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

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

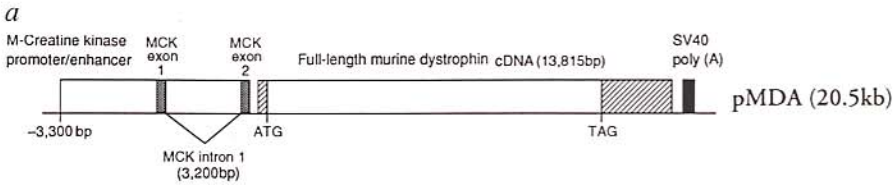
* Department of Human Genetics, † Institute of Gerontology, ‖ Human Genome Center, University of Michigan Medical School, Ann Arbor, Michigan 48109–0618, USA
‡ Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA
§ 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 dystrophin[1,2]. The *mdx* mouse, an animal model for DMD, has a mutation that eliminates expression of the 427K muscle and brain isoforms of dystrophin[1,3,4]. 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 disease[5,6]. 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. 1*a*. Tissue-specific expression of this full-length murine dystrophin cDNA[7] was controlled by regulatory regions of the mouse muscle creatine kinase (MCK) gene[8,9]. 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. 1*b*). The exogenous protein in transgenic *mdx* muscle comigrated with control dystrophin and migrated at a position corresponding to M_r ~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. 1*b*). 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 protein[1], 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. 1*b*).

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. 2*a*. Increased dystrophin expression was apparent from the increased immunofluorescence of transgenic *mdx* muscles compared with control muscles. Dys-

¶ To whom correspondence should be addressed.



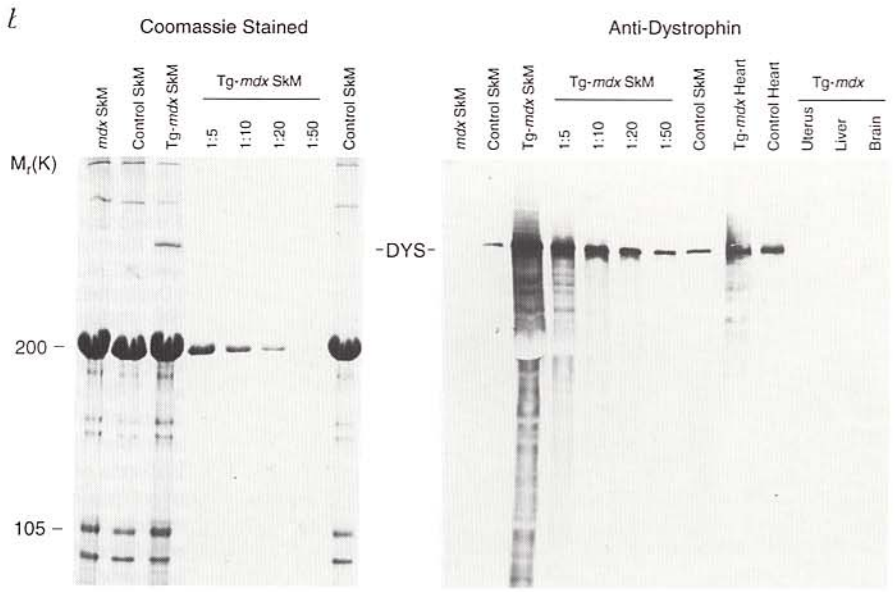
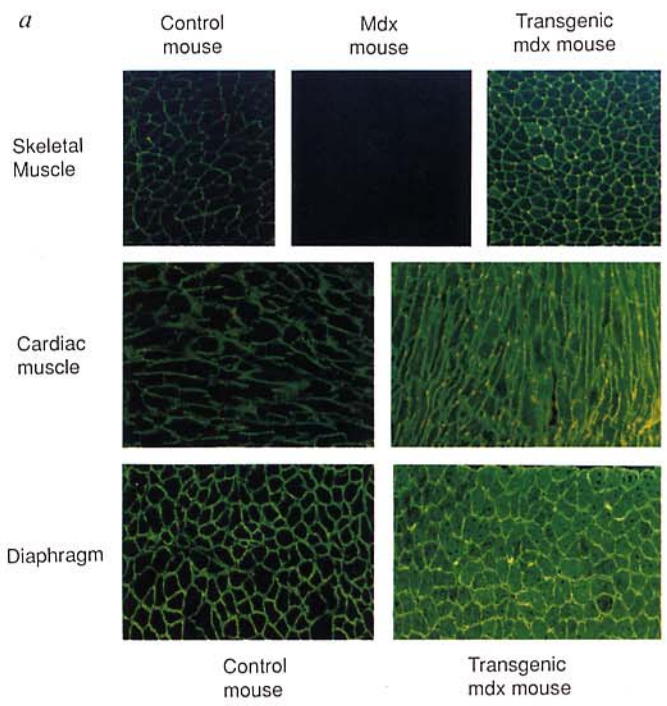


FIG. 1 *a*, Full-length dystrophin cDNA expression vector pMDA. The expression of the murine dystrophin cDNA[7] was regulated by 6.5 kb of MCK gene sequences, including −3,300 bp upstream of exon 1, the complete first intron, and a truncated exon 2 deleted just 5′ of the MCK initiator methionine[8]. A muscle specific enhancer is located 1,200 bp upstream of the transcription start site and a modulatory control element is located within the first intron[9]. The SV40 polyadenylation site was inserted 3′ of the dystrophin cDNA. Non-coding regions of dys-trophin cDNA are represented by hatched boxes. *b*, Coomassie-blue-stained 6% SDS–polyacrylamide gel and immunoblot analysis of dys-trophin (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 F_2 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 F_0 females were generated and upon mating with C57BL/10 *mdx* males, one of the F_0 mice transmitted the transgene to F_1 offspring. Transgenic mice on the *mdx* background were produced by breeding transgenic F_1 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[23]. 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 Dys-2 monoclonal antibody (Nova Castra), which recognizes the final 17 amino acids of dystrophin. Electrophoretic transfer to a membrane and immunostaining have been described[4].



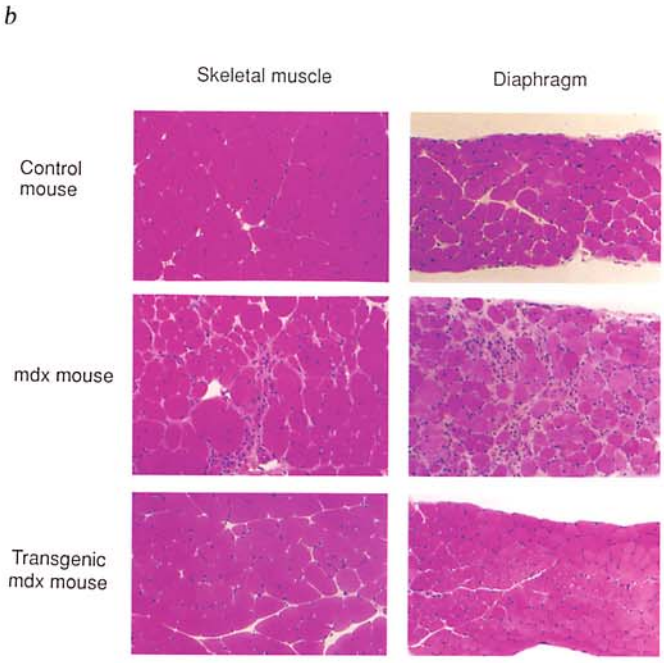


FIG. 2 Immunostaining and histological analysis of age-matched (6-month) C57BL/10 control, *mdx* and transgenic *mdx* muscle. *a*, Dystrophin immunostaining is localized to the sarcolemma of control quadriceps, cardiac and diaphragm muscles and is absent from *mdx* muscle, except for rare revertant dystrophin-positive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

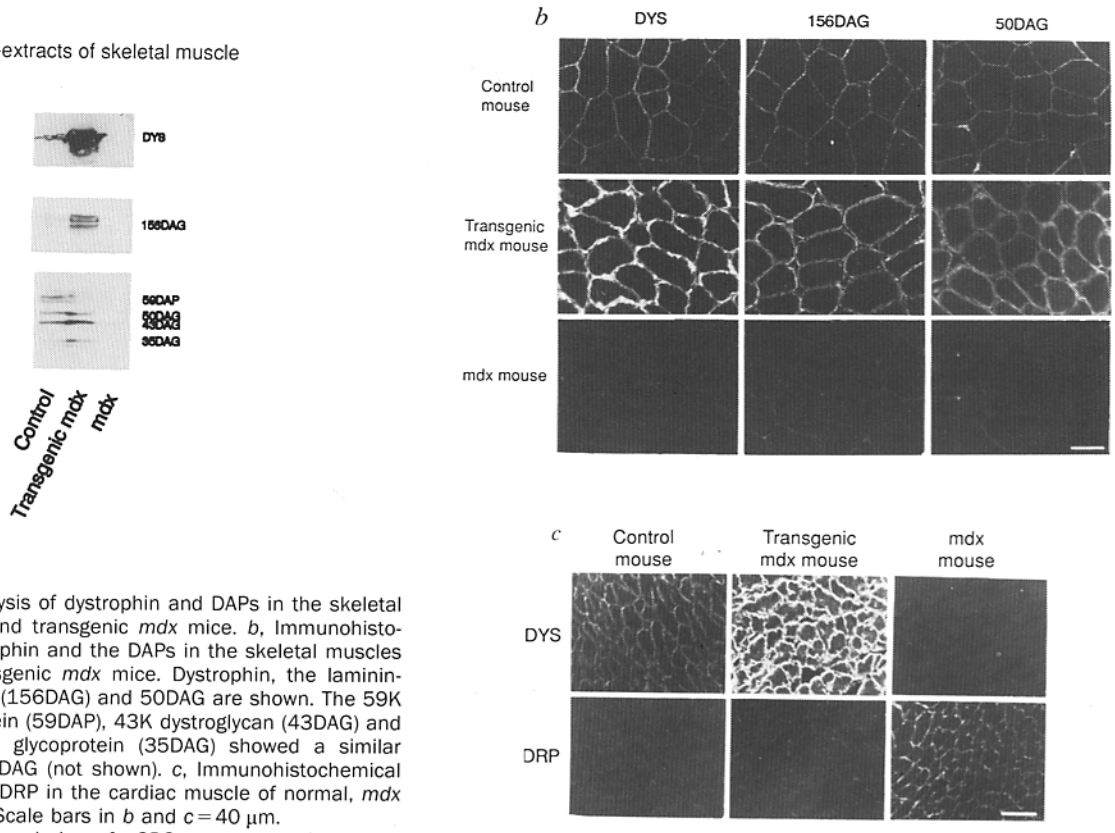


FIG. 3 *a*, Immunoblot analysis of dystrophin and DAPs in the skeletal muscles of control, *mdx* and transgenic *mdx* mice. *b*, Immunohistochemical analysis of dystrophin and the DAPs in the skeletal muscles of normal, *mdx*, and transgenic *mdx* mice. Dystrophin, the laminin-binding 156K dystroglycan (156DAG) and 50DAG are shown. The 59K dystrophin-associated protein (59DAP), 43K dystroglycan (43DAG) and 35K dystrophin-associated glycoprotein (35DAG) showed a similar staining pattern as the 50DAG (not shown). *c*, Immunohistochemical analysis of dystrophin and DRP in the cardiac muscle of normal, *mdx* and transgenic *mdx* mice. Scale bars in *b* and *c* = 40 μm.
METHODS. Immunoblot analysis of SDS extracts of muscle specimens[15,16] and immunohistochemistry of muscle specimens[13–16] have been described. Affinity-purified rabbit antibody against the last 10 amino acids of the C terminus of dystrophin (*a*), affinity-purified rabbit antibody against the first 15 amino acids of the N terminus of dystrophin (*b* and *c*), affinity-purified rabbit antibody against the last 12 amino acids of the C terminus of DRP (*c*), monoclonal antibody against 156DAG (*a*) and affinity-purified sheep antibodies against 156DAG, 59DAP, 50DAG, 43DAG and 35DAG (*a*, *b*) were used[13–16,24,25].

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. 2*b*). 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.

quadriceps, 11.6×; cardiac and diaphragm, 23×). *b*, Haematoxylin- and eosin-stained sections of transgenic *mdx* quadriceps and diaphragm muscles show corrected dystrophic *mdx* histopathology. Note the absence of fibrosis and centrally located nuclei in the control and transgenic *mdx* muscles (magnifications, 23×). The apparent difference in fibre size between transgenic and control muscle was not reproduced in further sections and is not a general feature of transgenic *mdx* muscle.
METHODS. Immunostaining of unfixed 7 μm muscle cryosections was done with a 1/1,000 dilution of a rabbit polyclonal antibody against the first 410 amino acids of dystrophin using conditions described previously[4]. Histological 4 μm sections were prepared from muscle tissues fixed in 4% paraformaldehyde and 1% gluteraldehyde, embedded in glycol methacrylate and stained with haematoxylin and eosin. Samples were photographed with brightfield illumination on a Nikon Optiphot-2 microscope equipped with a UFX-DX photomicrographic system.

Dystrophin is normally associated with a large oligomeric complex of sarcolemmal glycoproteins, including dystroglycan, which provides a linkage to the extracellular matrix component laminin[10,12]. In the absence of dystrophin in DMD patients and *mdx* mice, all of the dystrophin-associated proteins (DAPs) are reduced in the sarcolemma[13,14]. The specific deficiency of the 50K dystrophin-associated glycoprotein (50DAG) alone causes a muscular dystrophy with a DMD-like phenotype[15]. 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. 3*a*). Immunohistochemical analysis of limb (Fig. 3*b*) 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 3*a*, *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[16]. We were unable to detect DRP by immunohistochemical analysis of the sarcolemma of cardiac

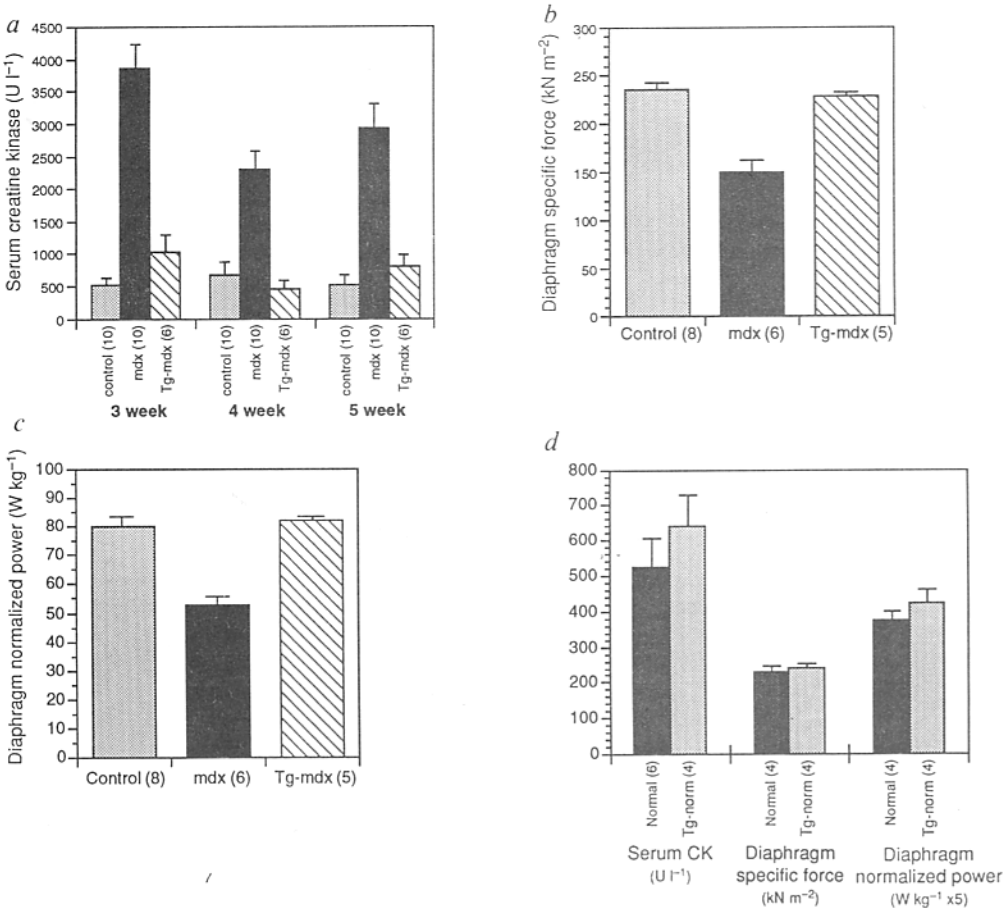


FIG. 4 Dystrophin expression in skeletal and cardiac muscles of transgenic *mdx* mice results in control levels of serum creatine kinase (CK). Serum samples were assayed at 3, 4 and 5 weeks of age in F_3 normal heterozygous (+/*mdx*) female, and hemizygous *mdx* and transgenic *mdx* (Tg-*mdx*) male littermates (26 animals in 3 F_3 litters). The serum CK levels of heterozygous (+/*mdx*) mice are not significantly different from wild type, enabling these littermates to be used as normal controls[17]. 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 F_3 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 (L_o) for the development of isometric force. Force was determined during maximum isometric tetanic contractions. Power output was determined by isovelocity shortenings from 100% L_o to 90% L_o 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[26,27]. 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 (W kg^{-1}).

muscles from transgenic *mdx* and control mice (Fig. 3*c*). In contrast, DRP was detected in the sarcolemma of cardiac muscles from the *mdx* mouse (Fig. 3*c*). 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. 4*a*). Heterozygous (+/*mdx*) female mice display an extremely limited pathology[17,18] 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 4*b* 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. 4*d*), 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 muscle[9]. Furthermore, MCK-regulated transgenes expressing other dystrophin isoforms have also given high levels of muscle expression (J.S.C. *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 reported[19]. 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 1*b* and 3*b*), 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 2*b* and 4*d*). 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 suboptimal infection frequencies might benefit from overexpression of dystrophin in portions of a multinucleated myofibre without concern for cytotoxicity[20]. Although progressive skeletal muscle weakness and wheelchair confinement are the most obvious symptoms of DMD, most patient deaths result from respiratory failure[21]. 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.

1. Hoffman, E. P., Brown, R. H. Jr & Kunkel, L. M. *Cell* **51,** 919–928 (1987).
2. Ahn, A. H. & Kunkel, L. M. *Nature Genet.* **3,** 283–291 (1993).
3. Sicinski, P. *et al. Science* **244,** 1578–1580 (1989).
4. Cox, G. A., Phelps, S. F., Chapman, V. M. & Chamberlain, J. S. *Nature Genet.* **4,** 87–93 (1993).
5. Partridge, T. *Neuropathol. appl. Neurobiol.* **17,** 353–363 (1991).
6. Stedman, H. H. *et al. Nature* **352,** 536–539 (1991).
7. Lee, C. C., Pearlman, J. A., Chamberlain, J. S. & Caskey, C. T. *Nature* **349,** 334–336 (1991).
8. Jaynes, J. B., Chamberlain, J. S., Buskin, J. N., Johnson, J. E. & Hauschka, S. D. *Molec. cell Biol.* **6,** 2855–2864 (1986).
9. Johnson, J. E., Wold, B. J. & Hauschka, S. D. *Molec. cell. Biol.* **9,** 3393–3399 (1989).
10. Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G. & Campbell, K. P. *Nature* **345,** 315–319 (1990).
11. Ervasti, J. M. & Campbell, K. P. *Cell* **66,** 1121–1131 (1991).
12. Ibraghimov-Beskrovnaya, O. *et al. Nature* **355,** 696–702 (1992).
13. Ohlendieck, K. & Campbell, K. P. *J. Cell Biol.* **115,** 1685–1694 (1991).
14. Ohlendieck, K. *et al. Neurology* **43,** 795–800 (1993).
15. Matsumura, K. *et al. Nature* **359,** 320–322 (1992).
16. Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. & Campbell, K. P. *Nature* **360,** 588-591 (1992).
17. Chapman, V. M., Miller, D. M., Armstrong, D. & Caskey, C. T. *Proc. natn. Acad. Sci. U.S.A.* **86,** 1292–1296 (1989).
18. Watkins, S. C., Hoffman, E. P., Slayter, H. S. & Kunkel, L. M. *Muscle Nerve* **12,** 861–868 (1989).
19. Wells, D. J. *et al. Hum. molec. Genet.* **1,** 35–40 (1992).
20. Ragot, T. *et al. Nature* **361,** 647–650 (1993).
21. Emery, A. E. H. *Duchenne Muscular Dystrophy* (Oxford Monog. Med. Genet. No. 15, Oxford Medical, 1987).
22. Hogan, B., Constantini, F. & Lacey, E. *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1986).
23. Chamberlain, J. S., Phelps, S. F., Cox, G. A., Maichele, A. J. & Greenwood, A. D. in *Molecular and Cell Biology of Muscular Dystrophy* (ed. Partridge, T.) 167–189 (Chapman & Hall, London, 1993).
24. Ohlendieck, K. *et al. Neuron* **7,** 499–508 (1991).
25. Ervasti, J. M., Kahl, S. D. & Campbell, K. P. *J. biol. Chem.* **266,** 9161–9165 (1991).
26. Faulkner, J. A., Zerba, E. & Brooks, S. V. *Am. J. Physiol.* **259,** 259–265 (1990).
27. Brooks, S. V. & Faulkner, J. A. *J. Physiol.* **436,** 701–710 (1991).

ACKNOWLEDGEMENTS. We thank K. Corrado, J. Rafael and J. Buskin for advice and discussions, and P. Mills, J. Wang, R. Brabec, 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.