomic DNA (not shown). Three of the fifteen mice (Nos. 4, 27, and 44) showed immunofluorescence staining of human laminin α2 in muscle tissue from the tail at 3 wk of age. Southern blot hybridization was performed to estimate the number of integrated copies of the transgene in these mice. Founder No. 44 had > 64 copies of the transgene, whereas Nos. 4 and 27 had 1–3 copies (Fig. 1 *B*). Immunoblotting was used to estimate the expression level of human laminin α2 protein. Leg muscle of No. 44 contained 10 times more human laminin α2 than Nos. 4 and 27 (Fig. 1 *C*). The amount of human laminin α2 relative to mouse laminin α2 cannot be evaluated with existing reagents. Three lines of homozygous transgenic mice were produced by breeding male and female heterozygous offspring of the founders. None of the lines exhibited any obvious abnormal phenotypic traits. Mice from lines Nos. 4 and 44 were selected for further analysis and for production of transgenic dy^W and dy^{2J} mice.

Characterization of expression of transgene and of localization and processing of transgene product. We next determined the time and site of expression and accuracy of posttranslational modification and processing of human laminin α2 in the transgenic mice from the No. 44 line. Immunofluorescence staining with the human-specific monoclonal antibody 5H2 showed a gradual increase in amounts of the human laminin α2 from barely detectable in occasional muscle fibers at 4 d after birth to strongly positive in all fibers at 4 wk. The human laminin α2 was mainly localized in the basement membrane zone of skeletal muscle (Fig. 2, *C* and *D*) and heart muscle (not shown). Some human laminin α2 was also present intracellularly in myofibers (Fig. 2 *K*), an unusual location for the laminin α2 chain that may be caused by the high expression level of the protein. Human laminin α2 was not present in the sciatic nerve, which is a normal site of expression of the endogenous chain (Fig. 2 *D*), or in brain capillaries, kidney mesangium, or intestinal crypts (not shown). Thus, the expression of the transgene was specific for striated muscle as expected from the MCK promoter (31).

The normal processing of laminin α2 involves a proteolytic cleavage into a 300-kD NH_2-terminal segment and an 80-kD COOH-terminal segment (32). An 80-kD band was detected in both transgenic and nontransgenic leg muscle extracts when using species–nonspecific Ab 804. The human-specific antibody 5H2 bound to an 80-kD band only in samples from transgenic mice (not shown), indicating that the human laminin α2 was processed correctly in the mice. The Ab 1301, antipeptide antibody, detects the 300-kD NH_2-terminal segment of mouse and human laminin α2 chain. In mature laminin, this segment is disulfide cross-linked to laminin β and γ chains resulting in a 700-kD protein in unreduced muscle extracts. More of the 700-kD protein was present in transgenic mice than in nontransgenic mice (Fig. 1 *C*), suggesting that the human laminin α2 in transgenic mice had assembled with endogenous laminin β and γ chains to a chimeric disulfide cross-linked protein. Small amounts of an Ab 1301-reactive, nondisulfide-bonded polypeptide of ~ 400 kD were also detected (not shown), most likely representing intracellular, unprocessed laminin α2 chain (Fig. 2, *G*, *H*, *L*, and *K*). We conclude that most of the transgenic laminin α2 was correctly processed and assembled into laminin molecules. Nos. 44 and 4 lines were bred to heterozygous $+/dy^W$ and homozygous dy^{2J}/dy^{2J} to ultimately produce mice heterozygous for the transgene and homozygous for the dy^W or dy^{2J} mutations, hereafter called transgenic dy^W (or dy^W[T]) and transgenic dy^{2J} (or dy^{2J}[T]). The human transgene was expressed in these lines in the same manner as in the wild-

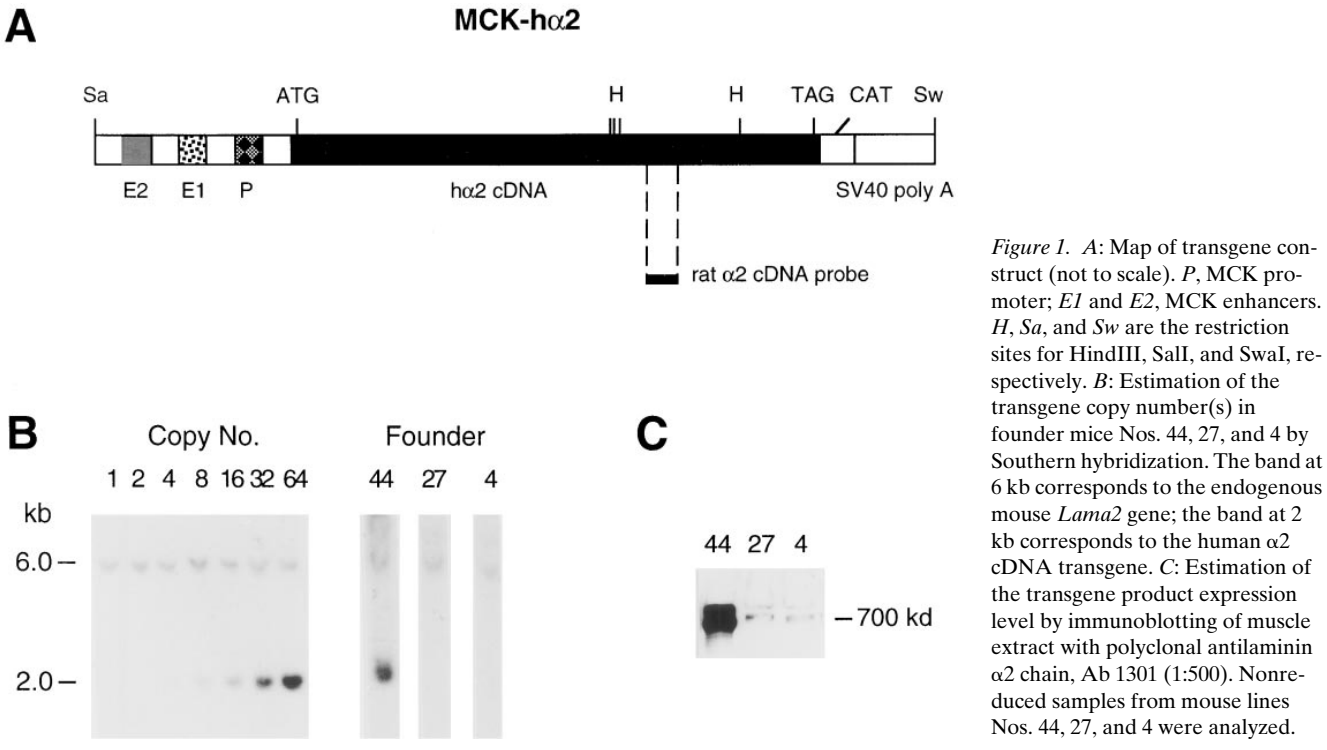

Figure 1. A: Map of transgene construct (not to scale). *P*, MCK promoter; *E1* and *E2*, MCK enhancers. *H*, *Sa*, and *Sw* are the restriction sites for HindIII, SalI, and SwaI, respectively. *B*: Estimation of the transgene copy number(s) in founder mice Nos. 44, 27, and 4 by Southern hybridization. The band at 6 kb corresponds to the endogenous mouse *Lama2* gene; the band at 2 kb corresponds to the human α2 cDNA transgene. *C*: Estimation of the transgene product expression level by immunoblotting of muscle extract with polyclonal antilaminin α2 chain, Ab 1301 (1:500). Nonreduced samples from mouse lines Nos. 44, 27, and 4 were analyzed.

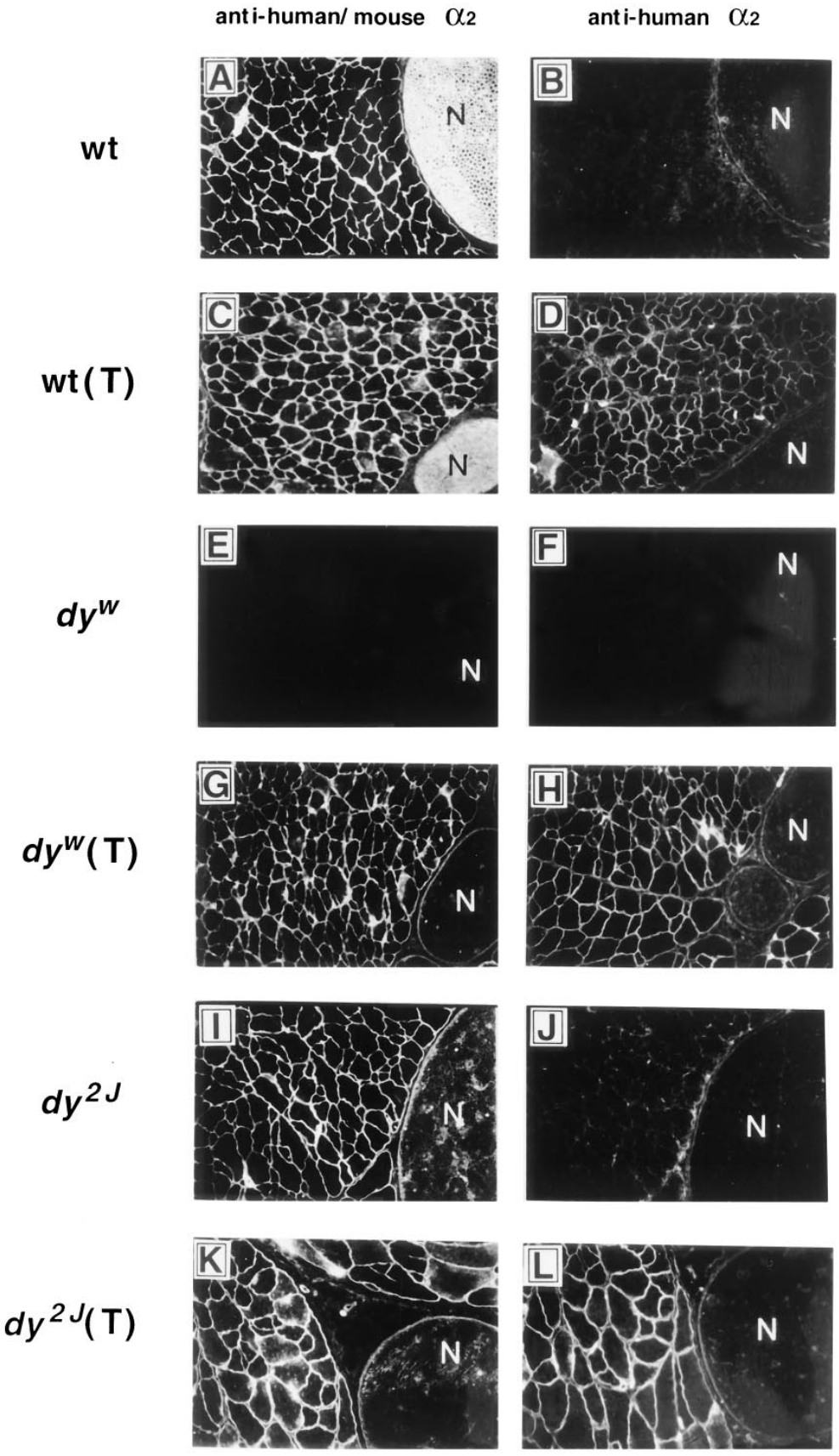

Figure 2. Expression and localization of mouse and human laminin α2 chain/merosin in skeletal muscle of mice with and without the human *lama2* transgene (*T*). All transgenic mice were from the No. 44 line. Immunofluorescence was performed with Ab 804 (10 μg/ml; *A, C, E G, I,* and *K*), which detects mouse and human laminin α2, and Ab 5H2 (hybridoma medium 1:1; *B, D, F, H, J,* and *L*), which is specific for human laminin α2. *A–D*: wild type; *E–H*: homozygous dy^{2J}/dy^{2J}; *I–L*: heterozygous *+/dy2J*. Bar, 60 μm. *N,* nerve. The human laminin α2 chain protein is only detected in transgenic mice and is restricted to skeletal muscle.

type mice (Fig. 2, *H* and *L*). Most of the data presented below were obtained with mice derived from the No. 44 high expresser line. Similar results were obtained with mice from the No. 4 lower expresser line unless otherwise noted.

Dystrophic mice with transgene are healthy and long lived. The expression of the transgene dramatically improved the general health of the dy^W mice as shown by appearance (Fig. 3), body weight (Fig. 4), and life span (Fig. 5). At first sight, the transgenic dy^W mice could not be distinguished from wild-type mice (Fig. 3); they appeared lively and well fed. Only upon closer inspection could they be identified based on characteristics detailed below. This is in contrast to the dy^W/dy^W mice without the transgene that are passive, small, and thin. The effect of the transgene on the dy^{2J} background was less dramatic, consistent with the dy^{2J}/dy^{2J} mice having a milder form of muscular dystrophy.

The body weight of transgenic dy^W mice were significantly higher than that of nontransgenic littermates; the average body weight of transgenic dy^W was close to that of wild-type mice at all ages and similar to that of nontransgenic and transgenic dy^{2J} mice (Fig. 4). The expression of the transgene had no effect on the body weight of wild-type mice, showing that the transgene had no effect on the general well-being of the mice. Transgene expression also had no effect on the weight of transgenic dy^{2J} mice relative to nontransgenic dy^{2J} mice (Fig. 4).

In contrast to the nontransgenic dy^W/dy^W mice, most of which die within 2–4 wk after birth, the transgenic dy^W mice continued to grow and thrive (Fig. 5). Aside from the death of a single transgenic dy^W mouse at 1 wk of age, none of over 50 transgenic dy^W mice produced has succumbed, and those that have not been used for experiments have remained in good health until at least 8 mo of age.

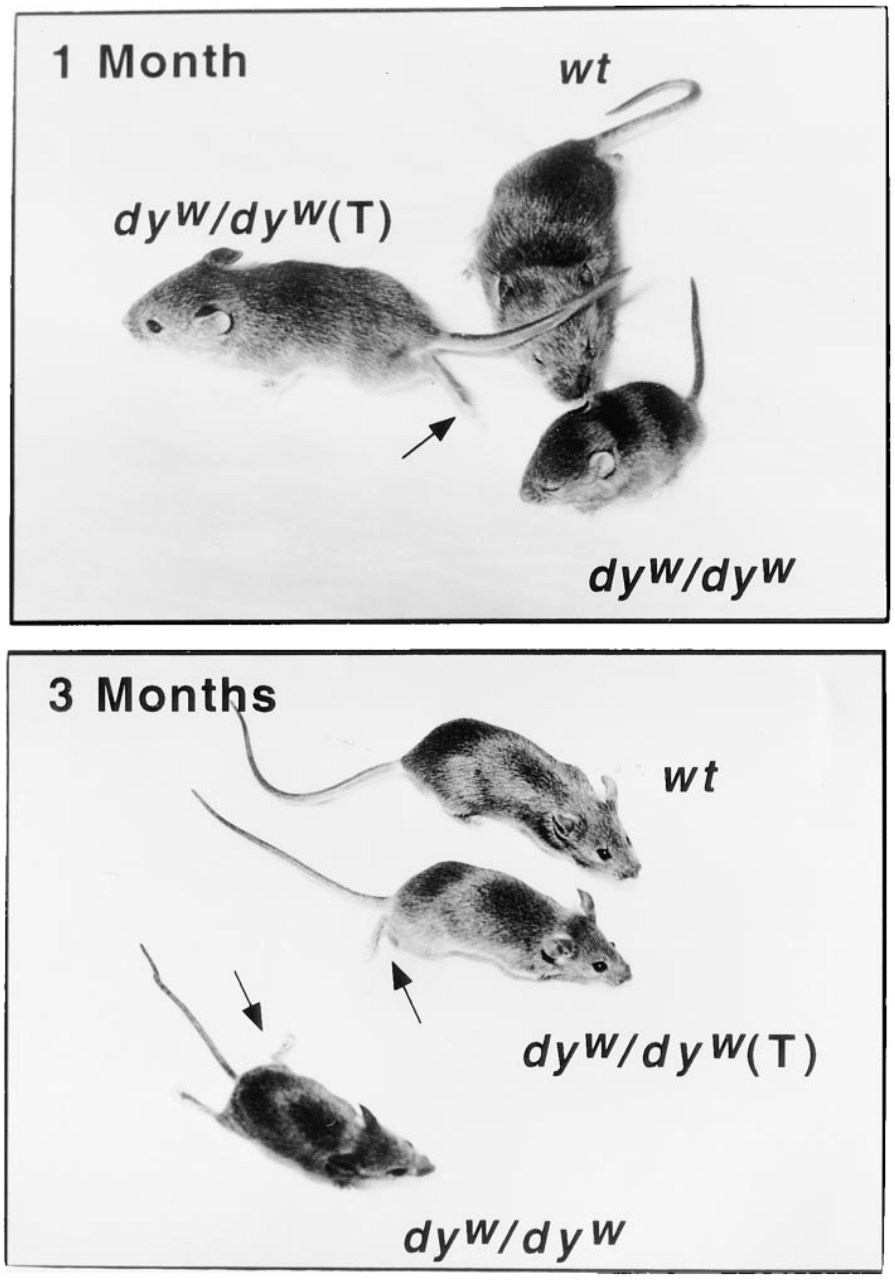

Figure 3. (*A*) 1-mo-old and (*B*) 3-mo-old littermates with different genotypes. The homozygous dy^W mice with transgene and their wild-type littermates are alert and lively with shiny coats and good muscle tone, whereas the homozygous dy^W mice are smaller (*A* and *B*) and emaciated (*B*). Contractures are obvious at 3 mo (*B*). dy^W mice with and without transgene show partial lameness of hindlegs (*arrows*).

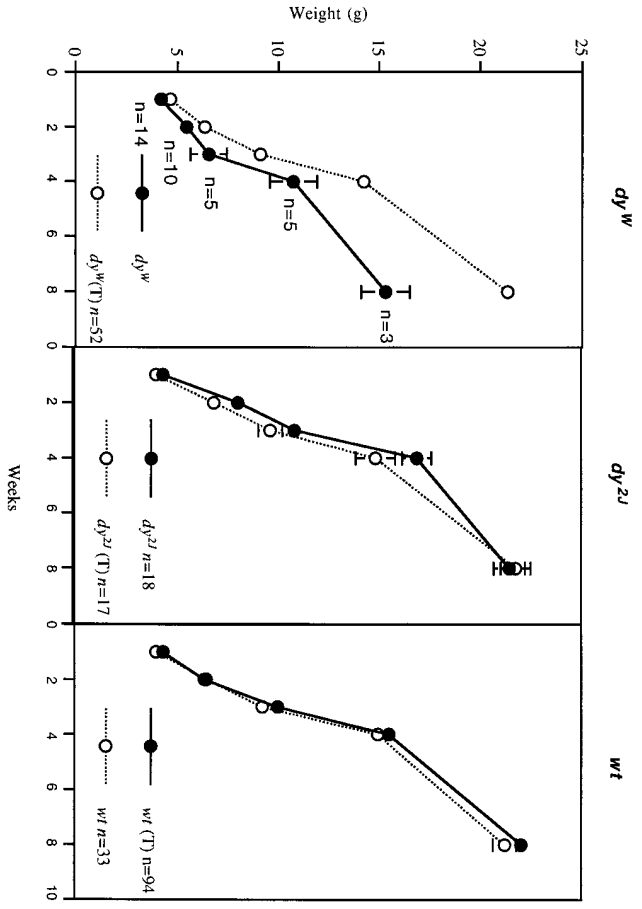

Figure 4. Growth curves for mice with or without transgene (*T*). With the expression of the transgene, the growth rates of transgenic wild type or dy^{2J} mice did not change significantly, but the growth rates of dy^{W} mice increased dramatically to become similar to those of dy^{2J} mice and close to those of wild type mice. Values are mean±SEM.

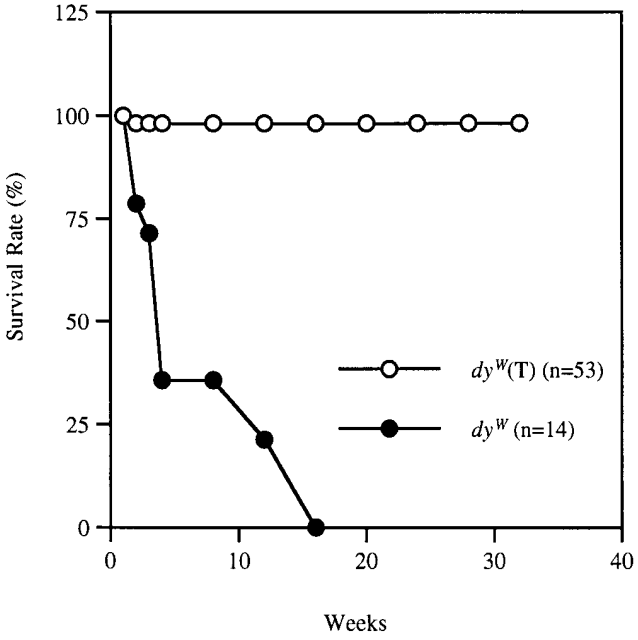

Figure 5. Longevity of mice with or without transgene (*T*). No death occured in the groups of wild-type (*wt*) mice with or without transgene ($n = 127$), or in homozygous dy^{2J} mice with or without transgene ($n = 35$). A single death occurred in the group of homozygous dy^{W} mice with transgene ($n = 53$), whereas most of dy^{W} mice without transgene ($n = 14$) died between 2–4 wk with a few surviving up to 3–4 mon.

Improved muscle morphology and integrity in transgenic dystrophic mice. The morphology of muscle from transgenic and nontransgenic mice was evaluated after routine fixation and staining (Fig. 6). Muscle from dystrophic dy^{W}/dy^{W} mice showed muscle fiber necrosis and occasional areas of regeneration, as judged by small fibers with central nuclei, as well as the pronounced fibrosis characteristic of MCMD (Fig. 6 *C*). Dystrophic dy^{2J}/dy^{2J} showed similar but less extensive changes (not shown). In contrast, the muscles from transgenic dy^{W} (Fig. 6 *D*) and dy^{2J} (not shown) mice of the same age had a near normal morphology. Only occasional areas of fibers with centrally located nuclei, suggestive of a mild myopathy, were seen (Fig. 6 *D*). Transgenic wild-type mice of the No. 44 high expresser line, but not of the No. 4 low expresser line (not shown), showed areas of similar myopathy, suggesting that excessive transgene expression may cause myopathic changes (Fig. 6 *B*).

Measurement of CK activity in mice at 1 mo showed that homozygous dy^{W}/dy^{W} mice had very high levels, homozygous dy^{2J}/dy^{2J} mice intermediate levels, and wild-type mice with or without transgene low levels of CK in serum (Fig. 7). Transgenic dy^{W} mice had dramatically lowered levels of CK compared to nontransgenic dy^{W} littermates, suggesting that the transgene product helped stabilize the muscle. The reduction of CK activity in transgenic dy^{2J} mice relative to nontransgenic littermates was less dramatic. The enzyme levels of the transgenic dy^{W} mice were intermediate between, and not statistically different from, those of transgenic and nontransgenic dy^{2J} mice. Thus, the muscle stability in transgenic dy^{W} mice is similar to that in dy^{2J} mice with or without the transgene.

Progressive lameness in transgenic and nontransgenic dystrophic mice. Although the morphology and integrity of skeletal muscle showed that the muscle greatly benefited from the expression of the transgene in dy^{W} and dy^{2J} mice, the transgenic mice still exhibited an abnormal trait, which is characteristic for the disease of merosin-deficient mice. From ~ 2–3 wk of age, transgenic and nontransgenic mice alike, from both dy^{W} and dy^{2J} strains, flexed hind legs to the trunk when lifted by the tail, a sign of a neurological problem. Moreover, the hind legs of the transgenic dy^{W} and dy^{2J} mice would become increasingly paralyzed with age and develop contractures similar to their nontransgenic counterparts (Fig. 3) (34).

Discussion

As MCMD may be the most frequent and severe congenital muscular dystrophy in humans (35–38), we thought it important to analyze the molecular and tissue consequences of the genetic defect in laminin α2 in this disease and to explore possible approaches to treatment. Our goal here was to examine the feasibility of gene therapy for the treatment of MCMD as well as to determine the relative contribution of the muscle defect to the overall disease in merosin deficiency. To accomplish

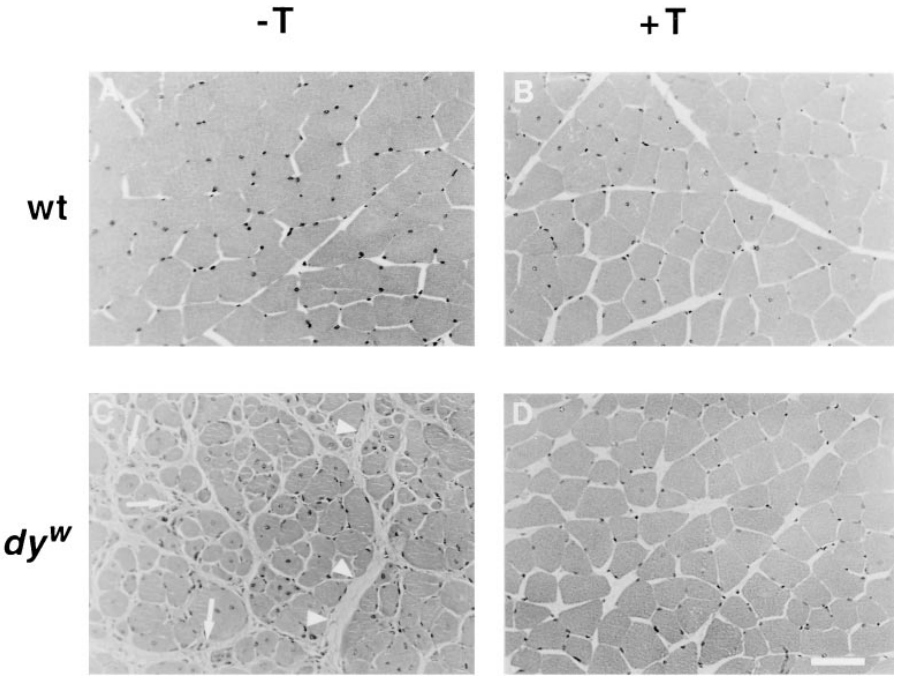

Figure 6. Hematoxylin and eosin staining of muscle tissue from 3-mon-old transgenic (*B* and *D*) and nontransgenic (*A* and *C*) wild-type (*A* and *B*) and homozygous dy^W (*C* and *D*) mice. Extensive degeneration (*arrows*) and fibrosis (*arrowheads*) are seen in the dystrophic dy^W muscle (*C*). The transgenic dy^W muscle (*D*) is indistinguishable from the transgenic wild-type muscle (*B*). Both show near normal morphology with occasional presence of centrally located nuclei, suggestive of mild myopathy. Bar, 60 μm.

this, we generated mice with tissue-restricted expression of a transgene on a null mutant background. Our results reveal novel aspects of the MCMD disease and suggest that correction of laminin α2 defects in muscle only would be an important treatment for MCMD but not a cure. Our data also add important insights into the biology of laminin.

The primary finding of this study is that the expression of a wild-type transgene in the muscle of *lama2* null mutant dy^W mice dramatically improved the general health of mice, which would be dystrophic without the transgene. This result suggests that somatic restoration of the *lama2* gene in muscle cells in severe MCMD may prolong life and greatly improve the quality of life of human patients, at least if performed early in the disease. The transgenic dy^W mice, aside from a single accidental death, seem to thrive, and they remain in good general health for up to 8 mon, the longest time of observation. The transgenic dy^W mice of all ages were in fact quite similar to wild-type mice in size and appearance. In contrast, the nontransgenic, null mutant dy^W mice develop a very severe disease, and few live beyond 2-4 wk.

The life-saving effects of muscle-specific restoration of laminin α2 in transgenic mice thus showed the importance of muscle phenotype to disease, as one might expect. However, the mice still developed the progressive lameness of hindlegs that is characteristic of mice from all *dy* strains. The lameness in transgenic mice is, thus, likely derived from the uncorrected deficiency of laminin α2 in peripheral nerves. It has been reported previously that the roots of motor nerves are poorly myelinated in homozygous *dy* and dy^{2J} mice (21, 23, 39, 40), pointing to a peripheral nerve defect as a result of lack/deficiency of laminin α2. Incomplete myelination and slow nerve conductance has been observed in MCMD patients (20, 23). It appears then that MCMD is not exclusively a muscle disease but has a significant neurological component. This important aspect of the disease can now be investigated with our mice and be taken into consideration in the analysis and future treatment of MCMD in humans.

The transgenic dy^W mice had improved muscle function as judged by their weight and longevity, but their muscle was not completely normal. The mice had signs of mild myopathy based on histology and elevated CK in serum. There are at least two potential causes of this myopathy. One is the uncorrected deficiency of laminin α2 in other tissues, indirectly af-

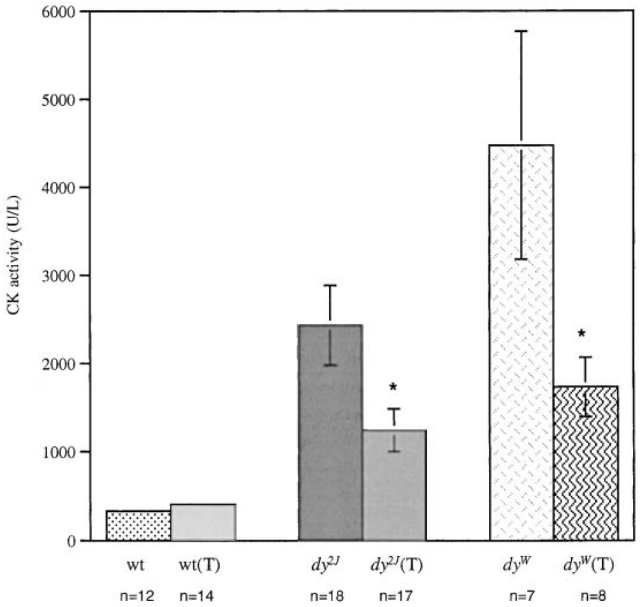

Figure 7. Serum CK activity in 1-mon-old wild-type, and homozygous dy^{2J} and dy^W mice with and without transgene (*T*). Bars show CK values (mean±SEM) for the indicated numbers of mice (*n*). The slight increase in CK activity of wild-type mice with transgene was not statistically significant. The differences in CK activity between dy^{2J} and dy^W mice with transgene and without were highly significant ($P < 0.05$). The differences between either $dy^{2J}(T)$ or dy^{2J} and $dy^W(T)$ were not significant.

fecting muscle. Another one is the use of a heterologous CK promoter leading to abnormal regulation of expression of the transgene. Muscle function depends on hormonal and trophic support from other tissues, particularly peripheral nerve and thyroid (41). As merosin is normally expressed in these tissues, at least part of the myopathy in transgenic dy^W and dy^{2J} mice may be due to the uncorrected deficiency in these tissues. It is possible that the merosin-deficiency in or of Schwann cells in MCMD cause inadequate nerve-derived trophic support for the muscle fibers. Besides merosin itself (30), ciliary neurotrophic factor and glial growth factor 2 are nerve-derived factors that have been shown to support growth and survival of muscle (42, 43). These, and other nerve-derived factors, may be lacking in the transgenic as well as in nontransgenic dy^W and dy^{2J} mice. Similarly, the lack of merosin in the thyroid may negatively affect thyroid function and secondarily affect the function of muscle. It will be most important to investigate the cellular and molecular consequences of the deficiency in nerves in MCMD, but the effects of merosin-deficiency in other tissues on the disease should also be considered.

Another cause of the observed myopathy in transgenic mice may relate to level or timing of transgene expression. Transgenic mice from the line with the highest expression of the transgene, but not from the low expresser line, showed a mild myopathy as evidenced by presence of myofibers with centrally located nuclei. Overexpression may lead to excessive basement membrane formation and muscle damage. Basement membrane thickening is a well known problem in diabetes. On the other hand, the overexpression of laminin may induce events that simulate myogenesis or muscle regeneration, which may not necessarily be harmful. In any event, it is possible that gene therapy with laminins and other basement membrane proteins will require a strict control of gene expression to avoid potential harmful side effects. This is in contrast to the situation for the expression of dystrophin transgene in *mdx* mice, in which overexpression was well tolerated (44). We cannot completely exclude at this point that the myopathy in transgenic mice was caused by the high expression of a xenogenic transgene used here, and that overexpression of a syngeneic transgene would be harmless. Although we showed that most of the human transgene product appeared to assemble correctly with the endogenous mouse laminin β and γ chains, some of it may be secreted in monomeric form (45), and it may in fact have a negative effect. In addition, we don't know if human laminin α2 may have a function slightly different from the endogenous mouse laminin α2. All this will need testing in the future. Some of the questions may be answered in in vitro studies. Our mice, and cell lines derived from them, may then be useful in qualitative and quantitative analysis of laminin biosynthesis and assembly, and basement membrane formation.

Our data also give insight into the function of an individual domain of laminin α2, the domain VI that is truncated in the dy^{2J} mice. The phenotypic resemblance of transgenic dy^W mice with dy^{2J}/dy^{2J} mice with or without the transgene was striking. As mentioned, the homozygous dy^{2J} mice have a much milder form of MCMD than homozygous null mutant and *dy* mice, and they live much longer. Although homozygous dy^{2J} mice in our colony do show the features of muscular dystrophy, including muscle necrosis and fibrosis, they are able to feed and maintain normal body weight, to breed and reproduce, and they have essentially a normal life span. The insignificant improvements in dy^{2J}/dy^{2J} mice upon expression of the transgene have lead us to speculate that perhaps the endogenous, truncated laminin α2 in these mice is relatively well functional in the muscle, and that the added wild-type transgene product makes little difference. The main clinical problem of the homozygous dy^{2J} mice is related to the progressive lameness and paralysis of their hind legs. As the lameness is likely a problem of neurological origin, this problem may be because the mutant laminin α2 is poorly functional in nerve. Interestingly, consistent with this hypothesis, there is abundant merosin present in muscle of dy^{2J} mice, but merosin is almost undetectable around Schwann cells (Fig. 2, *I* and *K*). Perhaps the protein is unable to assemble in nerve, because a nerve-specific assembly site in the defective domain VI is missing. We propose, based on these considerations, that the dy^{2J} mice suffer primarily from a neurological disease rather than a muscle disease and that the domain VI has a particularly important function in Schwann cells.

In summary, our data show that potential gene therapy of MCMD aimed exclusively at striated muscle is likely to provide an important treatment, but not a cure, owing to the prominence and importance of merosin in other tissues. Our data also suggest that regulation of times and levels of expression of the transgene may be important. Aside from the potential value of the present studies as models for treatments of human MCMD, the mice we have generated will be useful for further studies on the biology of laminins. The absence of laminin α2 chain in muscle causes a lethal muscle disease, and the early death has therefore precluded study of the function of laminin in nonmuscle tissues. As the muscle-specific expression of the transgene in the merosin null mutant dy^W mice dramatically improved the health of the mice, transgenic dy^W mice will survive long enough to allow the study of the function of laminins in peripheral nerve and in a number of vital tissues such as thymus, thyroid, and the central nervous system. It may now be possible to study the potential role of laminin α2 in processes such as immunity, metabolism, and neuronal plasticity in our transgenic mice.

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