

Adeno-associated virus vector gene transfer and sarcolemmal expression of a 144 kDa micro-dystrophin effectively restores the dystrophin-associated protein complex and inhibits myofibre degeneration in nude/*mdx* mice

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Duchenne muscular dystrophy is a severe life-threatening X-linked recessive disorder, caused by mutations in the dystrophin gene, for which currently there is no effective treatment. Because of the large size of the dystrophin cDNA (14 kb) this precluded it from being used in early adenovirus- or retrovirus-based gene therapy vectors. However, some therapeutic success has been achieved in *mdx* mice using adenovirus- and retrovirus-mediated transfer of a 6.3 kb recombinant mini-dystrophin cDNA. Despite this, problems with immunogenicity and inefficient transduction of mature myofibres make these vectors less than ideal for gene transfer to skeletal muscle. Adeno-associated viral (AAV) vectors overcome many of the problems associated with other vector systems. However, AAV vectors can only accommodate <5 kb of foreign DNA. For this reason we have produced a micro-dystrophin cDNA gene construct that is <3.8 kb. This construct, driven by a CMV promoter, was introduced into the skeletal muscle of 12-day-old nude/*mdx* mice using an AAV vector, resulting in specific sarcolemmal expression of micro-dystrophin in >50% of myofibres up to 20 weeks of age, and effective restoration of the dystrophin-associated protein (DAP) complex components. Additionally, evaluation of central nucleation indicated a significant inhibition of degenerative dystrophic muscle pathology. We have therefore shown that the current micro-dystrophin gene delivered *in vivo* using an AAV vector is not only capable of restoring sarcolemmal DAP complexes, but can also ameliorate dystrophic pathology at the cellular level.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe life-threatening X-linked disorder which affects 1/3500 males [one-third of which are sporadic cases with no family history (1)] and is characterized by a progressive loss of muscle mass and replacement of myofibres with connective and adipose tissue (2). This process leads to clinical symptoms of weakness, paralysis and breathing difficulties. The symptoms of the disease usually occur before 3 years of age, the patients are wheelchair bound by their early teens and death is normally at ~20 years of age.

The disease is caused by an absence of dystrophin, a 427 kDa cytoskeleton protein of the spectrin family (3,4). The DMD gene has one of the highest mutation rates reported so far, with two-thirds of disease-causing mutations being large deletions in the 5' and central portions of the gene. Interestingly, deletion size

has no influence over whether the phenotype is DMD or the much less severe form, Becker muscular dystrophy (BMD). Very mild forms of the disease, where ~50% or more of the gene has been deleted, have been described (5–8). Conversely, other individuals with only small deletions display a DMD phenotype. This apparent anomaly depends on whether the dystrophin open reading frame is maintained (BMD) or disrupted (DMD). At the molecular level, the DMD phenotype thus appears to be due either to the metabolic instability of the truncated mRNAs or proteins, or to a functional inactivity of the latter (9).

The dystrophin protein has four main structural domains; an N-terminal region, which binds to the F-actin of cytoskeletal structures, 24 triple-helical central rod repeats, interspersed with four hinge regions; and two C-terminal regions (CR and CT) which bind a complex of dystrophin-associated proteins (DAPs). These DAPs provide a molecular link between the

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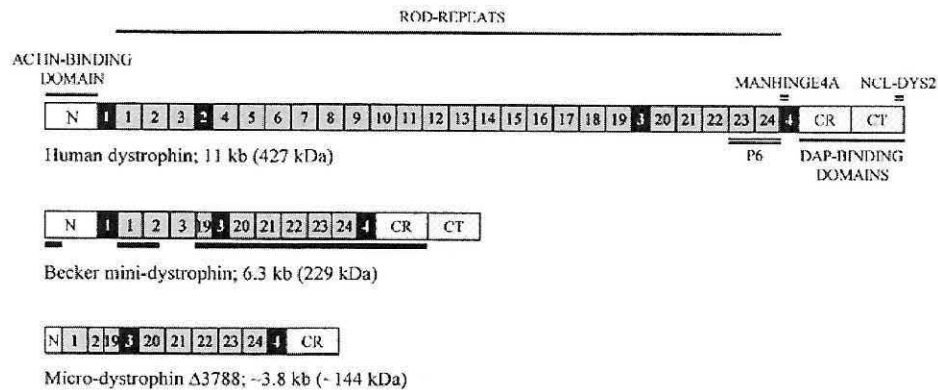


Figure 1. Diagrammatic representation of the coding regions of full-length human dystrophin, Becker mini-dystrophin and micro-dystrophin Δ3788 (not drawn to scale). Illustrated is the actin-binding domain of the N-terminus (N); hinge regions (black boxes); the rod-repeats (grey boxes); the dystrophin-associated protein (DAP) binding domains, C-terminal cysteine-rich region (CR) and distal C-terminus region (CT). Also shown are the binding sites of the three antibodies used (P6, MANHINGE4A and NCL-DYS2). Black lines under the 6.3 kb Becker mini-dystrophin indicate the regions that were PCR-amplified to produce micro-dystrophin Δ3788.

extracellular matrix and the internal cytoskeletal scaffolding of myofibres (10,11) and are thought to function by stabilizing the membrane against contraction-induced damage (12) and by mediating related cell signalling events (13,14).

Because of the size of the dystrophin cDNA (14 kb) this essentially precluded it from being used in early first and second generation adenovirus (Ad)- or retrovirus-based gene therapy vectors. Thus, as an initial step towards a gene therapy of DMD, researchers have employed smaller recombinant dystrophin minigenes based on BMD phenotypes, with the most commonly used cDNA being 6.3 kb (5,15). A number of *in vivo* studies using *mdx* mice (a DMD model) to assess the effectiveness of this minigene for DMD gene therapy have been quite successful (16–22). All observed the presence of dystrophin-positive fibres around the injection site, and two studies (16,20) noted corrected assembly of the DAP complex. The 6.3 kb minigene (both murine and human) has also been used to produce transgenic *mdx* mice, which developed almost completely free of dystrophic symptoms (23–25).

Two groups have evaluated further engineered reductions in the size of dystrophin molecules by creating a series of novel micro-dystrophin cDNA gene constructs that range in size from 3.1 to 4.2 kb (26,27). In the former study, four of the six constructs, carried in Ad vectors, were successfully expressed at the sarcolemma along with the DAPs, α -sarcoglycan (α -SG), β -dystroglycan (β -DG) and α -1 syntrophin. Similarly, in the study by Wang *et al.* (27), three minigenes transferred by adeno-associated viral (AAV) vectors were able to restore dystrophin expression as well as the DAPs α -, β - and γ -SG at the plasma membrane; however, it was the two smallest constructs that produced the highest percentage of dystrophin positive fibres. In addition, the products of these two smallest minigenes were able to ameliorate dystrophic pathology in *mdx* muscle, resulting in the restoration of normal myofibre morphology, histology and cell membrane integrity.

Apart from structural variations in the minigene constructs, the main difference between the studies by Yuasa *et al.* (26) and Wang *et al.* (27) was in the vector chosen to deliver the transgene; Ad versus AAV, respectively. A number of properties

of AAV have made this virus a very promising vehicle for therapeutic gene delivery. First, AAV vectors are able to infect a wide variety of human and animal cell types, both dividing and non-dividing, and are very efficient at transducing mature myofibres (27–31). Secondly, they can only productively replicate upon co-infection with a helper virus such as Ad (32). Thirdly, like third generation helper-dependent (guttled) Ad vectors, recombinant AAV (rAAV) vectors contain no viral genes, which may contribute significantly to their persistence in immune-competent hosts. Finally, AAV has never been associated with any human disease.

Based on the above observations we have designed, constructed and evaluated a new micro-dystrophin cDNA of <3.8 kb, encoding a product of ~144 kDa, for use in studies on AAV vector-mediated gene transfer and complementation of dystrophin deficiency.

RESULTS

Design rationale of micro-dystrophin gene construct Δ3788

The observation that large in-frame intragenic deletions of the rod domain of dystrophin (5–8) as well as deletions in the N-terminal actin-binding (33,34) and CT regions (35,36) resulted in only mild phenotypes allowed us to produce a micro-dystrophin cDNA that was only 3788 bp in length, encoding a protein product of 1247 amino acids, or ~34% of wild-type dystrophin.

The cDNA we constructed thus contains exons 1–2, 10–12 and 49–70 in their entirety (Fig. 1). At the 5' end, exons 3–9 (which constitute a large portion of the actin-binding domain) have been deleted. This section of the construct is based on two patients who had deletions spanning exons 3–7 (33). Although this deletion would normally constitute a frameshift mRNA, an alternatively spliced transcript linking exons 2 and 10 (which produces an in-frame product) was detected at low levels along with an appropriate protein product. This deletion of exons 3–7, which is the most frequent 5' deletion giving rise to BMD, is an exception to the 'reading frame' hypothesis, and the observed

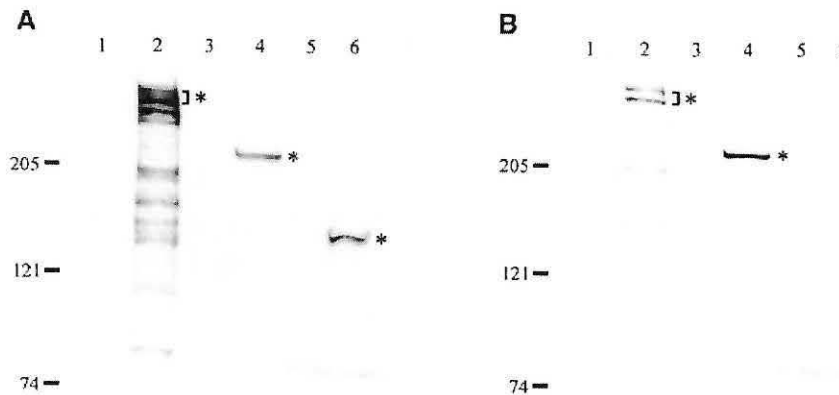


Figure 2. Western blot of mouse tissue and cell extracts probed first with dystrophin antibody MANHINGE4A (A) and then with NCL-DYS2 (B). (Note: blot was not stripped between stainings.) Lanes: 1, *mdx* muscle; 2, normal C57/B10 muscle; 3, C2C12 myoblasts; 4, C2C12 myoblasts transduced with a Becker mini-dystrophin Ad construct; 5, 293T cells transduced with helper Ad, Ad5dl312, only; 6, 293T cells transduced with rAAVΔ3788 and helper Ad, Ad5dl312. Dystrophin bands are marked with an asterisk. (Because of the numerous cross-reactive products in lane 2 only the largest two bands are labelled.) Molecular weight markers are in kDa.

Becker phenotype suggests that this segment of the N-terminal region is not absolutely essential for the function of the dystrophin molecule. Constructs containing deletions in this domain when introduced into transgenic *mdx* mice also produce a mild phenotype (34). The removal of exons 13–48 from the micro-dystrophin construct was based on two patient reports (6,7), where gene deletions corresponded to 50% of the coding region and up to 66% of the dystrophin rod domain (including hinge 2) resulted in a mild BMD phenotype. A similar mild phenotype was also observed when exons 17–48 (5) and 17–51 (8) were deleted. Finally, the rationale for the removal of exons 71–79, which span the penultimate CT domain of dystrophin, was based on two observations: (i) at the cDNA level, a naturally occurring alternatively spliced isoform of dystrophin without exons 71–74 is found in embryonic muscles and brain tissues (36), and (ii) a patient with a deletion removing exons 73–79 displayed only a mild form of BMD with absence of muscular pseudohypertrophy at 15 years of age (35). Thus, the nucleotide sequences retained in the micro-dystrophin cDNA are ~12% of the N-terminal actin-binding domain, six complete and two incomplete rod repeat units (of 24), two of the four hinge regions, and the entire CR region. So that the molecule retained some of its flexibility, the two hinge regions were separated by five rod repeats. This minigene was ligated into an AAV vector under the control of a CMV promoter and termed pAAVΔ3788. Prior to expression analysis, the integrity of the cDNA open reading frame was confirmed by sequencing (data not shown).

Expression of micro-dystrophin from pAAVΔ3788 and rAAVΔ3788 vectors in 293T cells

To confirm that the micro-dystrophin cDNA construct Δ3788 would functionally encode a polypeptide product of the predicted molecular weight, 293T cells were transfected with the plasmid pAAVΔ3788 and the cell lysate analysed by western blot. Using the polyclonal antibody P6 [specific for the C-terminal region of the dystrophin rod domain (Fig. 1) (37)], or the monoclonal antibody MANHINGE4A [specific for the

hinge 4 region (Fig. 1) (38)], two bands were observed (data not shown). The largest band was of the predicted size (~144 kDa); the other was between 105 and 110 kDa and presumably represents a dystrophin breakdown product since it was not detected in mock-transfected cells. rAAVΔ3788 particles, produced by transfection of pAAVΔ3788 and the helper plasmid pDG (39) into 293T cells, were transduced into 293T cells and the lysate analysed by western blot. To specifically identify the micro-dystrophin product, this blot was probed sequentially with two monoclonal antibodies; MANHINGE4A followed by NCL-DYS2 (Fig. 2) [specific for the C-terminus of the CT region (Fig. 1)]. Probing the blot with MANHINGE4A (Fig. 2A) revealed a single band in lane 6 (cells transduced with rAAVΔ3788 and the E1A⁻ mutant helper Ad, Ad5dl312) of ~144 kDa. As expected, this band was absent when the blot was probed with NCL-DYS2 (Fig. 2B, lane 6) as the cross-reacting region CT is deleted in this clone. Both MANHINGE4A and NCL-DYS2 detect full-length dystrophin (plus a number of breakdown/cross-reacting proteins) in normal C57/B10 muscle as well as the recombinant Becker mini-dystrophin [229 kDa (20)] expressed from Ad-infected C2C12 cells. No dystrophin expression was seen in *mdx* muscle with either antibody, neither were there any bands detected in cells infected with Ad5dl312 alone. A small amount of expression was observed in cells infected with rAAVΔ3788 alone when probed with MANHINGE4A (<10% of that observed with rAAVΔ3788 plus Ad5dl312) but not with NCL-DYS2 (data not shown). The micro-dystrophin AAV vector genome therefore seems to be packaged intact and expressing a product of the predicted size, which is approximately one-third the size of full-length dystrophin, or ~60% smaller than the Becker mini-dystrophin.

Expression of micro-dystrophin Δ3788 in the skeletal muscle of nude/*mdx* mice transduced with rAAV

To evaluate the expression of micro-dystrophin Δ3788 *in vivo*, we injected rAAVΔ3788 particles into the tibialis anterior (TA) muscle of 12-day-old nude/*mdx* mice and performed

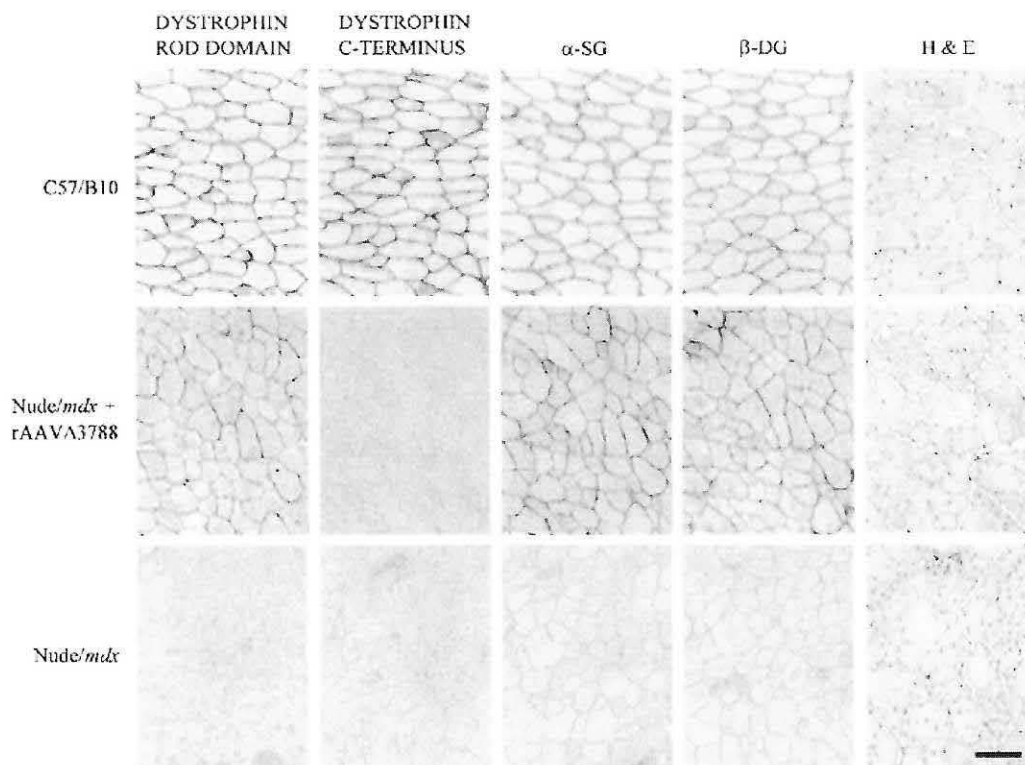


Figure 3. Immunohistochemical localization of dystrophin, the DAPs α -sarcoglycan (α -SG) and β -dystroglycan (β -DG), and haematoxylin and eosin (H&E) stained TA muscle sections of control and injected mice. Consecutive cryosections from a normal adult C57/B10 mouse, a 20-week-old nude/*mdx* mouse injected with rAAV Δ 3788, and the contralateral uninjected muscle were stained with dystrophin antibodies P6 (rod domain) and NCL-DYS2 (C-terminus; CT), and DAP antibodies NCL-a-SARC and NCL-b-DG which detect α -SG and β -DG, respectively. Tissue sections from the nude/*mdx* mouse injected with rAAV Δ 3788 and stained with antibodies P6, α -SG and β -DG all show positive staining at the sarcolemma (although at a somewhat lower level than is seen in C57/B10 sections). The NCL-DYS2 panel is negative in this muscle section as the CT region is absent in our construct. The C57/B10 muscle stains positive for the four antibodies, whereas the uninjected nude/*mdx* muscle is essentially negative (although there is a low level of staining with the antibodies to α -SG and β -DG). H&E staining of the dystrophin positive rAAV Δ 3788 injected muscle shows mostly peripherally nucleated fibres as is found in C57/B10 muscle with a more consistent myofibre size and little evidence of fibrosis. In contrast, the untreated dystrophin negative nude/*mdx* tissue displayed mostly centrally nucleated fibres which showed evidence of fibrosis and a less consistent myofibre size. Scale bar, 100 μ m.

immunohistochemical analysis on the muscle sections 20 weeks after vector injection. [Nude/*mdx* mice are immunodeficient and hence allow transduction efficiency to be evaluated in the absence of confounding cross-species immune responses to the recombinant human micro-dystrophin. They also yield a highly significant reduction in non-specific immunohistochemical staining with mouse monoclonal antibodies. It should be noted that the pattern of myofibre pathology in nude/*mdx* is similar to *mdx* mice apart from minor changes in deposition of interstitial fibrotic tissue (40).] Using the dystrophin antibody P6, mice which received a successful intramuscular injection showed marked positive staining of myofibres ($51.5 \pm 6.6\%$; $n = 5$) although the staining was not as intense as in the C57/B10 control muscle (Fig. 3). In contrast, the proportion of dystrophin-positive fibres found in the control nude/*mdx* skeletal muscle, which correspond to revertant fibres, was $1.1 \pm 0.18\%$ ($n = 11$). To confirm that the majority of the positive staining for dystrophin observed in rAAV Δ 3788 transduced muscles was due to the micro-dystrophin construct rather than to revertant myofibres, we probed the sections additionally with the anti-

body NCL-DYS2, which recognizes both the mouse and human dystrophin CT region. Because this region is absent in our construct, any positive fibres must, by definition, be revertants. As expected, the number of positive fibres was virtually identical to that found in P6 stained control nude/*mdx* skeletal muscle, suggesting that the staining observed with Δ 3788 rAAV transduced muscle was due almost exclusively to the micro-dystrophin (Fig. 3).

Recovery of DAPs at the sarcolemma

To evaluate the ability of Δ 3788 micro-dystrophin to restore components of the DAP complex at the sarcolemma we examined the expression of α -SG and β -DG in the TA muscles of nude/*mdx* mice after rAAV Δ 3788 injection by immunostaining of muscle sections. DAP expression exhibited a similar immunostaining pattern to that found on the adjacent sections stained for dystrophin (Fig. 3). As was the case for micro-dystrophin expression, the intensity of staining with α -SG and β -DG antibodies was less than in control C57/B10 muscle, indicating a

correlation between the two. The level of immunostaining for α -SG and β -DG in untreated control nude/*mdx* muscle was much less than that of C57/B10 or rAAV transduced myofibres. These results suggest that micro-dystrophin Δ 3788 is able to restore at least two components of the DAP complex at the sarcolemma despite being deficient in the CT domain.

Changes in central nucleation

The percentage of muscle fibres with centrally located nuclei is indicative of previous cycles of degeneration and regeneration and is inversely correlated with the ability of dystrophin to protect muscle from these cycles (23–25). In *mdx* mice the onset of this pathology starts at ~3 weeks of age. For this reason we chose to inject 12-day-old mice to evaluate the functional properties of the micro-dystrophin construct. Histological analysis of TA myofibres 20 weeks after rAAV treatment of nude/*mdx* mice showed that of the ~50% P6 antibody-positive fibres present, $92.9 \pm 3.6\%$ ($n = 5$) were peripherally nucleated and, like normal C57/B10 mice [which showed $99.4 \pm 0.19\%$ ($n = 8$) peripheral nucleation in dystrophin-positive fibres], exhibited more consistent myofibre size without evidence of fibrosis (Fig. 3). In contrast, in the untreated nude/*mdx* muscle, only $1.1 \pm 0.18\%$ ($n = 11$) of fibres stained positively with P6 and the majority were again peripherally nucleated, suggesting that the revertant dystrophin in these fibres is able to protect against cycles of degeneration and regeneration. Conversely, the remaining 98.9% of dystrophin-negative TA myofibres in these untreated nude/*mdx* mice exhibited $75.6 \pm 6.2\%$ ($n = 11$) central nucleation and showed additional signs of dystrophic pathology such as a wide variation of myofibre size and fibrosis (Fig. 3). These results suggest that the presence of the micro-dystrophin is preventing the onset of dystrophic pathology in AAV transduced fibres.

DISCUSSION

In order to utilize an AAV vector system to provide gene transfer based on complementation of dystrophin deficiency in muscle tissues *in vivo*, we have reduced the cDNA sequence of this gene down from 14 to ~3.8 kb to produce a micro-dystrophin cDNA. The rationale behind the rod-domain coding sequence deletions employed is based upon several studies reporting large intragenic in-frame deletions in this region of the DMD gene with patients exhibiting only a mild BMD phenotype (5–8). These observations suggest that the cognate region is at least in part dispensable for dystrophin function. Similar reports correlating deletion genotype to pathological phenotypes (33–36) allowed us to rationally reduce the molecule further by deleting coding sequences for part of the N-terminus actin-binding domain as well as the entire CT region of dystrophin. AAV vector-mediated delivery of the final Δ 3788 construct *in vitro* showed, by western blotting studies, that transduced 293T cells expressed a single protein of the predicted size. *In vivo*, the Δ 3788 micro-dystrophin was shown to be expressed at the sarcolemma of rAAV transduced myofibres and was correlated with the reappearance of DAP components at the sarcolemma in nude/*mdx* mouse muscle. Furthermore, sarcolemmal expression of Δ 3788 micro-dystrophin was able to largely prevent the centro-nucleation of myofibres, which is

associated with degenerative pathology in dystrophin deficient *mdx* mice.

There are several differences between our construct, which is based entirely on patient data, and those produced by other groups (26,27). In those studies the minigene constructs that were able to express dystrophin at the sarcolemma as well as restore the DAP complex all had intact N-terminus and CR coding regions. Six of the seven constructs had two hinge regions separated by one, two, three, five or six rod repeats, and one had three hinge regions separated by two and three repeats (27). The CT region was retained by Yuasa *et al.* (26) but deleted by Wang *et al.* (27). In contrast, our construct retains only 12% of the N-terminus, has two hinge regions, six complete and two partial rod domains, plus the entire CR region. Because expression at the sarcolemma and recovery of the DAPs was observed with all these constructs this suggests that a variety of different combinations of molecule can be tolerated whilst retaining significant functional activity. Variations in the levels of expression between engineered micro-dystrophin genes may be due to a variety of factors, such as differences in relative stability of transgene products (mRNA and protein), muscle type injected (TA versus gastrocnemius), age of mice at time of injection, promoter element used (CMV, CAG or muscle-specific MCK), amount of vector delivered, as well as the mode of delivery (Ad versus AAV). However, it is important to note that recovery of DAPs at the sarcolemma is not in itself sufficient to restore dystrophin function. Experiments on transgenic *mdx* mice expressing DP71 (a non-muscle product of the DMD gene which contains the CR and CT domains of dystrophin, but lacks the N-terminal actin-binding and central rod repeats) showed complete recovery of the DAPs, but failed to alleviate the dystrophic phenotype (41,42). In contrast to these findings, the results presented here show that not only were the DAPs restored at the sarcolemma of rAAV Δ 3788-treated nude/*mdx* mice, but also in these positive fibres the cycles of degeneration and regeneration seem to have been largely averted, as evidenced by the numbers of peripherally nucleated fibres present (~73% more than in untreated nude/*mdx* muscle, or ~93% of the level observed in control C57/B10 muscle).

Although the design of micro-dystrophin construct Δ 3788, described here, was based on BMD patient data, the observed functional activity is supported by transgenic studies examining interstitial dystrophin deletions in *mdx* mice (34,43,44). Several actin binding sites (ABS) have been identified in the N-terminal dystrophin domain [ABS1, amino acids 18–27; ABS2, amino acids 131–148; ABS3, amino acids 97–117 (45–49)]. The design of micro-dystrophin Δ 3788 was based on BMD patients with deletions of ABS2 and ABS3 (33,50–52) and thus retains only ABS1. In transgenic *mdx* mice bearing dystrophin transgenes deleted for essentially the same region (ABS2 and ABS3), but retaining ABS1, a significant reduction in dystrophic pathology was observed with residual phenotype proposed to reflect low expression levels rather than intrinsic dysfunction of the recombinant dystrophin (34). In the central rod domain of dystrophin, 24 triple-helical repeat and four hinge elements have been proposed to confer an extended but flexible rod structure to the molecule (53,54), which along with the N-terminal ABSs may also contribute to a large actin-binding interface (55). Micro-dystrophin Δ 3788 is based upon mild BMD phenotypes in patients with large central-rod

domain dystrophin deletions spanning exons 13–48 (5–8). This rod domain deletion has not been directly tested in *mdx* mice, but transgenic studies with exon 17–48 deletion constructs restored sarcolemmal DAPs, normalized serum creatine kinase levels and virtual elimination of dystrophic pathology was observed (24,25). In the case of the CR and CT domains of dystrophin, molecular and transgenic *mdx* mouse studies have found that deletions within the CR region interfere with β -DG binding, leading to disruption of the DAP complex and loss of function (43,44). In contrast, expression of CT region deleted dystrophins in transgenic *mdx* mice effectively prevents dystrophic pathology with sarcolemmal restoration of the DAP complex including neuronal nitric oxide synthase, syntrophin and dystrobrevin (43). This observation is in line with the limited number of BMD patient phenotype–genotype relationships on which again the current micro-dystrophin Δ 3788 construct was based (35,36). Thus, our results with micro-dystrophin Δ 3788 AAV are broadly in line with BMD patient phenotype and transgenic *mdx* studies. In conjunction with the previous gene transfer studies (20,26,27), our data thus indicates that to maintain significant functionality in highly engineered recombinant micro-dystrophins, retention of a minimal set of central rod domain repeats and hinges is required to confer vital flexibility to the molecule (26,27), along with critical portions of N-terminal and CR regions, presumably allowing interaction with the actin cytoskeleton and sarcolemmal recruitment of crucial DAP complex members, respectively (34,43,44).

Prolonged transgene expression is particularly important for DMD gene therapy where the target tissue is widespread and regular administration of the therapeutic agent would be unfeasible. A number of studies have shown that AAV-mediated gene delivery is not only able to efficiently transduce mature myofibres but also results in persistent expression of the transgene (27–31). Our observations suggest that micro-dystrophin Δ 3788 delivered *in vivo* using an AAV vector is stable over time although the level of expression of both dystrophin and DAPs appears to be lower than in the control C57/B10 mice. This may be due to the dose of rAAV particles injected, or to a reduced half-life of the micro-dystrophin protein itself. In the latter respect it may be of relevance that naturally occurring dystrophin proteins deleted for the ABS1 and ABS3 regions of the N-terminal domain are often expressed at levels below those in normal control muscle (6,50–52). It is thus important to consider how much of the recombinant protein is required for a therapeutic effect. Studies with transgenic animals have shown that a wide range of dystrophin gene expression levels can be tolerated and that the 427 kDa protein is protective when expressed at 20% of endogenous levels in the diaphragm, whereas higher levels are probably needed in quadriceps muscle (24). More important for this study is the finding that the 229 kDa mini-dystrophin appears to be protective when expressed at 20–30% of endogenous dystrophin levels (24,25). The observation of BMD patients who express dystrophin at 30% of endogenous levels but manifest only a mild phenotype (56,57) also supports the notion that a therapeutic benefit can be achieved with considerably less than 100% of endogenous dystrophin expression.

A number of *in vivo* approaches to the gene therapy of DMD are now being examined. In addition to those already mentioned, naked DNA (58,59), RNA–DNA oligonucleotide

(60,61), stem cell (62) and other viral vector [such as herpes simplex virus type 1 (63)] approaches are also being investigated. However, AAV vectors remain one of the most attractive systems for muscle gene transfer, and the studies described here further confirm the feasibility of using highly engineered micro-dystrophin AAV vectors as an approach towards gene therapy of DMD (26,27). Furthermore, recent observations have shown that AAV vectors based upon alternative capsid serotypes, particularly type 1 but also types 3 and 5, can increase levels of protein production by 100–1000-fold (64). Such an approach could greatly enhance the therapeutic potential of micro-dystrophin AAV vectors to complement dystrophin deficiency in a clinical setting. However, long-term studies in immunocompetent *mdx* mice as well as in larger animal models are now required to further evaluate the potential of this gene therapy approach in the context of the human disease.

MATERIALS AND METHODS

Construction of Δ 3788 micro-dystrophin cDNA and corresponding AAV plasmid vector

Construction of the Δ 3788 micro-dystrophin cDNA was accomplished by PCR cloning in three steps using the original pRSVDy- β , 6.3 kb mini-dystrophin cDNA plasmid (15) which incorporates deletions of exons 17–48 of the dystrophin coding sequence.

Deletion of exons 13–16 coding region

PCR products for exons 1–12 and 49–51 were produced using 3' or 5' primers (respectively) which incorporated exon 12/49 internally located junction sequences and 5' and 3' primers (respectively) incorporating endogenous *KpnI* sites. Following primer removal, mixing of the two amplification products and re-amplification with exon 1 and exon 51 primers generated a new PCR product incorporating exons 1–12 fused with 49–51 and flanked by *KpnI* sites. This fragment was cloned into *KpnI*-digested pRSVDy- β and correctly orientated clones were identified to generate pRSV μ Dy1 containing micro-dystrophin Δ 5732 lacking exons 13–48.

Deletion of exons 3–9 coding region

Plasmid pRSV μ Dy1 was used as a PCR template to generate this second N-terminal deletion construction. PCR products for exons 1–2 and exons 10–51 in pRSV μ Dy1 were produced using primers that incorporate exon 2/10 internal junction sequences, and external *KpnI* sites. Following primer removal, mixing of reaction products and re-amplification with the exon 1 and exon 51 primers generated a new PCR product incorporating exons 1, 2, 10–12 and 49–51, flanked by *KpnI* sites. This fragment was cloned into *KpnI*-digested pRSV μ Dy1 and correctly orientated clones identified to generate pRSV μ Dy2 containing micro-dystrophin Δ 4865 cDNA lacking exons 3–9 and 13–48.

Deletion of exons 71–79 coding region

Plasmid pRSV μ Dy2 was used as a PCR template to generate this third deletion construct. A PCR product from exon 67 (*BspEI* site) to the end of exon 70 was produced with the reverse primer incorporating additional codons for the penulti-

mate three amino acids of full-length muscle dystrophin plus sequence for two termination codons and flanking *EagI* and *Sall* sites. This fragment incorporating available 5' *BspEI* and 3' *Sall* sites was cloned into *BspEI*- and *Sall*-digested pRSV μ Dy2 to generate pRSV μ Dy3 containing micro-dystrophin Δ 3788 lacking exons 3–9, 13–48 and 71–79. To ensure the Δ 3788 product was error free the entire cDNA was sequenced in both directions.

Generation of pAAV Δ 3788 plasmid vector

The micro-dystrophin Δ 3788 cDNA fragment was digested intact from pRSV μ Dy3 using flanking 5' *NotI* and 3' *EagI* sites and cloned into a *NotI* digested AAV vector fragment from plasmid pNTC3-CMV β (65). The resulting AAV vector plasmid, pAAV Δ 3788, contains the Δ 3788 micro-dystrophin cDNA, 549 bp CMV promoter, SV40 polyadenylation signal and flanking inverted terminal repeats (ITRs) of AAV serotype 2.

Cells and production of rAAV Δ 3788 particles

Low passage number (passage 5–10) 293T cells were propagated in DMEM, supplemented with 10% (v/v) FBS and incubated at 37°C under 8% CO₂. To produce rAAV particles, the plasmid pAAV Δ 3788 was co-transfected with the helper plasmid pDG (39) into 293T cells, as described by Zolotukhin *et al.* (66). After 48 h incubation the cells were lysed and particles purified using iodixanol density centrifugation followed by heparin affinity chromatography columns (Sigma) (66). The titre, determined by dot-blot hybridization, was estimated at $\sim 2.4 \times 10^{12}$ viral particles per ml.

Cell transfection, SDS-PAGE and western blot analysis

Approximately 7.5×10^5 293T cells were plated 24 h prior to infection into a six-well dish. The micro-dystrophin plasmid construct, pAAV Δ 3788, was transfected into these cells using standard CaPO₄ co-precipitation and rAAV Δ 3788 particles were co-infected with the helper Ad, Ad5dl312, both at a multiplicity of infection of 100. Incubation was for 24 h using the conditions described above. After this time, cell cultures were washed twice with PBS and then collected in 200 μ l sample buffer (75 mM Tris pH 6.8, 10% SDS, 0.1% bromophenol blue, 20% glycerol, 100 mM DTT, 20 mg/ml PMSF, 60 μ g/ml aprotinin, 10 μ g/ml aprotinin, 0.5 μ g/ml leupeptin hemisulphate, 200 μ g/ml EDTA), passed three times through a 25G needle to shear the DNA and heated to 100°C for 3 min. TA muscle tissue samples were prepared from C57/B10 and *mdx* mice. The muscle was extracted and ground in frozen lysis buffer (1% NP-40, 137 mM NaCl, 20 mM Tris pH 7.4, 10% glycerol) and the lysate clarified by centrifugation at 12 000 g for 5 min in a micro-centrifuge. This clarified lysate was mixed with loading buffer and heat denatured as above. Denatured samples were electrophoresed through a 3–8% polyacrylamide Tris–Acetate gel (NuPAGE[®]; Invitrogen), and proteins blotted onto Hybond[™] ECL[™] nitrocellulose membranes (Amersham Pharmacia Biotech) using the NOVEX[®] Xcell II blotting apparatus (Invitrogen). Membranes were blocked in TBST [10 mM Tris pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20] containing 5% (w/v) non-fat milk powder, and primary and secondary antibodies were diluted in the same solution but with 2.5% (w/v) milk powder. Blots were

probed with either the polyclonal antibody P6 (1:3000) or monoclonal antibodies MANHNGE4A (1:100) and NCL-DYS2 (1:50) (Novocastra Laboratories Ltd). Goat anti-rabbit and goat anti-mouse HRP-conjugated IgG secondary antibodies (Jackson Laboratories) were used according to the manufacturer's recommendations. Detection was by the ECL[™] system (Amersham Pharmacia Biotech).

Animals and immunohistochemistry

Twelve-day-old nude/*mdx* mice were anaesthetized by intraperitoneal injection of a mixture of anaesthetics Hypnorm (fentanyl-fluonazone; Jansen Pharmaceutical) and Hypnovel (midazolam; Roche Laboratories) in sterile distilled water in the ratio of 1:1:2, according to Flecknell (67). The freshly prepared mixture was injected intraperitoneally at a level of 5 ml per kg of body weight. TA muscles were injected with 5 μ l of rAAV Δ 3788 in physiological saline ($\sim 1.2 \times 10^{10}$ AAV particles). The mice were culled after 20 weeks and both injected and contralateral uninjected TA muscles were excised and rapidly frozen in liquid-nitrogen-cooled isopentane. Cryosections, 10 μ m thick, were prepared and immunostained, without prior fixation, with four antibodies. P6 (1:400) and NCL-DYS2 (1:20) were used to detect different regions of the dystrophin molecule, and monoclonal antibodies NCL-a-SARC (1:100) and NCL-b-DG (1:50) (Novocastra Laboratories Ltd) were used to test for the presence of the DAPs α -SG and β -DG, respectively. Polyclonal antibody staining was carried out using the VECTASTAIN[®] ABC rabbit IgG kit, and monoclonal antibodies were HRP stained with the M.O.M.[™] kit (both from Vector Laboratories Ltd) according to the supplied protocols. For central nuclei counts, histological sections were stained with haematoxylin and eosin (H&E) in standard fashion. The percentage of centrally nucleated myofibres was determined by dividing the number of myofibres containing one or more centrally located nuclei by the total number of either dystrophin positive or dystrophin negative nucleated fibres. Myofibres with no nuclei in the plane of section were not counted. Approximately 1300 myofibres were counted for each different stain. Sections were photographed using a Leica DM IRB microscope.

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