Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity

Gregory A. Cox*, Nell M. Cole†, Kilchiro Matsumura†, Stephanie F. Phelps*, Stephen D. Hauschka§, Kevin P. Campbell‡, John A. Faulkner† & Jeffrey S. Chamberlain*†‡

* Department of Human Genetics, † Institute of Gerontology, ‡ Human Genome Center, University of Michigan Medical School, Ann Arbor, Michigan 48109–0618, USA
† Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA
§ Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA

Duchenne and Becker muscular dystrophy (DMD and BMD) are X-linked recessive diseases caused by defective expression of dystrophin1,2. The mdx mouse, an animal model for DMD, has a mutation that eliminates expression of the 427K muscle and brain isoforms of dystrophin3,4. Although these animals do not display overt muscle weakness or impaired movement, the diaphragm muscle of the mdx mouse is severely affected and shows progressive myofibre degeneration and fibrosis which closely resembles the human disease5,6. Here we explore the feasibility of gene therapy for DMD by examining the potential of a full-length dystrophin transgene to correct dystrophic symptoms in mdx mice. We find that expression of dystrophin in muscles of transgenic mdx mice eliminates the morphological and immunohistological symptoms of muscular dystrophy. In addition, overexpression of dystrophin prevents the development of the abnormal mechanical properties associated with dystrophic muscle without causing deleterious side effects. Our results provide functional evidence for the feasibility of gene therapy for DMD.

The dystrophin complementary DNA expression vector (pMDA) used to generate transgenic mdx mice is diagrammed in Fig. 1a. Tissue-specific expression of this full-length murine dystrophin cDNA3 was controlled by regulatory regions of the mouse muscle creatine kinase (MCK) gene3,5. Immunoblot analysis of transgenic mdx muscle tissue revealed that dystrophin expression from this transgene was ~50 times the level of endogenous dystrophin in control C57BL/10 muscles (Fig. 1b). The exogenous protein in transgenic mdx muscle comigrated with control dystrophin and migrated at a position corresponding to Mr ~400K. Dystrophin transgene expression was specific to skeletal and cardiac muscle tissues and was not detected in uterine smooth muscle or in non-muscle tissues such as liver and brain (Fig. 1b). A smear of dystrophin immunostaining is seen in the undiluted transgenic skeletal and cardiac muscle lanes and is presumably due to detection of excess dystrophin breakdown products. The overexpressed transgenic dystrophin, normally 0.002% of total muscle protein4, was also observed as a prominent 400K band in Coomassie-blue-stained SDS–polyacrylamide gels, but was not visible in control or mdx muscle extracts (Fig. 1b).

A variety of assays demonstrated that the high levels of dystrophin produced in the transgenic mice were sufficient to eliminate dystrophic symptoms from mdx muscles. The localization of dystrophin by immunofluorescence in control, mdx and transgenic mdx muscle is shown in Fig. 2a. Increased dystrophin expression was apparent from the increased immunofluorescence of transgenic mdx muscles compared with control muscles. Dys-

* To whom correspondence should be addressed.
**LETTERS TO NATURE**

**FIG. 1 a.** Full-length dystrophin cDNA expression vector pMDA. The expression of the murine dystrophin cDNA\(^2\) was regulated by 8.5 kb of MCK gene sequences, including \(-3,300\) bp upstream of exon 1, the complete first intron, and a truncated exon 2 deleted just 5' of the MCK initiator methionine\(^6\). A muscle specific enhancer is located 1,200 bp upstream of the transcription start site and a modular control element is located within the first intron\(^5\). The SV40 polyadenylation site was inserted 3' of the dystrophin cDNA. Non-coding regions of dystrophin cDNA are represented by hatched boxes. Coomassie-blue-stained 6% SDS-polyacrylamide gels and immunoblot analysis of dystrophin (DYS) expression in SDS-extracts of 6-month C57BL/10 normal control, mdx and transgenic mdx (Tg-mdx) mouse muscle. Molecular weight standards (\(\times 10^3\)) are indicated on the left. SKM: skeletal muscle.

**METHODS.** Transgenic mice were generated by microinjection of purified pMDA insert into F1 hybrid zygotes from C57BL/6J × SJL/J parents as described\(^{22}\). Positive transgenic mice were identified by the polymerase chain reaction (PCR) using primers specific for MCK intron 1 and the dystrophin cDNA. Two founder F1 females were generated and upon mating with C57BL/10 mdx males, one of the F2 mice transmitted the transgene to F2 offspring. Transgenic mice on the mdx background were produced by breeding transgenic F2 females heterozygous for the mdx mutation with C57BL/10 mdx males. The progeny were screened for the presence of the transgene by PCR and for the presence of the mdx point mutation by an allele-specific oligonucleotide assay using PCR primers flanking murine exon 23 as described\(^{22}\). Dystrophin expression was analysed by loading 100 µg of total protein from each tissue and 1/5, 1/10, 1/20 and 1/50 dilutions of transgenic mdx skeletal muscle extracts (as determined by the Coomassie-plus protein assay (Pierce)) onto identical 6% polyacrylamide gels. One gel was stained with Coomassie brilliant blue and the other transferred to nitrocellulose and stained with Dye-2 monoclonal antibody (Nova Castra), which recognizes the final 17 amino acids of dystrophin. Electrophoretic transfer to a membrane and immunostaining have been described\(^6\).

**FIG. 2 Immunostaining and histological analysis of age-matched (6-month) C57BL/10 control, mdx and transgenic mdx muscle. a, Dystrophin immunostaining is localized to the sarcolemma of control quadriceps, cardiac and diaphragm muscles and is absent from mdx muscle, except for rare revertant dystrophin-positive fibres. (Magnifications:**

726
trophin expressed from the transgene was localized correctly to the sarcolemma, although additional dystrophin staining in the sarcoplasm of transgenic mdx muscles was observed and may have been due to saturation of membrane-binding sites. Histological examination of control, mdx and transgenic mdx limb skeletal muscles and diaphragm muscles revealed a complete correction of dystrophic pathology in the transgenic animals (Fig. 2b). Features of mdx muscle pathology such as necrosis, large variation in fibre size, increased degeneration and regeneration with centrally located nuclei, and progressive degeneration and fibrosis of diaphragm muscle, were all absent from the transgenic mdx mice.

Dystrophin is normally associated with a large oligomeric complex of sarcolemmal glycoproteins, including dystroglycan, which provides a linkage to the extracellular matrix component laminin. In the absence of dystrophin in DMD patients and mdx mice, all of the dystrophin-associated proteins (DAPs) are reduced in the sarcolemma. The specific deficiency of the 50K dystrophin-associated glycoprotein (50DAG) alone causes a muscular dystrophy with a DMD-like phenotype. These findings suggest that all of the DAPs, in addition to dystrophin, must be restored in the sarcolemma for the transfer of the dystrophin gene to be successful in correcting the mdx phenotype. Immunoblot analysis of skeletal muscle extracts demonstrates the restoration of all of the DAPs in transgenic mdx skeletal muscles (Fig. 3c). Immunohistochemical analysis of limb (Fig. 3b) and diaphragm (data not shown) skeletal muscles reveals that both dystrophin and the DAPs co-localize to the sarcolemma in the transgenic mdx mouse. Interestingly, both immunoblot and immunohistochemical analysis (Figs 3a, b) indicate that the ratio of dystrophin to DAP expression is greater in transgenic muscle than in control muscle. Furthermore, immunoblot analysis of skeletal muscle membrane preparations reveals that the amount of dystrophin in the membranes appear to be the same for transgenic mdx and control mice (data not shown). These findings suggested that the sarcolemma of transgenic mdx mice was saturated with the DAPs and that a substantial fraction of the overexpressed dystrophin was not associated with the DAPs. The sarcolemmal expression of the dystrophin-related protein (DRP) has been reported to be upregulated in mdx cardiac muscle. We were unable to detect DRP by immunohistochemical analysis of the sarcolemma of cardiac

![Image of figure 3](image-url)
FIG. 4 Dystrophin expression in skeletal and cardiac muscles of transgenic mdx mice results in control levels of serum creatine kinase (CK). Serum samples were assayed at 3, 4, and 5 weeks of age in F1 normal heterozygous (+/mdx) female, and hemizygous mdx and transgenic mdx (Tg-mdx) male littermates (26 animals in 3 F1 litters). The serum CK levels of heterozygous (+/mdx) mice were not significantly different from wild type, enabling these littermates to be used as normal controls. The number of mice (n) in each group is shown in parentheses. b and c. Specific forces and normalized powers developed by diaphragm muscles from a sample of the same littermates as in a at 3 months of age. d. Comparison of serum CK levels, diaphragm specific force and diaphragm normalized power in phenotypically normal F1 heterozygous (+/mdx) female littermates with (Tg-norm) or without (normal) the dystrophin transgene. Serum CK values were based on an average value of each animal measured at 3, 4, and 5 weeks of age.

METHODS. Serum samples from mice were obtained from the retro-orbital sinus using heparinized capillary tubes. Serum CK measurements were determined using a coupled enzyme spectrophotometric assay (Sigma). Diaphragm strips 1 to 2 mm wide, including an adjacent section of a single rib and part of the central tendon, were cut from the central region of the lateral costal hemidiaphragm and immersed in an oxygenated bath containing mammalian Ringer solution (pH 7.4) at 25°C. Muscles were adjusted to the optimum lengths (L0) for the development of isometric force. Force was determined during maximum isometric tetanic contractions. Power output was determined by isovelocity shortenings from 100% L0 to 90% L0 during maximum muscle activation. Initiation of the isovelocity shortening ramp and stimulation of the muscle occurred simultaneously.

Stimulation was terminated at the end of the shortening ramp. Power output during a single contraction was calculated as the product of average force and velocity of shortening. The velocity of shortening and the frequency of stimulation were adjusted to elicit maximum power output. After measurements of power, the central tendon and rib bone were trimmed and the muscle was blotted and weighed immediately. Specific force (kN m−2) values were normalized to mean cross-sectional area. Power (W) was normalized by muscle mass (kW kg−1).

molecules from transgenic mdx and control mice (Fig. 3c). In contrast, DRP was detected in the sarcoplasm of cardiac muscles from the mdx mouse (Fig. 3c). The suppression of the upregulation of DRP in cardiac muscles from transgenic mdx mice was confirmed by immunoblot analysis (data not shown). The physiological effects of dystrophin expression on muscle pathology were assessed by measuring serum creatine kinase levels at 3, 4, and 5 weeks of age in control, mdx, and transgenic mdx littermates (Fig. 4a). Heterozygous (+/mdx) female mice display an extremely limited pathology17,18 and are thus useful as age-matched phenotypically normal controls. There were no significant differences in the serum creatine kinase levels of control and transgenic mdx littermates (t-test with unequal variances, P > 0.05), but serum creatine kinase was significantly higher in mdx mice than in the transgenic mdx littermates (P < 0.001) at all times tested. Functional correction of the mdx muscle pathology was demonstrated by comparing the contractile performance of in vitro bundles of diaphragm muscle fibres from control, mdx and transgenic mdx littermates at 3 months of age (Figs 4b and c). Compared with the mean values for control mice of 236 kN m−2 for specific force and 79.9 W kg−1 for normalized power, the values for muscles from transgenic mdx mice were not significantly different, whereas those for muscles from mdx mice were significantly lower at 63 and 66% respectively (P < 0.001). In addition, there were no differences in serum creatine kinase, and normalized force and power of the diaphragm muscle between normal (+/mdx) female littermates with and without the dystrophin transgene (P > 0.05; Fig. 4d), and so no toxic side effects resulted from overexpression of dystrophin.

We have demonstrated the functional correction of muscular dystrophy by tissue-specific expression of a full-length dystrophin cDNA clone in transgenic mdx mice. Although the high levels of dystrophin obtained may partially reflect the site of transgene integration, the MCK promoter is known to be highly active in skeletal and cardiac muscle19. Furthermore, MCK-regulated transgenes expressing other dystrophin isoforms have also given high levels of muscle expression (J.S. et al., unpublished). These studies indicate that MCK regulatory regions may be useful in the design of vectors for muscle gene therapy. A transgenic mdx mouse expressing extremely low amounts of a mutant dystrophin from a murine retroviral promoter has been reported19. These mice had their symptoms partially corrected, but it was unclear if the absence of a complete correction was due to low protein expression or to functional deficiencies of the truncated dystrophin clone. The correct size of the transgenic protein on immunoblots and detection with antibodies against the N terminus and C terminus (Figs 1b and 3b), indicate that our transgene encodes the entire dystrophin protein. Furthermore, overexpression of the full-length dystrophin transgene does not
result in an adverse phenotype (Figs 2b and 4d). This lack of toxicity suggests that tight control over dystrophin expression levels may not be necessary for correction of muscular dystrophy. Delivery of exogenous dystrophin by viral vectors with sub-optimal infection frequencies might benefit from overexpression of dystrophin in portions of a multinucleated myofibre without concern for cytotoxicity. Although progressive skeletal muscle weakness and wheelchair confinement are the most obvious symptoms of DMD, most patient deaths result from respiratory failure. The restoration of normal values for force and power development in transgenic mdx diaphragm muscles provides functional evidence supporting the feasibility of gene therapy for DMD.

Received 23 April; accepted 18 June 1993.


ACKNOWLEDGEMENTS. We thank K. Corrado, J. Rafael and J. Buskin for advice and discussions, and P. Milos, J. Wang, R. Brubec, A. Christensen and the Transgenic Animal Model Core, University of Michigan, for technical assistance. This work was supported by grants from the Muscular Dystrophy Association (U.S.A.) to J.S.C. and S.D.H.; by grants from The National Institute of Arthritis and Musculoskeletal Diseases (to J.S.C. and S.D.H.); The National Institute of Diabetes, Digestive and Kidney Diseases (to J.S.C.); The National Heart, Lung and Blood Institute (to S.D.H.); and The National Institute on Aging (to J.A.F.). NIH, G.A.C. is supported by an NSF graduate fellowship. K.M. is supported by a post doctoral fellowship from the Muscular Dystrophy Association (U.S.A.). J.S.C. is a recipient of a Basil O'Connor Starter Scholar Research Award from The March of Dimes Birth Defects Foundation; K.P.C. is an Investigator of The Howard Hughes Medical Institute.