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Expression of human full-length and minidystrophin in transgenic *mdx* mice: implications for gene therapy of Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive disorder with a high spontaneous mutation rate and no effective treatment, hence development of genetic based therapies is an important goal. We report that expression of a recombinant human minidystrophin cDNA, compatible with current viral vectors, can significantly reduce the myopathic phenotype in transgenic *mdx* mice, even when expressed at only 20–30% of endogenous dystrophin levels at the sarcolemma. To the extent that data obtained in mouse studies are applicable to DMD, the virtual elimination of morphological and biochemical abnormalities in the *mdx* mouse supports the use of this cDNA in somatic gene therapy protocols for DMD.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder with an incidence worldwide of 1 in 3,500 male births. Of these approximately one-third are due to *de novo* mutations in the 2.3 Mb dystrophin gene (1). No effective treatment exists for this lethal disease, nor can it be entirely eliminated by genetic counselling; hence development of genetic based therapies is an important goal. Viral systems offer the highest efficiency gene transfer into somatic tissues but cannot accommodate the complete dystrophin cDNA (2,3). Some patients with very mild forms of Becker muscular dystrophy (BMD) express dystrophin mRNAs and proteins of substantially reduced size due to large in frame deletions within the DMD gene (4). The mRNA corresponding to one such deletion of exons 17–48 has been cloned as a 6.3 kb cDNA that produces a 229 kDa protein localising to the muscle sarcolemma (5,6). This BMD-derived minidystrophin cDNA is sufficiently small to be accommodated by current adenoviral (2) and retroviral vectors (3) and has been successfully expressed with both systems in dystrophic *mdx* mouse (7) muscle *in vivo*. To assess the function of this recombinant protein and the quantity required for restoration of the normal muscle phenotype we have produced and examined *mdx* mice transgenic for the minidystrophin in comparison with transgenic mice carrying the full-length human dystrophin (8).

RESULTS

Generation of transgenic *mdx* mice expressing human dystrophin

Multiple transgenic lines were produced by microinjection of dystrophin expression constructs (Fig. 1a), utilising the human α-skeletal actin (HSA) (9) or the mouse M-creatine kinase (MCK) promoter (10), into the pronuclei of single celled fertilised ova of C57Bl/10×CBA/J mice (11). Transgenic lines were bred onto the *mdx* background by mating transgenic males with *mdx* females, and resultant male offspring were analyzed at 6 weeks, 3 months and 6 months for transgene expression and muscle pathology. Transgenic animals were always compared to non-transgenic littermates to eliminate any effects that might be due to minor differences in the genetic background. Southern blot analysis of F1 mice revealed copy numbers ranging from single copy to approximately 50 copies, with most lines being in the region of 2–5 copies. No

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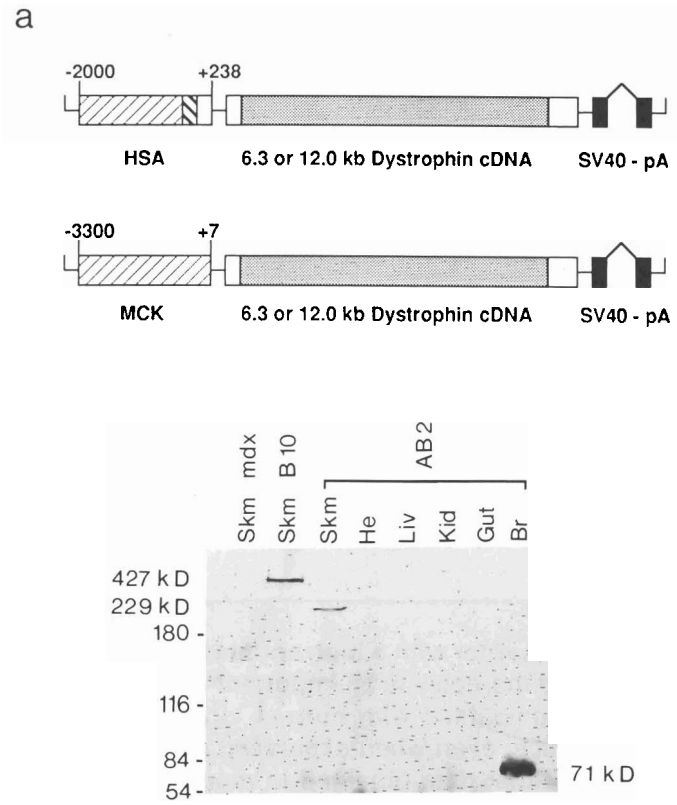

Figure 1. Production of human dystrophin transgenic mice. (**a**) Linear representation of the transgenic injection fragments, (i) using the human α-skeletal actin (HSA) promoter and (ii) using the mouse M-creatine kinase (MCK) promoter. Open boxes indicate untranslated regions, the hatched box indicates the first exon of α-skeletal actin and shaded boxes indicate the coding sequence of either the 6.3 kb minidystrophin or the 12 kb full-length dystrophin cDNA. The SV40 polyadenylation and splice site (black box) was inserted 3′ of the dystrophin cDNA. Nucleotide numbering of the promoter indicates position relative to the transcriptional start site of the gene of origin. (**b**) Immunoblot analysis of dystrophin expression in various tissues from control *mdx* and normal (B10) mice and a minidystrophin transgenic *mdx* mouse (AB2). Skeletal muscle and heart preparations were subjected to a low speed spin to remove myofibrils as myosin interferes with detection of the minidystrophin. All other lanes were run with whole tissue homogenates. Note the presence of the Dp71 protein in the brain that is produced from a promoter downstream of the stop codon in the *mdx* mutation and so detected with the C-terminal antibody used on this blot (Dy8/6C5).

correlation between expression level and copy number was apparent and expression of transgenes from the HSA promoter was confined predominantly to skeletal muscles with additional very low expression only in the heart (Fig. 1b). Similar results were obtained with the MCK promoter.

Analysis of transgenic *mdx* mice

Morphological changes in the dystrophic phenotype were initially assessed by examining haematoxylin- and eosin-stained sections of quadriceps from 6 week old transgenic *mdx* mice in comparison to their non-transgenic *mdx* littermates (Fig. 2a). Expression of either the human full-length or minidystrophin cDNA was able to effectively reduce or eliminate the dystrophic pathology, as judged by differences in the central nucleation of the muscle fibres, a marker of regeneration in rodents (12). Western blot analysis of microsomal fractions from skeletal muscle tissues demonstrated a range of transgene expression above and below the level of endogenous dystrophin in C57Bl/10 wild type mice. Even the expression of very small quantities of dystrophin produced a reduction in muscle dystrophic pathology. In lines which demonstrated normal or near-normal muscle morphology there was a corresponding decrease in serum creatine kinase to normal levels (Fig. 2b). Minor mismatches between the reduction in central nucleation and the serum creatine kinase levels likely reflect differences in the time of onset of any muscle necrosis relative to collection of blood samples. The degree of muscle pathology at 6 weeks of age was inversely proportional to the amount of recombinant dystrophin expressed at the muscle membrane, the latter being assessed by multiple Western blots and immunohistochemistry (Fig. 2c). Microsomal levels of 20–30% of endogenous were sufficient to correct the primary myopathic features of *mdx* skeletal muscle.

The *mdx* mouse has been criticised as a model of DMD since in most skeletal muscles, after an initial period of intense myopathy at 2–6 weeks, there is full regeneration and a lack of the progressive fibrosis seen in DMD patients (13). However, the diaphragm muscle does show the progressive damage and fibrosis typical of DMD (Fig. 3a), possibly as a consequence of its high work load in a rapidly breathing small mammal (14). Examination of the histopathology of the diaphragm at 3 and 6 months revealed a continued protection against the dystrophic phenotype in those lines that exhibited very little or no pathology at 6 weeks (Fig. 3b,c). In the case of full-length human dystrophin complete restoration of normal diaphragm histology has been obtained. However, with the minidystrophin transgenics occasional groups of centrally nucleated fibres have been observed at 6 months.

As previously described (6) both the human full-length and minidystrophin were localized to the sarcolemma in *mdx* muscle (Fig. 4). Utrophin, the autosomal homologue of dystrophin, which is expressed in adult *mdx* muscle (15) was decreased in the transgenics to normal control levels with the expected predominant accumulation at neuromuscular junctions. Both full-length and minidystrophin transgenics also demonstrated appropriate reactivated sarcolemmal expression of the 43 and 50 kDa dystrophin associated glycoproteins (DAG) and the 59 kDa dystrophin associated protein (DAP), which indicates stabilisation of the DAG complex (16) linking sub-sarcolemmal dystrophin to laminin and the extracellular matrix (17,18).

While both the full-length and minidystrophin proteins are clearly associated with the sarcolemma based on immunostaining, Western blot analysis using whole muscle and microsomal extracts revealed a marked difference between the full-length and minidystrophin proteins in the efficiency of association with the muscle membrane. When minidystrophin expression was examined on the wild-type background high levels, equivalent to or greater than endogenous mouse dystrophin, were observed in the whole muscle extracts (Fig. 5a). However, a much lower proportion of the recombinant minidystrophin purified with the microsomal fraction. This reduced association with the muscle sarcolemma was not due to species differences or competition with endogenous dystrophin as a similar relative pattern was noted when minidystrophin and

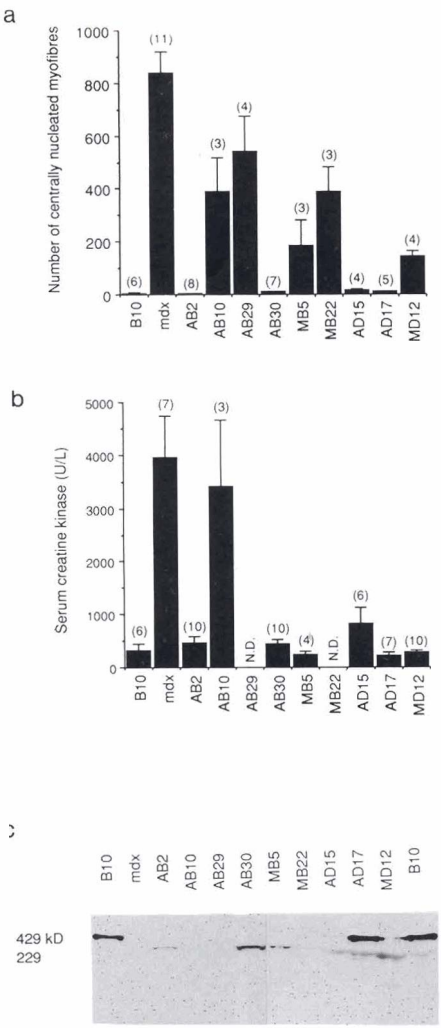

Figure 2. Correction of the myopathic phenotype in transgenic *mdx* mice. (**a**) Histogram showing the number of centrally nucleated fibres observed in the rectus femoris muscle of haematoxylin and eosin stained quadriceps sections for a range of independent minidystrophin (AB,MB) and full-length dystrophin (AD,MD) transgenic lines and C57Bl/10 and *mdx* control animals at 6 weeks of age. The number of centrally nucleated fibres across intact sections of rectus femoris muscles (approximately 1500 fibres) for a number of individual mice (number given in parentheses) were counted blind by two independent observers. (**b**) Histogram showing the level of creatine kinase (CK) present in the serum for 6 week old animals of the same transgenic and control lines shown in (a) demonstrating reduction of CK levels, in many cases to the normal range, in transgenic *mdx* mice. Similar results were obtained for samples of 4 and 5 week old animals. In both (a) and (b) the number of individuals examined is given in parentheses, the T-bars illustrates the S.E.M. and N.D. indicates **not determined.** (c) **Representative Western blot of** microsomal pellet preparations from the various transgenic and control lines examined in (a) and (b). The protein product of the full-length cDNA resolves at 429 kDa and that of the minidystrophin cDNA at 229 kDa. Dystrophin was detected with the C-terminal antibody (Dy8/6C5). Overexposure of this and other blots revealed dystrophin expression in all transgenic lines. Some cross-reactivity with contaminating myosin can be seen in the samples from full length transgenic mice.

human full-length expression were compared on the *mdx* background. Again the full-length protein was more highly concentrated in the microsomal fraction (Fig. 5b).

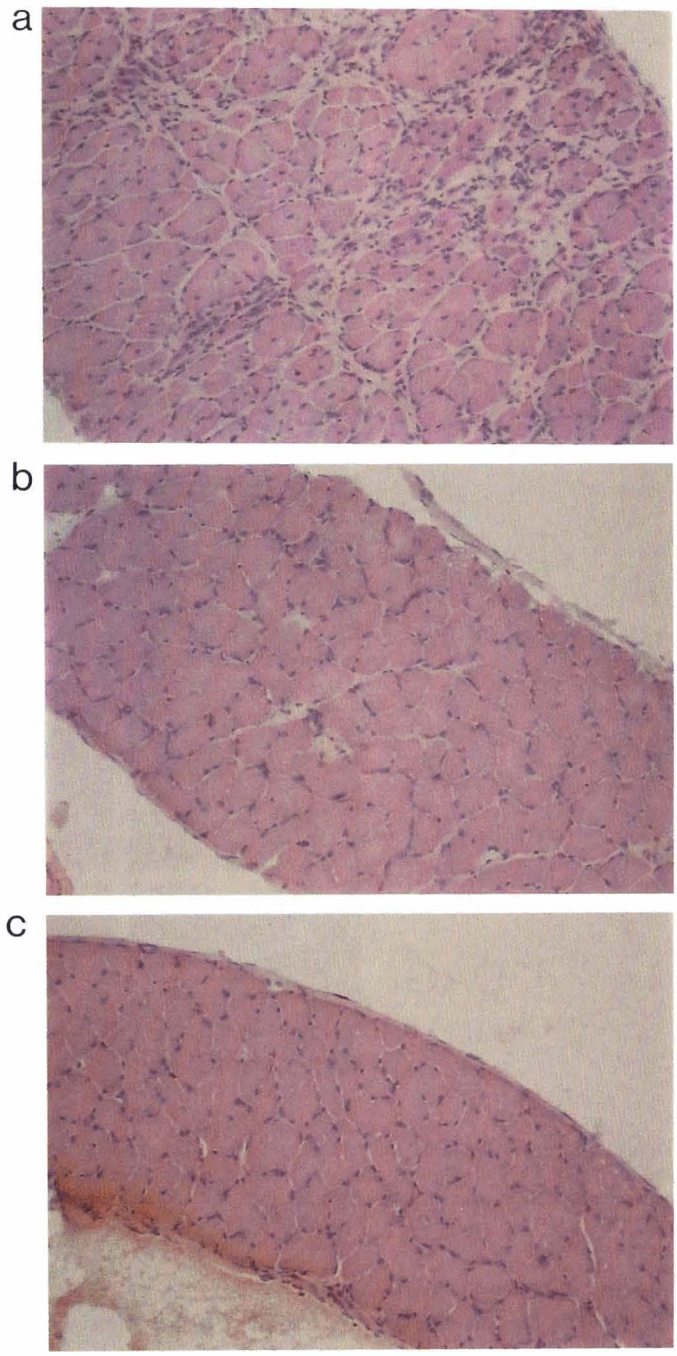

Figure 3. Diaphragm histomorphology at 6 months of age. Photomicrographs of representative haematoxylin and eosin sections of diaphragms from (**a**) 6 month old *mdx*, (**b**) AD17 full-length and (**c**) AB2 minidystrophin transgenic *mdx* mice. Diaphragm morphology is normal in both transgenic lines and does not show the severe degenerative changes typical of the *mdx* mouse.

DISCUSSION

Our data show that the 427 kDa protein product of the human full-length dystrophin cDNA can provide restoration of the normal muscle phenotype in the transgenic *mdx* mouse. More

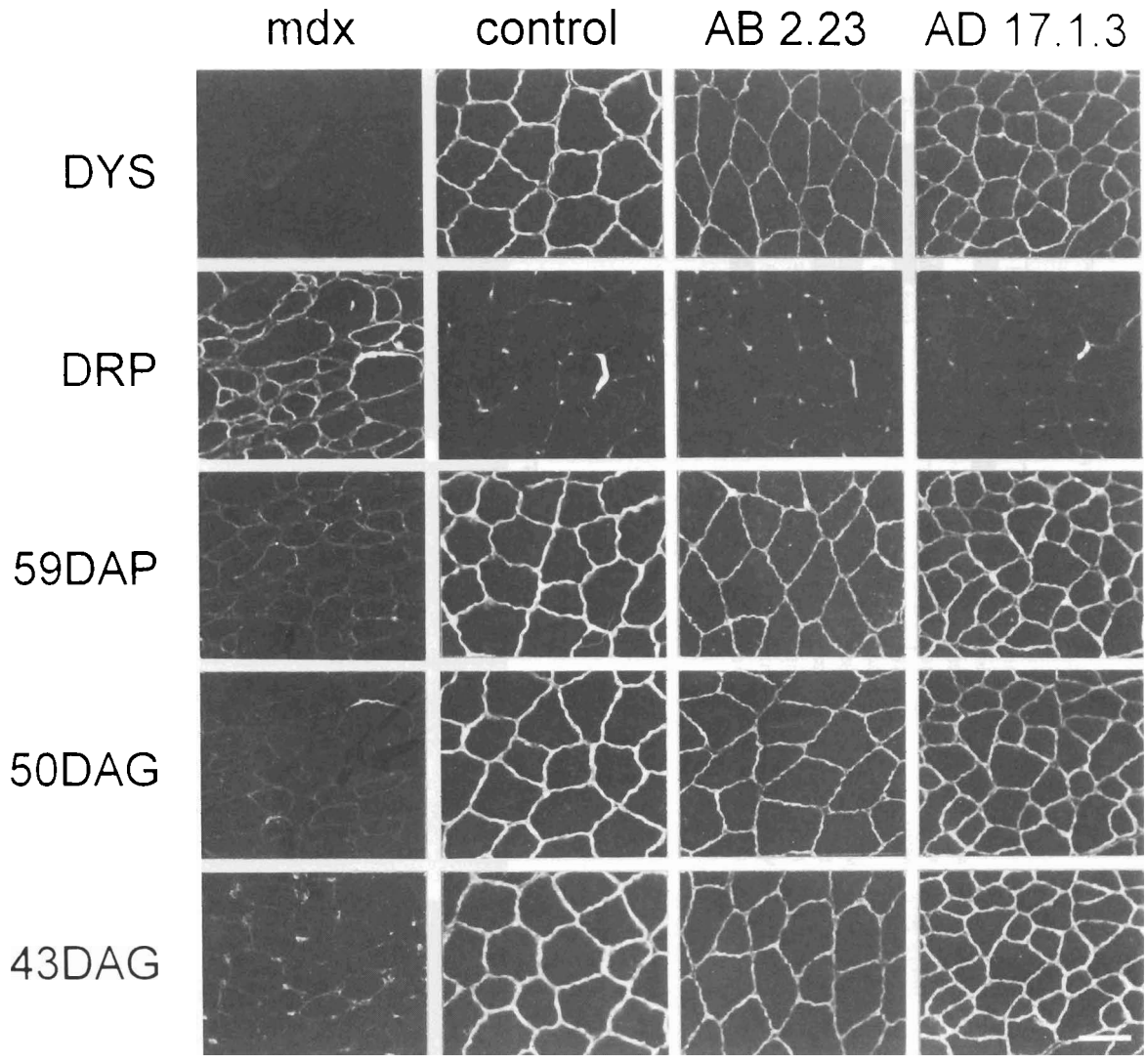

Figure 4. Immunostaining for dystrophin, utrophin and DAPs in skeletal muscle of age matched 6 week old C57Bl/10 control, *mdx*, minidystrophin (AB 2) and full-length (AD 17) transgenic *mdx* mice. Dystrophin immunostains localize to the sarcolemma in the control and both transgenic mice but is absent in the *mdx* mouse. Similar staining patterns are obtained with antibodies to the 59 DAP, 50 and 43 DAGs. In contrast, the dystrophin related protein, utrophin, which is strongly expressed in the *mdx* mouse is only expressed at the neuromuscular junctions in the control and transgenic mice. Scale bar represents 50 μm.

importantly, we have also demonstrated that the 229 kDa minidystrophin product of the human BMD-derived cDNA can provide substantial restoration of the normal muscle phenotype, including prevention of the severe fibrotic changes in the *mdx* diaphragm. Interestingly, expression of either protein product in the wild type background did not produce any pathology as a result of competition between transgene and endogenous dystrophin, suggesting the possibility that gene therapy using such constructs might be applicable not only to boys with DMD but also to manifesting carriers of DMD and boys with Becker muscular dystrophy. It seems likely that the minidystrophin product is less functionally effective at complementing dystrophin deficiency than that from the full-length cDNA and we have noted a relative instability in membrane association of the 229 kDa protein compared to the human 427 kDa protein. This implies it will be necessary to express higher levels of the minidystrophin relative to the full length dystrophin to achieve the same therapeutic effect. Nonetheless, as substantial protection is afforded even at low levels of minidystrophin expression at the muscle membrane, 20–30% of endogenous dystrophin, this result justifies the continued development of gene therapy protocols using currently available viral vectors that can accommodate this minidystrophin.

MATERIALS AND METHODS

Production of transgenic mice

The construct used for production of transgenic mice was assembled in pBS II (SK+)(Stratagene) by the following consecutive steps. A *Bam*H1 (Klenow repaired)/*Xho*1 fragment containing the SV40 early splice region and polyadenylation signals from pMSG (Pharmacia) was cloned into the *Apa*1 (T4 polymerase blunt ended)/ *Xho*1 cut pBS. A *Not*1 linker was inserted into the pBS polylinker *Kpn*1 site to provide a unique site at each end of the construct for final linear transgenic injection fragment preparation. The dystrophin

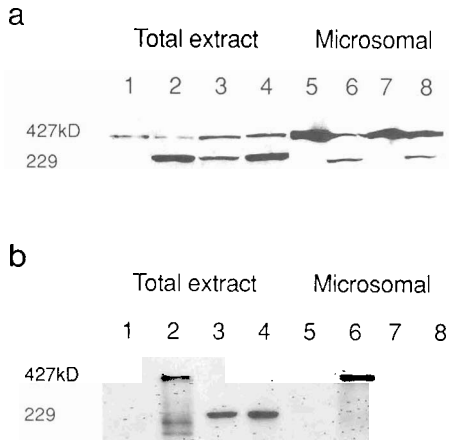

Figure 5. Membrane association of human dystrophins in transgenic *mdx* mice. (**a**) Immunoblot analysis of dystrophin expression in muscle extracts and microsomal pellet preparations of (1,5) C57Bl/10 control and (2,6) AB2, (3,7) AB29, (4,8) AB30 minidystrophin lines on the wild type C57Bl/10 background. (**b**) Similar preparations of (1,5) *mdx*, (2,6) AD17 full-length human dystrophin, (3,7) AB2 and (4,8) AB30 minidystrophin transgenic *mdx* mice. The human (AD17) dystrophin (427 kDa) fractionates predominantly with the muscle membrane but proportionally less of the 229 kDa minidystrophin protein is associated with the microsomal pellet. This differential effect was observed both in the presence and absence of endogenous full-length dystrophin suggesting that it is not due to saturation of available sites on the muscle membrane.

cDNAs (6,8) were inserted by directional cloning between the *Sma*I site and *Sal*I site as a blunt ended/*Sal*I fragment. Finally the *Hin*dIII promoter fragment was inserted into the *Spe*I site after two base fill in of insert and vector sites. Two promoters were used in this study to give a range of expression levels. The human skeletal actin promoter consists of 2 kb of 5′ flank (base −2000, *Hin*dIII), 90 bp of UTR, and 149 bp of IVS 1 to the *Dra*I site (*Hin*dIII linkered) at + 239 (9). The mouse muscle creatine kinase promoter consists of 3.3 kb of 5′ flanking sequence from −3300 to +7 *Bst*EII site (purified from pETCAT as a *Hin*dIII linkered fragment) (10). Lines produced with the HSA constructs are indicated by AB for the minidystrophin and AD for the full-length. With the MCK constructs lines are likewise denoted MB or MD.

Transgenic mice were produced by standard pronuclear injection of fertilised C57Bl/10×CBA/J F1 eggs using a 1–2 ng/μl solution of *Not*I linearised DNA which had been gel purified by electroelution, dialysed against 10mM Tris, 0.1 mM EDTA pH7.4 and filtered through 0.2 μm sterile filter. Two cell embryos were transferred into pseudopregnant females of the same background. Initial screening of offspring was carried out by PCR analysis using primer sets, forward 5′-CCTCTGACCCTACACGGAGC-3′, reverse 5′-CGGTAA-GTTCTGTCCAAGCCCG-3′ for the minigene lines and forward 5′-CCTCTG-ACCCTACACGGAGC-3′, reverse 5′-CAGTTATATCAACATCCAACC-3′ for full length lines. Founder animals were then bred onto the *mdx* background.

Serum creatine kinase analysis

Serum creatine kinase levels were measured using the Boehringer NAC-CK kit and controlled for run to run variation against Precinorm-U standard (Boehringer). The rate per minute was averaged over 10 min and calculated as U/l.

Western blot analysis

For Western blot analysis 0.4 g fresh skeletal muscle from the hindlimb was prepared as previously described (12). A low speed centrifugation was used to remove myofibrils as the position of myosin at 180 kDa may interfere with the detection of the minigene product at 229 kDa. High speed centrifugation was then used to pellet the microsomal fraction. Blots were stained with amido black to confirm equality of protein load and transfer. Blots were probed with the monoclonal antibody Dy8/6C5 which recognises an epitope at the C-terminus of dystrophin (19).

Immunohistochemistry and histology

The quadriceps of 6 week old transgenic and control mice were excised postmortem and rapidly frozen in liquid nitrogen cooled isopentane. Cryosections 7 μm thick were prepared and immunostained with affinity purified sheep polyclonal antibodies specific for dystrophin, 59 DAP, or β-dystroglycan (43 DAG) (20), or rabbit polyclonal antibodies against DRP (21) or adhalin (50 DAG) (22). For histological examination of muscle morphology frozen sections were cut at 7 μm and stained with haematoxylin and eosin in standard fashion. The number of centrally nucleated fibres across intact rectus femoris muscles (approximately 1500 fibres) were counted blind by two independent observers.

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