Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

Strategies for Molecular Therapy of Duchenne Muscular Dystrophy

von
Patrick Dunant
aus
Düsseldorf

2003

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Prof. Dr. Ernst-Ludwig Winnacker betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig ohne unerlaubte Hilfe erarbeitet.

München, am 6. Juni 2003

Patrick Dunant

Dissertation eingereicht am:	6. Juni 2003
1. Gutachter:	Prof. Dr. Ernst-Ludwig Winnacker
2. Gutachter:	Priv.-Doz. Dr. Hanns Lochmüller
Mündliche Prüfung am:	26. Juni 2003

Die vorliegenden Studien wurde im Zeitraum vom Mai 1997 bis April 2003 unter Anleitung von Herrn Priv.-Doz. Dr. Hanns Lochmüller am Genzentrum der Ludwig-Maximilians-Universität München durchgeführt. Teile dieser Arbeit wurden bereits veröffentlicht:

Dunant, P., Larochelle, N., Thirion, C., Stucka, R., Ursu, D., Petrof, B.J., Wolf, E., and Lochmüller, H. (2003)
Expression of mini-dystrophin driven by the 1.35 kb MCK promoter ameliorates muscular dystrophy in fast, but not in slow muscles of transgenic mdx mice.
In Druck in *Molecular Therapy*

Dunant, P., Walter, M.C. , Karpati, G., and Lochmüller, H. (2003)
Gentamicin fails to increase dystrophin expression in dystrophin-deficient muscle.
Muscle & Nerve **27**: 624-627.

Brun, C., Suter D., Pauli, C., **Dunant, P.**, Lochmüller, H., Burgunder J.M., Schümperli, D., and Weis, J. (2003)
U7 snRNAs induce correction of mutated dystrophin pre-mRNA by exon skipping.
Cellular and Molecular Life Sciences **60**: 557-566.

Volpers, C., Thirion, C., Biermann, V., Hussmann, S., Kewes, H., **Dunant**, **P.,** Von der Mark, H., Herrmann, A., Kochanek, S., and Lochmüller, H. (2003)
Antibody-mediated targeting of an adenovirus vector modified to contain a synthetic immunoglobulin Ig-binding domain in the capsid.
Journal of Virology **77**: 2093-2104.

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SUMMARY

During the 20th century revolutionary breakthroughs in medicine were achieved: e.g. the advent of antibiotics and vaccinations have resulted in overcoming many infectious diseases. In contrast, a large number of genetic diseases remain with no effective treatment in place. These disorders are caused by inherited or spontaneous mutations that result in the absence or dysfunction of an indispensable gene product. Cure may require the reconstitution of the missing function through a molecular treatment approach. The aim of this study was to investigate two strategies for the molecular therapy of Duchenne muscular dystrophy (DMD), an X-linked fatal muscle wasting disease. Specifically, the muscle creatine kinase promoter as a muscle specific gene expression element for dystrophin gene therapy and the use of gentamicin to induce translational "read-through" of dystrophin point mutations were studied in detail (see below). This work provided important insights into possible treatment strategies for DMD, which may not only be helpful for future basic research projects but also for upcoming clinical trials. Additionally, these findings may not only apply to the therapy of DMD but also to the treatment of other genetic diseases such as cystic fibrosis or haemophilia.

The strategy for gene therapy of DMD is to introduce an additional copy of the dystrophin gene/cDNA into skeletal muscle fibres to protect them from necrosis and to prevent their eventual loss that leads to muscle weakness. The most promising gene delivery vehicles, viral vectors, suffer from several limitations including immunogenicity, loss of therapeutic gene expression, and a limited packaging capacity. Therefore, various efforts were undertaken to use small therapeutic genes and to place them under the control of a strong and muscle-specific promoter. One aim of this study was to examine the effects of a mini-dystrophin (6.3 kb) under the control of a short muscle-specific promoter (MCK 1.35 kb) over most of the lifetime (4-20 months) of a transgenic mouse model.

Dystrophin expression remained stable and muscle-specific at all ages and greatly ameliorated the dystrophic phenotype. Importantly, muscle function in limb muscles was significantly improved not only in young but also in aged transgenic mice as compared to non-transgenic littermates. Interestingly, dystrophin expression was strong in fast-twitch skeletal muscles such as *M. tibialis anterior* and *M. extensor digitorum longus* but weak or absent in heart, diaphragm and slow-twitch muscles. Additionally, expression was strong in

glycolytic but weak in oxidative fibres of fast-twitch muscles. In conclusion, the MCK promoter may be well suited for certain applications which require long-lasting and strong muscle specific gene expression such as DNA-vaccination or the production of soluble proteins in muscle tissue for metabolic engineering. For the gene therapy of DMD and other muscular dystrophies, the MCK promoter may be most efficient in fast skeletal muscles but may not be adequate for use in tissues such as heart and diaphragm.

An alternative strategy for the restoration of functional dystrophin is aimed at overcoming the deleterious mutation by interfering with protein translation. A recent report suggested that aminoglycoside antibiotics may restore the expression of functional dystrophin to skeletal muscles of mdx mice, the animal model of DMD. This raised hopes that DMD may be treatable by a conventional drug, and several clinical trials were initiated. The proposed mechanism relies on the ability of aminoglycosides to interfere with translation and thereby permitting “read-through” of premature stop codons. Therefore, we investigated the effect of gentamicin treatment on dystrophin expression and force generation. For *in vitro* experiments, an immortalised myogenic mdx cell line was established, and cells were treated with gentamicin. Additionally, mdx mice received direct gentamicin injections. We did neither detect significant “read-through”-effects nor positive effects on muscle histology and function by gentamicin treatment. Therefore, we believe that additional preclinical experimentation is required to further evaluate the possibility of *in vivo* aminoglycoside therapy of DMD or other diseases.

Part I

Background

DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is a X-linked fatal progressive muscle wasting disease. With an incidence of ~ 1 in 3.500 male new-borns it is the most common muscular dystrophy and one of the most common fatal hereditary diseases. It was first described by Duchenne (Duchenne, 1868) and Gowers (Gowers, 1879). Affected boys appear normal at birth. First clinical symptoms of the disease develop between 2 and 5 years of age. They include a delay in walking, difficulties in climbing stairs and a waddling gait (Dubowitz, 1978). A typical behavioural adaptation (Gowers´s sign) is that patients use the arms to "climb" up the own thighs, when changing from a lying to a standing position. Calf muscles show a marked pseudohypertrophy while all proximal muscles become progressively weaker. On average the patients become confined to a wheelchair by the age of 12. Before mechanical ventilation was introduced, most DMD patients died from respiratory or cardiac failure in the second or third decade of life. Becker muscular dystrophy (BMD) is a milder allelic form of the disease (Becker and Kiener, 1955). Even though the clinical symptoms are similar to DMD they are significantly delayed. Some BMD patients remain ambulant until old age (England et al., 1990). Dystrophin is not only indispensable to guarantee muscle function, but also plays a role in the brain. This role is poorly understood presently, but absence of dystrophin in DMD may result in a mild cognitive impairment (Blake and Kroger, 2000; Mehler, 2000). Histological hallmarks of the disease in skeletal muscle are: fibre size variation, clusters of necrotic fibres, central nuclei in regenerating fibres, fibrosis, and infiltration of macrophages and CD4+ lymphocytes (Gorospe et al., 1990; McDouall et al., 1990; Blake et al., 2002). The progressive nature of the disease results from the inability of the affected muscle to efficiently replace damaged muscle fibres. This is mainly due to an exhaustion of the

potential to regenerate fibres from muscle progenitor cells, in concert with the gradual replacement of muscle fibres by connective tissue (Reimann at al., 2000). A useful, although unspecific diagnostic marker even before the onset of a clinical phenotype is the strongly increased serum creatine phosphokinase activity. A definite diagnosis for DMD is based either on the absence of dystrophin protein in a muscle biopsy or on the molecular identification of a mutation in the dystrophin gene. About 65% of dystrophin mutations are large deletions and duplications which occur preferentially at two mutation "hot spots" (Koenig at al., 1989). The remaining mutations are evenly distributed over the whole gene: 18% are nonsense mutations, 8% small deletions or insertions, 7% splice site mutations, and 2% are missense mutations (Roberts et al., 1994). According to the "reading frame theory" (Monaco et al., 1988) a deletion or duplication causing a frame shift leads to a premature termination of translation. The resulting dystrophin protein is truncated, not functional, and due to low mRNA and protein stability only present at very low levels. Patients with such mutations will develop DMD. Surprisingly, even these patients show a small subset of dystrophin positive fibres. The occurrence of so-called revertant fibres is directly associated with the nature of the mutation in individual patients. While some mutations allow revertant fibres others do not. A natural exon skipping mechanism was proposed to be the underlying molecular mechanism. Overall, revertant fibres are detected in approximately 50% of DMD patients and can account for 0.2 – 4 % of the total number of muscle fibres (Burrow et al., 1991; Nicholson et al., 1993).

In BMD, the mutations usually cause the loss of a small, non-essential part of the molecule while the reading frame stays intact. This allows the expression of smaller dystrophin molecules. The resulting dystrophins can have varying degrees of functionality, depending on the size and the position of the deleted sequence. This is reflected by the wide spectrum of the clinical severity of BMD patients. Female carriers are not or only mildly affected because they are normally able to compensate the defect via the functional dystrophin copy on their second X chromosome. Current treatment options for DMD and BMD are very limited. No effective pharmacological treatment is available so far while supportive therapies like artificial ventilation have moderately prolonged patients' survival but offer no cure (Scheuerbrandt, 1998).

DYSTROPHIN

The molecular cause of DMD is a defect in a single gene located at Xp21 (Murray et al., 1982). The dystrophin gene encompasses 2.4 Mb, contains 79 exons, and is one of the largest human genes (Coffey et al., 1992, Nobile et al., 1997). The full-length dystrophin protein has a molecular weight of 427 kDa and is translated from a 14kb mRNA. Expression is regulated by three independent promoters which have different activities e.g. in muscle and in brain (Boyce et al., 1991; Chelly et al., 1990; Klamut et al., 1990). These promoters use unique first exons which are spliced to the commonly used remaining 78 exons. Four additional internal promoters express smaller dystrophin molecules which lack large N-terminal parts (Fig. 1-1).

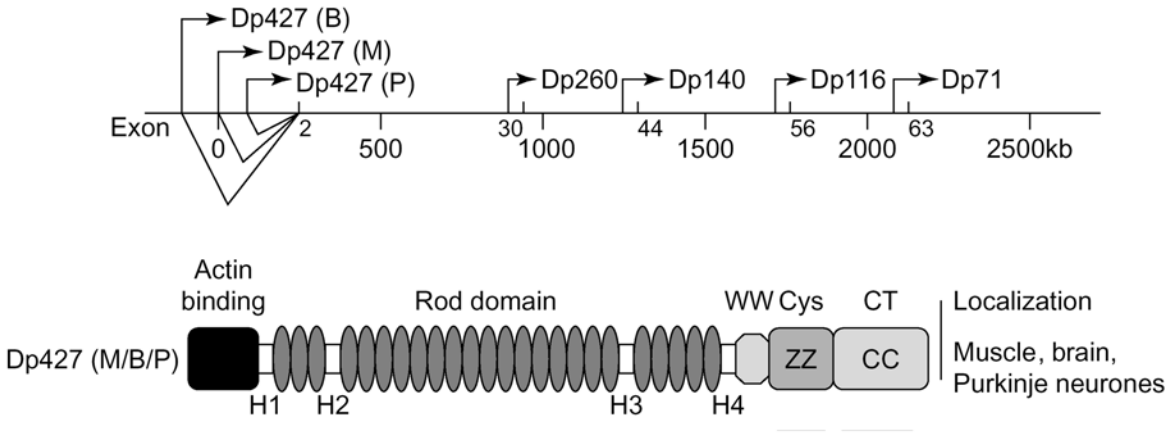


Fig. 1-1 Location of dystrophin promoters and organisation of functional domains. Three promoters (brain (B), muscle (M) and Purkinje neurones (P)) drive the expression of full-length dystrophin. Four additional internal promoters produce shortened dystrophins (Dp260, Dp140, Dp116, Dp71). The rod domain consists of 24 triple helical units and four hinge regions (H). Dystrophin contains a number of protein domains that form the binding sites for components of the dystrophin-associated protein complex. These are the WW domain (WW) the ZZ domain (ZZ) in the cysteine-rich region and the coiled-coil domain at the COOH Terminus (CT). (Adapted from Blake and Kroger, 2000.)

The most common short dystrophin isoform is Dp71 (71 kDa) which is found in many non-muscle tissues: e.g. in brain, lung, liver and kidney. However, Dp71 cannot replace full-length dystrophin in skeletal muscle (Cox et al., 1994). The number of isoforms is further

increased through alternative splicing at the 3'-end which may regulate the binding of several dystrophin-associated proteins (Crawford et al., 2000).

In skeletal muscle, dystrophin makes up approximately 0.002 % of the total protein and is located at the interior side of the sarcolemma. It is rod-shaped and contains 3685 amino acids which are organised into four different functional domains (Fig.1-1): the N-terminal actin-binding domain, the large rod domain, the cysteine-rich domain and the COOH-terminal domain. The N-terminal domain has an actin-binding site (amino acids 1-220) whose crystal structure has been resolved in detail (Norwood et al., 2000) and which is homologous to the structures of α-actinin and β-spectrin. The rod domain (amino acids 338-3,055) consists of 24 triple helical repeats of the spectrin type. These make up the majority of the total protein's size and are responsible for its rod shape (Koenig et al., 1988). It is believed that the rod domain acts as a spacer between the other domains. In addition to the repeat regions it contains four small proline-rich hinges which might confer some flexibility and a second actin-binding site (Rybakova et al., 1996; Koenig and Kunkel, 1990). The cystein-rich domain (amino acids 3,056-3,354) is crucial for the correct assembly of the complex of dystrophin-associated proteins. It contains several protein binding and regulatory motives: 1) A WW domain that binds proline rich substrates and is known from regulatory and signalling molecules (Bork and Sudol, 1994), 2) Two EF-hand motivs that resemble those in α-actinin and may bind intracellular Ca^{2+}, 3) A ZZ motive that resembles zinc finger motives and was shown to bind calmodulin in a Ca^{2+} dependent manner (Ponting et al., 1996). The fourth domain of the dystrophin molecule is the COOH-terminal region (amino acids 3,355-3,685). This region contains two α-helical coiled coil domains (Sadoulet-Puccio et al., 1997) and a region which is alternatively spliced in non-skeletal muscle tissues such as brain and cardiac muscle.

THE DYSTROPHIN-ASSOCIATED PROTEIN COMPLEX

In skeletal muscle, dystrophin is located at the cytoplasmatic site of the sarcolemma. Dystrophin binds to a complex of dystrophin-associated proteins (DAPC) which spans the sarcolemma. Together dystrophin and the DAPC provide a bridge between the extracellular matrix and the actin-based cytoskeleton. Dystrophin plays an essential part in the formation of the DAPC, because dystrophin deficiency leads to a loss of the DAPC. In the absence of dystrophin mRNA levels of DAPC components are normal, but the complex is not properly assembled and/or integrated into the sarcolemma or is degraded. A truncated dystrophin which contains the cysteine-rich domain, but not the N-terminal actin-binding site allows

the assembly of the DAPC. But importantly, when the restoration of the DAPC is mediated by this mutated dystrophin, the presence of the DAPC is not sufficient to prevent the DMD phenotype (Cox et al., 1994). This shows that both, the interaction with the DAPC and the connection to the actin-cytoskeleton via the actin-binding site are essential functions of the dystrophin molecule. The DAPC consists of three separate sub-complexes based on their localisation within the cell and their physical association with each other (Fig.1-2): the dystroglycan complex, the sarcoglycan complex and the cytoplasmatic complex (Blake et al., 2002).

The two most important components of the dystroglycan complex are α- and β-dystroglycan. They are both transcribed from a single dystroglycan gene and are subsequently proteolytically processed from a precurser protein (Ibraghimov-Beskrovnaya et al., 1993). α-dystroglycan is located on the extracellular site of the sarcolemma. It binds to several extracelluar matrix proteins like agrin or laminins. It is anchored to the membrane via a binding site to β-dystroglycan which has a single transmembrane domain and spans the sarcolemma. The COOH-terminus of β-dystroglycan binds to dystrophin. Crystal structure analysis of a β-dystroglycan/dystrophin complex revealed that β-dystroglycan interacts with the WW domain and the EF-hands of dystrophin (Huang et al., 2000).

The sarcoglycan complex consists of five transmembrane proteins: α-, β-, γ- and δ-sarcoglycan and sarcospan. The molecular interactions between the sarcoglycans and between them and the other components of the DAPC are not well understood. Mutations of the sarcoglycans are the cause of autosomal-recessive limb-girdle dystrophies (Straub and Campbell, 1997; Ozawa et al., 1998).

The third component of the DAPC is the cytoplasmatic complex which contains the syntrophins and the dystrobrevins among other proteins. Five members of the syntrophin family (Piluso et al., 2000) of proteins are known: α1, β1, β2, γ1 and γ2. They are differentially expressed in different muscles, but all syntrophins share similar functional domains: a pleckstrin type PH-domain, a intact PH-domain, a syntrophin unique region and a PDZ-domain. The PDZ-domain interacts with several other proteins e.g. ErbB4, Na-channels and nNOS (Brenman et al., 1996; Blake and Kroger, 2000).

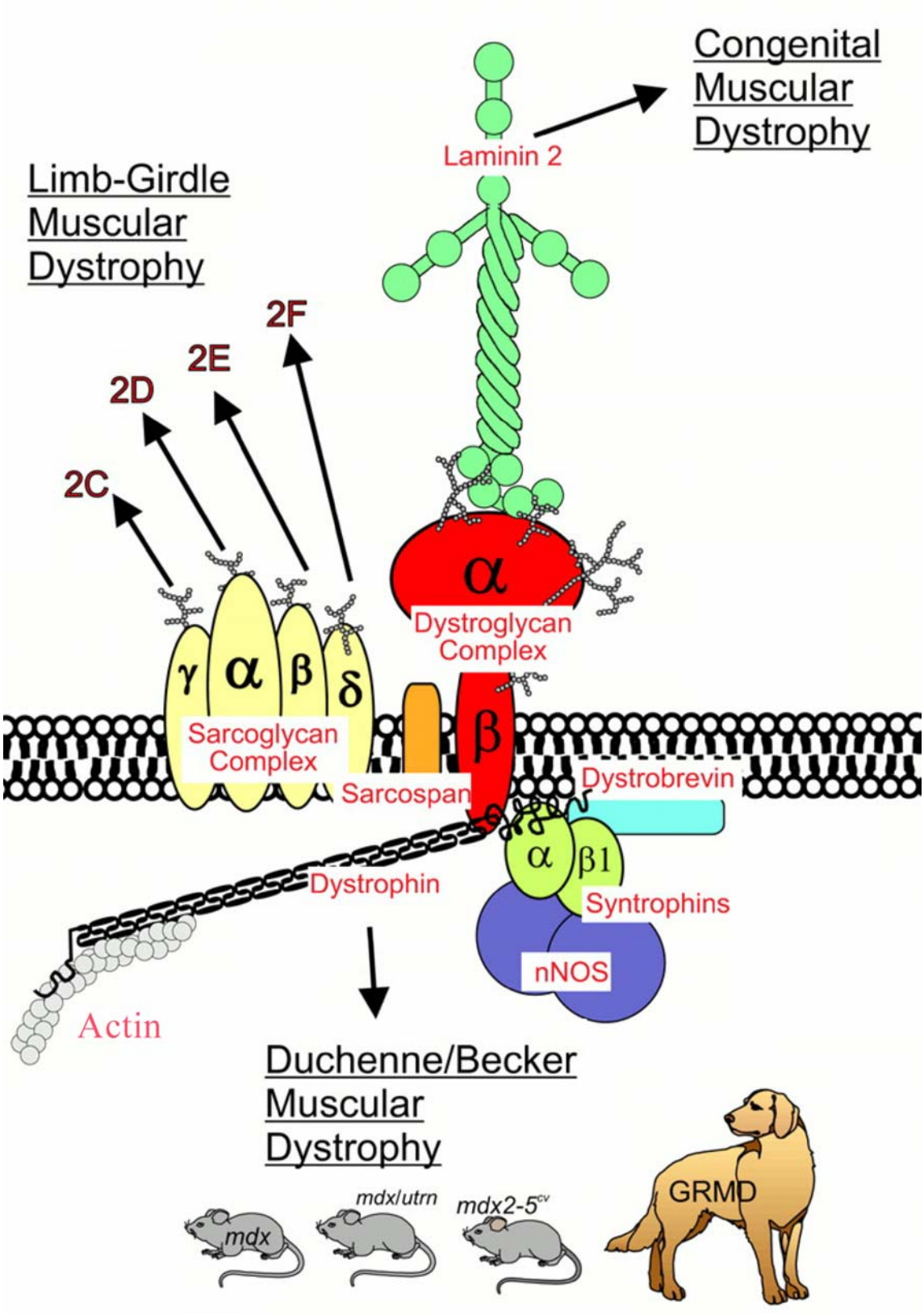


Fig. 1-2 **Organisation of the dystrophin-associated protein complex (DAPC) at the sarcolemma of skeletal muscle.** Muscular dystrophies that result from mutations in different parts of the complex are indicated. Currently used animal models for the gene therapy of Duchenne muscular dystrophy are also shown. (Adapted from Allamand and Campbell, 2000)

The dystrobrevins are dystrophin-related proteins as they have significant sequence homology to the COOH-terminal part of dystrophin (Wagner et al., 1993; Roberts, 2001) . There are two independent dystrobrevin genes each encoding several isoforms (Sadoulet-Puccio et al., 1996; Peters et al., 1997). α-dystrobrevins are expressed in muscle and the central nervous system while β-dystrobrevin is missing in muscle, but found in brain and other tissues, such as placenta and kidney. The exact function of α-dystrobrevins in skeletal muscle is unknown, but they may play a role in intracellular signal transduction.

UTROPHIN

Dystrophin and the dystrobrevins are not the only members of the dystrophin/dystrobrevin protein family. Vertebrates possess two other closely related proteins: utrophin and dystrophin-related protein 2 (Roberts et al., 1996). The utrophin gene is autosomally encoded (chromosome 6q24) and smaller than the dystrophin gene ~900 kb vs. 2,400 kb. Both genes share an almost identical intron-exon organisation, and it is very likely that they are paralogs that arose by duplication early in vertebrate evolution (Roberts and Bobrow, 1998).

The size and functional domain organisation of the two proteins are very similar. The utrophin protein has 3,433 amino acids and a molecular mass of 395 kDa. Most of the size difference to dystrophin results from a shorter rod-domain that contains 22 instead of 24 repeats (dystrophin repeats 14 and 18 are missing in utrophin). Utrophin expression is regulated by several promoters (Burton et al., 1999; Dennis et al., 1996). The expression pattern of utrophin is different from dystrophin. Utrophin is generally more widely expressed, hence it's name. It is found in non-muscle and in muscle cells. However, unlike dystrophin utrophin is not located at the sarcolemma, but at the neuromuscular and myotendinous junctions (Khurana et al., 1991). Utrophin knockout mice were normal except for reduced membran folding at the neuromuscular junction. Interestingly, dystrophin/utrophin double knockout mice showed a more severe phenotype (these mice die very prematurely at 20 weeks of age) than single knockout mice (Deconinck et al., 1997; Grady et al., 1997). These data suggest that dystrophin and utrophin can compensate for each other. It was also shown that overexpression of utrophin in muscle prevents the dystrophic phenotype in dystrophin deficient mice (Tinsley et al., 1996; Tinsley et al., 1998). Therefore, increased expression of utrophin at the sarcolemma may offer an

alternative therapeutic approach, and several strategies to achieve this goal are under development (Chaubourt et al., 1999; Corbi et al., 2000).

FUNCTION OF DYSTROPHIN

In depth studies of natural and intentionally induced mutations of dystrophin were undertaken to elucidate the function of the whole molecule, the functions of individual domains and interactions with other molecules. Because most gene therapy vectors have packaging size limitations for the therapeutic gene, a shortened dystrophin would be highly desirable. The size of dystrophin would need to be minimised without compromising functionality. Therefore, various experiments have been undertaken to answer the following question: what are the essential parts of the dystrophin protein and which parts might be dispensable and could be removed? The N-terminal domain and the cysteine-rich domain were shown to contain vital elements for optimal dystrophin function (Corrado et al., 1996). In contrast, the COOH-terminal region may be removed without major negative consequences (Rafael et al., 1996; Crawford et al., 2000) The region with the biggest potential for significant size reduction is the rod domain which makes up 74% of the full-length molecule. Natural occurring large deletions of the rod domain were identified in BMD patients with very mild phenotypes (Love et al., 1991; Winnard, 1993; Mirabella et al., 1998). A shortened dystrophin with a large in-frame deletion in the rod-domain (exons 17-48) based on a mutation found in such a patient was widely used in gene therapy trials (England et al., 1990). It is 46% shorter than the full-length dystrophin and is referred to as mini-dystrophin. In animal studies, it almost completely restored a normal muscle phenotype in the absence of full-length dystrophin (Cox et al., 1993; Wells et al., 1995). Recently, dystrophin constructs with even larger deletions in the rod domain and deletion of the COOH-terminus were developed (Harper et al., 2002; Scott et al., 2002). Some of these micro-dystrophins contained only four of the inverted terminal repeats. Their size was reduced by 74% when compared to full-length dystrophin. Even though micro-dystrophins correct most abnormalities related to dystrophin-deficiency, they do not restore full muscle strength. Therefore, the rod domain may not be completely removed to allow optimal function of dystrophin.

Surprisingly, the exact role of dystrophin and the mechanisms by which its absence leads to the observed phenotype remain enigmatic. Several hypotheses have been proposed. And a

combination of different mechanisms may contribute to the pathogenesis including increased membrane permeability, elevated Ca^{2+} levels, proteolysis, oxidative damage, inflammation, apoptosis, regeneration, and fibrosis (Fig. 1-3).

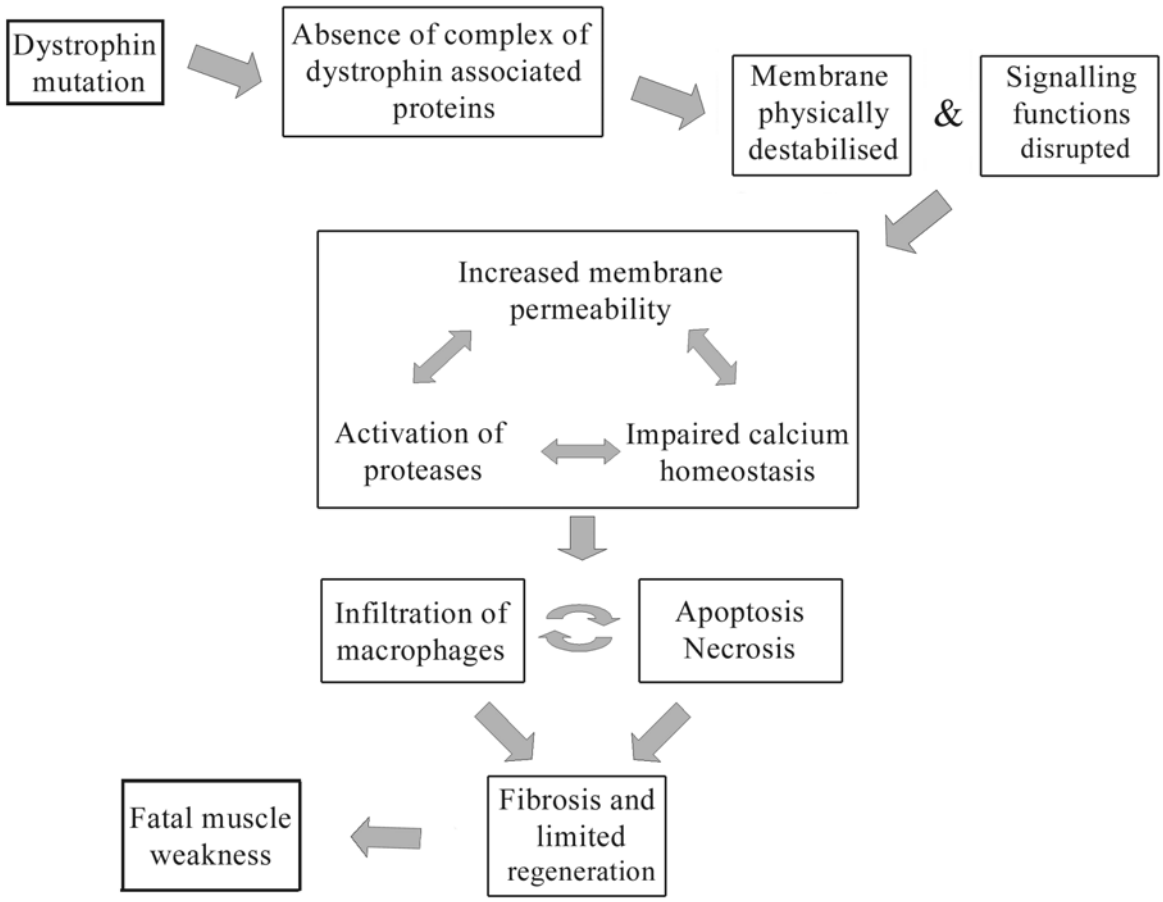


Fig. 1-3 The pathophysiology of Duchenne muscular dystrophy. Dystrophin deficiency results in the absence of the complex of dystrophin-associated proteins (DAPC). Muscle fibres become vulnerable to mechanical stress through the loss of mechanical stability and/or the loss of some so far unknown signalling capability. Increased membrane permeability, an impaired calcium homeostasis and activation of proteases contribute to increased levels of cell death. In the long term, insufficient regeneration and progressive fibrosis lead to an ultimately fatal muscle weakness.

One of the crucial mechanisms shall be shortly discussed: Membranes of dystrophin deficient fibres have an increased permeability to macromolecules. This was shown in experiments where exogenous vital dyes such as Evans blue or Procion orange normally

excluded from muscle cells were internalised in dystrophin deficient cells (Bradley and Fulthorpe, 1978; Petrof et al., 1993; McArdle et al., 1994).

This membrane permeability increases when the muscle is exercised. This may be an indicator of a structural weakness of the membranes which cannot withstand the physical forces which arise with muscle exercise. This idea is supported by several observations: heavy exercise leads to increased levels of muscle cell necrosis and infiltration of macrophages in muscles of DMD patients while inactivity reduces myonecrosis (Kimura et al., 1998). In the mouse model for DMD a susceptibility of muscles to contraction-induced stress was also observed. The first histological evidence of muscle damage in dystrophin-deficient mice is found 2-3 weeks after birth when the mice start to move around to find there own food. In contrast, immobilisation or denervation of muscles prevents the development of muscle damage in mice.

Alternatively a not yet identified signalling role of dystrophin together with the increased permeability may disrupt the normal balance of molecules inside and outside the cell. Elevated Ca^{2+} levels in muscle fibres of DMD patients may be of particular interest (Cullen and Fulthorpe, 1975). Calcium homeostasis plays an important regulatory role in muscle (Berchtold et al., 2000). Ca^{2+} levels may be increased not only by the increase in membrane permeability but also through leaky calcium channels (Carlson and Officer, 1996).

ANIMAL MODELS FOR DMD

Animal models have provided valuable clues to the understanding of DMD pathogenesis and offer good opportunities for the development and testing of therapeutic approaches (Allamand and Campbell, 2000). Several species of animals have been identified in which mutations in the dystrophin gene (murine, canine and feline) lead to the absence of dystrophin and to the development of dystrophy with varying degrees of severity. The mdx mouse (murine dystrophy x-linked) is the most common murine model for DMD. A point mutation in the dystrophin gene (position 3185) results in a premature stop codon (**C**AA -> **T**AA) (Sicinski et al., 1989). Skeletal muscle fibres are therefore dystrophin-negative with the exception of a small number of revertant fibres (about 1%). Surprisingly, the clinical phenotype in mdx mice is much more benign than in human DMD patients (Gillis, 1999). In captivity these animals have a nearly normal life expectancy and move normally at old ages. The reason for this marked difference in pathology between human and murine dystrophin deficiency is not understood. The mild phenotype of mdx mice leads to two

problems: firstly, it is more difficult to access the success of a therapeutic intervention, and secondly, one should be cautious to extrapolate results from mdx mice to human patients. Some features of DMD are also observed and can be evaluated in mdx mice. Creatine kinase activity levels are highly elevated. Necrosis-regeneration circles of muscle fibres start at 15-21 days of age and are still present in animals older than 2 years, peaking between the ages of 45 and 60 days. There is also a pronounced inflammatory reaction with invasion of immune cells into the muscle. Generally, some muscles are more affected than others. Histopathological signs and muscle force deficits are especially pronounced in diaphragm. On the contrary, in muscles which are composed mainly of fibres of small diameter such as extra-ocular muscles (Karpati and Carpenter, 1986) or smooth muscle (Boland et al., 1995) pathological signs are absent. A typical feature of regenerated muscle are centrally located nuclei. Under normal conditions nuclei return to the periphery of the muscle fibre after a regeneration process is completed. In mdx muscles very high levels of central nuclei were observed over the entire lifespan. It is not known why the nuclei remain central in mdx mice, but central nucleation is an easily measurable and well accepted indicator of muscle pathology in mdx mice.

The dog model of DMD is golden retriever muscular dystrophy (GRMD). It is caused by a point mutation in the consensus splice acceptor site in intron 6 of the canine dystrophin gene. The mutation results in the deletion of exon from the mRNA thereby introducing a frame shift which leads to a premature stop codon. Affected pups gain weight slower than unaffected siblings. Hind limbs become increasingly affected and the animals often advance them simultaneously (bunny hopping). Many of the affected dogs die within days or months (Cooper et al., 1988). The severe clinical phenotype, the accompanying histopathological progression and the larger size of the animals make the GRMD dogs a good model for DMD (Howell et al., 1997; Cozzi et al., 2001). However limitations arise from observations that these dogs are difficult to breed and that there are large variations in the development of the phenotype e.g. some of the dogs live for 6 and more years. Hypertrophic feline muscular dystrophy (HFMD) has been described in dystrophin deficient cats (Carpenter et al., 1989; Gaschen and Burgunder, 2001). Hypertrophy is the most notable effect in affected animal. An abnormal gait and some necrosis are present but the overall phenotype is benign.

MOLECULAR THERAPY FOR DMD

DMD is the most common fatal muscle disease and affects hundreds of thousands patients world-wide. Because no effective treatments are available, great efforts are underway to develop a cure. Once an effective approach is established it might be applicable with modifications in large numbers of other heritable diseases. Several different strategies could be envisioned for the treatment of DMD (Fig. 1-4).

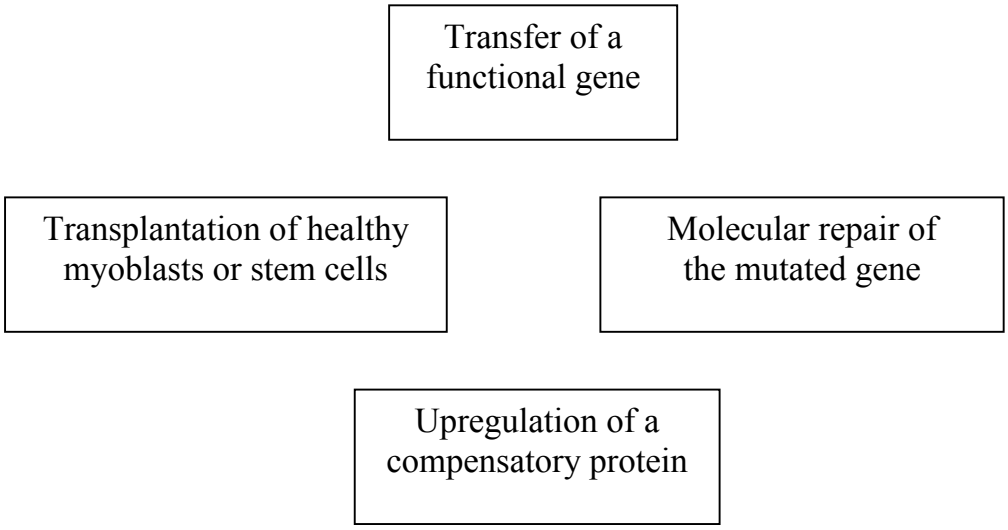


Fig. 1-4 Treatment strategies for muscular dystrophies.

For an optimal effect, a therapy has to fulfil the following requirements: 1) all affected cells should be reached, 2) the treatment should completely cure the phenotype while having no side-effects, 3) the treatment should work over the whole life time of a patient.

Reaching all affected cells may prove a great challenge in DMD: firstly because of the sheer mass of the affected cells and secondly because of the relative inaccessibility of several muscles such as diaphragm, heart, and brain. Optimal function could be restored either by replacing the defect dystrophin with a functional copy or by "repairing" the mutated dystrophin (see below). Alternatively, other molecules could take over missing dystrophin functions. Possible candidates to stabilise the membrane are utrophin or the extracellular matrix protein $\alpha 7\beta 1$ integrin (Tinsley et al., 1998; Burkin et al., 2001). To guarantee a life long effect the treatment either needs to be long-lasting or allow for easy readministration.

One avenue for the successful treatment of DMD would be to substitute the defect dystrophin gene with a functional therapeutical gene. Methods for *in vivo* gene delivery to skeletal muscle fibres are either based on naked DNA transfer or on virally mediated gene transfer. High-level and long-lasting gene expression has been reached by the development of improved naked DNA transfer techniques: intravascular injections with high pressure (Budker et al., 1998), optimised electroporation conditions (Vicat et al., 2000) and use of ultrasound (Danialou et al., 2002). Several viral vector systems for gene therapy of muscle diseases are currently under development: adenovirus (Ad), adeno-associated virus (AAV) retroviruses, and herpes simplex virus (HSV). They all have distinct advantages and disadvantages which will be shortly discussed for the two most important vectors Ad and AAV.

Ad vectors have a large cloning capacity, can be produced at high titers (1012-1013) and infect muscle fibres relative efficiently (Yang et al., 1998). The latest generation of Ad vectors are devoid of any viral sequences (Kochanek et al., 1996). This allows a maximal cloning capacity of >30 kb. It was envisaged that these "gutted" Ads will elicit a weaker immune response than earlier Ad vectors which still contain viral genes. Ad gene therapy vectors do not integrate into the genome of the target cells. This is an advantage because of safety considerations but it is unclear whether long term gene expression (> 1 year) can be achieved.

AAV vectors hold great promise, because they infect muscle well, they show little immunogenicity, they can be produced at high titers (1012-1013) and they have the potential of site specific integration (Xiao et al., 1996; Fisher et al., 1997). Unfortunately, they have a relatively small packaging capacity of maximum 5 kb. It is not yet clear if micro-dystrophins of this size are completely functional (Harper et al., 2002).

An alternative to the delivery of genes into muscle fibres is the transplantation of myogenic cells. These could be either myoblasts or myogenic stem cells. Myoblast therapy was already attempted in clinical trials, but the results were disappointing (Partridge et al., 1998). It is hampered by the poor survival of the injected myoblasts. Only about 1% of the transplanted cells contribute to the formation of new muscle (Beauchamp et al., 1999). The poor survival is partly due to an immune response against the myoblasts and the therapeutic

protein (Guerette et al., 1997). Immunsuppresion of mdx mice has resulted in prolonged survival and even some functional improvement after myoblast transfer (Brussee, 1998). Recent advancements in stem cell research raised the hope that it might be possible to identify muscle progenitor cells that could not only be used for local injection but also for systemic application. Two reports describe that bone marrow transplantation of stem cells resulted in low level transgenic dystrophin expression in skeletal muscle (Bittner et al., 1999; Gussoni et al., 1999). Clinical therapy of DMD would presumably require two steps prior to the application of the cells. Firstly, suitable stem cells would have to be isolated from the patient. And secondly, the therapeutic gene would need to be integrated into these stem cells. Lentiviral vectors could be used for this purpose because they efficiently infect a variety of stem cells (Van den Driessche et al., 2002).

The goal of gene repair is not to replace the dysfunctional gene, but to allow the production of a functional protein through molecular manipulations which overcome a deleterious mutation. Therefore, this techniques will be limited to patients with mutations where all the functional domains of dystrophin are preserved, but the mutations prevent correct expression. It is estimated that up to 30-50% of DMD patients fall into this category. Depending on the nature of the mutation, one of the following strategies might be suitable: single base pair repair, targeted exon skipping or manipulations of the translation machinery.

The repair of point mutations in the dystrophin gene has recently been demonstrated in cultured mdx cells and in muscles of mdx mice (Bertoni and Rando, 2002) This single base pair repair is mediated by so called chimeraplasts, which are single stranded RNA/DNA oligonucleotides. They bind in a first step to the targeted sequence and then in a second step trigger the repair of the mutation through the endogenous DNA repair machinery (Bartlett et al., 2000) In theory, this method would allow a permanent correction of all kinds of point mutations and therefore is a very general and promising approach. However, efficiencies have to be significantly improved because so far only 1-2% of all fibres per muscle were repaired which is not enough for clinical improvement.
A different approach to overcome point mutations which introduce a premature stop codon is the manipulation of the translation machinery. This strategy will be examined in detail in Chapter IV.

Another possibility is using the mechanism of RNA-processing, e.g. to skip a stop codon mutation or to restore an open reading frame after a frame shift mutation. Even though the resulting proteins are predicted to be slightly smaller than the full-length dystrophin, they may nevertheless be highly functional, comparable to the functionality of Becker type dystrophins. A similar phenomenon occurs naturally *in vivo*: a small amount of dystrophin positive (revertant) fibres is regularly found in muscles of DMD patients. The exact mechanism of reversion is not understood, but a detailed study of revertant fibres in mdx muscle indicated that exon skipping was involved: several alternatively spliced dystrophin mRNA species were found which lacked the mdx premature stop codon in exon 23, while the stop codon could be still detected in the genomic dystrophin sequence of the dystrophin positive fibres (Wilton et al., 1997; Lu et al., 2000). To specifically induce exon skipping of mutations in DMD patients antisense oligonucleotides (AOs) were used. Successful skipping of the mdx stop codon (in exon 23 of the mouse dystrophin) mutation has already been demonstrated *in vitro* and *in vivo* when AOs specific for the junction of intron 22 and exon 23 were applied (Dunckley et al., 1998; Mann et al., 2001).

An alternative to the repair of the mutated dystrophin or the transfer of a therapeutical gene is to upregulate a compensatory protein in affected cells. A natural candidate for such a purpose is the dystrophin paralog utrophin (Tinsley and Davies, 1993). Utrophin is already moderately upregulated in dystrophin deficient muscle. This upregulation was also detected in muscles of mdx mice. It was shown that utrophin can functionally replace dystrophin in numerous transgenic and viral-gene transfer studies (Tinsley et al., 1996; Wakefield et al., 2000). To facilitate the improvement of utrophin expression in mature muscle fibres its regulatory elements were studied in great detail. This led to the discovery of a novel additional utrophin promoter (Burton et al., 1999). Large-scale screenings for small molecules that may specifically upregulate utrophin are currently under way.

IMMUNE REACTIONS AGAINST TRANSGENE AND VECTOR

Presently, immune reactions either against the vector system or the transgene itself are among the major obstacles which slow down the development of new therapies for DMD and genetic disorders in general. They can block or reduce the extent of gene transfer, hinder reapplication, result in the clearance of already cured cells, and cause systemic

reactions that in severe cases can lead to death (Yang et al., 1996; Morral et al., 2002). Several strategies may help to minimise these adverse immune reactions (Fig. 1-5).

It is important to note that the dystrophin protein itself is potentially immunogenic in DMD patients. There is even concern that forced expression of "non-self" epitopes associated with therapeutic dystrophin may not only compromise long-term dystrophin expression, but even worsen muscle function by stimulating a cytotoxic T-cell mediated destruction of myofibres which express dystrophin (Ebihara et al., 2000). Depending on the kind and number of revertant fibres which might reduce dystrophin immunogenicity there might also be variations between different patients. Several studies reported an immune response in mdx mice against human dystrophin constructs (Ferrer et al., 2000; Braun et al., 2000). Another study showed that also murine dystrophin acted as a transplantation rejection antigen in mdx mice (Ohtsuka et al., 1998). One method to circumvent this problem may be the use of therapeutic utrophin constructs which are not immunogenic due to the normal or even increased utrophin expression in dystrophin deficiency.

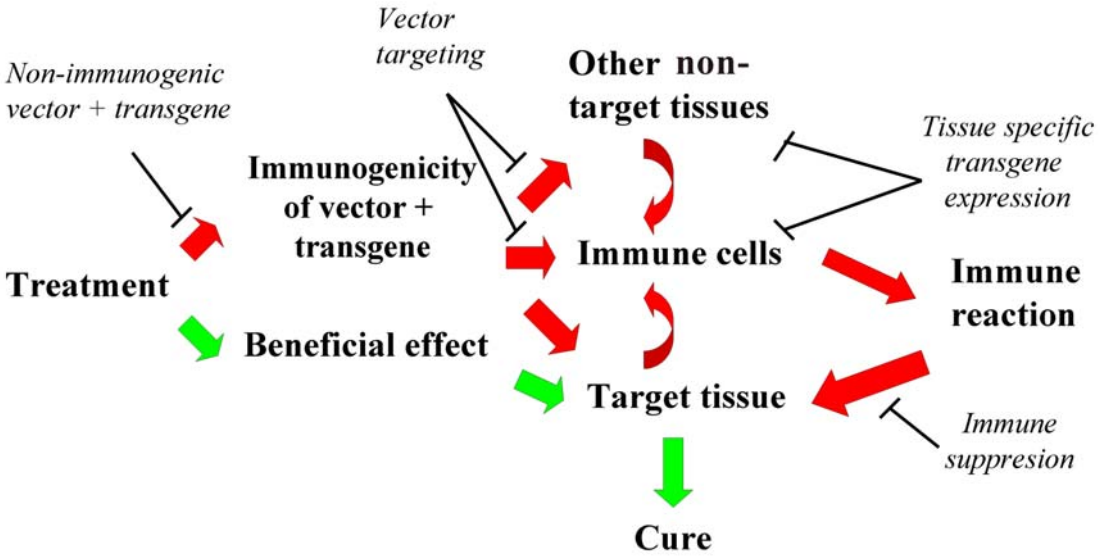


Fig. 1-5 Immune reactions and counter measures.

The immune response against viral proteins of the vector may be very strong, but is usually transient, especially when AAV and gutted Ad vectors are used which contain no viral genes. Consequently there is no *de novo* viral protein synthesis and only for a short time the immune system is exposed to the viral proteins of the delivering viral vectors. Therefore, a transient immune suppression might be sufficient to avoid an immune response against the vector (Chamberlain, 2002).

The immune response can be divided in innate and acquired defense mechanisms. Innate immune responses are thought only to be directed against certain vectors and delivery routes (i.e. high doses of systemically injected adenoviral vectors) while not affecting other applications. Acquired immune responses can be differentiated in humoral (B-cell) and cellular (T-cell) pathways. A humoral immune response is based on circulating antibodies against newly introduced epitopes. While transgene persistence is normally not compromised by these antibodies, they can prevent successful readministration (Fisher et al., 2001). This problem may be circumvented by switching to different viral serotypes for successive administrations (Morral et al., 1999). The cellular immune response seems to be the main culprit responsible for the rapid declines of transgene expression in transfected muscle that has been observed in many gene transfer studies. The destruction of dystrophin-expressing cells in mdx mice is mediated through cytotoxic CD8+ cells. To become fully active, these cells require presentation of the antigen via the MHC I pathway. Because muscle fibres themselves only have low levels of MHC I and also lack important costimulatory molecules they require the help of professional antigen presenting cells e.g. dendritic cells for maximal activation of cytotoxic T cells. Ads readily infect macrophages and dendritic cells (Jooss et al., 1998). But AAV vectors (which have a low tropism for dendritic cells) evoked a cellular immune response against the transgene (Cordier et al., 2000). It seems that the APCs receive antigen from transfected non-muscle cells or myofibres (Fig. 1-5) and then are able to present antigens on MHC I molecules via a cross-priming pathway (Ulmer et al., 1996; Sarukhan et al., 2001). These effects may be avoided or diminished by the use of a muscle specific promoter which limits expression of the transgene to muscle cells.

Not only the nature of the vector and the transgene influence the strength of the immune reaction but also the condition of the targeted muscle. The immunogenicity associated e.g. with AAV was more pronounced in dystrophic muscle than in normal muscle. In dystrophic muscles leaky or dying muscle fibres may release increased amounts of cytoplasmatically localised antigen and immune effector cells are present at elevated levels (Chamberlain, 2002; Yuasa et al., 2002).

PART II

Materials and Methods

GENERATION OF DYSTROPHIN TRANSGENIC MDX MICE

All animal experiments were conducted in accordance with recommendations of the institutional animal care committee and in agreement with provincial and federal law. A *Sac*II/*Cla*I fragment from plasmid pAdMCKBecker (Larochelle et al., 2002) was microinjected into pronuclei of B6D2F2 zygotes using standard procedures (Hogan et al., 1989). This fragment contains a previously described (Larochelle et al., 1997) short version of the MCK promoter and enhancer (1.35 kb; from nucleotide position –1354 to +1; transcription initiation site at np 0), a "Becker-type" mini-dystrophin with a large in-frame rod deletion (6.3kb), and the minimal rabbit beta-globin poly-A-signal (110 bp) (Jani et al., 1997). DNA was extracted from mouse-tails, and founder animals were identified by PCR using the primer set: 5´-CCATGGGCAAACTGTAT and 5´-GGTAAGTTCTGTCCAAGC. After 2 min at 94°C the thermocycler was programmed for 3 cycles of 94°C x 1 min, 55°C x 1 min, 72°C x 2 min, then 30 cycles of 94°C x 50 s, 55°C x 50 s, 72°C x 50 s. Primers correspond to dystrophin sequences adjacent to the 5'- and 3'- breakpoints of the large rod deletion. A 506 bp fragment is amplified from mini-dystrophin but not from full-length dystrophin.

To ascertain whether the MCK promoter was integrated and intact in transgenic mice, a 1.6 kb fragment was amplified from genomic DNA using the primers 5´-CTGGTGCGGGTCTCATCGTA and 5´-CAGTCCTCTACTTCTTCCCA. After 2 minutes at 94°C the thermocycler was programmed for 35 cycles of 94°C x 1 min, 54°C x 2 min, 72°C x 3 min. The primers correspond to transgene sequences that flank the 1.35 kb MCK promoter/enhancer; they do not amplify sequences from the endogenous MCK

promoter/enhancer. The 1.6kb PCR fragment was sequenced using additional internal primers.

Breeding of the transgene onto the mdx background was established by crossing dystrophin transgenic male founders (F0) with mdx females. Transgenic male offspring (F1/N1) were bred with female mdx to generate male and female offspring (N2). These animals are negative for wild-type dystrophin and were used in the experiments described below. To eliminate any effects that might be due to minor differences in the genetic background, transgenic animals were always compared to non-transgenic littermates.

DYSTROPHIN IMMUNOBLOTTING

Frozen tissue samples were homogenised in RIPA buffer (Bulman et al., 1991). Protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A). Equal amounts of total protein were loaded onto 5.5% polyacrylamide gels and transferred to a nitro-cellulose membrane. Dystrophin expression was detected using an affinity-purified, polyclonal antiserum raised against 17 amino acids at the C-terminus of dystrophin (dilution 1:50; kindly provided by Dr. Paul Holland, Montreal) and visualised with anti-rabbit secondary antibody linked to horseradish peroxidase (Dianova, Hamburg, Germany; dilution 1:100) and chemiluminescence (Pierce). This antiserum detects normal dystrophin (at approximately 420 kDa) as well as transgenic mini-dystrophin (at approximately 220 kDa).

HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Muscle samples were embedded in Tragacanth (Sigma, St Louis, MO) and frozen in melting isopentane. Serial cross sections (5 μm) were cut on a cryostate and used for staining. For dystrophin detection, a polyclonal antiserum was used (same as described under immunoblotting; dilution 1:50). Biotinylated secondary antibody (Dianova, Hamburg, Germany; dilution 1:100) was applied and visualised by Cy3-conjugated streptavidin (Dianova, Hamburg, Germany; dilution 1:1000). To visualise cell nuclei the sections were incubated with 12μg/ml bisbenzimide (Sigma, Taufkirchen, Germany) in PBS for 10 min. In a final step, sections were stained with the NADH-tetrazolium reductase method to distinguish between oxidative and glycolytic fibres: Sections were incubated for 30 min at 37°C in 0.2 M Tris (pH 7.4), 1.5 mM NADH and 1.5 mM Nitroblue Tetrazolium (all from Sigma, Taufkirchen, Germany). After incubation, slides were rinsed 3 times with

distilled water. Fibre counts and determination of fibre area were performed using the UTHSCSA Image Tool program (University of Texas, Health Science Center, San Antonio, U.S.A.). Fibre scoring results are from seven *M. tibialis anterior* (TA) and *M. extensor digitorum longus* (EDL) muscles from 7 month-old MCK1.35 and mdx mice. For EDL, all fibres of an entire muscle cross-section were counted. For TA, five fields of 50-100 fibres were randomly chosen and evaluated. More than 300 fibres were scored for each TA and EDL muscle. Overall, more than 2500 fibres were scored per group.

For sarcoglycan detection on muscle sections, a polyclonal antiserum raised against β-sarcoglycan was used (dilution 1/1000; kindly provided by Dr. Carsten Bönnemann, Philadelphia; Bönnemann et al., 1996).

ISOLATION OF IMMORTALISED MYOGENIC CELL LINES

Two male homozygous H-2Kb-tsA58 (immorto) mice were obtained from Charles River (UK Limited, Margate, Kent, UK). These mice contain the thermolabile tsA58 mutant of the SV40 large T antigen under the control of the H-2Kb promoter. Isolated cells of this mouse strain are easily immortalised by growing them under permissive conditions: a temperature of 33 °C and the presence of INF-γ (interferon-γ). To breed the SV40 large T gene onto the mdx background, advantage was taken from the fact that the dystrophin gene is localised on the X-Chromosome. The male immorto mice were bred with female mdx mice. F1 male offspring received one copy of the SV40 large T antigen gene and a Y-Chromosome (that does not contain the dystrophin gene) from the immorto father and the mdx dystrophin point mutation on the X-chromosome from the mdx-mother. Therefore male F1 offspring are heterozygous for the SV40 large T gene and also dystrophin negative. Leg muscles from 6 week old animals were then removed under sterile conditions under a flow hood. The muscles can then be kept for up to 5 days at 4°C in SolA (10 mM glucose, 30 mM HEPES, 130 mM NaCl, 3.0 mM KCl, and 0.003 mM phenol red (all from Sigma), 50 µg/ml gentamicin (Gibco BRL, Invitrogen, Karlsruhe, Germany) pH 7.6,) without loss of proliferative capacity of the muscle progenitor cells. Isolation of single myoblasts and satellite cells was based on a procedure previously described (Shoubridge et al., 1996).

Muscle pieces (~150–300µg) were transferred into a tissue culture dish and washed repeatedly with SolA. They were then cut into smaller pieces (<1mm3) with two scalpels in a volume of 5 ml of SolA+Trypsin+EDTA (SolA + 0.05% (w/v) crystalline Trypsine (Gibco) + 0.02% (w/v) EDTA) and transferred into a 15 ml Wheaton trypsinising flask

(Wagner und Munz, München, Germany) containing a magnetic stirrer bar. To completely transfer all muscle bits the tissue culture dish was rinsed twice with 5ml SolA+Typsin+EDTA, each time the remaining tissue fragments and the solution were transferred to the Wheaton flask.

The muscle was now further disaggregated to isolate single cells by vigorous stirring for 15 min on a magnetic stirrer. To keep the temperature at 37°C the Wheaton flask was kept in a heated water bath. The tissue debris was then allowed to settle on the ground for 1 min. The solution containing the dissociated cells was decanted into a 50 ml centrifuge tube containing 15 ml of washing medium (Dulbecco's modified Eagle Medium (DMEM,) + 15% fetal calf serum (FCS,) + 50µg/ml gentamicin (all from Gibco)). Myoblasts that remain with the debris were isolated by stirring two more times for 15 min in 15 ml SolA+Trypsin+EDTA and decanting.

The cells were then centrifuged down at ~ 500 for 10 min and the supernatant was discarded. The cells were now re-suspended in 2ml of Supplemented Growth Medium (SGM, PromoCell, Heidelberg, Germany).

The goal of the next steps was to receive immortalised clones which originate from single cells. Therefore, the cell suspension was diluted 1/10, 1/50 or 1/100 in SGM+INF-γ (20U/ml Interferon-γ (murine, rekombinant) Life Technologies, Invitrogen, Karlsruhe, Germany). Then each dilution was plated in two 24 wells. Cells were incubated in a humidified incubator (5 % CO_2) at 33°C. After two days the medium was changed to remove fragments of erythrocytes and disrupted myofibres. After 4-5 days small colonies of dividing myoblast could be observed using a inverted phase contrast microscope. Single wells which originally received more than one cell could be identified because they contain more than one clone. After 1-2 weeks the cells were transferred to 6 wells and later to tissue culture dishes. After the first week SGM was replaced with DMEM + 15 % FCS + Glutamax1Supplement (Gibco). Clones that consist of small myofibre like single cells which show good division rates were then selected. Aliquots were frozen for later use and to save these lines. Myoblasts from these clones were then tested for the ability to fuse to myotubes. Fusion conditions were: a cell density of ~ 80%, DMEM + 2% Horse Serum (Gibco) and a temperature of 37°C. Two lines which divided and fused nicely were cultivated for over 1 year to ensure the immortalisation of these myogenic cell lines. If the cells grow to 90-100% confluency they start to fuse spontaneously even in the presence of high serum concentrations. This can impair their ability to amplify and to fuse. Therefore, the cell lines were always splitted when they reached 70-80% confluency.

FORCE MEASUREMENTS ON ISOLATED MUSCLES

The goal of a therapy of a muscular dystrophy is to re-establish normal muscle function in the affected patients. The best test to evaluate the effectiveness of a treatment in an animal model are therefore direct force measurements. One aim of this study was to develop and establish the necessary methodology for this kind of measurements in our laboratory. Suitable equipment was selected, purchased, interlinked and thoroughly tested before the experiments were started. Then force measurements on isolated mouse muscles were carried out basically as described (Petrof et al., 1993).

Mice were first anesthetised with ketamine (130 mg/kg) and xylazine (20 mg/kg) to achieve a loss of deep pain reflexes. Entire muscles were then carefully removed from tendon to tendon. Animals were killed while still anesthetised. Isolated muscles were anchored on one end while the other end was connected to a force transducer (model Grass FT03; Astro-Med, West Warwick, RI, U.S.A) coupled to a signal amplifier (model MIO 0500; FMI, Seeheim, Germany). During measurements the muscle was kept in a bath at 30°C in Ringer solution which was perfused with 95% O2 and 5% CO2. Muscle length and forced muscle lengthening were adjusted by a computer-controlled servomotor (model x.act LT 50; Linos, Göttingen, Germany). Electrical field stimulation was induced via platinum electrodes on both sides of the muscle (Fig. 2-1).

Supramaximal stimuli with a monophasic pulse duration of 1 ms were delivered using a computer-controlled electrical stimulator (model ISG-8834/1-S; FMI, Seeheim, Germany). Signals were converted by an analog/digital converter (model Digidata 1200B; Axon Instruments, Union City, California, U.S.A.). Data recording and measurement protocol execution were handled by a custom-made software. After an initial adjustment time of 10 min the optimal muscle length Lo (Lo is the length at which maximal twitch force is achieved) was determined and defined as the starting length. Measurements started 30 min after the muscle was removed. The following muscle parameters were evaluated: twitch force, tetanic force and resistance to eccentric contraction.

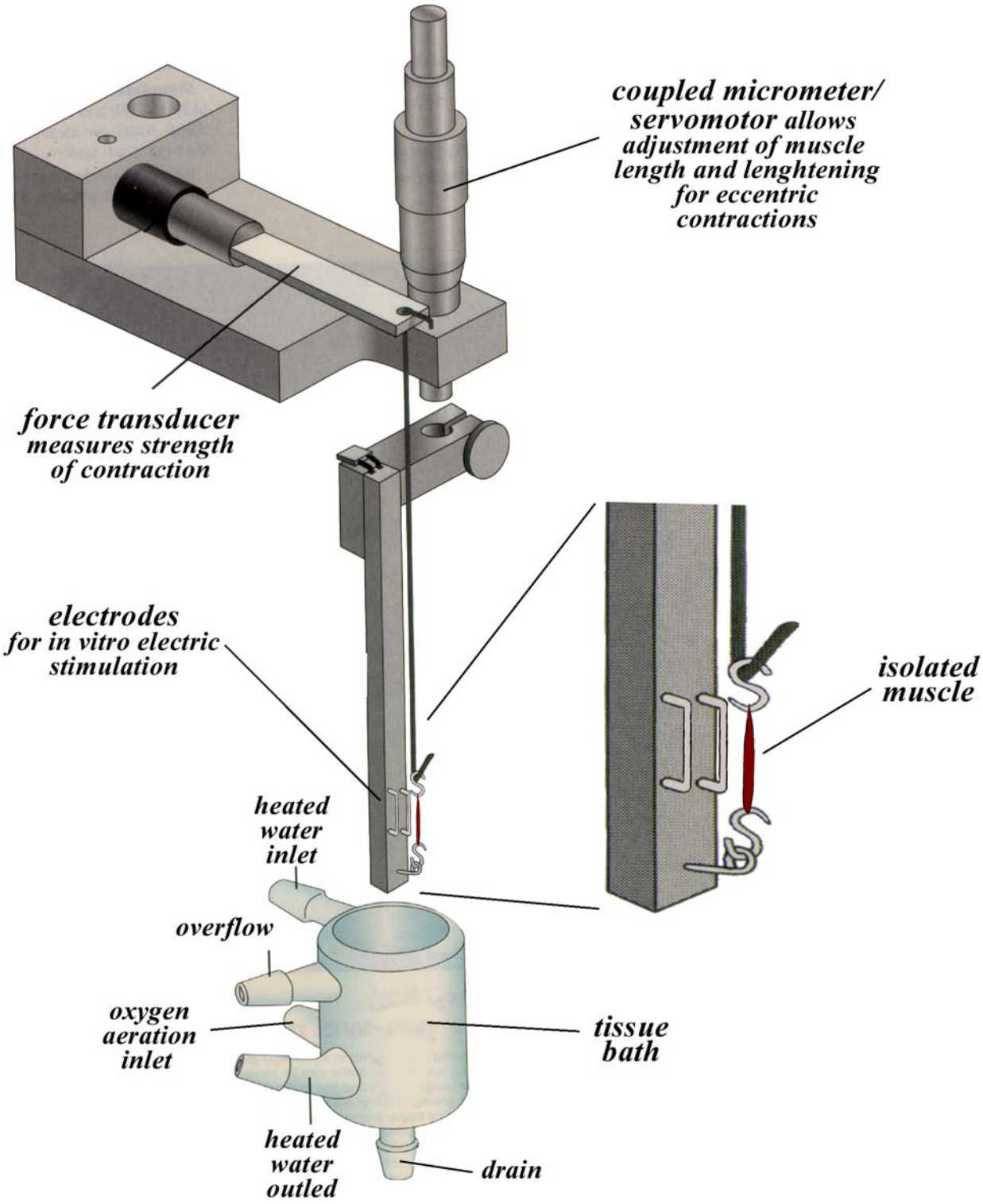


Fig. 2-1 Equipment for force measurement of isolated muscle. (adapted from World Precision Instruments, Berlin, Germany 1999)

Three twitch stimulations were recorded and the mean value was used to calculate maximal isometric twitch force. Maximal isometric tetanic force was then measured by stimulating the muscle at 125 Hz for 175ms, allowing a clear plateau in force to be attained. Specific force was calculated by normalising maximum tetanic force to total muscle cross-sectional area (CSA). CSA was determined by dividing muscle mass (milligram) by the product of fibre length (millimeter) and the average density of mammalian skeletal muscle taken as 1.06 mg /mm3.

Muscle of dystrophin-deficient mice (mdx) is especially susceptible to eccentric contraction which consists of forced muscle lengthening during maximal tetanic contraction. Successive eccentric contractions result in a more pronounced force drop in mdx muscle compared to wild type muscle. Measurements for eccentric contraction were carried out basically as described (Petrof et al., 1993). Briefly, the isolated muscle was stimulated at 125 Hz for 700 ms; the muscle was held at Lo for the initial 200 ms and was then additionally stressed by lengthening (speed: 3 mm/s) of 10% Lo during the final 500 ms. A total of five contractions was recorded, each separated by a two min recovery period at Lo. The decline in maximal isometric force at Lo, obtained from the tetanic plateau reached during the first 200 ms of the first measurement of muscle stimulation, was compared to the value of the fifth measurement and used as an index of eccentric contraction induced muscle damage.

GRIP STRENGTH OF LIVING ANIMALS

Grip strength of living animals was determined as described previously (Connolly et al., 2001) . In brief, mice were allowed to grasp a small metal trapeze which was attached to a force transducer (same as described above). The mice instinctively try to hold onto the trapeze. They were then pulled backwards until they released their grip (Fig. 2-2). The maximal grip strength was measured by a force transducer and recorded by a computer.
The protocol for each mouse consisted of 10 repeated pulls separated by rest periods of 8 sec.

The mean value of the three highest measurements was taken as the maximal grip strength. To evaluate if the animals showed different resistances to fatigue induced by repeated pulls we divided the mean of the three strongest pulls from measurements 1 - 5 by the mean of the three strongest pulls from measurements 6 - 10. These values were then expressed as percentages. A value for resistance to fatigue of 100% indicates that the animals grip strength remained on the same level during the course of 10 pulls while a value of 0% indicates that the animal would not hold on any more during the last five measurements.

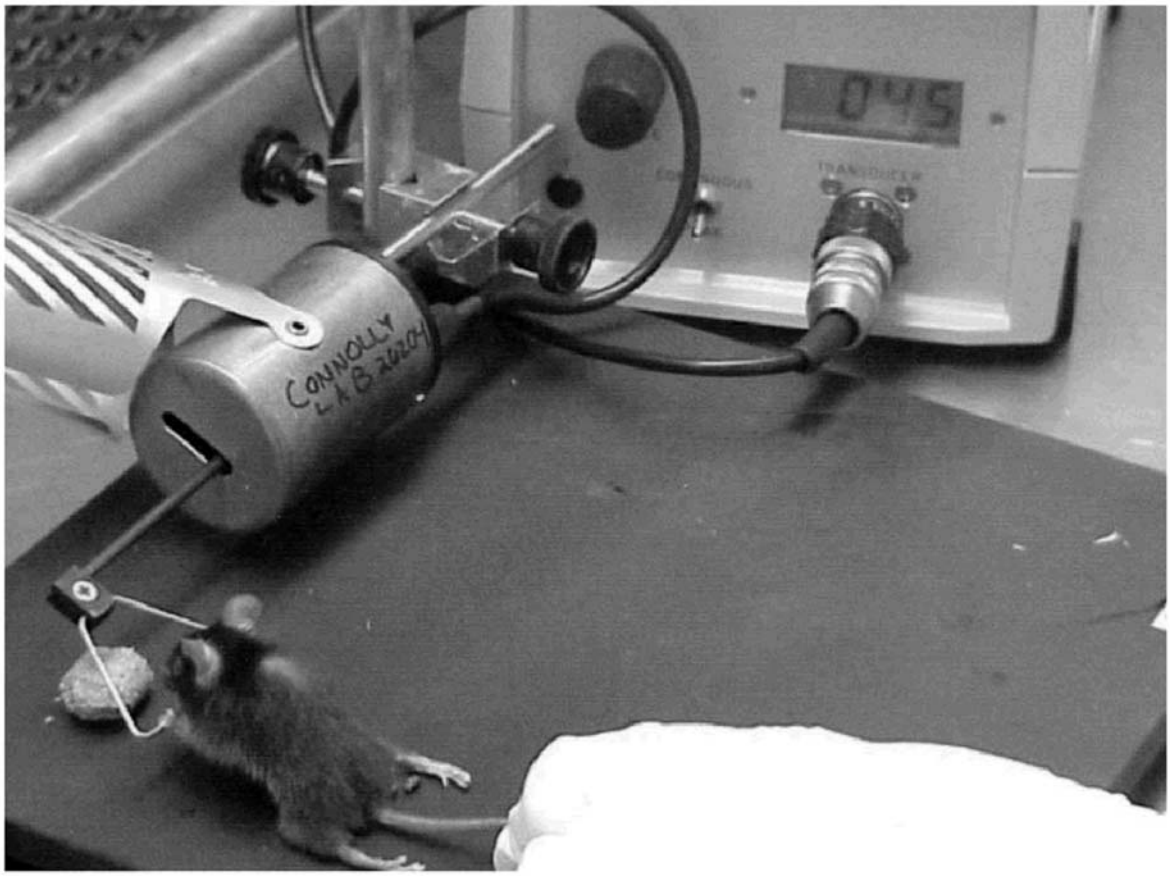


Fig. 2-2 Grip strength measurement. After it has gripped the trapeze the mouse is gently pulled backwards until it releases its grip. Grip strength is measured by a force transducer and recorded by a computer. (after Connolly et al., 2001)

STATISTICS

Force measurement data were first statistically evaluated by an analysis of variance. The difference between two groups was further evaluated by the Tuckey test. Histological results (central nuclei, dystrophin and NADH counts) were compared between groups using

Students *t* test. Chi-square tests were used to test the hypothesis that adjacent dystrophin-positive fibres decrease the probability of central nucleation in dystrophin-negative fibres.

PART III

Muscle specific gene expression in MCK 1.35kb mini-dystrophin transgenic mdx mice

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common primary muscle wasting disease in humans. Because of its high incidence, well characterised genetics, the complete lack of effective treatments and its severe progression with a fatal outcome, DMD has been considered a prime candidate for gene therapy (Hoffman and Dressman, 2001). Even though the concept of supplementing dystrophin-deficient muscle cells with a functional copy of the dystrophin gene is simple, its realisation has proven difficult (Somia and Verma, 2000). Viral vectors capable of transferring therapeutic genes to a high number of cells in clinically relevant muscles such as the heart, diaphragm and limb muscles are under development. Ideally, several requirements should be met by the regulatory elements that drive expression of the therapeutic gene in the target tissue such as: 1) being strong enough to produce sufficient quantities of the therapeutic protein; 2) remaining active for the lifetime of the patient; 3) restricting expression to the target tissue; and 4) being small enough to fit into viral vectors with a limited packaging capacity (Walther and Stein, 1996; Cordier et al., 2001). It is noteworthy that strong constitutive viral promoters such as the CMV promoter are prone to cytokine-induced gene silencing and thus might not ensure long-term, stable expression (Harms and Splitter, 1995). Moreover, viral promoters are active in many tissues other than skeletal muscle, which may also impair the safety, tolerability, and longevity of gene transfer (Ferrer et al., 2000; Pinto et al., 2000). Indeed, the immune response against the vector and transgene product is aggravated when antigen-presenting cells (APC) are infected by the vector and the transgene is expressed by APC (Hartigan-O'Connor et al., 2001; Yuasa et al., 2002). Therefore, regulatory elements, which are selectively active in the target tissue, but not in cells of the immune system, are increasingly recognised as being preferable for the purpose of somatic gene therapy (Weeratna et al., 2001).

The muscle creatine kinase (MCK) promoter is currently considered a good candidate to achieve the goal of muscle-specific gene expression in DMD. The MCK enzyme is abundantly expressed in skeletal muscle (Welle et al., 1999), and its regulatory regions have been well characterised *in vitro*, in transgenic animals and in gene transfer experimentation (Haecker et al., 1996; Deconinck et al., 1996; Yang et al., 1998; Hauser et al., 2000; Wang et al., 2000). The regulatory elements of the MCK promoter/enhancer are located within a 6.5kb sized region (Fig. 3-1).

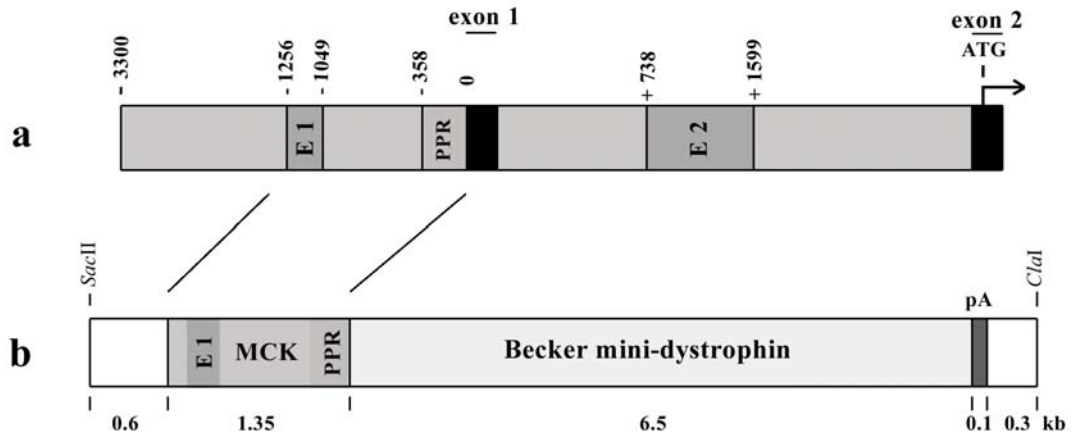


Fig. 3-1 MCK Promoter and MCK1.35dys construct. A) Depiction of the 6.5 kb full-length murine muscle creatine kinase regulatory region. Sites that contain major regulatory elements are indicated: the enhancers 1 and 2 (E1 and E2) and the proximal promoter region (PPR). Translation is initiated at the ATG codon. B) MCK1.35dys transgenic mice were produced by micro-injection of a 9.5 kb *Cla*I/*Sac*II fragment, which contained a 1.35kb MCK promoter, a 6.5kb mini-dystrophin, and a poly-A signal (pA).

About half of this region is located upstream of the first exon within the 5'-untranslated region and the other half downstream of the first exon within intron1. Directly 5' of the first exon lies a proximal promoter region (PPR) which has a size of approximately 385 bp. In promoter deletion studies the PPR was found to be indispensable for high level muscle specific gene expression. It contains at least one muscle specific regulatory element namely a MEF2 site. Two additional elements were identified that are important for optimal gene expression, they were called the MCK enhancer region 1 and 2 (E1 and E2). The E2 region lies in the first MCK intron 738bp downstream from the transcription initiation site (+738bp to +1598). It increases the activity of the basal promoter in skeletal muscle but not in the heart. The 206 bp E1 region (-1258bp to -1049bp) directs strong expression in

skeletal muscle and somewhat weaker expression in the heart (Johnson et al., 1989) Multiple transcriptional regulatory elements have been identified in this region: Two E-boxes, MEF-2, AT-rich, and CArG sites, and Trex sites (Fabre-Suver and Hauschka, 1996; Hauser et al., 2000). An overview of the factors that bind to the different sites is given in Table 3-1.

Table 3-1 Regulatory sites in the MCK Enhancer 1 (E1)

MCK E1 sites	DNA binding factors	
E-boxes	Members of MyoD family	Lassar et al., 1989
MEF-2	MEF-2	Yu et al., 1992
AT-rich	MHox, MEF-2, Oct-1	Cserjesi et al., 1992
CArG	Serum response factor (SRF)	Shore and Sharrocks, 1995
Trex	Trex-binding factor	Fabre-Suver and Hauschka, 1996

Virus mediated gene therapy requires packaging of the therapeutic construct consisting of the transgene and a promoter into the viral vector. However, the full-length MCK promoter/enhancer (6.5 kb) exceeds the limited packaging capacity of some of the most promising vectors for somatic gene therapy, such as adeno-associated virus and lentivirus based vectors (Scott et al., 2002). Therefore, several short versions of the MCK promoter/enhancer have been developed and tested in transgenic mice or gene transfer experimentation. Unfortunately, most of these studies were limited by their short-term nature and/or were based upon analysis of reporter genes rather than therapeutic gene expression. In addition, although previous studies have suggested unequal activity of MCK promoter/enhancer elements in slow-twitch and fast-twitch skeletal muscles, this issue has not been examined in detail.

To address the aforementioned issues we created a transgenic mouse line which expresses a 6.5kb mini-dystrophin under the control of a short muscle specific promoter. In the study mentioned above we have determined the efficacy of the short MCK 1.35 kb promoter/enhancer driving mini-dystrophin expression in multiple muscles for the entire lifetime of transgenic mice. Furthermore, we have evaluated whether there is any selectivity for expression within fast-twitch glycolytic fibres under these conditions. Since large glycolytic muscle fibres are more prone to undergo necrosis in dystrophin-deficiency than

small oxidative fibres (Karpati and Carpenter, 1986), sufficient transgene expression in this subset of fibres might be of particular clinical relevance.

Our model provides important insights into the scenario that may be anticipated following systemic delivery of a viral vector containing MCK regulatory elements to drive therapeutic transgene expression in DMD.

RESULTS

Generation of dystrophin transgenic mdx mice

We identified two founder animals that were positive for transgene sequences. Both founders were found to transmit the transgene to their offspring thus establishing two independent dystrophin transgenic lines. To ensure integrity of the promoter and promoter/dystrophin boundaries the complete MCK 1.35 kb promoter/enhancer region and adjacent parts of mini-dystrophin were sequenced for both lines. No mutations or rearrangements were found (data not shown). Both lines showed very similar expression patterns and expression levels for dystrophin once crossed onto the mdx background. If not otherwise stated the presented data are from transgenic line 2.

Tissue-specific expression of dystrophin in transgenic mic

In normal mice, expression of full-length dystrophin driven by endogenous promoters is limited to skeletal muscle, heart, and brain, and is very low or absent in other tissues. In contrast, muscles of mdx mice do not contain dystrophin except for a few so-called revertant fibres.

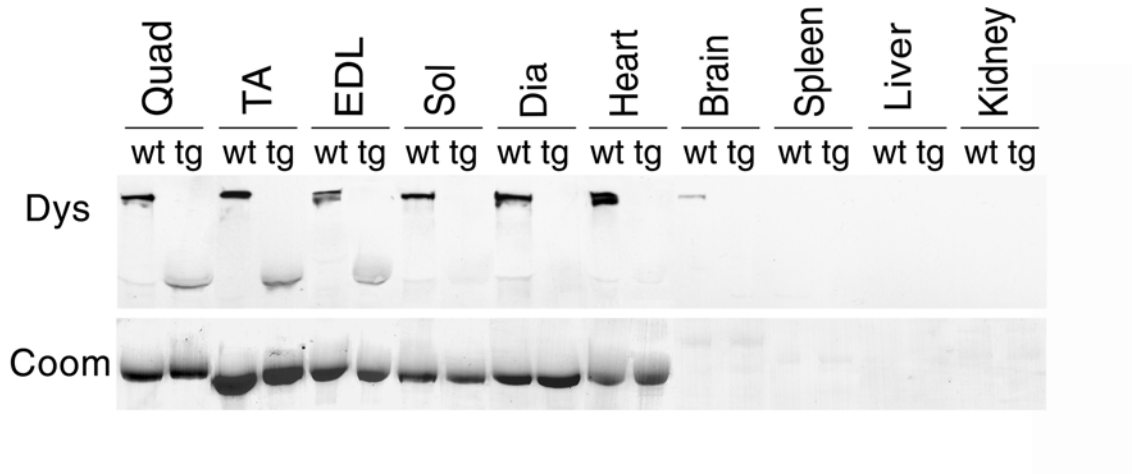


Fig. 3-2. Immunoblot analysis of full-length (427 kDa) and mini-dystrophin (220 kDa) in various muscles and other tissues of wild-type (wt; C57BL/6) and transgenic (tg) mice. Muscle samples show a prominent band (myosin at 200 kDa) on the post transfer Coomassie gel (loading control). Dystrophin expression in transgenic mice is muscle-specific. It is strong in fast-twitch muscles but weak or absent in slow-twitch muscles, diaphragm and heart.

Western blot analysis of MCK1.35dys transgenic mice showed a muscle-specific, but more restricted pattern of dystrophin expression as compared to wild-type mice

Expression was absent in non-muscle tissues, but varied considerably among different muscles. In detail, mini-dystrophin expression was 1) strongest in fast muscles such as *M. tibialis anterior* (TA), 2) intermediate in *M. extensor digitorum longus* (EDL) which is composed of fast-twitch and slow-twitch fibres and 3) absent or very weak in muscles containing mainly slow-twitch fibres such as *M. soleus*; and not detectable in the heart. This is in contrast to wild-type mice where expression levels of endogenous dystrophin are equally strong in all muscles and do not depend on muscle type (Fig. 3-2)..

Muscle histochemistry and immunohistochemistry of transgenic mice

Immunohistochemistry of transgenic mice revealed that even for dystrophin-positive muscles expression was not uniform and equal in all fibres, but a mosaic of dystrophin-positive and dystrophin-negative fibres was observed. This pattern was seen in both transgenic lines, it was characteristic of a certain muscle (such as TA), and did not change in aged animals (Fig. 3-3).

As dystrophin-positive fibres were larger than negative fibres we measured individual fibre cross sectional areas. This allowed us to compare the contribution of different fibres to the total muscle cross sectional area (CSA). In transgenic mice, 56% (TA) and 48% (EDL) of the CSA consisted of dystrophin-positive fibres (Table 3-2). Moreover, fibrosis in TA and EDL in 21-month-old dystrophin transgenic mice was slightly reduced if compared to non-transgenic littermates (data not shown). In accordance with the immunoblot results, almost no dystrophin staining was observed in *M. soleus* and diaphragm of dystrophin transgenic mice. In these muscles, very few fibres were dystrophin-positive and their number did not exceed the number of revertant fibres in non-transgenic mdx mice of the same age.

In addition, fibrosis of *M. soleus* and diaphragm in dystrophin transgenic mice were not reduced in comparison to non-transgenic littermates (data not shown).

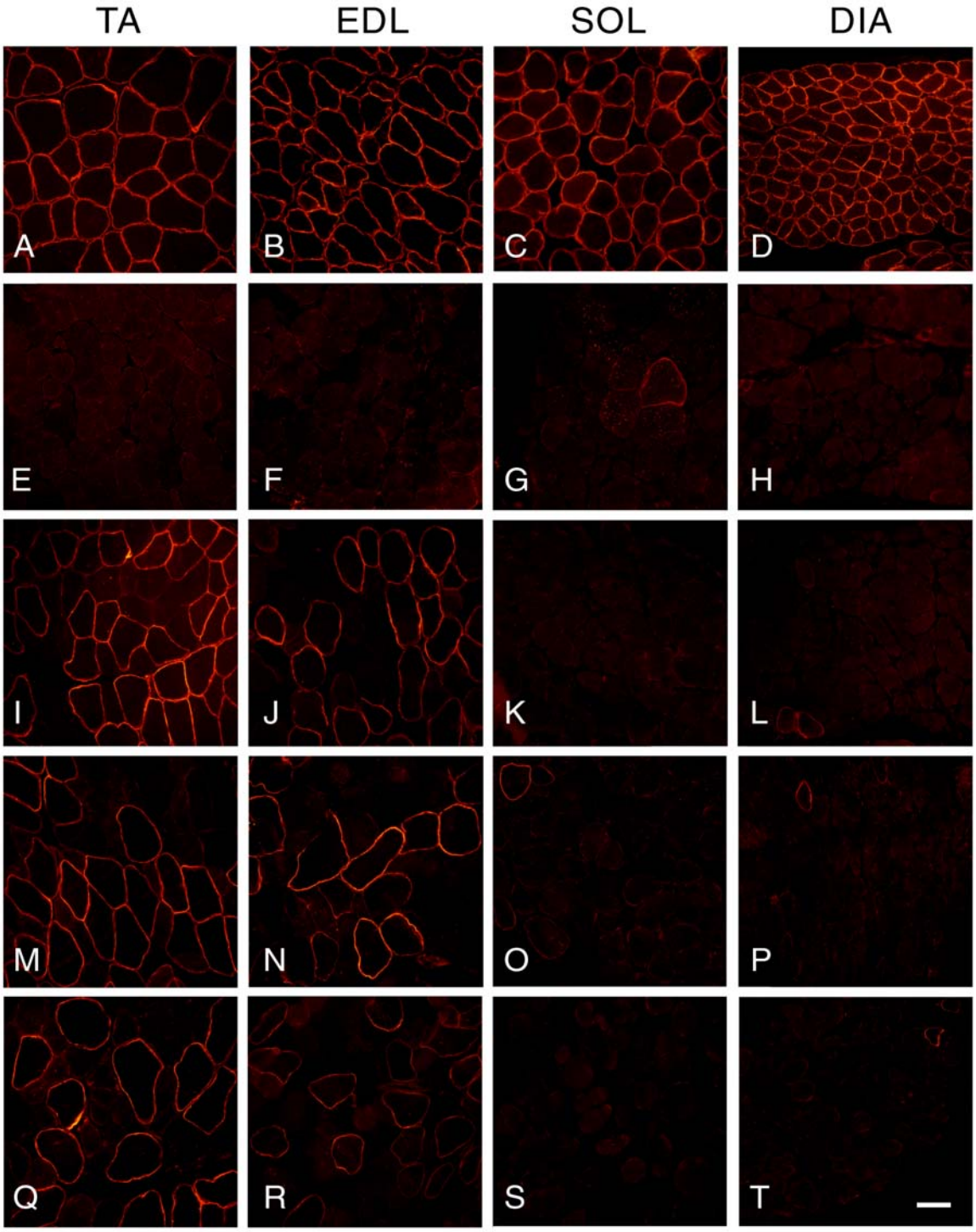


Fig. 3-3 Immunofluorescent localisation of dystrophin in muscles from control and transgenic mice. TA (A, E, I, M, Q), EDL (B, F, J, N, R), *M. soleus* (C, G, K, O, S), and diaphragm (D, H, L, P, T). Panels (A-P) are from mice at the age of 7 months, panels (Q-T) from mice at the age of 20 months. Note the regularity of dystrophin expression in C57BL/6 (A-D), its absence in mdx (E-H) and the mosaic pattern in adult muscle from both transgenic lines: line 2 (I-L) and line 1 (N-P) at the age of 7 months, and line 2 at the age of 20 months (Q-T). Bar = 50 µm.

Table 3-2 Comparison of dystrophin expression, central nuclei and metabolic fibre type between transgenic and mdx mice

		MCK1.35dys (tg)	mdx
Dystrophin-positive fibres	TA	56.3 ± 4.4	0.9 ± 0.7
% of total area	EDL	48.2 ± 10.6	1.2 ± 0.7
Fibres with central nuclei	TA	33.2 ± 8.5	82.8 ± 2.8
% of total area	EDL	25.5 ± 3.5	86.7 ± 5.3
Glycolytic fibres	TA	64.6 ± 6.0	57.8 ± 8.0
% of total area	EDL	61.7 ± 7.2	53.2 ± 5.0

TA and EDL from transgenic mice have a significantly higher percentage of dystrophin - positive fibre area and less central nucleation as compared to mdx ($P < 0.001$). Transgenic EDL has a moderately increased percentage of glycolytic fibre area ($P < 0.05$). Mean ± standard deviation is shown.

Each muscle has a characteristic composition of fibre types, which corresponds to the metabolic requirements of the muscle. A subset of fast-twitch muscle fibres depends mainly on glycolytic energy metabolism, whereas slow-twitch fibres depend on oxidation. The metabolic fibre type was visualised by the NADH reductase reaction, which is strong (dark) for oxidative fibres, and weak (light) for glycolytic fibres. In transgenic mice, a moderate increase in the number of glycolytic fibres was observed if compared to non-transgenic mdx mice (Table 3-2).

To characterise the frequency, distribution and effect of MCK driven mini-dystrophin in TA and EDL muscles of transgenic mice (age 7 months), dystrophin expression, central nucleation and metabolic fibre type were assessed on the same sections (Fig 3-4).

We observed a strong correlation between dystrophin expression and metabolic fibre type in transgenic mice: almost all dystrophin-positive fibres were large and glycolytic (98.5% for TA; 94.7% for EDL), whereas dystrophin-negative fibres were mostly small and oxidative (Table 3-3).

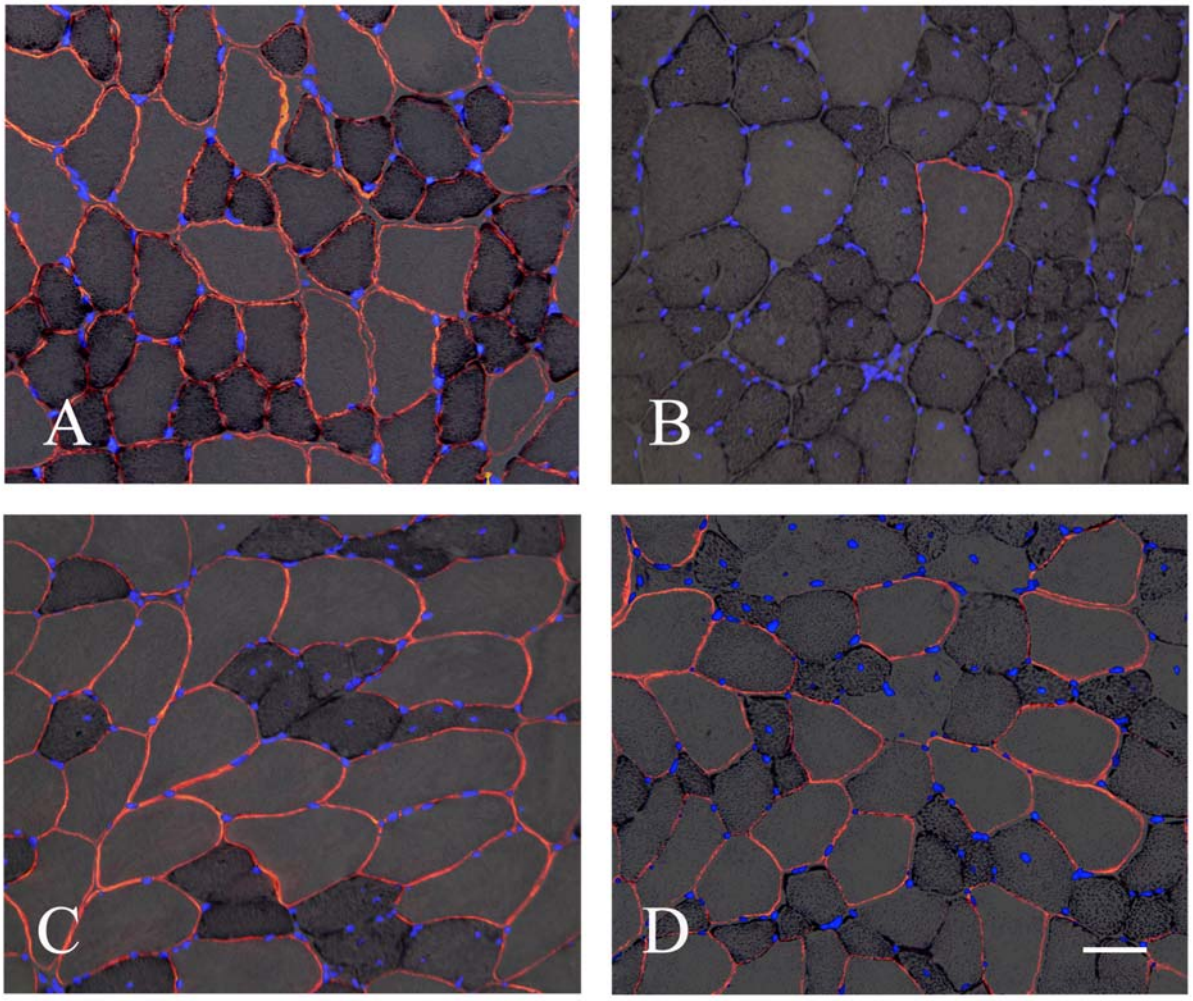


Fig. 3-4 Correlation of dystrophin expression, central nucleation and metabolic fibre type. Sections from 7-month-old C57BL/6 TA (A), mdx TA (B), transgenic TA (C), and transgenic EDL (D). Dystrophin immunostaining (red); nuclear bisbenzimide staining (blue); NADH-tetrazolium reductase (glycolytic fibres light; oxidative fibres dark). In transgenic muscles, dystrophin-positive fibres are large; central nuclei are nearly absent (C, D). In contrast, dystrophin-negative fibres are smaller, mostly oxidative and often have central nuclei (C, D). Interestingly dystrophin expression depends on muscle fibre type. Dystrophin is nearly exclusively found in fast glycolytic fibres. Also note the central nucleus in the revertant (dystrophin-positive) fibre in the mdx muscle (B). Bar = 50µm.

A high rate of central nuclei is typically found in dystrophin-deficient muscle (mdx) and indicates ongoing cycles of necrosis and regeneration. Central nucleation increases with age and reaches 70-80 % in 3-6-month-old mdx mice. In transgenic TA and EDL, the area with centrally nucleated fibres was reduced by about 50 % if compared to mdx. This proportion remained stable even in aged mice (20 months). Muscles of transgenic mice that did not

express dystrophin, such as *M. soleus* showed similarly high levels of central nucleation as muscles of mdx mice.

Table 3-3 Correlation between dystrophin expression, central nuclei and metabolic fibre type in transgenic mice.

		MCK1.35dys (tg)
% Dystrophin-positive fibres	TA	5.6 ± 7.8
with central nuclei	EDL	2.7 ± 1.9
% Dystrophin-negative fibres	TA	68.6 ± 10.3
with central nuclei	EDL	45.0 ± 20.6
% Dystrophin-positive fibres	TA	98.5 ± 1.1
which were glycolytic	EDL	94.7 ± 6.7
% Dystrophin-negative fibres	TA	78.4 ± 5.5
which were oxidative	EDL	69.5 ± 4.7

Fibres expressing dystrophin show a significant reduction of central nucleation. Note that almost all dystrophin expressing fibres are of the glycolytic fibre type. In contrast, dystrophin-negative fibres are mostly oxidative. Mean ± standard deviation is shown.

Dystrophin-positive fibres showed considerably less central nucleation as compared to dystrophin-negative fibres (Fig. 3-4). In dystrophin-positive fibres of transgenic mice, central nucleation was strongly reduced to 5.6 % in TA and 2.7% in EDL (Table 3-3). Therefore, mini-dystrophin expression appeared to be sufficiently protective against fibre degeneration. In dystrophin-negative fibres of transgenic mice, central nucleation was moderately reduced to 69% in TA and 45% in EDL when compared to corresponding mdx muscles. Interestingly, dystrophin-negative fibres with peripheral nuclei were frequently found directly adjacent to dystrophin-positive fibres. Central nucleation of dystrophin-negative fibres not in contact with dystrophin-positive fibres was significantly higher than central nucleation of dystrophin-negative fibres surrounded by dystrophin-positive fibres (80.9 % versus 7.0 %; $P < 10^{-6}$; Table 3-4).

Table 3-4 Neighbouring dystrophin-positive fibres reduce the rate of central nucleation in dystrophin-negative fibres of transgenic mice

	number of fibres	central nuclei (%)
surrounded by dystrophin-positive fibres	57	7.0
up to 75 % of total fibre surface area in contact with dystrophin-positive fibres	255	18.4
up to 50 % of total fibre surface area in contact with dystrophin-positive fibres	443	36.1
up to 25 % of total fibre surface area in contact with dystrophin-positive fibres	529	61.2
no contact with dystrophin-positive fibres	482	80.9

Dystrophin-negative fibres of transgenic mice were scored for central nucleation according to their proximity with dystrophin-positive fibres. Contact with dystrophin-positive fibres led to a gradual reduction of central nucleation. Seven transgenic EDL muscles of 7-months-old mice were scored. $P < 10\text{-}6$ for all groups if compared to the group of fibres with no contact with dystrophin-positive fibres.
In contrast, revertant (dystrophin-positive) fibres of mdx muscles showed a high percentage of central nucleation (73%; TA; 7 months; n = 102) which indicates that revertant dystrophin is not fully protective.

Force measurements on isolated muscles

To compare the muscle function of transgenic, mdx and C57BL/6 mice in detail force measurements on isolated muscles are required. Three important parameters were measured: specific twitch force, specific tetanic force (Quinlan et al., 1992) and resistance to eccentric contraction.

Twitch and tetanic contractions were measured under isometric conditions, i.e. the length of the muscle remained unchanged during contraction. A twitch is a short contractions in response to a single action potential that can be induced by a short (1 ms) electrical stimulation. Since the mechanical response lasts for about 100ms it is possible to induce a second action potential during the ongoing mechanical activity of the first stimulus. Therefore, when a muscle is stimulated by many rapidly successive electric pulses the level of tension increases until a maximum, tetanic force, as it is called, is reached (Fig. 3-5).

A protocol which emphasises the marked susceptibility of dystrophin deficient muscles to muscle damage consists of forced lengthening of the muscle during tetanic contractions. A series of these so-called eccentric contractions results in a dramatic force drop in mdx muscle, whereas normal muscle is more resistant to mechanical stress. In Fig. 3-5 data from tests of an EDL muscle are displayed, which demonstrated that the muscle force measurement worked as intended.

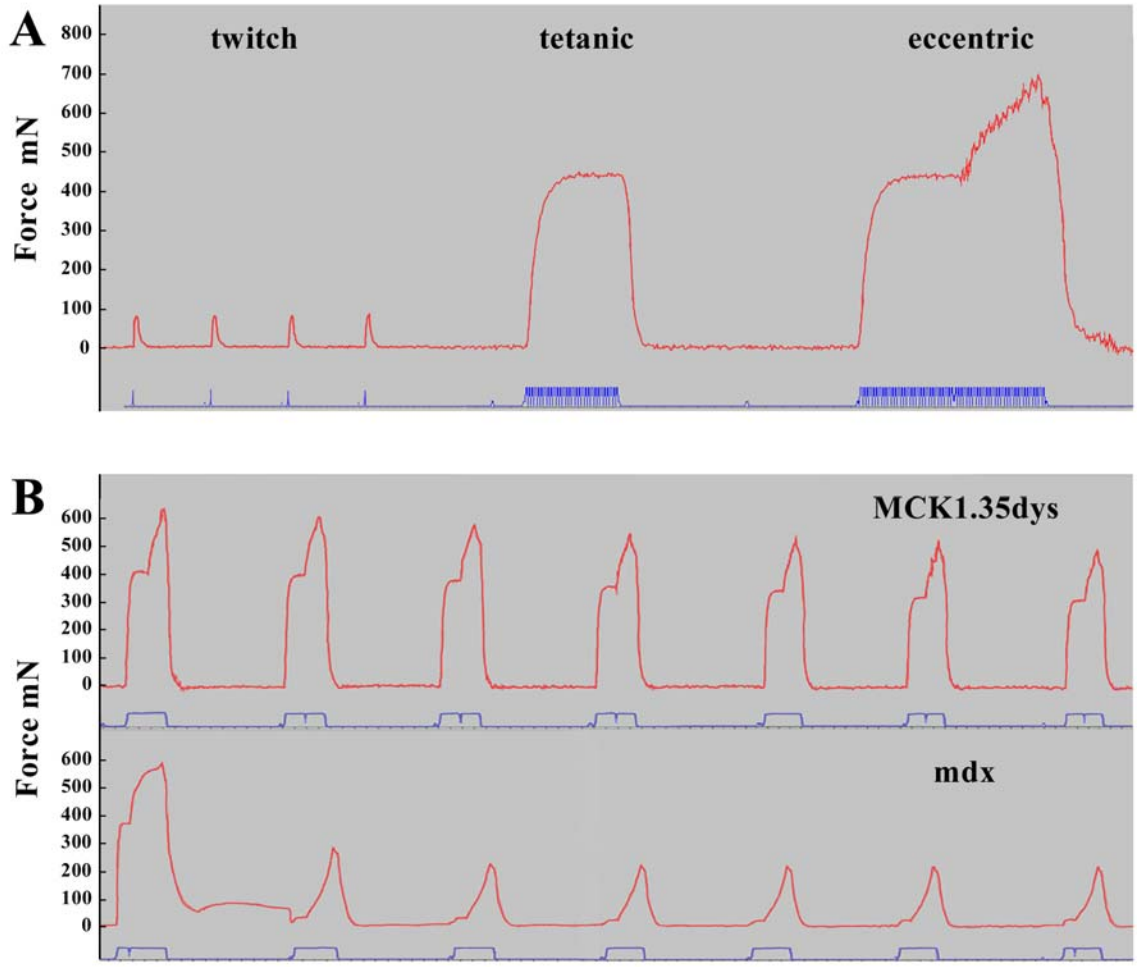


Fig. 3-5 Measurement of muscle functions (Forces: red; electrical stimulation: blue)
(A) single electric stimuli produce short "twitches" of the muscle. A rapid series of electric stimuli produces a tetanic contraction. For an eccentric contraction the muscle is stretched when a tetanic contraction has reached its maximal force, which increases the strain on the muscle. (B) Comparison between transgenic and mdx muscle when subjected to a series of successive eccentric contractions. Note the great susceptibility of the mdx muscle and the resistance of the transgenic muscle.

To monitor the long-term function of MCK-driven dystrophin expression force measurements were carried out in isolated muscles of transgenic mice at the ages of 3.5, 7, 14, and 20 months and compared to non-transgenic littermates (mdx) and wild-type mice (C57BL/6). No difference in muscle function between dystrophin transgenic and mdx mice was found for muscles such as *M. soleus* and diaphragm that did not show significant dystrophin expression (data not shown). The EDL of transgenic mice showed a mosaic pattern of dystrophin expression (see above) and revealed an improved function if compared to EDL of age-matched, non-transgenic littermates (Fig. 3-6). Isometric force of transgenic mice was restored to wild-type levels.

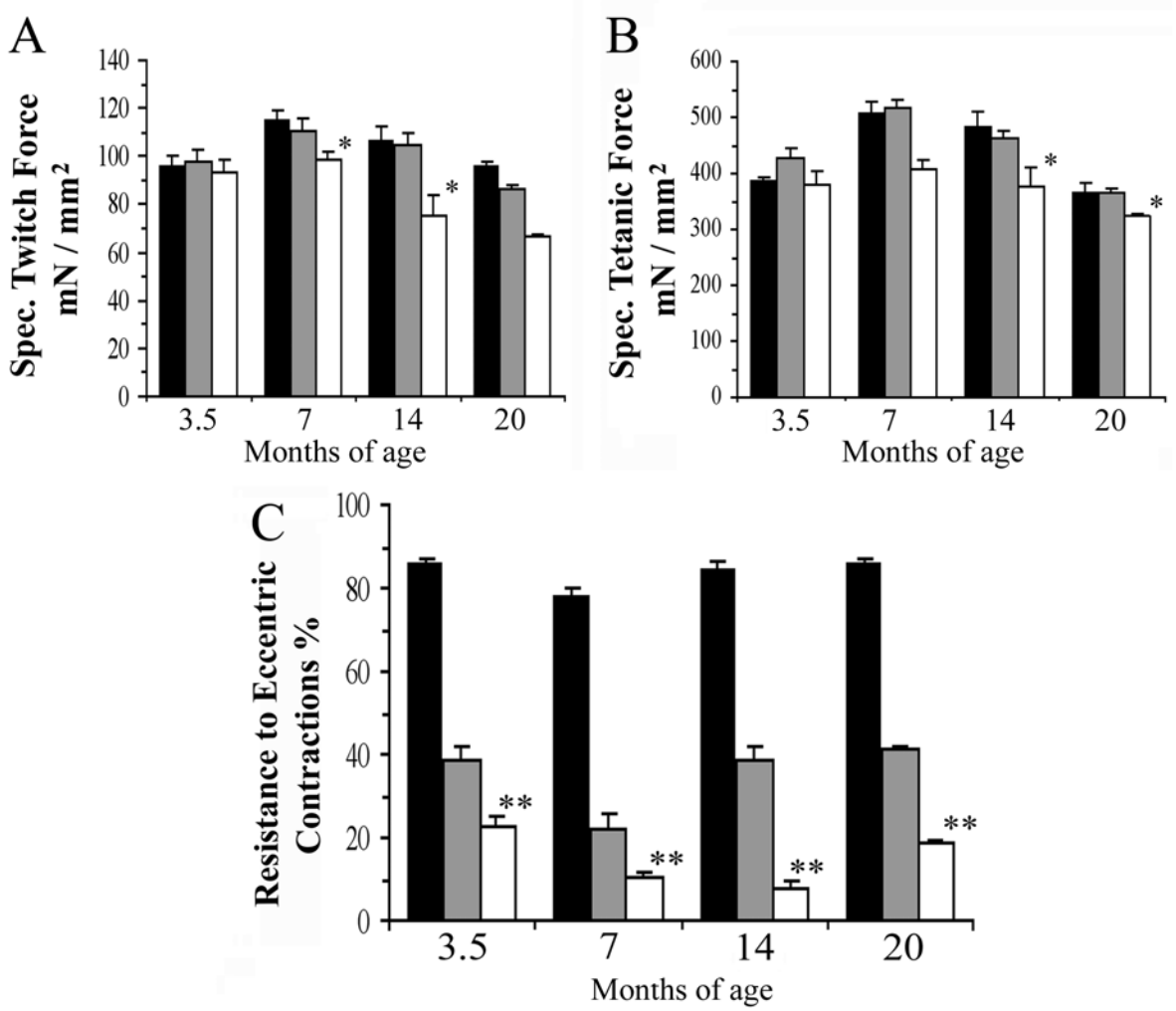


Fig. 3-6 Mechanics of isolated EDL muscle. Evaluation of specific twitch force (A), specific tetanic force (B), and resistance to eccentric contractions (C) for female wild-type (black), transgenic (grey) and mdx (white) mice. Between 5 and 18 muscles were analysed in every group. Data are presented as mean ± s.e.m. Statistically relevant differences between transgenic and mdx mice are indicated (* $P < 0.05$; ** $P < 0.01$). Differences between wild-type and mdx mice are highly significant for eccentric contraction ($P < 0.001$; not indicated).

After twitch and tetanic force measurements the muscle were subjected to an eccentric contraction protocol. For all mouse lines tested the force drop remained fairly constant at all ages. Dystrophin transgenic EDL showed a significantly improved performance as compared to non-transgenic littermates (mdx; $P < 0.01$), but did not reach levels of wild-type mice. The partial resistance to eccentric contractions in transgenic EDL corresponded well with the percentage of dystrophin-positive fibres.

Grip strength of living animals

Grip strength was measured to evaluate an overall beneficial effect of limited, transgenic mini-dystrophin expression at different ages. No significant difference between transgenic and non-transgenic (mdx) mice was detected at ages up to 14 months (data not shown). In contrast, wild-type mice were significantly stronger at all ages, and the difference in strength increased with age (data not shown). At 17 months of age, transgenic mice were significantly stronger than non-transgenic (mdx) mice ($P < 0.05$; Fig. 3-7).

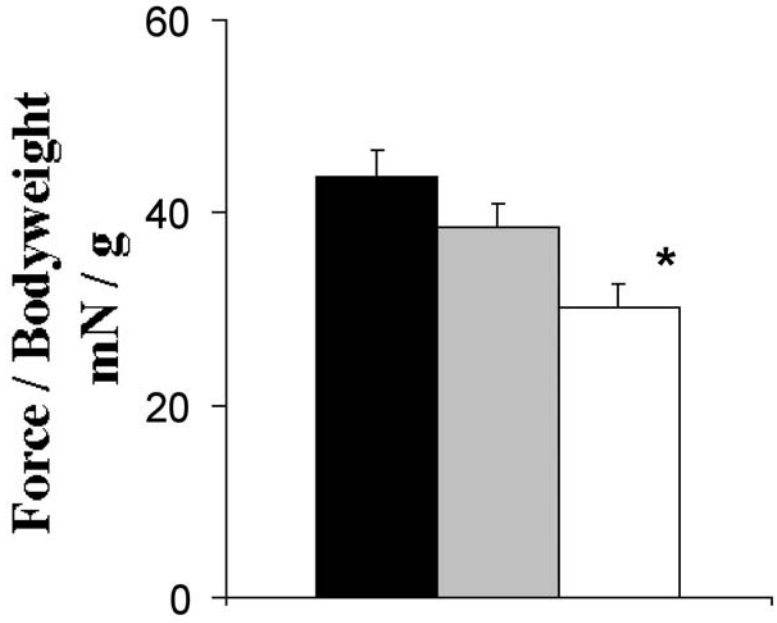


Fig. 3-7 Grip force measurements. 17-month-old female C57BL/6 (black), transgenic (grey) and mdx (white) mice. Between 8 and 10 mice were tested in every group. Data are presented as mean ± s.e.m. Statistically relevant differences between transgenic and mdx mice are indicated (* $P < 0.05$).

Moreover, we measured the resistance to fatigue against repeated grip strength pulls. There was no difference between mdx and transgenic mice, but they both were significantly less resistant to fatigue than wt mice (Fig. 3-8). While wt mice could maintain a constant force over 10 repeated pulls, the force dropped strongly for the other two models. After the measurement, wt mice immediately started to move in the cage. In contrast, mdx and transgenic mice made no attempts to move around and also had a much higher breathing frequency.

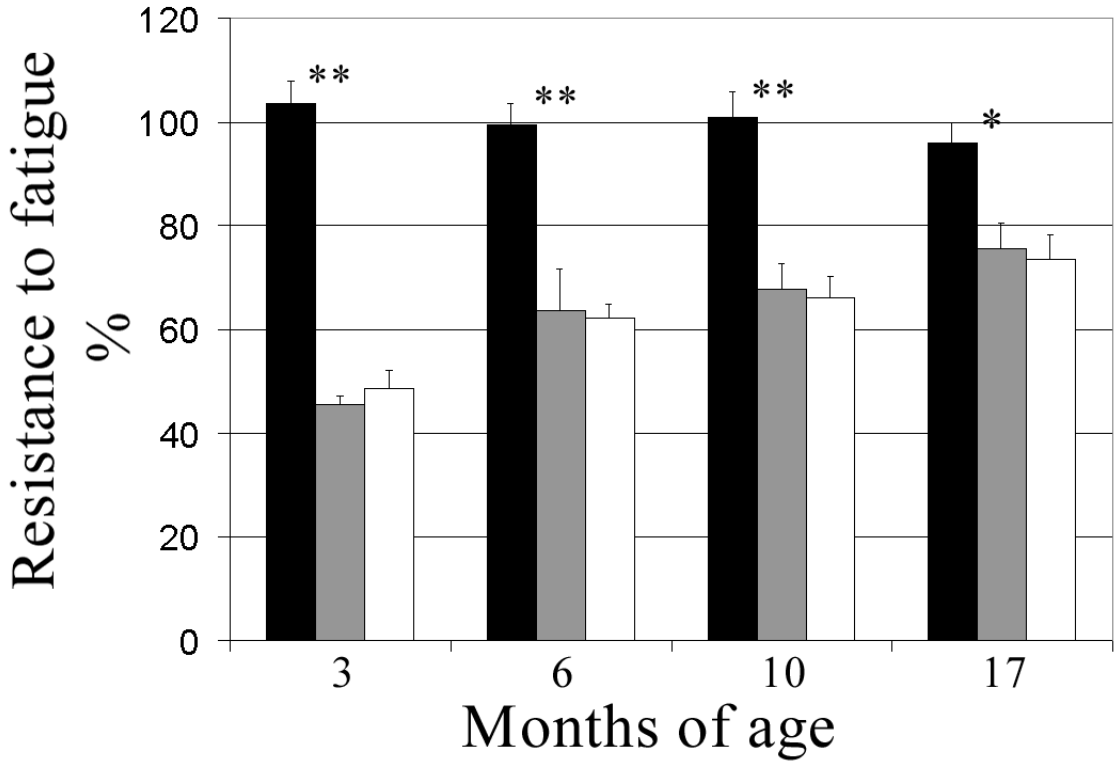


Fig. 3-8 Resistance to fatigue. Female C57BL/6 (black), transgenic (grey) and mdx (white) mice aged between 3-17 months . 8 - 10 mice were tested in every group. Data are presented as mean ± s.e.m. Differences were not significant between mdx and transgenic mice. But both were significantly different when compared to C57BL/6 mice at all ages (** $P < 0.001$; * $P < 0.05$).

DISCUSSION

In this study we demonstrate that transgenic expression of mini-dystrophin driven by a 1.35 kb muscle creatine kinase (MCK) promoter partially protects skeletal muscle throughout the lifetime of dystrophin-deficient (mdx) mice. Interestingly, we observed a preference of transgenic dystrophin expression for glycolytic fibres in fast skeletal muscle of two independent mouse lines. Our detailed histological and functional analysis of several muscles provides new insights into the therapeutic effects of mosaic transgene expression.

Ideally, somatic gene therapy of muscular dystrophy will result in adequate transgene expression in every muscle and in every muscle fibre of the body. However, current gene delivery protocols suffer from several limitations: skeletal muscle is distributed throughout the body and accounts for more than 40% of total body mass, many muscles such as heart and diaphragm are difficult to reach directly, and systemic vector delivery implies additional risks such as increased toxicity and immunogenicity (Kay et al., 2001). Therefore, gene delivery is likely to be incomplete and to result in unequal or mosaic transgene expression for different muscles or muscle fibres. So far, few attempts have been made to determine the level and distribution of transgene expression that would permit functional improvement. Phelps et al. compared several lines of transgenic mdx mice with varying levels of dystrophin expression and fibre-by-fibre mosaics. They concluded that uniform dystrophin expression at a level of 20-30% in all fibres efficiently corrects the dystrophic phenotype. In contrast, high expression levels restricted to a smaller number of fibres were not equally protective (Phelps et al., 1995). Interestingly, no negative effect was observed in transgenic mice that strongly overexpress dystrophin in all tissues by an ubiquitously active viral promoter (Cox et al., 1993).

Most studies use central nucleation as an endpoint in gene transfer and transgenic experimentation and correlate the overall levels of central nucleation and dystrophin expression in entire muscles. Our approach to score large numbers of individual fibres in different muscles allowed for additional conclusions: central nucleation in transgene expressing fibres is reduced to almost wild-type levels (5.6 % for TA and 2.7 % for EDL). Therefore, the 6.3 kb mini-dystrophin may be fairly protective even if it is not strongly overexpressed. This is in accordance with the original observation of this mini-dystrophin in a very mildly affected patient (England et al., 1990). However, precise phasing of the dystrophin rod deletion may lead to functionally improved mini-dystrophin molecule

(Harper et al., 2002). In contrast, dystrophin-positive, so-called revertant fibres of non-transgenic mdx mice showed high levels of central nucleation close to levels in dystrophin-negative fibres. Revertant dystrophins are thought to arise from somatic deletions or post-transcriptional exon skipping that excise the mdx stop codon (Uchino et al., 1995; Wilton et al., 1997; Lu et al., 2000; Crawford et al., 2001). Our findings may indicate that the majority of naturally occurring revertant dystrophin is dysfunctional.

Interestingly, even dystrophin-negative fibres in muscles with mosaic transgene expression show a moderately reduced rate of central nucleation if compared to non-transgenic littermates (69% in transgenic TA compared to 82% in mdx TA; 45% in transgenic EDL compared to 86% in mdx EDL). We cannot rule out that "dystrophin- negative" fibres express low amounts of dystrophin that are below the detection threshold of our immunohistochemical analysis. However, it appears unlikely that such very low transgene levels would protect muscle fibres from degeneration. Moreover, we observed lower levels of central nucleation mostly in dystrophin-negative fibres that were in close vicinity to dystrophin-positive fibres. Therefore, we hypothesise that dystrophin-negative fibres may benefit from a positive bystander effect. Under these conditions, dystrophin-positive fibres may carry the main mechanical burden, thereby leading to reduced stress and cell death of the adjacent, more vulnerable dystrophin-negative fibres. Moreover, fibre necrosis in dystrophic muscle is accompanied by inflammation which results in further deterioration of the muscle (Porter et al., 2002; Spencer et al., 2001). Therefore, an overall reduction of inflammation may contribute to a further prolonged survival of all muscle fibres.

The reduction of central nucleation is a well-accepted histological outcome measure of therapeutic or transgenic intervention in muscular dystrophies. However, the primary and most clinically relevant goal is the improvement of force generation. The mdx mouse has been criticised as an animal model for human dystrophin-deficiency since caged mdx mice do not reveal severe functional impairment on first sight. Maximum tetanic force is not compromised in several hindlimb muscles of mdx mice. This is because a decrease in maximum tetanic force per muscle cross sectional area (specific force) is compensated by a gain in muscle mass and diameter (Coulton et al., 1988; DelloRusso et al., 2001). However, specific forces and resistance to eccentric contractions are clearly impaired in mdx muscle if compared to controls. These deficits are due to the lack of dystrophin and worsen with

age. To further evaluate the effect of transgenic mini-dystrophin expression we ascertained functional parameters in isolated EDL muscles of young adult and aged mice.
For specific twitch and tetanic forces as well as the resistance to eccentric contractions, we found significant improvements in transgenic mice as compared to their non-transgenic littermates. This improvement was most pronounced in aged mice, which is in agreement with previous results (DelloRusso et al., 2001; Lynch et al., 2001). Resistance to eccentric contractions was the most sensitive parameter, showing significant differences for the EDL muscle in all age groups (Moens et al., 1993).

It is an interesting question why skeletal muscles of mdx mice are highly susceptible to eccentric contraction while other mechanical functions are not substantially affected.
Two additional observations in mdx mice support this notion: The diaphragm is most severely affected, and intensive downhill running results in increased membrane leakage. Both are naturally occurring models for eccentric contractions, i.e. muscle lengthening during contractile work. Apparently, mdx muscle can sustain stress connected with normal muscle contractions, but cannot cope with the additional physical stress during eccentric contractions.

Grip force measurements are non-invasive tests, which allow for longitudinal studies without killing the mice. However, grip force appeared to be a less sensitive parameter if compared to isolated muscle measurements, since a significant difference between transgenic mice and non-transgenic littermates was only detected in aged mice. Similarly, the degree of muscle fibrosis was only reduced in EDL and TA of aged transgenic mice as compared to their non-transgenic littermates.
In other studies mdx mice had to complete exercise protocols such as swimming or running (Hayes and Williams, 1998; Granchelli et al., 2000; Vilquin et al., 1998). It was noted that mdx mice show earlier and stronger signs of exhaustion than normal controls. Apparently, dystrophin-deficiency in mice results in a phenotype that allows for normal movement and behaviour of caged animals, but additional exercise reveals physical limitations. To quantify increased fatigue we measured grip strength of 10 successive pulls (see above). Interestingly a highly significant fatigue in mdx mice was detected when compared to wt mice. This protocol is a fast, non-invasive, repeatable and sensitive method to evaluate the overall physical performance of mdx mice. Importantly, this method allowed a sensitive characterisation of the mdx phenotype already at 3 months of age. However, this method

does not allow to distinguish among a number of possible causes for increased fatigue such as limb muscle weakness, heart insufficiency, influence on behaviour, or breathing insufficiency. However the increased breathing rate that we observed in the transgenic animals and mdx mice after repeated grip strength measurements may be caused by the very pronounced phenotype that is found in the diaphragm of these mice. Stedman et al. have argued that the eccentric mode of action and a high respiratory work load in rodents are the reasons why the mdx diaphragm is more severely affected than other muscles (Stedman et al., 1991). When the resistance to fatigue of MCK1.35dys mice was measured, no improvement was detected in comparison to mdx mice. Since dystrophin was not expressed in heart and diaphragm of the transgenic mice, an impairment in cardio-respiratory function may have been responsible for the increased overall fatigue.

Another finding of interest in our study was a mosaic pattern of transgene expression in several muscles within two independent transgenic mouse lines, which suggests that this pattern is not due to a position effect of a specific integration site in the mouse genome, but rather an intrinsic property of the MCK promoter. We hypothesise that the MCK promoter shows higher activity in fast-twitch muscles and glycolytic fibres as compared to slow-twitch muscles and oxidative fibres. Evidence from several studies of the function and distribution of muscle creatine kinase, from MCK promoter analysis and from *in vivo* expression studies support this notion. Indeed, muscle type creatine kinase is mainly located at sites of high ATP consumption and participates in the regeneration of ATP from phosphocreatine (Bessman and Carpenter, 1985; Wallimann et al., 1992). Therefore, muscle creatine kinase plays a pivotal role in fibres that depend on glycolysis for energy generation. Accordingly, fast-twitch glycolytic muscles contain higher levels of MCK-mRNA and MCK-activity in comparison to slow-twitch oxidative muscles (Andres et al., 1990; Yamashita and Yoshioka, 1991; Tsika et al., 1995).

Regulatory elements of the MCK promoter/enhancer that are responsible for muscle-specific expression and for differential expression in fast-twitch, slow-twitch and cardiac muscles have been identified (Johnson et al., 1989; Shield et al., 1996). MCK1.35 used in this study contains two major regulatory elements: a muscle-specific enhancer (E1) located 1.1 kb upstream of the transcription start site and the proximal promoter region. The intronic enhancer (E2) is not included. MCK promoter/enhancer constructs have been used in several studies to achieve muscle-specific gene expression in transgenic animals, and

include variants of 6.5kb, 3.3kb and 570bp (Johnson et al., 1989; Hauser et al., 2000). Most of these studies focused on transgene expression in fast-twitch skeletal muscles and reported strong variations in MCK-driven expression: 1) between different transgenic lines; and/or 2) between different muscles; and/or 3) a mosaic pattern of expression in muscles (Lee et al., 1993). Although strong transgene expression has been demonstrated in diaphragm in some exceptions (Cox et al., 1993; Squire et al., 2002), expression levels are usually highest in fast-twitch skeletal muscles and considerably lower in slow-twitch skeletal muscle, diaphragm and heart (Phelps et al., 1995; Harper et al., 2002; Johnson et al., 1989; Shield et al., 1996; Wells et al., 1995; Donoviel et al., 1996; Corrado et al., 1996; Ahmad et al., 2000).

As with our study, most *in vivo* studies on MCK promoter/enhancer function are based upon observations in transgenic animals. While it appears theoretically possible that a different expression pattern could result after viral gene transfer (particularly when the transgene does not stably integrate in the host genome), it is noteworthy that MCK-driven transgene expression remained muscle-specific after adenoviral gene transfer (Larochelle et al., 2002; Scott et al., 2002). Previous gene transfer studies have mainly examined fast-twitch muscles or failed to directly compare expression in fast-twitch and slow-twitch muscles. However, MCK-driven luciferase expression was high in fast-twitch muscle and poor in heart if normalised to activity of the RSV promoter (42% for skeletal muscle and 0.7% for heart) (Larochelle et al., 1997).

This comprehensive study allows for the first time a direct correlation between dystrophin expression, metabolic fibre type and central nucleation in transgenic mice using a MCK promoter/enhancer construct. In conclusion the MCK promoter/enhancer provides not only muscle-specific expression, but also different levels of expression depending on the metabolic characteristics of the muscle and its component muscle fibres. The MCK promoter seems to be highly appropriate in applications in which transgene expression should be restricted to skeletal muscle and high expression in a limited number of muscle fibres is sufficient. This may include DNA immunisation (Gebhard et al., 2000), metabolic engineering for systemic delivery of non-muscle proteins (Bou-Gharios et al., 1999) and experiments in which expression of a transgene - specifically in glycolytic fibres - is desirable (Lin et al., 2002). Since large, glycolytic fibres are especially vulnerable to the dystrophic process in DMD, high levels of transgene expression in these fibres is likely to

be essential (Karpati and Carpenter, 1986). However, therapeutic efficacy of the MCK promoter might still be limited in gene therapy of muscular dystrophies, since a more widespread and even expression of the transgene is probably needed. On the other hand, it may be possible to overcome these limitations by modifying the MCK promoter/enhancer or by coupling MCK with regulatory elements from other muscle-specific or ubiquitous promoters. As for now, *in vivo* expression analysis of such constructs has focused solely on fast-twitch skeletal muscle (Hauser et al., 2000; DelloRusso et al., 2002). Further studies in a larger variety of muscles of dystrophic animals will be required to fully ascertain the therapeutic efficacy and fibre type specificity of such constructs in the context of muscular dystrophies.

These experiments discussed above focussed on tissue specific gene expression achieved through the use of a tissue specific promoter in a transgenic mouse model. In a therapeutic gene therapy approach the tissue specificity may not only be determined through the control of gene expression, but also through the specificity of the applied gene transfer system for the target tissue. To accomplish optimal tissue specificity it may be therefore beneficial to combine a tissue specific promoter with a tissue specific method of gene delivery. The high synergy of such an approach was demonstrated in a system for lung specific gene expression after adenoviral gene transfer. By combining transductional targeting to a pulmonary endothelial marker and transcriptional targeting the selectivity of transgene expression for the lung was largely improved (Reynolds et al., 2001). For the DMD therapy a muscle specific promoter should be combined with a delivery method that targets all affected muscle tissues.

Direct injections into the muscle would allow some level of specificity. Unfortunately, transgene expression is restricted to the injection site and the near vicinity (O'Hara et al., 2001). For human muscular dystrophies this approach would require hundreds of intramuscular injections. Recently, improved uptake of naked DNA, lipid coupled DNA and viral vectors into muscle was reported by electrotransfer (Cappelletti et al., 2003), ultrasound (Danialou et al., 2002), and the modulation of Starling forces (Cho et al., 2000). To reach all muscles including the heart and the diaphragm, systemic delivery through intravascular routes would be favourable. For systemic delivery, several obstacles need to be overcome: capillaries may transport the therapeutic agent to many non-muscle tissues,

and the therapeutic agent has to pass the blood vessel walls and to enter the muscle cells with high efficiency.

Use of Ads for gene therapy of adult skeletal muscle is restricted by low levels of the primary virus attachment receptor, the Coxsackie- and adenovirus receptor (CAR), on the surface of mature myofibres (Bergelson et al., 1998; Nalbantoglu et al., 1999). Especially after systemic injection the majority of Ad is not found in muscle but in other tissues that express higher levels of CAR, most notably hepatocytes. Retargeting of Ad therefore depends on a twofold strategy (Thirion et al., 2002). First, the ablation of the national tropism by removing the CAR binding site and other sites which still have to be identified that allow interactions with non-muscle cells (Alemany and Curiel, 2001); and second, the introduction of binding moieties that allow to specifically target muscle fibres (Fig. 3-9).

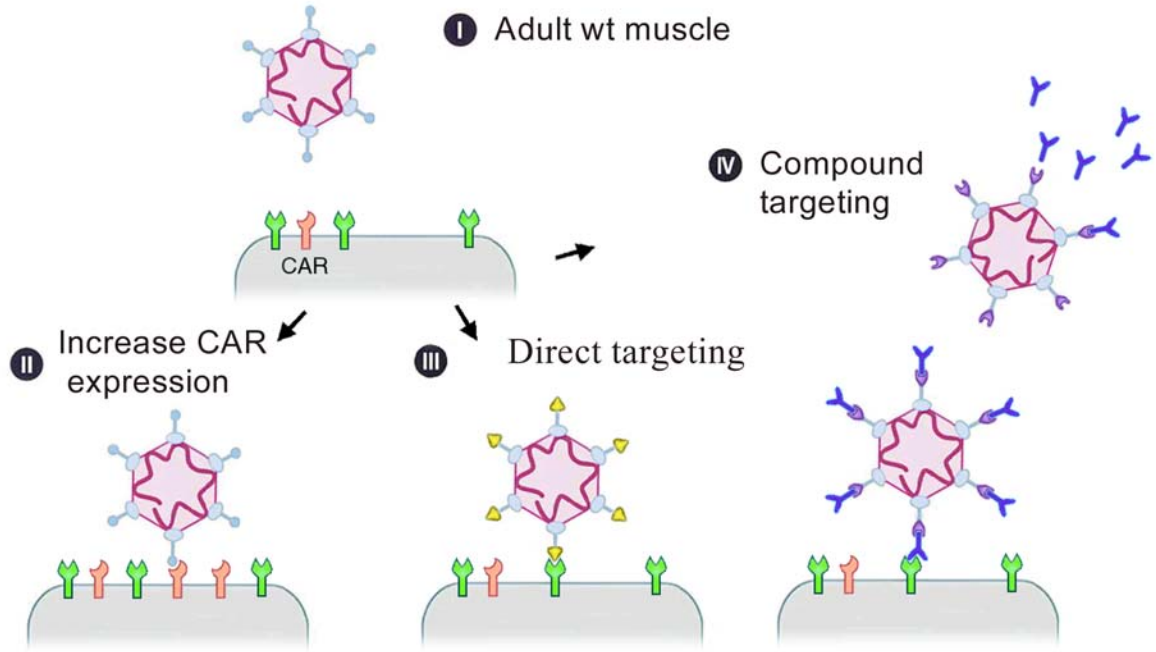


Fig. 3-9 Strategy for adenovirus targeting and enhanced gene delivery into the targeted tissues. (I) In wild-type adult skeletal muscle Ad gene-transfer is limited by the low amount of CAR on myofibres. This low tropism could be overcome by: (II) an increase of CAR expression on the target tissue surface, (III) direct targeting through genetic engineering, e.g. insertion of targeting ligand into the Ad fibre, fibre-swab between Ad-serotypes, or modifications of the penton or hexon sub-units of the viral capsid, (IV) coupling of targeting compounds such as antibody- or bispecific protein-adapters.

Recently, we have demonstrated that antibody-mediated targeting of Ad resulted in muscle specific targeting. A synthetic 33-amino-acid immunoglobulin G (IgG) binding domain derived from staphylococcus protein A was inserted into the Ad fibre (Volpers et al., 2003). The modified fibre bound IgG with high affinity and retained the ability to assemble as fibre-trimers to the viral capsid. Preincubation with monoclonal antibodies against muscle surface molecules like neuronal cell adhesion molecule (NCAM) or α_7-integrin resulted in an up to 77-fold increase in reporter gene transfer in differentiated primary human myoblasts.

This IgG-binding Ad vector holds promise to allow a) easy systematic screenings for the identification of new tissue specific surface receptors, b) targeted gene transfer to a wide variety of cell types without laborious specific modifications of the capsid for each individual target through the use of different antibodies, and c) a double targeting strategy for skeletal muscle through the combination of a muscle specific gene control element with targeted gene transfer.

PART IV

Gentamicin Induced Read-Through of Stop Codons

INTRODUCTION

For decades aminoglycoside (AG) antibiotics have been used to fight bacterial infections. The structural and biochemical properties of AG explain both the therapeutic and side-effects of these drugs. AG interact with ribosomal RNA's (rRNA) inducing errors of the cellular translation machinery .They bind to a highly conserved decoding region of rRNA. The structure of the RNA complexed to gentamicin was recently resolved (Yoshizawa et al., 1998). The affected RNA region normally mediates the precise codon-anticodon pairing. When aminoglycosides are bound to this region the rRNA conformation is modified and the accuracy of the translation process is reduced. Although this interaction with rRNA takes place in various organisms, bacterial translation appears to be more sensitive to AG than mammalian translation (Lynch and Puglisi, 2001). Furthermore, mitochondrially encoded rRNA may be more vulnerable to AG than nuclear encoded rRNA, which may explain some of the side-effects, i.e. ototoxicity and nephrotoxicity (Guan et al., 2000).

Several *in vitro* experiments indicated that AG-related interference with translation may be used for therapy of inherited disorders. A certain proportion of these disorders is caused by nonsense mutations, i.e. a single nucleotide exchange replacing a codon for an amino acid by a stop codon resulting in premature termination of the respective amino acid chain. The resulting truncated proteins are normally not functional and are rapidly degraded.

Several independent groups have shown in cellular models of cystic fibrosis, Hurler's disease and DMD, that AG are capable of inducing “read-through” of such nonsense mutations generating full-length protein that is otherwise deficient (Howard et al., 1996; Howard et al., 2000; Keeling et al., 2001). These full–length proteins may carry an amino

acid substitution as a consequence of the incorporation of an amino acid at the site of the stop codon but in most cases will be nevertheless functional (Fig. 4-1).

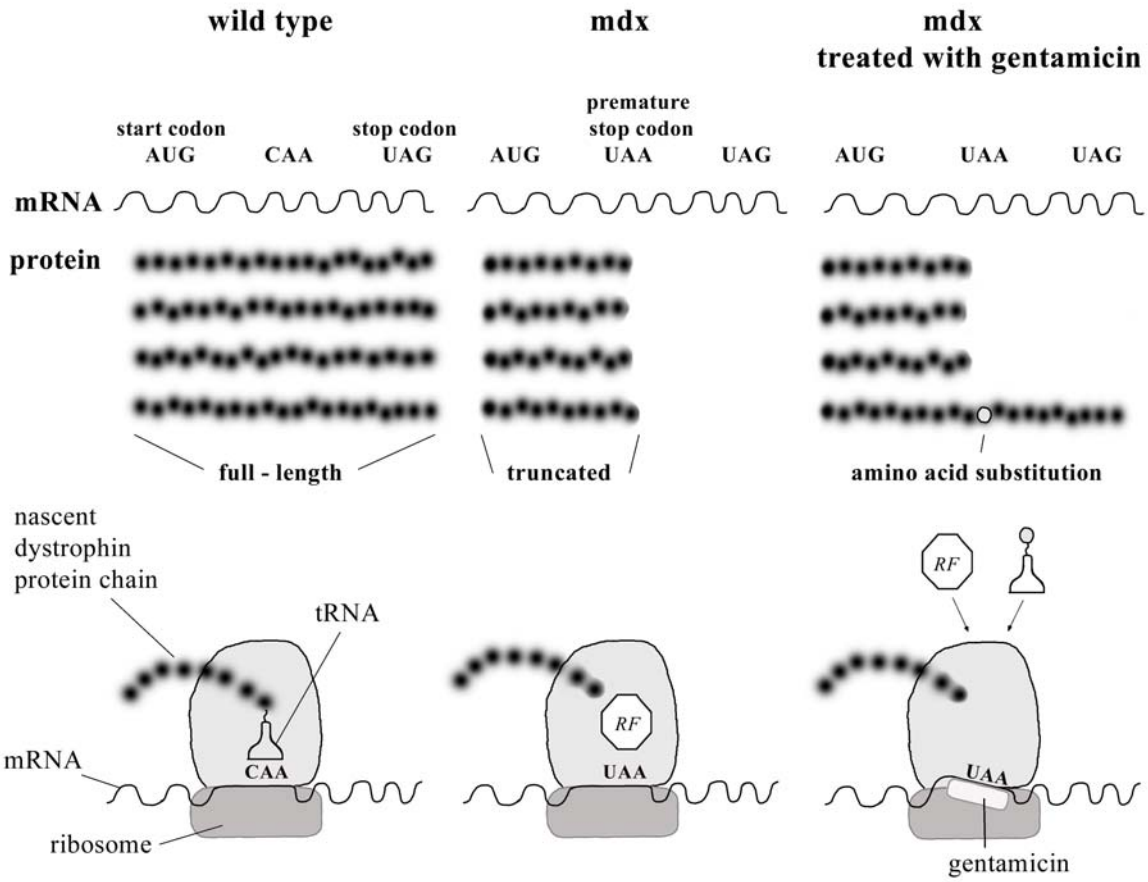


Fig. 4-1 Gentamicin induced "read-through". The wt mRNA encodes the full-length dystrophin protein. At the ribosome a glutamine-carying tRNA normally binds to the corresponding CAA sense codon. In the mdx mouse mRNA a single base transition (CAA > UAA) codes for a premature stop codon which is recognised by release factor proteins (RF). The RFs bind to the mRNA and terminate the translation. Gentamicin binds close to the ribosomal tRNA recognition site and interferes with correct codon recognition. Now, not only RFs bind to the mRNA but occasionally also a tRNA carrying an amino acid. Therefore, full-length dystrophin can be assembled carrying an amino acid substitution if another amino acid than glutamine is incorporated. (Adapted from Mankin and Liebman, 1999)

However, the amount of protein generated was dependant on several factors such as the dosage and composition of the AG as well as the exact sequence of the mutation, and even in the best case scenario it did not reach the levels of wild type cells. Moreover, *in vivo*

application of subtoxic doses of AG has resulted in little if any functional benefit (Clancy et al., 2001).

Therefore, the findings of Barton-Davis et al. reporting that AG can restore functional dystrophin and associated proteins in mdx mice came as a welcome surprise for the scientific community and the lay public (Barton-Davis et al., 1999; Mankin and Liebman, 1999). A number of arguments in favour of AG therapy led to the rapid initiation of several clinical trials for DMD: 1. DMD is a fatal disorder for which other therapies are not really effective. 2. Molecular therapeutic approaches such as gene or stem cell therapy may be years ahead and/or may not live up to the expectations. 3. Gentamicin can be systemically administered and thereby potentially reach all affected muscles. 4. It can be easily produced in sufficient quantities 5. It is a widely used AG, an approved, conventional drug with a long-standing record in safety and tolerance. While a restoration of dystrophin to normal levels was not anticipated, even levels as low as 20% of the normal level may prevent muscle fibre necrosis and improve muscle function (Ahmad et al., 2000; Yang et al., 1998). The majority of mutations of the dystrophin gene, such as large deletions, are unlikely to be beneficially influenced by gentamicin treatment. However, it was estimated that 5- 15% of all DMD cases are due to nonsense mutations of the dystrophin gene, and such cases would be candidates for gentamicin therapy (Mendell et al., 2001).

Up to the present time, results of three clinical trials using gentamicin in DMD/BMD patients carrying nonsense mutations of the dystrophin gene have been reported; two in abstract form and one as a full communication (Dubowitz, 2002, Serrano et al., 2001, Wagner et al., 2001). The fully reported trial by Wagner et al. did not reveal an increase of dystrophin expression in four gentamicin-treated DMD/BMD patients (Wagner et al., 2001), neither did the trial by Serrano et al. in twelve patients. By contrast, G. Nigro in a special workshop reported an immunohistochemical increase of dystrophin expression for one out of four gentamicin-treated DMD patients, but immunoblotting data were not presented (Dubowitz, 2002). The latter trial consisted of 3 cycles of daily gentamicin infusions for one week each, while in the first two trials, a 14-day protocol of daily infusions, very similar to the mouse study (Barton-Davis et al., 1999), was applied. Interestingly, a decrease in creatine kinase (CK) levels during gentamicin treatment was noted in several patients although an increase in dystrophin expression was not detected. The clinical significance of this finding is unclear at the moment. In summary, in contrast

to the reported mouse data (Barton-Davis et al., 1999), clinical trials in DMD patients with short courses of subtoxic gentamicin infusions did not result in clinical benefit. Therefore, independent experimentation in the mdx mouse was carried out to evaluate the effect of gentamicin treatment.

RESULTS

Because a treatment for DMD is in urgent need, treatment of mdx mice with gentamicin was simultaneously tested in two laboratories. The author conducted the work in the laboratory of Hanns Lochmüller which is described here in detail. Supporting data from the trial in Montreal are marked as such.

In the Montreal-study (Laboratory of George Karpati, Neuromuscular Research Group, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada), gentamicin was applied as described in reference 2 (data not shown). Groups of 6 mdx mice were used. Starting at age 4-6 days mice were injected subcutaneously with gentamicin sulfate (34mg/kg or 17 mg/kg; Sigma) or normal saline daily for 14 days.

In the Munich-study effects of gentamicin on dystrophin expression were tested *in vitro* and *in vivo*.

Establishment of immortalised dystrophin negative cells

For *in vitro* experiments, myogenic cells were needed that are dystrophin deficient due to a nonsense mutation of the dystrophin gene (mdx cells). However, cultures of primary murine myoblasts are very heterogeneous, and the cells cannot be propagated for an extended time in tissue culture. Therefore, we generated immortalised lines of dystrophin deficient myogenic (mdx) cells. We used the H-2Kb-tsA58 mouse system which allows quick isolation of immortalised cells from different tissues. Cell lines were isolated from normal and mdx mice that were cross-breeded with “immorto” mice. The resulting cell lines divide indefinitely when cultivated at 33°C and in the presence of INF-γ. They have the additional advantage that the immortalising SV40 large T antigen can be shut down when the cells are cultivated at 37°C and INF-γ is withdrawn. Clones from single muscle progenitor cells of mdx × H-2Kb-tsA58 mice were isolated and characterised. Several clones had the appearance of myoblasts when grown at low densities. Under fusion conditions the cells fused to form elongated multinucleated myotubes (Fig. 4-2).

Fusion started after 36 hours and the majority of cells fused after 3-5 days. Importantly, most myotubes displayed spontaneous contractions after 5-7 days which is a very good

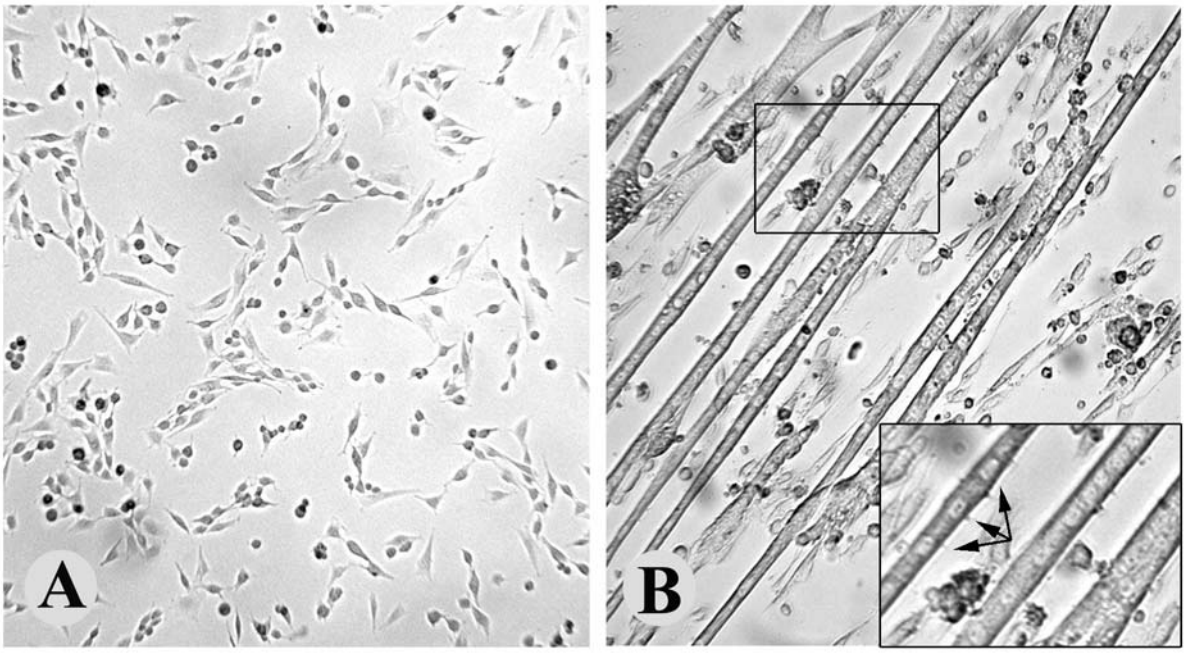


Fig. 4-2 Immortalised dystrophin negative cells. (A) At low densities the myoblasts divided rapidly. (B) At higher densities and fusion-conditions the cells fused to multinucleated myotubes which contracted spontaneously. Arrows in the magnified window indicate the position of single nuclei.

indicator that cells had the potential of differentiated muscle cells. Because human myoblasts whose life span were prolonged by the expression of viral tumor genes (Lochmüller et al., 1999) and primary murine myoblasts show signs of senescence after multiple cell divisions we wanted to assess that our clones were truly immortalised. Two independent clones were continuously cultivated for more than 12 months. The speed and the total number of cell divisions were measured based on cell counts and splitting rates. Overall, cells divided approximately every 22 hours. More than 400 cell divisions were monitored. To ensure that the cultures kept their myogenic features they were regularly tested for fusion and contraction competence. The cells also withstood repeated freezing and thawing without losing any of their characteristic features, which allows convenient storage and distribution of these cell lines. As a control, we isolated an immortalised cell line from a dystrophin positive wild-type mouse with the same method. Western blot analysis revealed that dystrophin was expressed in fused and unfused cells from the immortalised control cell line but was absent in cultured mdx cells (data not shown).

In vitro gentamicin treatment

Dystrophin negative cells were treated with different concentrations (200-500µg/ml) of gentamicin sulfate from two different suppliers. Treatment was continued for 14 days to ensure that dystrophin could accumulate to detectable levels. Cells were either cultivated under normal growing conditions or under fusion conditions. High concentrations of gentamicin sulfate resulted in lower fusion rates and deattachment of the cells (Table 4-1). Therefore, it seems that too high levels of gentamicin interfere with the eukaryotic translation machinery and thereby block proliferation and differentiation.

Table 4-1 *in vitro* effect of gentamicin treatment on cultivated mdx cells (fusion conditions)

Immortalised Cell line	Control	mdx	mdx	mdx	mdx	mdx
Gentamicin (µg/ml)	/	/	200	300	400	500
Fusion to myotubes	+++	+++	+++	++	++	+
Presence of deattached cells	-	-	-	+	++	++
Dystrophin staining	+++	-	-	-	-	-

A dystrophin signal could not be detected either in directly stained myotubes (data not shown) nor in western blot analysis of cell lysates (Fig. 4-3).

***In vivo* gentamicin treatment**

For *in vivo* experiments, groups of 4 mdx mice were used. Mice were 5 weeks old at the beginning and 12 weeks old at the end of the experiment. Three courses of daily intraperitoneal gentamicin sulfate injections were given for one week (8.5 mg/kg for female and 34mg/kg for male mdx mice). Gentamicin sulfate was purchased from GibcoBRL (Batch 3034117; composition according to the supplier: C1 - 28%; C1a - 20%; C2a and C2 - 52%). The 3 courses were separated by 2-week intervals. On the day following the last injection, the resistance to eccentric muscle contractions was measured (Yang et al., 1998) and muscles were collected for immunohistochemistry and immunoblotting. No fatality was noted during the treatment and no obvious side-effects were observed.

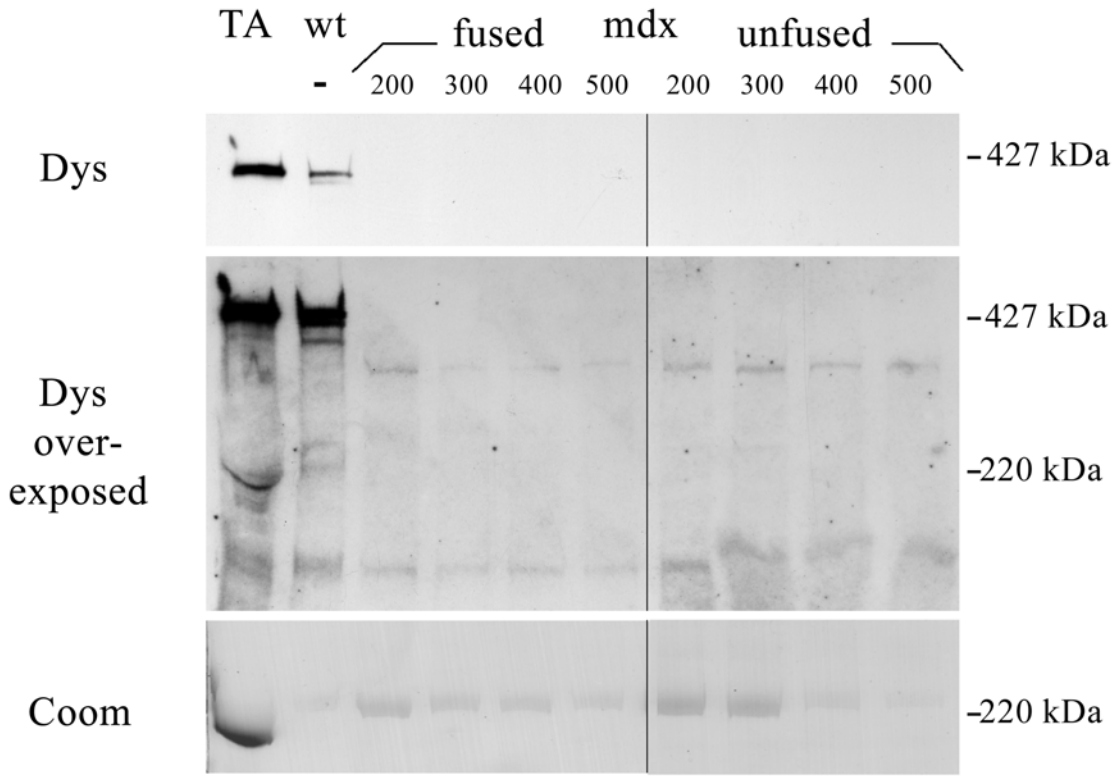


Fig. 4-3 Dystrophin expression in immortalised mdx myotubes that were treated *in vivo* with different concentrations of gentamicin (200-500µg / ml). Two exposures of the dystrophin immunoblot and the corresponding post-transfer Coomassie blue stained gel are shown. Lane 1, protein lysate of *tibialis anterior* muscles from a dystrophin positive wt mouse and lane 2, lysate of immortalised cells of a wt mouse - as positive controls. Note that no dystrophin was detected in the cell lysates of gentamicin-treated immortalised mdx cells even when the blot was overexposed.

Muscle function measurements

Resistance to eccentric muscle contraction was measured for EDL muscles (*M. extensor digitorum longus*) according to standard protocols (Yang et al., 1998). Gentamicin-treated mdx mice were not different compared to untreated control mdx mice in contrast to the very good resistance of age matched C57BL/6 muscles (Table 4-2).

Table 4-2 Resistance to eccentric muscle contraction was measured for EDL muscles.

	male mice		female mice	
	force drop	SD	force drop	SD
C57BL/6 (control)	20.1 %	4.1 %	14.2 %	3.8 %
mdx (untreated)	68.1 %	9.7 %	65.0 %	12.2 %
mdx (gentamicin-treated)	67.9 %	12.7 %	61.6 %	8.9 %

The maximum force at the first tetanic contraction is compared to the maximum force at the first eccentric contraction. The force drop is given as a percentage +/- standard deviation. A high force drop is observed in muscles with decreased membrane stability such as dystrophin deficient muscle. Gentamicin-treated mdx mice were not different compared to untreated control mdx muscles in contrast to the very good resistance of age matched C57BL/6 muscles.

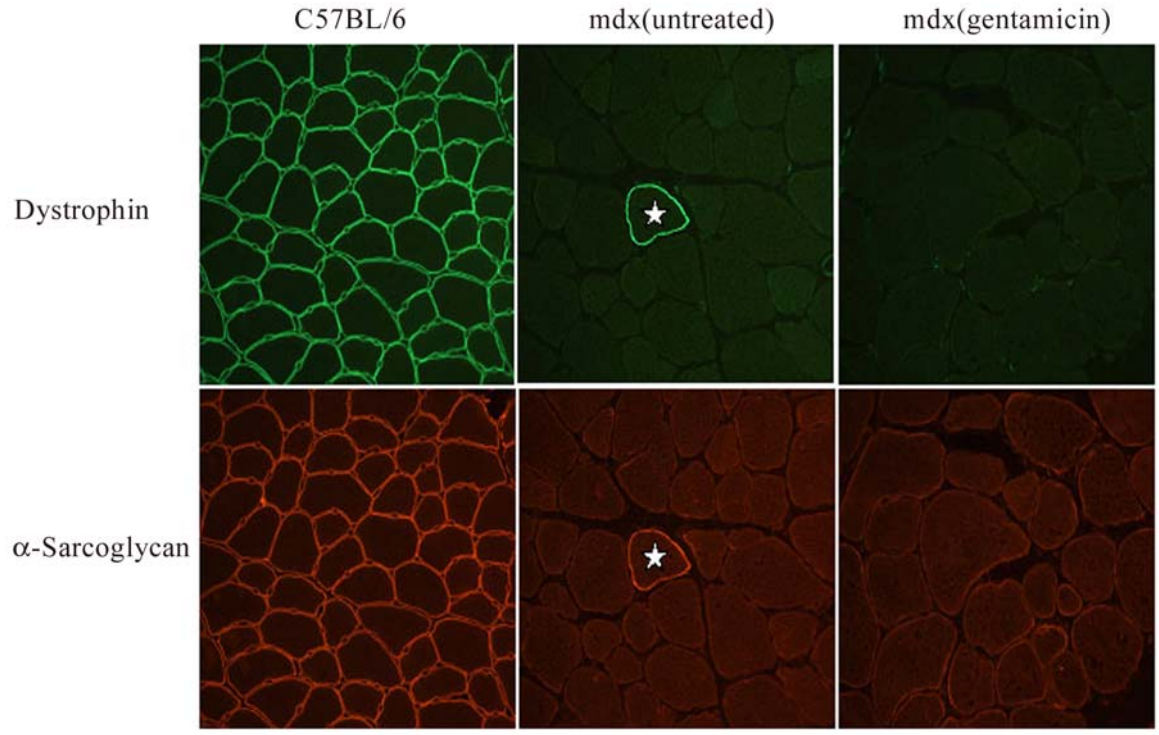


Fig. 4-4 Immunohistochemistry for dystrophin and β-sarcoglycan. Representative results (*M. tibialis anterior*) of dystrophin-positive control mice (C57BL/6), of gentamicin-treated mdx mice and of untreated mdx mice are shown. Revertant fibres are indicated by an asterisk. In muscles of gentamicin-treated mdx mice an increase of dystrophin or sarcoglycan was not detected.

Detection of dystrophin expression

Sections of various skeletal muscles (*M. tibialis anterior, M. soleus, M. quadriceps femoris, M. extensor digitorum longus, diaphragm*) were analysed immunohistochemically for the expression of dystrophin and sarcoglycans. Muscle sections of mdx and C57BL/6 mice were used as controls. An increase of dystrophin or sarcoglycans in muscles of gentamicin-treated mice was not observed (Fig. 4-4).

Western blot analysis of dystrophin does not give information about the localisation and distribution of the protein of interest, but is very sensitive and also allows an estimate of the size of the detected protein. In the case of a gentamicin induced read-through mechanism full-length dystrophin (427kDa) would be expected. Muscle protein lysates of *M. tibialis anterior* and heart from gentamicin-treated mdx mice were analysed for dystrophin expression on western blots. Lower amounts (50% and 17%) of muscle protein from dystrophin-positive C57BL/6 mice were used as positive controls, and blots were overexposed. We estimated that our method could detect dystrophin levels as low as 3 % of the normal. However, no specific signal for dystrophin was present in gentamicin-treated mdx mice (Fig. 4-5).

In conclusion, gentamicin treatment failed to lead to a significant increase of dystrophin expression as ascertained by both immunohistochemistry and immunoblotting.

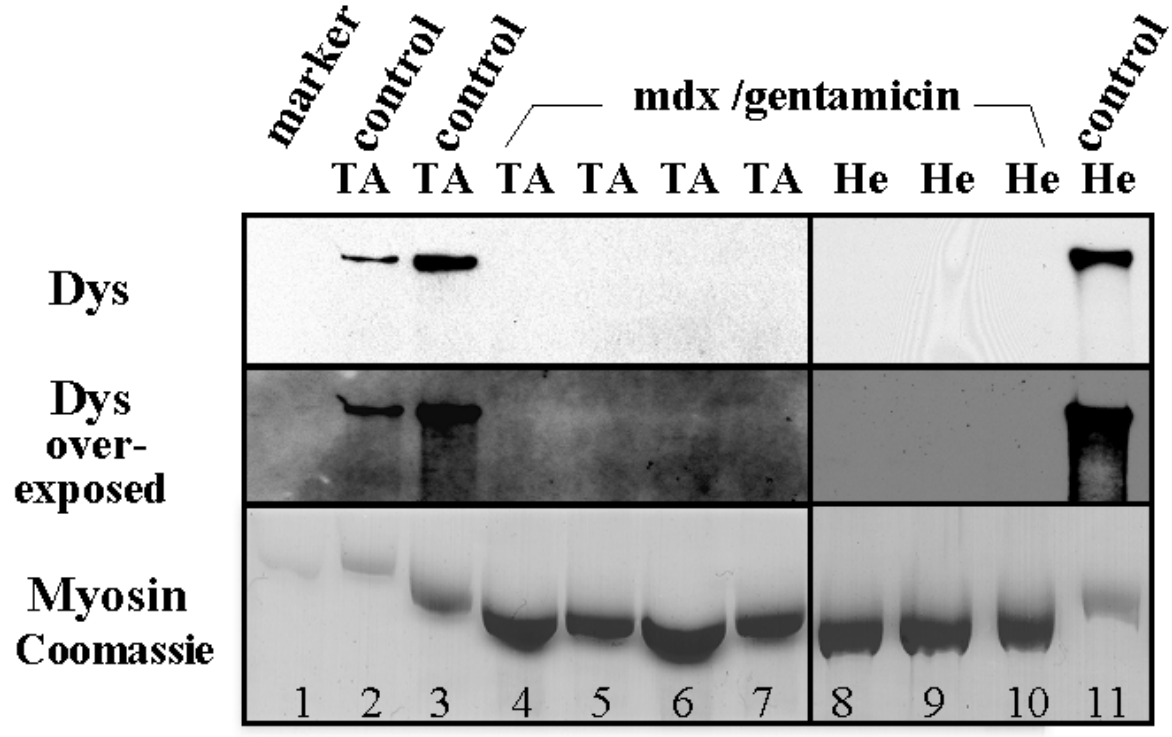


Fig. 4-5 Dystrophin expression in muscles from mdx mice that received gentamicin injections. Two exposures of the dystrophin immunoblot and the corresponding post-transfer Coomassie blue stained gel are shown. In lane 1, a molecular size marker was loaded (myosin, 200 kDa). Lanes 2,3 are protein lysates of *M. tibialis anterior* (TA), lane 11 cardiac muscle of dystrophin-positive control mice (C57BL/6). Note that only 17% (lane 2) and 50% (lane 3,11) total protein were loaded for controls if compared to gentamicin-treated mdx mice. Lane 4-8 are protein lysates of *tibialis anterior* muscles, 9 and 10 of cardiac muscle of gentamicin-treated mdx mice (2-6 and 9 - female mdx mice; lanes 7,8,10 - male mdx mice). In muscle lysates of gentamicin-treated mdx mice dystrophin was not detected even when the blot was overexposed (panel B).

DISCUSSION

The urgent need of treatment for DMD, the *in vitro* proof that aminoglycoside antibiotics can overcome premature stop codons and an initial report that claimed substantial gentamicin induced dystrophin expression and improvement of muscle function in mdx mice led to the rapid initiation of clinical trials. Unfortunately, DMD patients treated with gentamicin did not show any benefit and dystrophin expression was not restored.

In clinical trials, potential side-effects have to be carefully balanced with potential benefits. Long term use of gentamicin, as would be required for the therapy of DMD, may be associated with damage especially to the renal tubes (nephrotoxicity) and to the hair cells of the inner ear which can result in hearing loss (ototoxicity). Gentamicin also has a weak neuromuscular-junction-blocking activity among other less common side-effects. The exact cause of these effects is not known. But it seems reasonable that the same mechanisms which effect general translation and allow read-through in the case of the mdx point mutation also may promote the misreading in other proteins. This may result in detrimental function losses or gains in the affected proteins. Alternatively, proteins which do not fold properly due to alterations may form toxic aggregates. Aminoglycosides were shown to interfere with a number of RNA functions such as self splicing of group I introns (Von Ahsen and Noller, 1993) and hammerhead ribozyme cleavage (Stage et al., 1995). They can also destabilise plasma membranes and interfere with phosphatidylinositol mediated signal transduction. Mitochondria may be also involved in aminoglycoside toxicity because mitochondrial translation is closer related to procaryotic translation and therefore more vulnerable than eucaryotic translation. Because of the widespread use of and the frequent side-effects attempts were undertaken to reduce the toxicity of aminoglycosides. Iron chelators (Song et al., 1998) such as dihydoxybenzoate (DHB) and substances that reduce the production of reactive oxygen species (McFaddden et al., 2003) attenuated ototoxicity in guinea pigs which support the idea that iron and free radicals play a critical role. Ototoxicity was also reduced by leupeptin, a calpain inhibitor (Ding et al., 2002). The calpains are a family of calcium-activated proteases that participate in protein degradation and cell death. Hair cells could be also protected through inhibitors of the c-Jun-N-terminal kinase (JNK) signalling pathway (Bodmer et al., 2002). Even though these studies are still at a pre-clinical stage they may lead to the development of a protocol that reduces gentamicin induced side-effects. An alternative option to reduce the toxicity of aminoglycosides is to target the drug to the most affected tissue. In the case of DMD it

would be sufficient if the compound would reach the muscle. This may reduce damage of the more sensitive organs, i.e. the ear and the kidneys. Drug targeting to skeletal muscle may be achieved through coupling of the aminoglycoside to a compound that binds preferentially to muscle fibres. Similarly, drugs were targeted to the liver by coupling to galactose derivates (Hashida et al., 2001; Ulrich, 2002).

Even if the side-effects of gentamicin could be controlled and were tolerable, a significant improvement of muscle function related to the treatment needs to be shown in DMD patients. Initial clinical trials in DMD did not reveal functional improvement or even a small raise in dystrophin levels. This was in contrast to results that had been reported on successful gentamicin treatment in the mdx mouse (Barton-Davis et al., 1999), and a species-specific difference in gentamicin action was discussed. Therefore, the aim of our experiments was to reevaluate gentamicin treatment *in vitro* and in the mdx mouse model.

In vitro experimentation has the advantages that it can be rapidly conducted, is ethically unproblematic when compared to animal or clinical trials, and that gentamicin application is straightforward in tissue culture. Even though a functional improvement of muscle force can not directly be ascertained in cultivated cells, a gentamicin induced read-through that results in *de novo* dystrophin expression should be easily detectable in mdx cells. Moreover, *in vitro* tests may allow the rapid screening of different forms and concentrations of aminoglycosides.

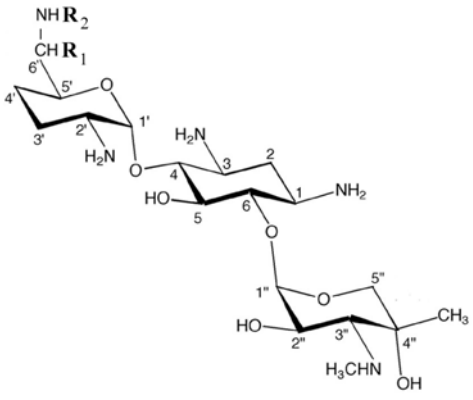
Therefore, we generated immortalised, myogenic mdx cells that yielded reproducible results. Moreover, these cells can easily be differentiated to fused myotubes. Initial tests ascertained that in contrast to control myotubes the mdx myotubes did not produce dystrophin. Immortalised mdx cells were then incubated for 14 days with gentamicin from two different suppliers at different concentrations. At the highest concentrations gentamicin was clearly toxic to the cells because an inhibitory effect on myoblast division and myotube formation was observed. Moreover, treatment with gentamicin did not lead to significantly increased dystrophin expression. It should also be noted that immortalised mdx cells were successfully used by us to express dystrophin in other therapeutic experiments:

Immortalised mdx cells were used in experiments in which AOs were applied to overcome a mutation in the dystrophin gene by splicing out the affected exon (see introduction). Clinical use of AOs would require an repeated administration to obtain a lasting effect. A refinement of this strategy could be the *in situ* production of antisense RNA. This would

require the transfer of the AOs sequences into the affected tissues. But because the AOs are very small (~25 bp) this might be more easily achieved than the transfer of the much longer dystrophin protein-constructs. In a recent study, Weis and colleagues in co-operation with our group proved the feasibility of this concept by demonstrating that expression of small antisense U7 snRNAs could restore dystrophin expression in immortalised mdx myoblasts (Brun et al., 2003).

We then conducted gentamicin experiments in mdx mice. However, no increase in dystrophin expression and no improvement of muscle force was observed. Therefore, neither clinical trials in DMD/BMD nor our studies in mdx mice revealed a significant increase of dystrophin expression in skeletal or cardiac muscle in response to gentamicin treatment. Subtoxic doses of systemically administered gentamicin did not reveal therapeutic efficacy in dystrophin deficiency. This is at variance with the original positive report (Barton-Davis et al., 1999). A number of reasons may account for this discrepancy such as the chemical composition of the drug, the duration of treatment, the method of application, the exact sequence of the mutation, etc. (Karpati and Lochmüller, 2001).

An important issue may be the composition of the gentamicin sulphate used. A given batch of commercially available gentamicin usually contains 3 major components (gentamicin C1, C1a and C2) that are structurally identical except for the different methylation patterns at the 6` position (Fig. 4-6).



	R_1	R_2	%
Gentamicin C1	CH_3	CH_3	25-50
Gentamicin C2	CH_3	H	15-40
Gentamicin C1a	H	H	20-55

Fig. 4-6 Structure of gentamicin components. Commercially available gentamicin is a mixture of 3 components. The composition of the individual components varies from batch to batch.

Interestingly, the three components differ significantly in their propensity to induce misreading in bacterial and eukaryotic ribosomes (Loveless et al., 1984; Yoshizawa et al., 1998). It was shown that gentamicin C_2 induced the highest rate of misreading for poly (U)-directed protein synthesis (Loveless et al., 1984) while gentamicin C_1 was virtually ineffective. The gentamicin used in our experiments contained a relative high amount of the C_2 component (52%) and may not account for the failure of dystrophin induction. It may be necessary to test pure compounds for their ability to induce read-through of point mutations. Additionally, subtle alterations of the stereochemistry at the 6' position of gentamicin may lead to drugs that combine the optimal induction of stop codon read-through with a low overall toxicity. Alternatively, other aminoglycosides such as G-418 and paromomyxin that were also demonstrated to induce read-through (Howard et al., 2000) could be used.

The dosage, the form of application and the duration of the gentamicin treatment may be critical, especially as there may be only a narrow window that allows for efficient dystrophin production (Barton-Davies et al., 1999). According to the original report, gentamicin dosage was critical and differed among female and male mdx mice (Barton-Davies et al., 1999). However, we used the same dosage and application method as originally reported (Barton-Davies et al., 1999). Therefore, it appears unlikely that these factors are responsible for the divergent results obtained by us if compared to the previously reported study (Barton-Davies et al., 1999).

Nevertheless, the assessment of an optimal dosage would prove a great challenge in the putative treatment of DMD patients, becaus of a) differences in individual sensitivity to gentamicin toxicity b) different drug metabolism rates and c) different point mutations (see below). This may necessitate long term optimisation of dosing for individual patients. This optimisation would be further complicated since mild improvement would be difficult to seize: quantification of dystrophin protein in muscles would necessitate repeated muscle biopsies. Criteria that do not depend on invasive methods such as improvement of muscle function (e.g. force) may require long time expression. Surrogate markers of muscle damage such as serum creatine kinase levels may not yield a realistic assessment of muscle function. For example, gentamicin treatment of DMD patients resulted in a decrease of the serum creatine kinase relative to pre-trial values, while dystrophin expression and muscle function were not improved (Wagner et al., 2001).

Effective treatment of DMD may require the long term persistence of dystrophin in affected muscles. In theory, dystrophin production may be strongest after single peaks of aminoglycoside application. Therefore, it is important to determine the half-life of the dystrophin protein and the duration of the protective effect. Ahmad et al. (2000) demonstrated in a transgenic mouse model which allowed inducible dystrophin expression, that dystrophin persisted for more than 6 months and was accompanied with a functional protection of the muscle once it was associated with the sarcolemma. These observations indicate that it may be possible to develop treatment regimes where short time periods of treatment alternate with rest periods to reduce possible side-effects. We also conclude that the combined 3 weeks of gentamicin treatment in our mdx trial should have been a sufficient time period to accumulate enough dystrophin to allow its detection.

Previous studies of aminoglycoside treatment have indicated that the efficiency of read-through depends strongly on the sequence context and on the different termination efficiencies of the 3 possible stop codons. This may be of particular interest because individual DMD patients carry different stop codons in varying parts of the dystrophin gene. Different stop codons have low and somewhat different rates of naturally occurring read-through (UGA > UAG > UAA), i.e. in the absence of a drug. Moreover, it was recently shown that efficiency of translation termination correlates inversely with the ability to allow aminoglycoside induced read-through (Howard et al., 2000). UAA is the strongest translation terminator and allows the lowest rate of read-through in the presence of gentamicin. The context of 5' and 3' sequences also modifies the stop codon efficiency. The most pronounced effect has the nucleotide immediately after the stop codon (+4) (Brown et al., 1990; McCaughan et al., 1995). For UGA the order of bases at +4 that allowed the highest read-through in *in vitro* assays were C > U > A ≥ G. In general, purines may mediate a stronger termination signal at this position than pyrimidines. Approximately 90% of strong house keeping genes in mammals have a purine right after the stop codon (Tate and Mannering, 1996). Interestingly, the naturally occurring stop codon read-through can reach levels that ameliorate the progression of a genetic disease even in the absence of aminoglycosides: a UGA stop codon mutation in the gene that causes cystic fibrosis (CFTR) led to a milder phenotype than other types of missense mutations (Cuppens et al., 1990; Cuttting et al., 1990). The same mutation allowed a read-

through of approximately 10% in the corresponding gene of a yeast model (Fearon et al., 1994).

Even in the most positive therapeutic scenario, it can be predicted that dystrophin will not reach normal wt levels. Therefore, additional questions need to be answered : What are the maximal levels of functional dystrophin that can be expected by gentamicin treatment? What are the minimal levels of dystrophin that bring about functional improvement? First, induction of high dystrophin levels is in direct competition with the impairment of general protein translation and increased levels of wrongly incorporated amino acids. Aminoglycoside concentrations that may allow high levels of dystrophin read-through may therefore at the same time compromise normal translation termination. In addition, aminoglycosides may lead to the introduction of missense mutations that will impair the function of dystrophin and other proteins.
A second limitation may be the availability of dystrophin mRNA. It has been shown that the dystrophin mRNA is strongly reduced in muscles of mdx mice to approximately 20% of control levels (Chamberlain et al., 1988). This decrease of dystrophin mRNA may be mediated through the nonsense-mediated mRNA decay pathway which degrades specifically messages containing premature stop codons (Hentze and Kulozik, 1999; Frischmeyer and Dietz, 1999; Byers, 2002). Therefore, low levels of the prerequisite dystrophin mRNA may also reduce maximal translational read-through. It should be noted though that low-levels of aminoglycoside induced read-through may on the other hand be sufficient to reduce the level of nonsense-mediated decay which may result in elevated dystrophin mRNA levels. An example for the latter possibility has been described: read-trough of a UGA stop codon, induced by selenocystein incorporation reduced nonsense-mediated mRNA decay of the selenium-dependent glutathione peroxidase I gene (Moriarty et al., 1998).

When gentamicin was not effective in the clinical trials it seemed possible that differences between humans and mice were responsible. Since our experiments in mice do not show a positive effect of gentamicin treatment in mice, the difference between species is probably not a key factor. The data that were represented in this study clearly indicate that additional tissue culture and *in vivo* mouse experimentation is required to identify factors or circumstances that may render AG effective in treating DMD/BMD. While gentamicin thus far did not show efficacy in DMD, the recent clinical trials yielded several useful data: 1.

new methodology has been developed to identify nonsense mutations in dystrophin-deficient patients, not only large-scale deletions (Dolinsky et al., 2002; Mendell et al., 2001); 2. an awareness was raised that different molecular defects of the same gene in the same disease may require different modalities of molecular treatments ; and therefore 3. molecular treatment may need to be custom-tailored for individual patients (Dubowitz, 2002; Walter and Lochmüller, 2001).

Part V

Appendix

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ABBREVIATIONS

°C	degrees Celsius
AAV	adeno-associated-virus
AG	amino glycoside
APC	antigen presenting cell
AO	antisense ologonucleotides
Ad	adenovirus
Ads	adenoviruses
BMD	Becker muscular dystrophy
bp	base pair
CAR	coxsackie adenovirus receptor
cDNA	complementary (to mRNA) DNA
CFTR	cystic fibrosis transmembrane regulator
CMV	cytomegalovirus promoter
COOM	Coomassie
CSA	cross sectional area
DAPC	dystrophin-associated protein complex
DHB	dihydoxybenzoate
Dia	diaphragm
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
Dys	dystrophin
EDL	*M. extensor digitorum longus*
EDTA	ethylendiamintetraacetate
EF-hand	intracellular calcium binding motif
e.g.	*exempli gratia* (Lat. = for instance)
et al.	*et alii* (Lat. = and others)
FCS	fetal calf serum
Fig.	figure
GRMD	golden retriever muscular dystrophy
g	gram
HEPES	N-(2-Hydroxyethyl)-piperazine-N´-2-ethansulfonacid
HFMD	hypertrophic feline muscular dystrophy
HSV	herpes simplex virus
Hz	Herz
i.e.	*id est* (Lat. = that is to say)
IgG	immunoglobulin G
INF-γ	interferon-gamma
JNK	c-Jun-N-terminal kinase
kb	kilo (103) bases

kDa	kilodalton
l	liter
Lo	optimal muscle length
M	molar
M.	musculus
Mb	mega (106) bases
MCK	muscle creatine kinase
MEF2	muscle enhancer factor 2
mdx	murine dystrophy X-linked
MHC	major histocompatibility complex
min	minute
mRNA	messenger RNA
N	newton
nNOS	neuronal nitric oxide synthase
NCAM	neuronal cell adhesion molecule
nt	nucleotide
NADH	nicotineamide adenine dinucleotid
pA	poly-A signal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPR	proximal promoter region
QU	*M. quadriceps*
RF	release factor
RNA	ribonucleic acid
RSV	rous sarcoma virus
s	second
SD	standart deviation
s.e.m.	standart error
Sol	*M. soleus*
SV40	Simian virus 40
TA	*M. tibialis anterior*
tg	transgenic
tRNA	transfer RNA
v/v	volume per volume
w/v	weight per volume
WB	western blot
wt	wild-type
WW domain	domain that contains 2 conserved tryptophans and binds proline rich proteins

Curriculum vitae

PERSONAL INFORMATION

Date of birth: 26th of December 1970

Place of birth: Düsseldorf

Citizenship: British, Swiss

Marital status: Married

EDUCATION

1997– 2003 **Genzentrum, Ludwig-Maximilians-Universität, München**
Ph.D. at the department of chemistry and pharmacy
„Strategies for molecular therapy of Duchenne muscular dystrophy“ Supervisors Prof. Dr. Winnacker and Priv. Doz. Dr. Lochmüller

Spring 1999 **McGill University, Montreal, Canada**
Training in muscle force measurement

1990 – 1997 **Westfälische Wilhelms-Universität, Münster**
Diplom Biologie
„Molekularbiologische und biochemische Untersuchungen an Pathogenitätsfaktoren von A. rabiei.“ Supervisor Prof. Dr. Barz

1994 – 1995 **University of Sevilla, Spain, *Auslandssemester***

1981 – 1990 **Städtisches Meerbusch Gymnasium, Meerbusch, *Abitur***

1977 – 1981 **Städtische Martinus Grundschule, Meerbusch**

SCHOLARSHIP

2001 – 2003 **Predoctoral scholarship from „*aktion benni & co.“***
(Duchenne Parent Organisation)

Acknowledgements

I would like to express my sincere gratitude to the people, who contributed over the last years in making this work not only a scientific success but also a part of my life that I will always happily remember.

Prof. Dr. Ernst-Ludwig Winnacker, who kindly accepted me into the PhD program and provided our group with excellent research facilities at the gene centre. The exquisite state of the art equipment proved to be as valuable as the close neighbourship to many excellent multidisciplinary research groups.

Priv. Doz. Dr. Hanns Lochmüller, who devoted great interest and efforts to these projects. I am also very grateful to Hanns for having confidence in me, having offered me the possibility to join the laboratory in Munich, and especially for his friendship.

Prof. Dr. Eckhart Wolf and his group, especially Ingrid Renner-Müller and Petra Renner, who took expertly care of the research animals.

Prof. Dr. Rudolf Grosschedl, who supported me in acquiring the muscle force measurement equipment.

My colleagues Angela Abicht, Ursula Klutzny, Nancy Larochelle, K-Kris Orlopp, Rolf Stucka, Carolin Schmidt, and C-Chris Thirion, who contributed to the productive and enjoyable atmosphere in our lab. Ursula Klutzny and Eva Schmidtmeier for valuable technical assistance.

Daniel Ursu, who helped to develop the software, which controlled the muscle force measurements.

The staff of the gene centre, especially Petra Fulde, Siegi Kastenmüller, Manfred Schülein and Michi Till.

A special acknowledgement to the Duchenne Parent Organisation “aktion benni & co”, Germany, for the financial support in the form of a pre-doctoral scholarship.

I also thank my family, who always encouraged and supported me.

My final acknowledgement goes to Sabine, because without her love, support, and comprehension, none of this would have been possible.

I sincerely wish that this work has contributed to the advancement of the formulation of a therapy for Duchenne muscular dystrophy.