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Two lines of transgenic *mdx* mice have been generated that express a 71 kD non-muscle isoform of dystrophin (Dp71) in skeletal muscle. This isoform contains the cysteine-rich and C-terminal domains of dystrophin, but lacks the N-terminal actin-binding and central spectrin-like repeat domains. Dp71 was associated with the sarcolemma membrane, where it restored normal expression and localization of all members of the dystrophin-associated glycoprotein complex. However, the skeletal muscle pathology of the transgenic *mdx* mice remained severe. These results indicate that the dystrophin C terminus cannot function independently to prevent dystrophic symptoms and confirms predictions based on patient data that both the N and C-terminal domains are required for normal dystrophin function.

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Duchenne and Becker muscular dystrophy (DMD and BMD) are severe X-linked degenerative diseases caused by mutations in the dystrophin gene[1]. Although the gene was cloned in 1986 (ref. 2), the precise function of the dystrophin gene product remains unknown. Dystrophin is a 427 kD sarcolemma-associated protein that is expressed as a 14 kilobase (kb) mRNA from three different promoters in skeletal muscle and brain (reviewed in ref. 3). Two additional internal promoters have been identified that express 5.5 and 4.8 kb mRNAs encoding C-terminal proteins of 116 kd (Dp116 or apodystrophin-2) and 71 kD (Dp71 or apodystrophin-1) in Schwann cells and in non-muscle tissues respectively[4,5].

Based on homologies to α-actinin and spectrin[6] and *in vitro* binding studies[7-9], the N terminus of dystrophin is thought to bind actin in the subsarcolemmal cytoskeleton. Dystrophin also binds to a multisubunit dystrophin-associated glycoprotein complex at the sarcolemma membrane that includes α-dystroglycan which has been shown to bind to laminin in the extracellular matrix[10,11]. Dystrophin-binding studies *in vitro* suggest that the cysteine-rich and unique C-terminal domains of dystrophin are responsible for the interaction of dystrophin with the complex of dystrophin-associated proteins (DAPs)[12,13]. A structural model of dystrophin function based on its homology to other cytoskeletal proteins and specific protein interactions has been proposed in which dystrophin links the subsarcolemmal cytoskeleton to the extracellular matrix through its N and C-terminal protein interactions and thus reinforces the sarcolemma membrane to minimize damage from the forces of contraction[11].

That these two domains of dystrophin are primary in the function of dystrophin is supported by the existence of mild BMD patients with large deletions that remove the central spectrin-like repeat domain but preserve the N and C-terminal domains intact[14,15]. Patients with in-frame deletions[16] and one identified with a point mutation[17] in the N-terminal actin-binding domain of dystrophin typically display an intermediate to DMD phenotype, although some patients with severe alterations of the N terminus have a mild phenotype[18]. The description of a severely affected DMD patient with a deletion of the entire C terminus who expresses a truncated dystrophin on the sarcolemma suggests that expression of the N terminus and spectrin repeat domains alone is not sufficient for dystrophin function[19,20]. However, the potential functional independence of the C-terminal domain of dystrophin in skeletal muscle has not been directly examined.

We have analysed the structure and function of the C-terminal and cysteine-rich domains of dystrophin encoded by the Dp71 cDNA in the absence of endogenous dystrophin in transgenic *mdx* mice. The *mdx* mouse is an animal model of DMD that contains a point mutation that eliminates expression of the 427 kD isoforms of dystrophin in muscle and brain[1,21]. As the absence of dystrophin leads to a reduction of all of the dystrophin-associated glycoproteins[22,23] and because the C terminus of dystrophin is expressed as a natural isoform in non-muscle tissues, we wanted to (i) determine if expression of the C terminus alone in *mdx* muscle was sufficient to restore the DAPs on the membrane and (ii) determine if restoration of the complex in the absence of the dystrophin actin-binding domain could alleviate some or all of the

symptoms of muscular dystrophy, thus suggesting independent functions for the N and C termini and possibly anon-structural role for dystrophin. Our analyses of two independent transgenic *mdx* lines ectopically expressing Dp71 indicate that the sequences necessary for sarcolemmal localization and association with the DAPs *in vivo* are contained within the Dp71 transcript but that restoration of this complex is not sufficient to prevent muscular dystrophy.

Dp71 expression and modification

The Dp71 cDNA expression vector (pMCA) used to generate two lines of transgenic *mdx* mice (MCA-1 and 2) is shown in Fig.1*a*. The specific Dp71 cDNA isoform used to construct MCA transgenic mice includes all dystrophin exons from 63 to 79 and includes the unique Dp71 exon one encoding seven N-terminal amino acids but differs from the originally identified isoform of Dp71 which had exons 71 and 78 spliced out of the mRNA[5]. Reverse transcriptase PCR of non-muscle tissues indicates that this is a naturally occurring isoform of Dp71 (J.S.C., unpublished results) that has the added benefit of mimicking the predominant C-terminal structure of the full-length 427 kD dystrophin protein. Dp71 is normally expressed in non-muscle tissues and in undifferentiated myoblasts but is down regulated during muscle differentiation as the full-length 427 kD isoform begins to be expressed[24]. The normal function of Dp71 in non-muscle tissues and in myoblasts is unknown, however it may associate with members of the complex that are not muscle specific such as dystroglycan (43 and 156 DAG)[25] or help to pre-assemble the complex during muscle differentiation to facilitate binding to dystrophin.

Muscle specific expression of the Dp71 cDNA was controlled by regulatory regions of the mouse muscle creatine kinase (MCK) gene[26,27]. The MCA transgene directs expression of Dp71 in skeletal muscle when the endogenous Dp71 gene is normally shut down during muscle differentiation. Immunoblot analysis oftransgenic *mdx* muscle tissues revealed similar levels of expression from both lines in skeletal and cardiac muscles but no Dp71 expression was evident in control muscles (Fig. 1*b*). The transgene-expressed Dp71 protein comigrates with the endogenous Dp71 from non-muscle tissues in control mice. Interestingly this protein, which is encoded by a single cDNA isoform in transgenic *mdx* mice, does not migrate as a single band on SDS polyacrylamide gels but as a doublet or triplet. The endogenous non-muscle Dp71 also migrates as multiple bands (Fig. 1*b* and *c*)[28] but since the C terminus of dystrophin is known to be alternatively spliced[29,30] it was unclear if these bands were due to postranslational modification or alternative splicing. The structural similarity and sarcolemmal localization of dystrophin to other cytoskeletal proteins such as α-actinin and β-spectrin that are known to be phosphorylated[31] and the recent demonstration that dystrophin is a substrate for endogenous and *in vitro* protein kinases[32-34] suggested that Dp71 might also be phosphorylated.

To explore the potential phosphorylation status of Dp71, the pMCA cDNA expression vector was transfected into an *mdx* myoblast cell line. Immunoprecipitation of Dp71 from fused, stably transfected cells metabolically labelled with ^{32}P-orthophosphate revealed that the upper band from the doublet observed in western blots was phosphorylated (Fig. 1*c*, lanes c and d). In addition, expression of a second isoform of Dp71 (pMCA-alt C) with a deletion of exon 78 that encodes a unique C terminus was also observed to migrate as a doublet with the upper band being phosphorylated (lanes e and f). Treatment of the immunoprecipitated Dp71 with calf

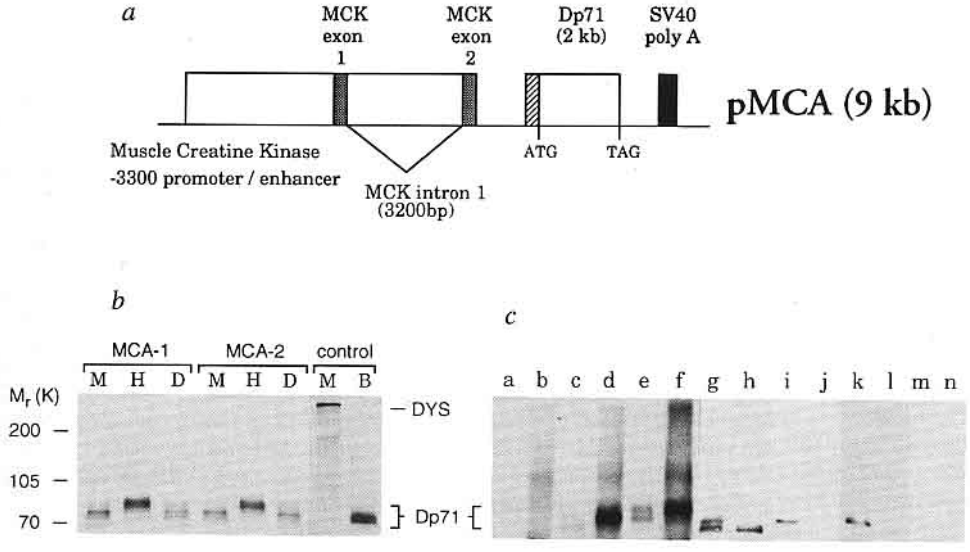

Fig. 1 *a*, Dp71 cDNA expression vector pMCA. Expression of the murine Dp71 cDNA was regulated by 6.5 kb of MCK gene sequences, including 3,300 bp upstream of exon 1, the complete first intron and a truncated exon 2 deleted just 5' of the initiator methionine. The SV40 polyadenylation site was inserted 3' of the Dp71 cDNA. The 5' untranslat2ed region is indicated by the hatched box; the 3' end of the Dp71 cDNA corresponds precisely to the alternate, downstream stop codon of the murine dystrophin mRNA, although this cDNA utilizes the upstream stop codon[30,60]. *b*, Immunoblot analysis of Dp71 expression in three-month-old C57BL/10 control and MCA-1 and MCA-2 transgenic *mdx* tissues. No Dp71 immunostaining is observed in control muscles although the 427 kD muscle dystrophin is visible(DYS).Note that the transgene encoded Dp71 and the endogenous non-muscle Dp71 migrate as doublets or triplets at 70-72 kD. Molecular weight standards (M_r) in kilodaltons (kD) are shown. M, skeletal muscle; D, diaphragm; H, heart; B, brain. *c*, Phosphorylation of Dp71 in myoblast cell culture and in MCA transgenic *mdx* muscle. Immunoprecipitated proteins from metabolically labelled ([^{32}P]orthophosphate) *mdx* myoblasts stably transfected with: a & b, mock transfected; c & d, pMCA; e & f, pMCA-alt C were separated by 10% SDS PAGE, transferred to nitrocellulose, exposed to x-ray film (lanes b, d and f) and immunostained with monoclonal antibody Dys-2 (lanes a, c, g and h) or rabbit polyclonal antibody 18-4 (lane e). Note that the upper band of each doublet observed by immunostaining (lanes c and e) is phosphorylated in the corresponding autoradiograph (lanes d and f). Phosphatase treatment of the immunoprecipitated Dp71 reduced the doublet at 70 and 72K to a single 70K band (lanes g and h, respectively). The specificity of phosphorylation was determined with monoclonal antibodies specific for phosphoserine (lanes i and j), phosphothreonine (lanes k and l) or phosphotyrosine (lanes m and n) and specific immunostaining was eliminated by pretreatment of the proteins with CIAP (lanes j, l and n).

intestinal alkaline phosphatase (CIAP) reduced the doublet to a single band migrating at the position of the lower band and confirmed that phosphorylation was responsible for the differential migration of the proteins (lanes g and h). To determine the specificity of phosphorylation on serine, threonine, or tyrosine residues, immunoblot analyses with monoclonal antibodies specific for each phosphorylated amino acid were performed. As shown in Fig. 1*c* (lanes i-n), immunoprecipitated Dp71 that had been phosphorylated in vivo in transgenic *mdx* muscle bound antisera against phosphorylated serine and threonine but not tyrosine residues and this immunostaining was eliminated by pretreatment of the proteins with calf intestinal alkaline phosphatase (CIAP), consistent with in vitro phosphorylation studies[32,33].

Immunolocalization and histopathology

Examination of the intracellular localization of Dp71 by immunohistochemistry in skeletal muscle cryosections revealed that it is expressed at levels similar to endogenous dystrophin in control muscles and that it is localized to the sarcolemma (Fig. 2*b*). Also, immunoblot and immunohistochemical analyses demonstrate that Dp71 is sufficient for the restoration of all of the members of the dystrophin-associated glycoprotein complex to control levels (Fig. 2*a* and *b*). However, dystrophin-related protein (DRP or utrophin) expression remains upregulated in transgenic *mdx* muscles and uniformly stains the sarcolemma similar to that observed in *mdx* muscles (Fig. l*a* and *b*). The inability of Dp71 to reduce the elevated levels of DRP at the sarcolemma in the transgenic *mdx* mice was presumably due to the potential interaction of actin with the DRP N terminus, which is highly homologous to the dystrophin N-terminal actin-binding domain and which the Dp71 protein lacks[35]. The intracellular localization of DRP is normally restricted to the neuromuscular junction (NMJ) in control muscle but in the absence of dystrophin its expression is elevated and becomes localized to the entire sarcolemma membrane[36,37]. In contrast, dystrophin is normally localized to the sarcolemma but it is also observed to be concentrated at the NMJ[38,39]. However, the role of dystrophin at the NMJ is unknown and no adverse effects on the function of the NMJ have been observed in the absence of dystrophin in *mdx* muscles[40]. Immunofluorescence detection with both an antibody specific for the C terminus of dystrophin (rabbit antibody 18-4) and rhodamine conjugated α-bungarotoxin which binds acetylcholine receptors (AChR) demonstrate that Dp71 is also localized to the NMJ similar to the endogenous 427 kD dystrophin in control muscle (Fig. 3). These results indicate that sequences necessary for the localization of dystrophin and the DAPs at the sarcolemma and the NMJ are contained within the Dp71 cDNA.

The effect of ectopic expression of Dp71 on transgenic *mdx* skeletal muscle pathology was determined by examination of control, *mdx*, and transgenic *mdx* histological sections (Fig. 4). The dystrophic pathology of *mdx* limb skeletal muscle is characterized by necrosis, a large variation in fibre size and centrally located nuclei in regenerated fibres[41]. Young adult transgenic *mdx* mice (both MCA-1 and 2) expressing Dp71 appeared to have a greater degree of focal necrosis and cellular infiltration than nontransgenic littermates. This was particularly apparent at three months of age when *mdx* muscle necrosis becomes less severe. The more severe dystrophic muscle pathology observed in three-month-old transgenic *mdx* mice was not progressive as degeneration and regeneration patterns were similar for one-year-old transgenic and nontransgenic *mdx* mice (Fig. 4). In addition, the degree and age of onset of the degeneration and regeneration were similar in young mice (four-week-old MCA-1 littermates are shown. Fig. 4). There were also no obvious differences in diaphragm or cardiac muscle pathology between transgenic and nontransgenic *mdx* littermates (data not shown). Histopathology of transgenic mice on a wild-type (non-*mdx*) background was examined to determine if ectopic expression of Dp71 in skeletal muscle

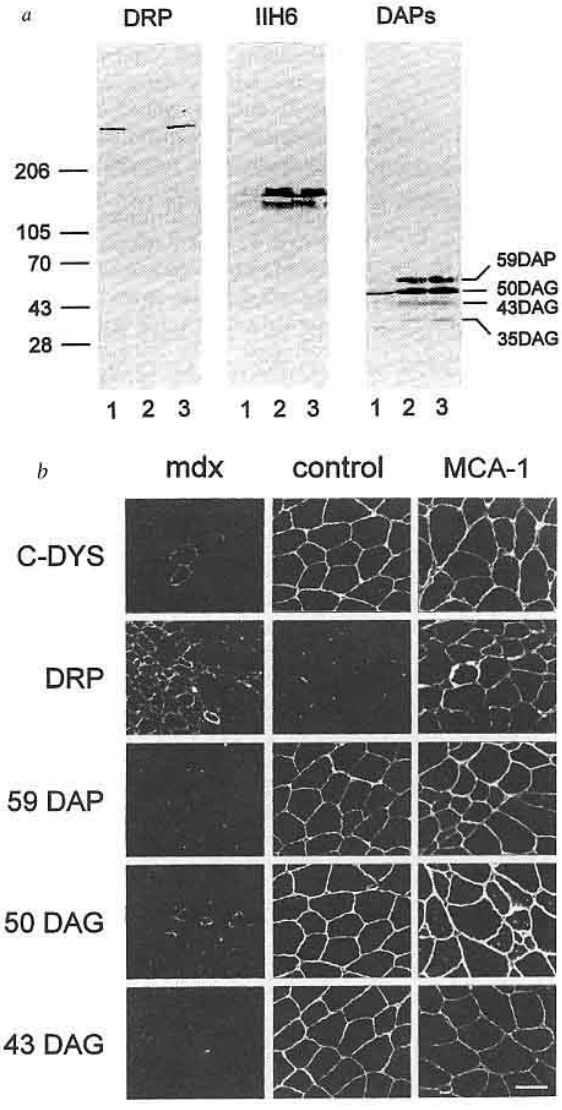

Fig. 2 *a*, Immunoblot analysis of DRP and the dystrophin-associated proteins in skeletal muscles from *mdx* (lane 1), control (lane 2), and MCA-1 transgenic *mdx* (lane 3) mice. *b*, Immunostaining of skeletal muscle cryosections with antibodies against the dystrophin C-terminal domain, DRP, 59 DAP, adhalin (50 DAG), and β-dystroglycan (43 DAG). MCA-1 transgenic *mdx* skeletal muscle showed control levels of dystrophin-associated protein expression, however DRP expression was still upregulated similar to *mdx* muscles. Bar indicates 50 μm.

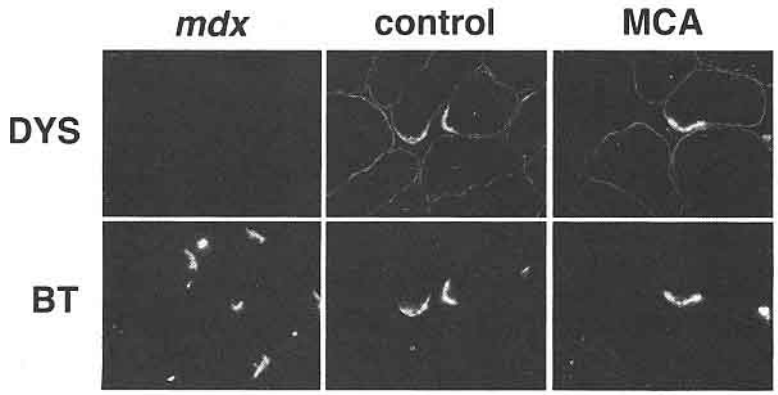

Fig. 3 Dual label immunofluorescent staining of dystrophin (FITC) and α-bungarotoxin (Rhodamine) in cryosections from *mdx*, control C57BL/10 and MCA-2 transgenic *mdx* skeletal muscle. Dystrophin and Dp71 immunostaining are localized to the sarcolemma and are concentrated at the neuromuscular junctions (NMJ). DYS, Dystrophin immunostaining with affinity purified rabbit polyclonal antibody 18-4 which reacts with both dystrophin (427 kD) and Dp71 but does not cross-react with DRP as seen by the absence of signal in *mdx* muscle. BT, Rhodamine labelled α-bungarotoxin staining. All exposure times and magnifications were identical.

had a dominant-negative effect in mice also expressing the full-length 427 kD dystrophin. No dystrophic pathology was evident in these transgenic animals with either our MCA-1 or MCA-2 transgenes (data not shown).

Discussion

We have generated two independent lines of transgenic *mdx* mice that ectopically express Dp71 in skeletal and cardiac muscle. The phenotypic effects ofDp71 expression on the dystrophic pathology of these mice has allowed the function of the C-terminal domain of dystrophin to be examined independently from the rest of the protein. The Dp71 expression vector used to create transgenic mice contains all C-terminal exons of dystrophin from exon 63 to 79 and mimics the C terminus of the full-length skeletal muscle dystrophin. The Dp71 protein expressed from this single cDNA isoform in transgenic *mdx* mice migrates as multiple bands on SDS polyacrylamide gels which are due to phosphorylation of serine and threonine residues of the C terminus of dystrophin in cell culture and in transgenic mice. This is consistent with recent results described for *in vitro* phosphorylation of a dystrophin C-terminal bacterial fusion protein with $p34^{cdc2}$ kinase[33] and phosphorylation of the full-length dystrophin by several endogenous protein kinases[32]. The reduced rate of migration of Dp71 on western blots in cardiac muscle compared to skeletal muscle and brain was consistently observed (Fig. 1*b*) and is presumably due to tissue-specific differences in the level of phosphorylation or of the action of different kinases in each tissue. The specific protein kinases responsible for this postranslational modification *in vivo* and the role of phosphorylation on dystrophin function are unknown but it may act in regulating protein-protein interactions with the DAPs or in directing correct intracellular localization within skeletal muscle. An endogenous Ca^{2+}/calmodulin-dependent kinase has recently been shown to copurify with the dystrophin glycoprotein complex (DGC) and specifically phosphorylates dystrophin and a 59 kD component of the complex on serine residues[34]. (It is unclear though whether this kinase is a member of the DGC or due to a contaminating activity.)

Although expression of Dp71 in skeletal muscle restores the DAPs on the sarcolemma membrane, it is unable to prevent the dystrophic pathology of the transgenic *mdx* mice. Interestingly, the phenotype of young adult transgenic *mdx* mice (three month) appears to be more severe than in nontransgenic littermates but the differences are not apparent in older animals (one year). It is unclear why ectopic expression of Dp71 in transgenic *mdx* skeletal muscle might lead to a more severe phenotype than in nontransgenic *mdx* littermates. The severe necrosis apparent in *mdx* mice at three to five weeks of age that resolves to a milder nonprogressive myopathy is also observed in the MCA transgenic *mdx* mice but persists until at least 12 weeks of age (Fig. 4). An autoimmune response to the Dp71 protein seems unlikely as *mdx* mice normally express Dp71 in non-muscle tissues[28] and the differences detected in the severity of the disease subside in older animals. The complex formed by Dp71 and the DAPs may be functionally defective in skeletal muscle and could by itself be deleterious. Additionally, ectopic expression of Dp71 may interfere with the normal function of homologous proteins in skeletal muscle. DRP and the 87 kD postsynaptic membrane protein are both highly homologous to the C terminus of dystrophin and both proteins, and Dp71 (Fig. 3), are localized to the NMJ[37,42]. We were unable to detect any dominant-negative effects of Dp71 expression in MCA-1 or 2 transgenic mice with a wild-type (non-*mdx*) dystrophin gene, however we experienced enormous difficulties in generating transgenic mice expressing Dp71 and the highest levels of expression achieved were only approximately equal to control levels of dystrophin (only four out of 120 pups born were positive for the transgene and of those, only two expressed detectable levels of Dp71, see Methodology). This is significantly lower than other transgenic lines prepared using the same promoter that have expressed up to 50X higher than control levels of dystrophin[43-44]. It is possible that dominant negative effects of Dp71 expression would be evident at higher levels of expression and may explain the difficulties we experienced in generating transgenic mice using this normally highly transcriptionally active promoter[26,27,43,44].

Various forms of muscular dystrophy arise when any number of alterations of dystrophin or its associated proteins occur. Mutations of dystrophin lead to DMD and BMD, both of which are associated with reduced expression of members of the DGC[23]. Aberrant expression of adhalin, the 50 kD dystrophin-associated protein, leads to severe childhood autosomal recessive muscular dystrophy (SCARMD)[45] and the absence of merosin, the isoform of laminin that binds the DGC in the extracellular matrix of skeletal muscle, is associated with a certain form of congenital muscular dystrophy[46,47]. In each of these types of muscular dystrophy, expression of or binding to one or more members of the DGC is abnormal, suggesting that normal expression of the dystrophin-DAP-merosin complex may be the most critical element required to prevent muscular dystrophy. In contrast, our results demonstrate that restoration of the DAPs and presumably the C-terminal linkage to merosin in the extracellular matrix is not sufficient for the prevention of dystrophic symptoms. Similar results have been obtained in transgenic

mdx mice using the originally identified isoform of Dp71 which is deleted for the alternatively spliced exons 71 and 78 (see accompanying paper, ref. 48). Together with our data, these results fail to support an independent function or activity for the C terminus of dystrophin and are consistent with a structural model of dystrophin function in which both the actin-binding N-terminal domain and the C-terminal domain are required to link the subsarcolemmal cytoskeleton to the extracellular matrix via the dystrophin-associated proteins. The relatively high abundance of dystrophin at the sarcolemma membrane (5% of sarcolemma-associated proteins)[49] and its homology to the cytoskeletal proteins α-actinin and spectrin[6] also suggest that dystrophin is a structural component of the sarcolemma membrane. The potential implications of this hypothesis on the design of a gene therapy treatment for DMD are testable in an animal model such as the *mdx* mouse and include determining the number of fibres and the level of expression that would be necessary to restore normal muscle function. In addition, transgenic *mdx* models will remain crucial for the structure and function analyses of the various domains of dystrophin to determine the minimal essential sequences required *in vivo* for full function.

Methodology

MCA transgenic mice. The murine Dp71 expression vector pMCA was constructed as described for the full-length dystrophin expression vector pMDA[43] with the Dp71 cDNA (containing the unique Dp71 first exon in-frame with exons 63 to 79 of dystrophin)[28] under the transcriptional control of the 6.5 kb muscle creatine kinase promoter, enhancer and first intron and SV40 polyadenylation signal. Transgenic mice were generated by microinjection of purified pMCA insert into F_1 hybrid zygotes from C57BL/6J X SJL/J parents essentially as described[50]. Of the 456 embryos transferred and the 120 live pups screened, only four founder F_0 animals (3.3%) were positive for the transgene, all of which had low copy number transgene insertions (between one and five copies, not shown), and only two of which expressed detectable levels of Dp71 on western blots and were examined further. Transgenic mice on the *mdx* background were produced by mating the F_0 MCA-1 male with C57BL/10 *mdx* females. The transgene was maintained on the *mdx* background by backcrossing the F_1 males and all subsequent positive progeny with C57BL/10 *mdx* mice. To increase the copy number and expression of the transgene, homozygous MCA-1 mice were identified by Southern blot from the progeny ofheterozygous MCA-1 incrosses. Transfer of the MCA-2 transgene onto the *mdx* background was accomplished by crossing the F_0 MCA-2 female with C57BL/10 *mdx* males. Two positive F_1 males were identified by PCR and these F_1 males were backcrossed with C57BL/10 *mdx* females. All of the resulting female progeny (11) and none of the male progeny (6) from the backcross were positive for the transgene indicating that the transgene had integrated onto the X Chromosome. To separate the transgene from the wild-type dystrophin gene on the X Chromosome, transgenic females heterozygous for the *mdx* mutation were backcrossed with C57BL/10 *mdx* males. The resulting progeny were screened for the presence of the transgene by PCR and for a crossover event between the transgene and the wild-type dystrophin gene by an allele specific oligonucleotide (ASO) assay to identify the *mdx* point mutation using PCRprimers flanking murine exon 23 as described[51]. Acrossover event was identified in three out of the 24 transgene positive progeny screened. These three recombinants were backcrossed with C57BL/10 *mdx* mice to maintain the transgene on the *mdx* background. All studies of MCA-2 transgenic *mdx* mice were performed with male mice to ensure that the transgene was expressed in all myofibre nuclei and was not reduced due to X Chromosome inactivation in approximately 50% of the myofibre nuclei. No differences were observed in the pathology of either the homozygous MCA-1 or the hemizygous MCA-2 animals.

Antibody generation. A -*Not*I-*Nru*I restriction fragment of the murine cDNA[52] encoding the C-terminal 315 amino acids of dystrophin (3364-3678) was cloned in-frame into the maltose binding protein fusion vector pMAL (New England Biolabs). pMAL-EF fusion protein was purified on amylose resin as described[53]. Antiserum in rabbit 18 was raised using complete Freund's adjuvant (Sigma) for the initial injection and incomplete Freund's adjuvant (Sigma) for booster injections[54]. High titre antiserum (10^{-5} dilution using alkaline phosphatase secondary antibodies (Fig. 1*c*) or 10^{-6} dilution using Enhanced Chemiluminescence (ECL, Amersham) secondary antibodies (data not shown) on skeletal muscle western blots was obtained at bleed 4. Cross-reacting antibodies against dystrophin-related protein (DRP, or utrophin) on immunohistochemistry were eliminated by affinity purification against a pMAL fusion protein encoding the highly homologous murine DRP C-terminal 305 amino acids corresponding to amino acids 3123-3427 of human utrophin[35]. Serum 18-4 was diluted 10^{-3} in PBS and passed three times over an Acti-Disk separation and purification cartridge (FMC) with 1 mg of purified pMAL-DRP fusion protein bound to the disk. The flow-through was used undiluted for immunohistochemistry (Fig. 4).

Western blots. Total cellular proteins were extracted from fresh transgenic *mdx*, *mdx*, and C57BL/10 tissues as described[55]. Protein concentrations were determined by the Coomasie Plus colorometric assay (Pierce). 100μg of total protein (Fig. 1*b* and *c*) or 800 μg of total microsomes (Fig. 2*a*) from each tissue were separated by electrophoresis on 10% and 3-12% SDS polyacrylamide gels in the presence of 1% 2-mercaptoethanol, respectively. Proteins were electrophoretically transferred to nitrocellulose using the Trans-Blot Cell (Bio-Rad) for 130 $V\text{-}h^{-1}$. Blots were immunostained with Dys-2 monoclonal antibody (Fig. 1*b* and *c*, NovaCastra Labs) that recognizes the final 17 amino acids of dystrophin at a 10^{-2} dilution,

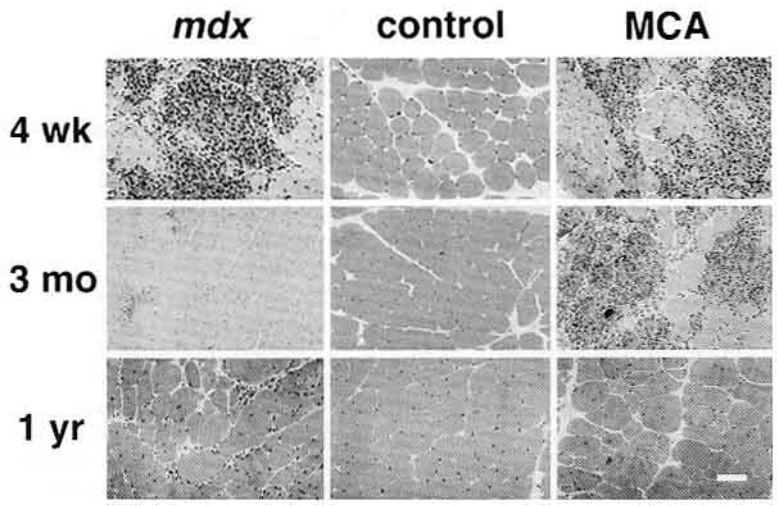

Fig. 4 Histological analysis of age-matched *mdx*, control C57BL/10 and MCA transgenic *mdx* quadriceps muscles at four weeks, three months and one year of age. Three month MCA-1 transgenic *mdx* muscles reveal the persistence of focal necrosis and cellular infiltration similar to that seen in transgenic and nontransgenic *mdx* MCA-1 littermates at four weeks of age. Differences in the pathology between *mdx* and transgenic *mdx* muscles are resolved by one year of age. Sections were stained with haematoxylin and eosin. Bar indicates 100 μm.

rabbit antibody 18-4 (Fig. 1*c*) generated against the final 315 amino acids of murine dystrophin at a 10^{-5} dilution, affinity-purified rabbit antibody against the last 12 amino adds of the C terminus of DRP[37] (Fig 2*a*), monoclonal antibody IIH6 against α-dystroglycan (156 DAG)[10] (Fig. 2*a*) or a mixture of affinity-purified sheep antibodies against the 156 DAG, 59 DAP, 50 DAG, 43 DAG and 35 DAG (Fig. 2fl)[56].

Immunohistochemistry. Characterization of dystrophin expression on the sarcolemma and at the NMJ was conducted by immunofluorescence staining of unfixed frozen C57BL/10, *mdx*, and transgenic *mdx*, skeletal muscle cryosections (7 µm). Samples were immunostained with affinity-purified sheep polyclonal antibodies specific for the dystrophin C-terminus, α-dystroglycan, 59 DAP, β-dystroglycan (43 DAG), or 35 DAG[56], or rabbit polyclonal antibodies against DRP[37], or adhalin (50 DAG)[57] (Fig. 2*b*),or affinity-purified rabbit antibody 18-4 (Fig. 3). Incubations were in Hartman's phosphate-buffered saline (20 mM potassium phosphate, 160 mM NaCl, pH 7.4) with 0.2% gelatin (Sigma) as a nonspecific blocking agent (PBSG). Sections were washed with PBSG, incubated for 1 h with a 1/200 dilution of biotin conjugated goat anti-rabbit IgG (Pierce), washed in PBSG and incubated 1 h with a 1/300 dilution of FITC conjugated streptavidin (Pierce). NMJ were visualized by incubation of cryosections for 1 h at 25 °C with 5 µg ml^{-1} tetramethylrhodamine conjugated α-bungarotoxin (Molecular Probes, Inc.) in PBSG. Dystrophin and NMJ staining were visualized and photographed using a Nikon Optiphot-2 fluorescence microscope and UFX-DX photomicrographic system.

Myoblast transfections and metabolic labelling. An *mdx* myoblast cell line (S. Hauschka, unpublished results) was cotransfected with 1 µg of pRc/RSV (Invitrogen) containing the neomycin resistance gene and 10 µg of pMCA or 10 µg of pMCA-alt C (deleted for exon 78 [32 bp] and encoding the alternate 32 amino acid C terminus of dystrophin[29,30]) by calcium phosphate-mediated transfection[58]. Stable transfectants were selected with 200 µg ml^{-1} G418 for two weeks. For metabolic labelling, two plates each of mock transfected, pMCA transfected, and pMCA-alt C transfected *mdx* myoblast pooled stable clones were grown to near confluence in Ham's F10 (Sigma) with 15% horse serum and 2 ng ml^{-1} fibroblast growth factor (R&D Systems) and then switched to Ham's F10 with 5% horse serum (differentiation medium). The cells were allowed to differentiate for five days then were rinsed once with HEPES-buffered saline and incubated for 18 h in phosphate free Dulbecco's minimal essential medium (Sigma) and 10% dialyzed fetal bovine serum (BRL) with 1 mCi ml^{-1} [^{32}P]orthophosphate (Amersham). The plates were rinsed twice in ice cold Tris-buffered saline (TBS) and the cells were lysed for 15 min on ice with 1 ml of RIPA lysis solution with protease and phosphatase inhibitors (50 mM Tris (8.0), 150 mM NaCl, 1% NP-40, 0.1%SDS, 0.5% sodium deoxycholate, 1 mM Na ortho vanadate, 50 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 U ml^{-1} Aprotinin, and 1 µg ml^{-1} leupeptin). Lysates were treated with 50 µg ml^{-1} RNaseA for 30 min to reduce nucleic acid-derived background[59] and were clarified by centrifugation at 14,000 RPM for l5 min at 4°C. Immunoprecipitation of the lysates were carried out in a volume of 1 ml with 5 µl of a 10^{-1} dilution of rabbit 18-4 antiserum and precipitation ofthecomplex with protein A-Agarose (BRL) essentially as described[54]. Immunoprecipitated proteins were resuspended in 50 µl of SDS sample loading dye. 25 µl of each sample was loaded onto a 10% SDS polyacrylamide gel and transferred to nitrocellulose as described above prior to exposure to X-ray film and immunostaining with Dys-2 monoclonal antibody (Fig. 1*c*, lanes a, c, g and h), a monoclonal antibody specific for phosphoserine ((Sigma), Fig. 1*c*, lanes i andj), a monoclonal antibody specific for phosphothreonine ((Sigma), Fig. 1*c*, lanes k and 1), a monoclonal antibody specific for phosphotyrosine ((UBI), Fig. 1*c*, lanes m and n) or C-terminal rabbit polyclonal antibody 18-4 (Fig. 1*c*, lane e). One-half of the immunoprecipitated proteins were treated with 50 U of calf intestinal alkaline phosphatase (Boehringer) for 1 h at 37 °C and the reactions were stopped by the addition of 25 µl of SDS sample loading dye.

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