

# Utrophin in Therapy of Duchenne Muscular Dystrophy

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A thesis submitted for the degree of Doctor of Philosophy

Supervisor

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Dedicated to my father: Yeoh Ho Huat,  
my mother: Tan Kim Choo,  
my husband: Steve,  
and my children: Julie and Peter.

## Declaration

The work presented in this thesis was initiated at the Genetics Unit, Biochemistry Department, Oxford University, South Parks Road, Oxford and completed at the MRC Functional Genetics Unit, Department of Human Anatomy and Genetics, South Parks Road, Oxford. Except where acknowledgement is made, the work is my own and has not been submitted for any other degree in this or any other University or Institute of learning.

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## Abbreviations

AAV	adeno-associated virus
ABD	actin binding domain
ABS	actin binding site
ACh	acetylcholine
AChR	acetylcholine receptor
ALDP	adrenoleukodystrophy
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BMD	Becker muscular dystrophy
bFGF	basic fibroblast growth factor
bp	base pairs
[Ca <sup>2+</sup> ] <sub>i</sub>	intracellular calcium ion concentration
cDNA	complementary DNA
CK	creatine kinase
CMD	congenital muscular dystrophy
CMS	congenital myasthenic syndrome
CMV	cytomegalovirus
CNS	central nervous system
CO <sub>2</sub>	carbon dioxide
CoA	co-enzyme A
C-terminal	COO <sup>-</sup> - terminal domain
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
DPC	dystrophin associated-protein complex
Dpx	Synthetic resin mountant. Deflandre 1947.
DRP	dystrophin related protein
dy	dystrophic muscularis
E1-E3	early genes of adenovirus
ECG	electrocardiogram
EDL	extensor digitorum longus
EDTA	ethylenediamine tetra-acetic acid
eNOS	endothelial NOS
FGF	fibroblast growth factor
FITC	fluorocein isothiocyanate
GRMD	golden retriever muscular dystrophy
H&E	haematoxylin and eosin staining

H <sub>2</sub>	hydrogen
HCG	human chorionic gonadotrophin
HCl	hydrochloric acid
HRP	horse radish peroxidase
HSA	human skeleton $\alpha$ -actin
i.p.	intra-peritoneal
IGF	insulin growth factor
iNOS	inducible NOS
iu	international unit
kb	kilobase pairs
kDa	Kilo-Dalton, the approximate mass of a biological polymer in units equal to approximately 1000/12 x the molecular mass of a <sup>12</sup> C atom. 1kDa=1.66 x 10 <sup>-24</sup> kg.
LGMD	limb-girdle muscular dystrophy
mdm	muscular dystrophy with myositis
<i>mdx</i> mouse	dystrophin-deficient mouse
MHC	myosin heavy chain
min	minute
mm	millimetre
mRNA	messenger RNA
msec	millisecond
MTJ	myotendinous junction
NMJ	neuromuscular junction
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
OSI	Onco Science Inc.
PCR	polymerase chain reaction
LAMA2	Alpha2-chain of laminin 2
FSH	Follicle stimulating hormone
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSC	standard saline citrate
TA	Tibialis Anterior
TE	Tris-EDTA
TE buffer	Tris-HCl EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
SCARMD	severe childhood autosomal recessive muscular dystrophy
ORF	open reading frame

PDZ	PSD95, disc large and zona occludens
TGF- $\beta$	transforming growth factors- $\beta$
tk	thymidine kinase
TRIS	Tris(hydroxymethyl)aminomethane
UL <sup>-1</sup>	unit per litre
VLCFA	very long chain fatty acids

# Abstract

## Utrophin in Therapy of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an inherited, severe muscle wasting disease caused by the loss of the cytoskeletal protein, dystrophin. Patients usually die in their late teens or early twenties of cardiac or respiratory failure. Currently there is no cure for this devastating disease. In healthy skeletal muscle, dystrophin is localised to the cytoplasmic surface of the sarcolemma, where it is involved in a series of molecular interactions forming a mechanical linkage between the cytoskeletal actin and the extracellular matrix through the dystrophin-associated protein complex (DPC). The absence of dystrophin in DMD results in the loss of DPC and muscle degeneration.

Utrophin is the paralogue of dystrophin. It has similar functional domains and binding partners. Utrophin is ubiquitously expressed. It is localised to the cytoplasmic face of the neuromuscular and myotendinous junctions in normal adult skeletal muscle; it is found at the sarcolemma of regenerating and developing muscle. The structural similarity between the two proteins led to the postulation that utrophin may be able to replace dystrophin in DMD.

In this thesis, mice transgenic for a truncated utrophin minigene and a full-length utrophin gene were generated to test the hypothesis that utrophin can replace

dystrophin in *mdx* (animal model of the disease) skeletal muscle. Utrophin transgenic expression re-established the DPC to the sarcolemma and restored strength to the muscle in the *mdx* mouse. Ubiquitous over-expression of full-length utrophin in a broad range of tissues was shown not to be detrimental in the *mdx* mouse. This suggested that tight control over utrophin expression might not be necessary for the correction of muscular dystrophy. Therefore, up-regulation of the endogenous utrophin by drugs to compensate for dystrophin deficiency is a possible therapy in the treatment of DMD.

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# Chapter 1

## Introduction

### 1.1 Duchenne muscular dystrophy (DMD)

#### 1.1.1 *Clinical symptoms*

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive progressive muscle wasting disease affecting approximately one in three thousand boys and it is caused by mutations in the DMD gene encoding the protein dystrophin (Gowers, 1879; Emery, 1993). It is the most common genetic muscular degenerative disease. There is now excellent prenatal diagnosis but as yet there is no effective treatment for this devastating disease. Clinically infants are normal at birth. Affected boys may be hypotonic or have delayed motor development. The first symptoms are often related to locomotor problems developing around the age of three with the affected child “unable to run properly”, walking unsteadily with waddling gait, walking on toes and having difficulty in climbing stairs (Emery, 1993).

Pseudohypertrophy of the calf muscles is the most obvious feature in the early stages of the disease and it is occasionally found in other affected muscles as well. Pseudohypertrophy is mainly due to the accumulation of adipose and connective tissues. In general, the lower limbs are more affected by muscle wasting than the

upper limbs and the proximal muscles more than the distal muscle. At the early stage of the disease, certain muscles are predominantly affected and highly selective. For example, the quadriceps is more affected than the hamstrings, triceps more than biceps, wrist extensors more than flexors and neck flexors more than extensors. This differential muscle involvement becomes less clear as the disease progresses. Weakness of the knee and hip extensors results in the classical Gower's manoeuvre: "the child climbs up his thighs" using his hands to push himself up to standing position from lying (Gowers, 1879).

As the disease progresses lumbar lordosis becomes more exaggerated and the waddling gait increases. Shortening of the Achilles tendon becomes more marked. The patients are usually wheel chair bound by the age of twelve. Upper limb weakness follows: it is progressive and disabling. Contractures increasingly develop particularly flexion contracture of the elbows, knees and hips. Progressive kyphoscoliosis results in thoracic deformity that restricts adequate pulmonary airflow caused by intercostal muscle and diaphragmatic weakness, in addition to causing discomfort and disfigurement. The patients usually die in their late teens or early twenties due to heart and respiratory failure.

### **1.1.2 Skeletal muscles involvement**

The disease is characterised by repeated cycles of skeletal muscle fibres degeneration and regeneration. This results in necrotic patches undergoing phagocytic infiltration and regenerating fibres of varying fibre size with centrally located nuclei. Eventually, fibrocollagenous and adipose tissues replace the dead fibres, giving rise to the clinical appearance of pseudohypertrophy followed by severe atrophy. The type 1 fast twitch muscle is affected predominantly initially followed by the slow twitch type 2 muscle

at later stages (Webster et al., 1988). However, extraocular and pharyngeal muscles are not affected (Khurana et al., 1995b; Porter et al., 1998; Andrade et al., 2000). Rarely there is mild facial weakness.

### **1.1.3 Involvement of other tissues**

DMD is a multi-systems disease and a variety of tissues other than skeletal muscle can be affected. Some patients suffer from bladder paralysis, paralytic ileus and gastric dilatation (Robbin and Falewski, 1963). Detailed autopsy studies revealed that the smooth muscle of the intestinal tract often showed variation in fibre size, atrophy and fibrosis. These morphological changes are similar to those seen in affected skeletal muscle (Huvos and Pruzanski, 1967).

There is overwhelming evidence from clinical, pathological and physiological studies that cardiac muscle is involved in DMD (Hunter, 1980). The affected cardiac muscle resembles that seen in skeletal muscle with variation in fibre size, fragmentation of muscle fibres, replacement by connective and fatty infiltration. Myocardial fibrosis is an important feature and progressive cardiac failure is clinically apparent in about fifteen percent of the patients (Boland et al., 1996). Dilated cardiomyopathy is seen in about twenty four percent of patients (Melacini et al., 1996b). The cardiac dysfunction in DMD is further complicated by the presence of pulmonary hypertension in some patients with nocturnal respiratory problems (Melacini et al., 1996b). One case of affected vascular system leading to multiple thrombosis in the brain in a Becker patient has been reported (Higuchi, 1999). Thymus hyperplasia has been seen in some cases but the significance of this is not clear (Huvos and Pruzanski, 1967).

Many patients have mild mental retardation with a lowered IQ but Dubowitz and Crome could not detect any consistent pathological abnormality in the brain (Dubowitz and Crome, 1969; Emery, 1993). Dystrophin (discussed in Section 1.2) is found in neurons and is concentrated in post-synaptic membranes. Some patients suffer from cognitive impairment and this is thought to be due to an abnormality in the neuronal membrane that is caused by the absence of dystrophin (for review see Blake and Kroger, 2000).

#### **1.1.4 Allelic variants**

##### **1.1.4.1 Becker muscular dystrophy**

Becker muscular dystrophy (BMD) is the milder form of the disease that results from the mutation of the dystrophin gene (Becker and Keiner, 1955). The patients have a later and milder clinical onset and some of them have a normal life span.

##### **1.1.4.2 X-linked cardiomyopathy**

Despite normal muscle phenotype, some X-linked dilated cardiomyopathy is caused by mutations in the dystrophin gene. The full-length dystrophin is expressed in the skeletal muscle but not in the heart due to the activity of the muscle promoter (see section 1.2.4.3). This phenotype shows progressive ventricular wall dysfunction, dilated cardiomyopathy and cardiac failure (for review see Ferlini et al., 1999).

## **1.2 The DMD gene and the dystrophin protein**

### **1.2.1 The gene**

The dystrophin-associated gene was first identified by Kunkel's group in 1986 (Monaco et al., 1986). It maps to Xp21 and is expressed as a 427 kDa protein which was called dystrophin by Kunkel and colleagues since its absence causes muscular

dystrophy (Hoffman et al., 1987; Koenig et al., 1987). This remarkably large gene has a genomic region of 2.5 Mb and encodes a 14 kb mRNA transcript which contains a minimum of 85 exons including seven independent promoters (Brockdorff et al., 1987; Burghes et al., 1987; Cross et al., 1987; Koenig et al., 1987; Monaco et al., 1987; Koenig et al., 1988; Coffey et al., 1992; Monaco et al., 1992; Roberts et al., 1992; Roberts et al., 1993). This unusually large gene may account for the high mutation rate observed for this disease. The gene with an 11 kb open reading frame (ORF) is transcribed in the centromere to the telomere direction.

The different independent promoters at 5' end of the dystrophin gene specify the transcription of their respective alternative first exons in a cell-specific and developmentally controlled manner (Boyce et al., 1991; Gorecki et al., 1992; for review see Ahn and Kunkel, 1993). The 427 kDa full-length dystrophin is expressed predominantly in skeletal muscle, cardiac and smooth muscles with lower levels in the brain (Chamberlain et al., 1988; Nudel et al., 1988). The muscle promoter (M-promoter) is active in skeletal muscle, cerebral glial cells and cardiomyocytes (Barnea et al., 1990; Chelly et al., 1990). The cerebral neuronal promoter (C-promoter) is active in cortical neurons (Barnea et al., 1990; Chelly et al., 1990; Boyce et al., 1991). The Purkinje cell promoter (P-promoter) is active in cerebellar Purkinje cells and skeletal muscle (Gorecki et al., 1992). Figure 1 shows the schematic representation of the dystrophin promoters.

The transcription of the dystrophin gene is very complex and its mechanisms are not well understood. The core muscle promoter, an 850 bp fragment upstream from the cap site confers muscle specific regulation during myogenesis (Nudel et al., 1989; Chelly et al., 1990; Hoffman et al., 1988). This core muscle promoter contains Mef-1,

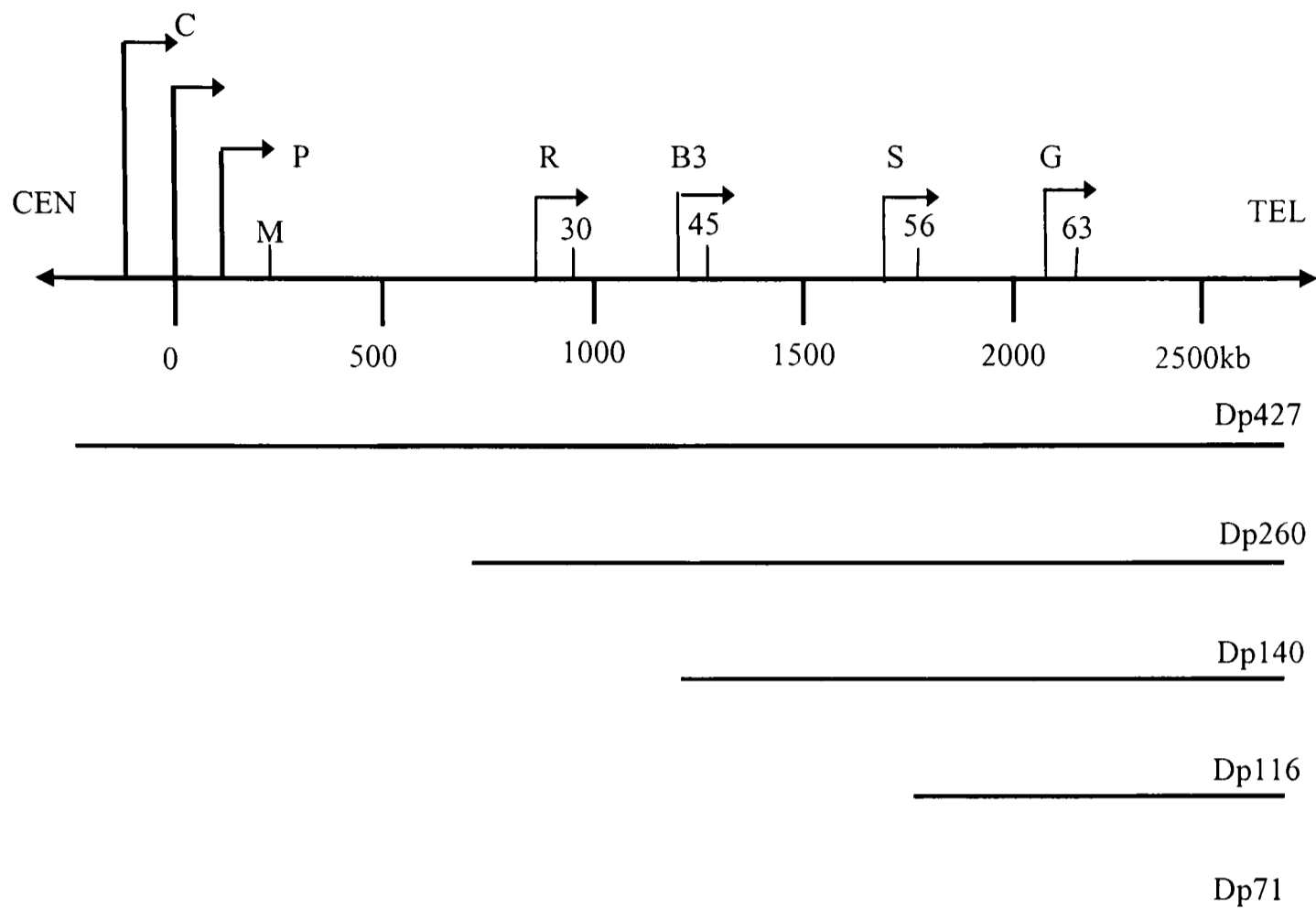


Figure 1 Schematic representation of the dystrophin promoters.

Dystrophin and the approximate positions of the seven dystrophin promoters and the shared exon into which each unique first exon splices [Im, 1996 #534]. Horizontal bars representing each of the five size classes of transcripts that arise from the seven promoters. (C, brain cortical; M, skeletal muscle; P, Purkinje cell; R, retinal; S, Schwann cell; B3, brain; G, ganglia).

The full-length dystrophin is initiated at 3 independently regulated 5' promoters that transcribed unique first exons that are spliced to a common second exon (2).

The shorter isoforms are transcribed by internal promoters located at the C-terminus. Dp260 is controlled by a promoter located in intron 29, Dp140 by a promoter in intron 44, Dp116 by a promoter in intron 55 and Dp71 by a promoter in intron 62. The functions of the shorter isoforms are mainly unknown.

2, 30, 45, 56 and 63 denote exons; 0 to 2500kb denote genomic regions. The diagram is not to scale.

Mef-2 [Mefs belong to the family of the transcription factors that play a key role in the regulation of many muscle specific genes (Gossett et al., 1989; Molkentin et al., 1995)], E-box (a helix-loop-helix factor binding site) and a CArG box binding site, but only the latter one is functionally essential. It also contains other ubiquitous transcription factors binding sites, for example, Sp1, Ap1 and Ap2, SRF (serum response factor), YY1 (zinc finger nuclear factor) and DPBF (dystrophin promoter bending nuclear factor). All these transcription factors interact in a complex manner between cis-acting regions through the CArG box to confer a muscle preference of expression (Nudel et al., 1989; Gilgenkrantz et al., 1992).

In terms of its muscle-specific expression, dystrophin transcription are found at approximately the same levels in skeletal and cardiac muscles but at a lower level in smooth muscle tissues, about 2 to 10% of that in skeletal muscle (Chelly et al., 1988). This suggests that skeletal muscle cells express alternative regulatory factors which interact in a different way with the same cis-acting muscle specific sequences which use alternative domains within this region of the gene (Klamut et al., 1990).

Regions outside the core muscle promoter, that is upstream and distal to the 850 bp fragment, may play important roles in regulating the specific expression of dystrophin gene. For example, the mouse dystrophin intron-1 enhancer element, located approximately 8.5 kb downstream from the muscle dystrophin gene muscle promoter, contains four E boxes (E1-E4), Mef-2 and a serum response element. Site-directed mutagenesis studies showed the E-boxes 1, 2 and 3 and SRE are required for enhancer activity specific to differentiating myoblast (Marshall et al., 2001). This regulatory region also involves in the cardiac muscle specific expression of dystrophin. Normally

the mouse dystrophin muscle promoter targets expression in the right ventricle of the heart only, suggesting that other sequences are needed to target other compartments of the heart. Since myogenic differentiating factors are absent from the heart, Mef-2 may also interact with factors such as SRE (Belaguli et al., 2000) or GATA-4 (Morin et al., 2000) to activate transcription in the cardiac muscle.

In addition, there are a number of internal promoters at the C-terminus region, giving rise to shorter transcripts with unique N-terminus that encode truncated C-terminus isoforms of dystrophin. These isoforms are as follows: Dp260 is a 260 kDa protein whose transcription is controlled by a promoter located in intron 29 (D'Souza et al., 1995). It is expressed in retina, central nervous system (CNS) and heart. Dp140 is a 140 kDa protein localised to the CNS and is controlled by a promoter in intron 44 (Lidov et al., 1995). Dp116 is a 116 kDa protein and is exclusively detected in Schwann cells of the peripheral nervous system and cultured glial cells (Byers et al., 1993). It is transcribed by a promoter located between exons 55 and 56. Finally, Dp71 is a 71 kDa protein controlled by a promoter located between exons 62 and 63 (Lederfein et al., 1993). It is expressed in a wide range of non-muscle tissues, for example brain, liver, kidney and lung (Cox et al., 1994; Sarig et al., 1999). Figure 2 shows the schematic representation of dystrophin and its shorter isoforms. The functions of these shorter dystrophin isoforms are mainly unknown. They may be involved in the stabilisation of the dystrophin-associated protein complex in non-muscle tissues since the isoforms share identity with the carboxy-terminus (C-terminus) of the full-length dystrophin and therefore contain the necessary binding sites for interaction with the components of the dystrophin-associated protein complex. Recent report that Dp71 knockout mice study suggests that Dp71 has a

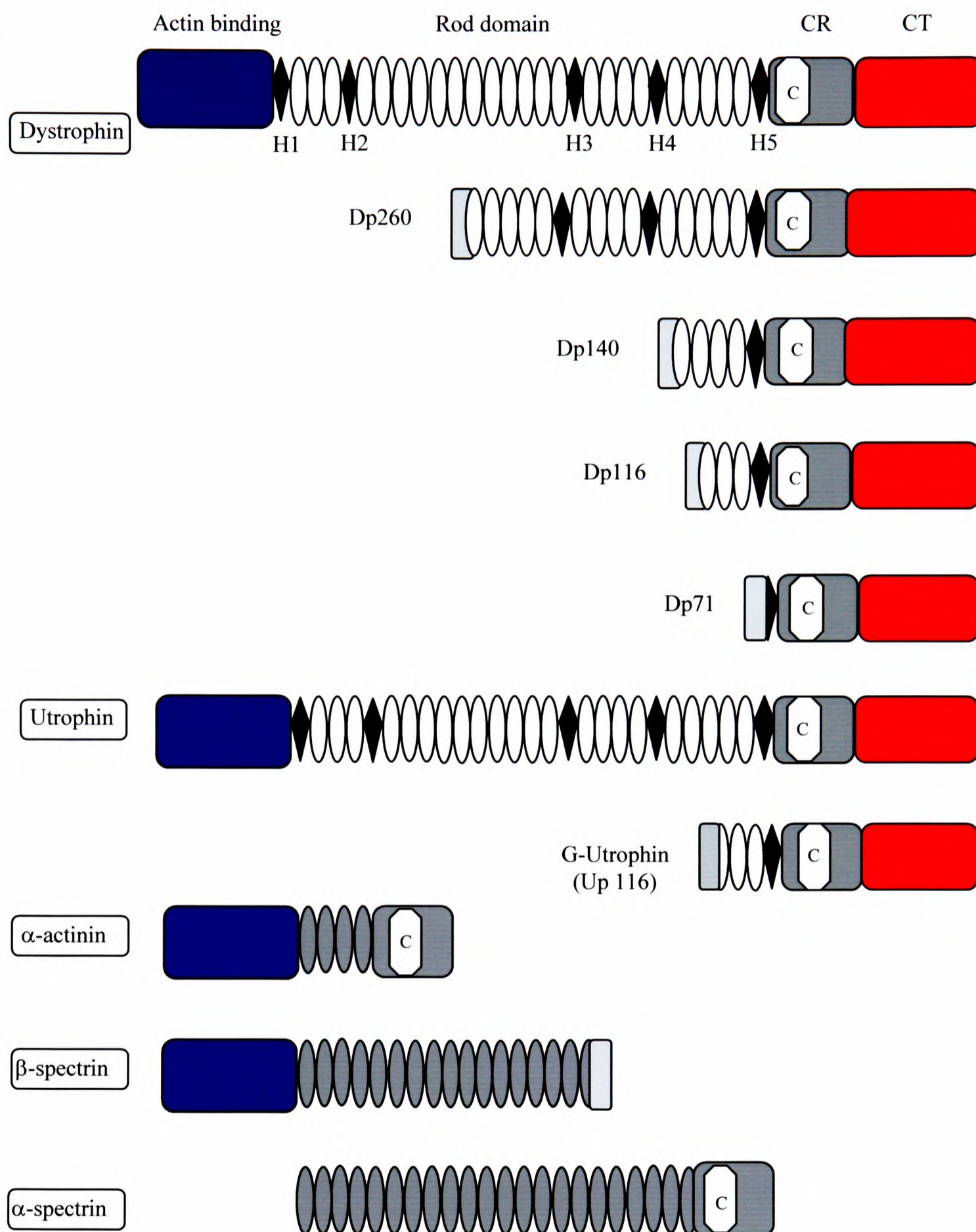


Figure 2 Schematic representation of dystrophins and their relatives

Dystrophin is comprised of an amino-terminus actin-binding domain, a central rod-domain containing 24 spectrin-like repeats and 5 proline-rich hinges (H) and a carboxy-terminus region (CT). CT region is predicted to contain a WW domain, calcium binding EF-hands (c), a cystein-rich region (CR), two ZZ domains and coiled-coil regions at the C-terminus.

The actin-binding domain (blue) is shared among a large number of actin-binding cytoskeletal proteins, for examples  $\alpha$ -actinin and  $\beta$ -spectrin. The C-terminus (red) is shared by the dystrophin and utrophin.

functional role in differentiated tissues (Lumeng et al., 1999). For example, Dp71 is found in neurons of inner nuclear layer and inner plexiform layer where it may involve in retinal signal transmission. It is also found in kidney tubular network of adult mouse kidney where it may maintain contacts between epithelial cells and the basal lamina for the normal function of kidney.

### **1.2.2 The dystrophin protein**

Dystrophin protein, containing 3685 amino acids, is structurally related to the cytoskeletal protein  $\alpha$ -actinin and  $\beta$ -spectrin. Dystrophin is a cytoskeletal protein localised to the cytoplasmic face of the skeletal muscle sarcolemma (Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988). It is enriched at myotendinous junctions (MTJ) and the post-synaptic membrane of neuromuscular junctions (NMJ) (Samitt and Bonilla, 1990; Byers et al., 1991). In normal brain, dystrophin is localised to the post synaptic regions of neurons in the cerebral and cerebellar cortex (Lidov et al., 1990). Dystrophin is localised in the sarcolemma of cardiomyocytes in the heart and the cell membranes of Purkinje fibres (Tanaka et al., 1990; Bies et al., 1992).

The N-terminus of dystrophin shows a strong homology to the actin-binding domain of  $\alpha$ -actinin and binds to F-actin (Hemmings et al., 1992; Rybakova et al., 1996). The central rod domain consists of twenty four coiled-coil spectrin-like repeats separated by five proline-rich “hinge” regions which probably makes the molecule a flexible rod-shape structure (Koenig et al., 1988; Brown and Lucy, 1997). The C-terminus contains several domains involved in protein-protein interactions, which are discussed in the following section.

### **1.2.3 *Dystrophin-associated protein complex (DPC)***

The transmembrane proteins associated with dystrophin are dystroglycans, sarcoglycans, sarcospan and the cytoplasmic dystrophin-associated syntrophins and dystrobrevins, collectively known as the dystrophin-associated protein complex (DPC, see Figure 3). In muscle, the assembly of this complex is dependent on the presence of dystrophin. In the absence of dystrophin, there is a secondary reduction of the DPC components in the sarcolemma of the muscle (Ervasti et al., 1990; Campbell, 1995). These alterations of the DPC are thought to contribute to the pathology in dystrophin-deficient muscle (discussed in Sections 1.3 and 1.4).

The dystroglycans consist of  $\alpha$ - and  $\beta$ -dystroglycan sub-units. The C-terminus of dystrophin binds to  $\beta$ -dystroglycan (Campbell and Kahl, 1989; Yoshida and Ozawa, 1990; Suzuki et al., 1992; Suzuki et al., 1994; Sunada and Campbell, 1995). Laminin-2 (merosin), laminin-4, perlecan and agrin in the extracellular matrix bind to  $\alpha$ -dystroglycan (Matsumura and Campbell, 1994; Gesemann et al., 1998; Peng et al., 1998; Montanaro et al., 1999; Colognato and Yurchenco, 2000).  $\beta$ -Dystroglycan, which is a transmembrane protein, binds to  $\alpha$ -dystroglycan, giving rise to the molecular linkage between the muscle cytoskeleton and the extracellular matrix (Montanaro et al., 1999; for review see Winder, 2001).

The interaction of dystrophin with  $\beta$ -dystroglycan is dependent upon the WW domain and the EF-hand motifs in the cysteine rich domain of dystrophin (Rentschler et al., 1999). In addition, the proline-rich domain of  $\beta$ -dystroglycan binds to the adapter protein, growth-factor-receptor-bound protein 2 (Grb2) through both of its SH3

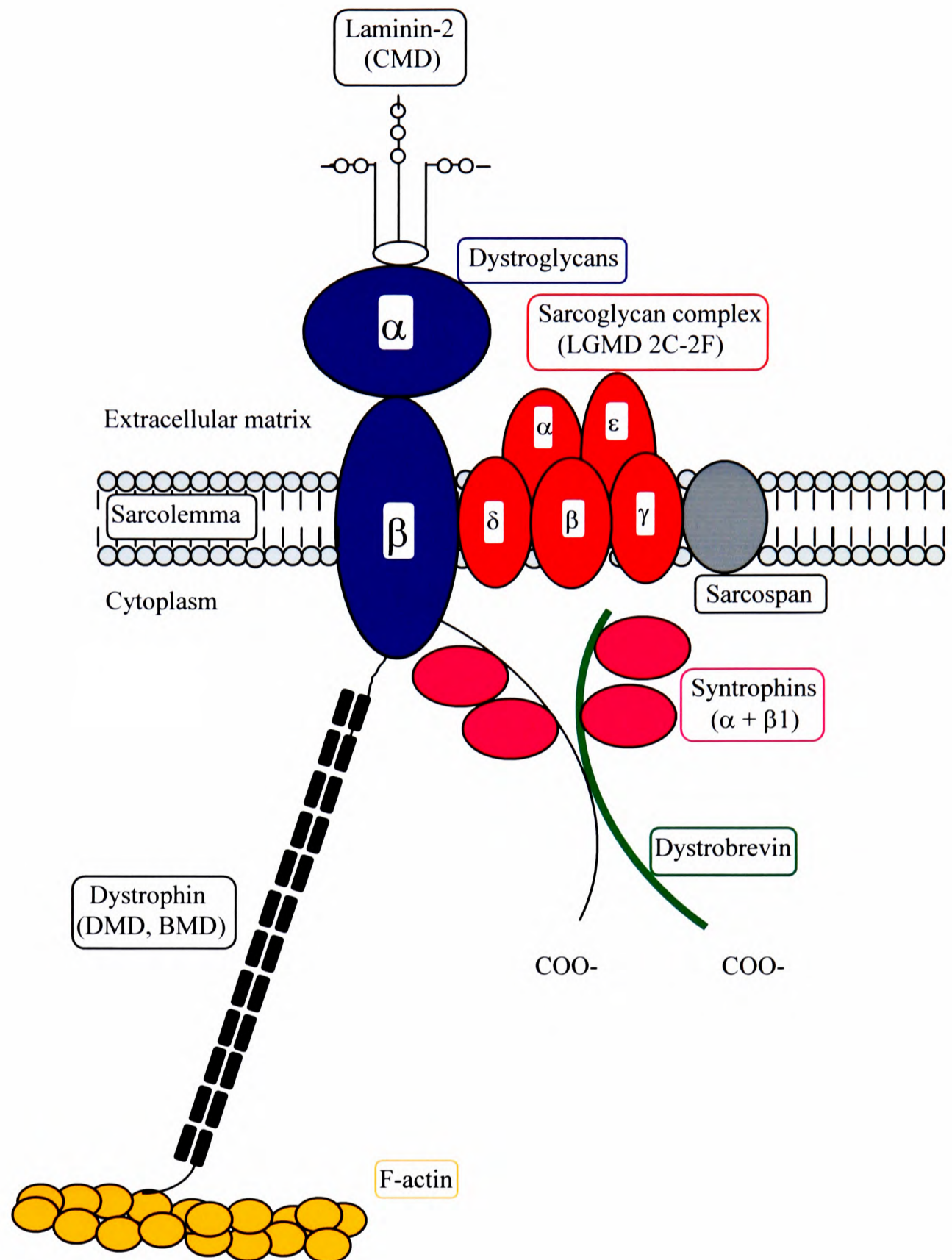


Figure 3 Schematic representation of the molecular organisation of dystrophin and its associated proteins (DPC) at the Sarcolemma.

In normal skeletal muscle, the amino-terminus of dystrophin binds to F-actin and the C-terminus binds to the DPC.  $\alpha$ -dystroglycan binds to laminin-2, linking the intracellular actin cytoskeleton to the extracellular matrix. Disruption of this link causes DMD and BMD. Mutations of the sarcoglycan genes cause different forms of limb girdle muscular dystrophies (LGMD) where expression of the sarcoglycan proteins are reduced or lost from the sarcolemma. Mutations in the  $\alpha 2$  chain of laminin-2 (merosin) causes congenital muscular dystrophy (CMD).

Other proteins that associate with dystrophin like calpain-3, myotilin, dysferlin, caveolin-3, integrin complex, filamin-2, nNOS etc are not shown.

domains (Yang et al., 1995). Grb2 is involved in signal transduction. Grb2 binds  $\beta$ -dystroglycan on a site which overlaps the dystrophin binding site, it is possible that Grb2/ $\beta$ -dystroglycan complex can be established only under some physiological conditions, suggesting that the Grb2/ $\beta$ -dystroglycan complex formation is highly dynamic (Russo et al., 2000). This specific dystroglycan-Grb2 interaction might have a role in extracellular-matrix-mediated signal transduction, suggesting that the role of dystrophin is not structural entirely but also involve in signal transduction. In bovine brain synaptosomes, protein kinases and Grb2 associate with the dystrophin complex. Grb2 may mediate the interaction of  $\beta$ -dystroglycan with focal adhesion kinase p125<sup>FAK</sup> (FAK), a non-receptor tyrosine kinase that involves in the intracellular transduction pathway triggered by integrins and neurotransmitter receptor activation, possibly involved in signal transduction at synapses (Cavaldesi et al., 1999).

The sarcoglycan complex is composed of five glycoproteins,  $\alpha$ - (50 kDa),  $\beta$ - (43 kDa),  $\gamma$ - (35 kDa),  $\delta$ - (35 kDa) and  $\epsilon$ - (43 kDa) (Tinsley et al., 1994; Ettinger et al., 1997; Holt and Campbell, 1998; for review see Hack et al., 2000). All the sarcoglycans are expressed in skeletal, smooth and heart muscles; the mRNA of  $\beta$ -sarcoglycan is found in brain;  $\epsilon$ -sarcoglycan is expressed in many different tissue types and is highly related to  $\alpha$ -sarcoglycan (McNally et al., 1998; Barresi et al., 2000; for review see Hack et al., 2000).

Another new member of the DPC, called sarcospan (25 kDa) does not bind directly to dystrophin but preferentially associates with the sarcoglycan complex and this interaction is critical for stable localisation of sarcospan to the sarcolemma, NMJ and

MTJ. In addition, the sarcoglycan-sarcospan subcomplex stabilises  $\alpha$ -dystroglycan to the muscle plasma membrane (Crosbie et al., 1997; Crosbie et al., 1999). The localisation of the sarcoglycan complex to the sarcolemma is dependent upon the co-synthesis of all the sarcoglycan components (Holt and Campbell, 1998). The functions of these transmembrane proteins are unclear but mutations of the sarcoglycan genes cause different forms of limb girdle muscular dystrophy (LGMD) collectively called sarcoglycanopathies (for review see Hack et al., 2000). Sarcoglycanopathy is an autosomal muscular dystrophy with a phenotype similar to DMD and BMD. Mutation of the alpha2-chain of the laminin 2 (LAMA2) gene causes congenital muscular dystrophy (Allamand et al., 1997). Deficiency of adhalin, a 50 kDa transmembrane protein that associates with DPC in the extracellular matrix leads to severe childhood autosomal recessive muscular dystrophy (SCARMD) (Matsumura et al., 1992b; Fardeau et al., 1993; Matsumura and Campbell, 1993; Higuchi et al., 1994). All these dystrophies have a similar phenotype to DMD and BMD.

Absence of dystrophin results in the loss of the DPC from the sarcolemma, implying that dystrophin is required to maintain the localisation of the DPC (Ervasti et al., 1990). In normal muscle, dystrophin links the actin cytoskeleton of the muscle cell to laminin in the extracellular matrix through the DPC. This linkage confers strength and stability to the muscle system (Ibraghimov-Beskrovnya et al., 1992). Disruption of this linkage occurs in DMD with the loss of dystrophin and the DPC, leading to muscle degeneration and necrosis (Ervasti et al., 1990). The restoration of normal levels of DPC in Dp71 transgenic-*mdx* mice did not alleviate the pathology of the disease. Intact DPC is required but is not sufficient for normal muscle function

indicating that both the N- and C-termini of dystrophin are required for normal muscle function (Cox et al., 1994; Greenberg et al., 1994). However, recent findings showed that the dystrophin C-terminus is not required for the assembly of the DPC (Crawford et al., 2000). Figure 3 shows the schematic representation of the molecular organisation of dystrophin and its associated proteins at the sarcolemma.

The coiled-coil domain in the C-terminus of dystrophin forms the binding site for the cytoplasmic components of the DPC (Blake et al., 1995b). The syntrophins (Adams et al., 1993; Ahn et al., 1994; Ahn and Kunkel, 1995; Peters et al., 1997a), dystrobrevins (Blake et al., 1996a; Peters et al., 1997b; Blake et al., 1998; Nawrotzki et al., 1998; Peters et al., 1998; Holzfeind et al., 1999) and their associated proteins bind to this region. There are three different syntrophin proteins,  $\alpha$ ,  $\beta$ 1 and  $\beta$ 2, that can bind directly to dystrophin,  $\alpha$ -dystrobrevin and utrophin (discussed in Section 1.5) in muscle.  $\alpha$ -Syntrophin also binds to neuronal nitric oxide synthase (nNOS) in muscle through PDZ (PSD95, discs large, and zona occludens) domains. In dystrophin-deficient muscle, nNOS is lost which is consistent with it being a dystrophin-associated protein.

There are several isoforms of dystrobrevin in muscle, which are the products of the same alternatively spliced gene (Ambrose et al., 1997; Nawrotzki et al., 1998).  $\alpha$ -Dystrobrevin 1 and  $\alpha$ -dystrobrevin 2 bind directly to dystrophin through the coiled-coil region (Sadoulet-Puccio et al., 1997; Nawrotzki et al., 1998; Peters et al., 1998). A complex of two syntrophin proteins associated with dystrophin and  $\alpha$ -dystrobrevin is formed at the sarcolemma of the muscle (Peters et al., 1997a; Newey et al., 2000).

The importance and functions of syntrophins and dystrobrevins are largely unknown, although they may be involved in cell signalling pathways (Bredt, 1999; Grady et al., 1999).

#### **1.2.4 Mutations of the dystrophy gene**

##### **1.2.4.1 Duchenne muscular dystrophy (DMD)**

The clinical features of DMD and BMD patients are due to mutation of different parts of the same dystrophin gene. The translational open reading frame (ORF) theory is based on intragenic deletions and their effects on the translation of triplet codons into amino acids of the dystrophin protein (Monaco et al., 1988). About seventy percent of BMD and DMD patients have deletions that shift the ORF of triplet codon for amino acids (out-of-frame shift). This results in a semi-functional dystrophin protein in the BMD cases, whereas DMD patients produce a severely truncated dystrophin protein that would be unstable (Hoffman et al., 1987; Monaco et al., 1988; Baumbach et al., 1989; Koenig et al., 1989; Beggs et al., 1990). The remaining one-third of cases of DMD and BMD are associated with small mutations. Missense mutations are extremely rare and the vast majority of DMD point mutations result in a premature stop codon (for review see Roberts et al., 1994).

About a third of mutations in the DMD gene are spontaneous new mutations and the normal screening of known affected families will miss this one-third of new mutations. It is not clear why the new mutation rates in DMD is so high. It was thought to be due to the large dystrophin gene which is particularly susceptible to mutations.

#### **1.2.4.2 Becker muscular dystrophy (BMD)**

The BMD patients have milder phenotype and later onset of the disease. They have a normal lifespan and can lead quite a normal life style with support management. Mutations that cause in-frame deletions, that is, the translational ORFs are maintained and produce a shorter dystrophin protein are associated with BMD. The patients produce partially functional truncated dystrophin protein in varying amounts (Monaco et al., 1988; Baumbach et al., 1989; England et al., 1990).

#### **1.2.4.3 X-linked cardiomyopathy**

X-linked dilated cardiomyopathy results from mutations that abolish the cardiac expression of dystrophin, while expression in the skeletal muscle is not affected. Several mutations have been described, one example is a mutation in the muscle promoter which abolishes the production of the full-length M isoform of dystrophin from the heart, while skeletal muscle synthesises dystrophin via exon skipping or alternative splicing (for review see Ferlini et al., 1999).

### **1.3 Pathophysiology of dystrophin deficiency**

The precise pathophysiological events that result in dystrophin-deficient muscle degeneration are not fully understood. There is evidence to support the hypothesis that the absence of dystrophin alters the physiological properties of the sarcolemma, resulting in membrane stiffness and increased susceptibility to contraction-induced sarcolemmal rupture compared to normal controls (Carpenter and Karpati, 1979; Petrof et al., 1993; Pasternak et al., 1995; Gillis, 1996; Deconinck et al., 1997c; Petrof, 1998; Tinsley et al., 1998). This hypothesis most probably provides the most likely explanation of the pathogenesis of dystrophin-deficient muscle because the loss

of DPC components due to the absent of dystrophin results in gaps in the sarcolemma (Ervasti et al., 1990). These gaps could be the initiation sites of membrane damage, resulting in calcium influx through damaged ion channels. This hypothesis also ties in with the second hypothesis that dysregulation of calcium homeostasis results in muscle degeneration.

Second hypothesis postulates that the dysregulation of the intracellular calcium ion homeostasis, leads to calcium-dependent protease activities resulting in the degeneration of the dystrophin-deficient muscle (Fong et al., 1990; Emery, 1993; Turner et al., 1993; Gillis, 1996; Leijendekker et al., 1996; Carlson, 1998; Tutdibi et al., 1999; Williams and Bloch, 1999; Robert et al., 2000; Alderton et al., 2000; for review see Burton and Davies, 2000).

Finally, the association of dystrophin with signal transduction proteins at the sarcolemma has led to the postulation that abnormal signalling and other biochemical abnormalities in dystrophin-deficient muscle may play a role in muscle degeneration (Brenman et al., 1995; Brenman et al., 1996; Duggan et al., 1997; Peters et al., 1997a; Crosbie et al., 1998; Gee et al., 1998; Grozdanovic and Baumgarten, 1999). This hypothesis provides a likely explanation for abnormal signalling because of the cocktail of chemicals released from damaged/regenerating muscle fibres.

The absence of dystrophin appears to initiate a complex pathophysiological response depending on species determines the actual clinical phenotype that develops. Degenerating or necrotic muscle fibres are characteristically seen in DMD patients and the *mdx* mouse, and the necrotic fibres are often seen in clusters or as group necrosis, implying that other extracellular factors may play a role. The cycles of

degeneration and regeneration result in progressive connective tissue proliferation and the deposition of collagen is believed to result from wound repair (Beranek, 1991). It then follows a complex pathological pathway modulated by multi-functional growth factors and cytokines (for review see Kingsnorth and Slavin, 1991).

The consequences of dystrophin deficiency leads to a two-phase disease. The necrotic/hypertrophic Phase 1 stage is similar in man and other species; that is, all have elevated creatine kinase (CK) levels, group myofibre necrosis, muscle hypertrophy and variable fibre size. The Phase 2 stage is when the normal muscle repair mechanism fails and turns pathological, leading to progressive muscle death and fibro-infiltration.

During the Phase 1 stage, the immediate consequences of dystrophin deficiency result in the failure to maintain the integrity of the plasma membrane resulting in abnormal function of ion channels. The hypotheses of the “leaky membrane theory” and the “calcium influx theory” are currently used to explain the muscle necrosis in dystrophin-deficient muscle (Fong et al., 1990; Turner et al., 1993). Myotubes that lack dystrophin have an abnormal calcium ion channel resulting in a markedly increased level of intracellular free calcium concentration at rest and this leads to an increased rate of muscle protein degradation (Turner et al., 1988; Franco and Lansman, 1990; Turner et al., 1993; Alderton and Steinhardt, 2000; McCarter and Steinhardt, 2000). Calcium influx is caused by the “leaky” state of a normal type of mechano-transducing ion channel that could provide a pathway for calcium ions to leak into the cell. Calcium-dependent proteases (calpains) have been implicated as the proteases responsible for muscle necrosis in human DMD muscles. The increase in calpains level is due to post-translational regulation and it plays a role in both the

degenerative and regenerative aspects of *mdx* dystrophy (Spencer et al., 1995). Interestingly, LGMD type 2A, mapped to chromosome 15q15, is caused by mutations in the gene specific for the muscle-specific calpain-3 enzyme (Richard et al., 1995). Loss of function of calpain-3 generates dystrophy (Bushby, 1999). This is an interesting paradox, on one hand; calpain is involved in degeneration and regeneration of dystrophic muscle and on the other hand, its absence causes dystrophy.

During the Phase 2 stage, the long-term consequences of dystrophin deficiency lead to (1) progressive failure of muscle regeneration, (2) progressive fibre loss and (3) progressive fibrosis. These three lead to progressive muscle wasting and result in premature death in man.

There are inflammatory cells (T cells, macrophages and mast cells) at the perimysial and endomysial sites of the muscle biopsies of DMD patients (Arahata and Engel, 1984; McDouall et al., 1990). Inflammatory cells could participate in Phase 1 (group necrosis) or Phase 2 (fibrosis and wasting) or both. A high concentration of mast cells is invariably observed in areas of group necrosis (Gorospe et al., 1994). If the “leaky membrane” theory is correct, this may explain the higher concentration of mast cells in dystrophin-deficient muscle: release of cytoplasmic contents is chemotactic to mast cells. Other factors like biological activities of the cells, other inflammatory molecules, growth factors (transforming growth factors 1, TGF- $\beta$ 1; basic fibroblast growth factor, bFGF) and cytokines may precipitate group necrosis (Phase 1) leading to the pathogenesis of progressive fibrosis (Phase 2) (Davidson et al., 1985). Mast cells may be involved in fibro-proliferation in the Phase 2. Mast cells secrete heparanase, which can degrade the basement membrane of muscle fibres and also histamine, tryptase, TGF- $\beta$  etc, which regulate the function of fibroblasts.

Dystrophin plays an important role in the cardiac conduction system. In the heart, dystrophin is localised to the plasma membrane and the transverse tubules of the cardiac muscle and the membrane of the Purkinje fibre (Knudson et al., 1988; Yarom et al., 1992). Purkinje fibres are specialised subendocardial muscle fibres forming part of the conducting system of the heart. Dystrophin is an important molecule for membrane function in the Purkinje fibre conduction system of the heart. Dystrophin colocalised with  $\beta$ -sarcoglycan at the membrane of each fibre in the Purkinje Bundle would permit cell-cell adhesion and communication, as in internal synaptic connections (Rivier et al., 1999). This supports the hypothesis that defective dystrophin expression contributes to cardiac conduction disturbances in DMD/BMD patients (Bies et al., 1992).

Following muscle fibre necrosis caused by dystrophin-deficiency, the regeneration process is initiated (Brown and Lucy, 1997). Polymorphonuclear leucocytes and macrophages invade the necrotic cytoplasm, digest and remove the debris. The release of chemotactic factors like interleukins and IGF-1 from the damaged fibres, attract macrophages and facilitate the rapid removal of necrotic tissues, is a key event in muscle regeneration (Robertson et al., 1993). The quiescent or resting satellite cells which are the myoblast stem cells become activated and start to divide, giving rise to myoblasts that differentiate into functional skeletal muscle, using the original basal laminae as scaffolding.

The response of satellite cells to injury occurs within four to eight hours as shown by the expression of MyoD and myogenin which are specific early markers of activated satellite cells (Grounds et al., 1992; Rantanen et al., 1995). The activation of satellite cells after injury is probably influenced by various factors from the extracellular

matrix, growth factors and electrical activity. Basic fibroblast growth factor (FGF) stimulate proliferation but depresses differentiation, insulin-like growth factors-1 (IGF-1) stimulates differentiation and beta-transforming growth factor ( $\beta$ -TGF) depresses proliferation and initiates differentiation (Allen and Boxhorn, 1989). Recent studies show IGF-1 induced muscle hypertrophy via activation of satellite cells which leads to increased muscle regeneration (Barton-Davis et al., 1999b). Thus, by altering these factors, satellite cells can be stimulated to proliferate, differentiate or to remain quiescent. There is considerable interest in the motility of satellite cells now. They migrate along the length of their fibres and between fibres and could migrate through injured or intact basal laminae in normal muscle. However, in well-established DMD muscle, it would be more difficult or impossible to move through the fibrous connective tissues between fibres. The epimysial connective tissue stops the migration of satellite cells between muscle fibres (Shultz et al., 1986). However, Watt et al. (1994) showed that implanted mononuclear myoblasts migrate from distant sites to regions of muscle injury.

The role of dystrophin and its associated proteins in muscle regeneration *in vivo* is not clear. Dystrophin is shown to incorporate into the plasma membrane at four days post injury and two days later than the dystroglycans following muscle damage by the snake toxin, notexin (Vater et al., 1992).

However, regeneration is not sufficient to compensate for the muscle breakdown in DMD and BMD. There is a two to three fold increase of the number of satellite cells in DMD patient muscle (Wakayama and Schotland, 1979). There is no difference in their ultra-structure and they are capable of mitosis (Watkins and Cullen, 1986). However, only six percent of cultured DMD myoblasts are capable of fifty doublings

that are characteristic of the average satellite cells from a five year old control (Webster and Blau, 1990). Therefore, the increase in satellite cells alone is not sufficient to sustain regeneration in DMD patients.

#### **1.4 The *mdx* mouse, an animal model**

There are several naturally occurring animal models for DMD. These are the *xmdl* dogs (Cooper et al., 1988; for review see Valentine et al., 1992), the muscular dystrophy cats (Carpenter et al., 1989; Lin et al., 1998) and the *mdx* mouse (Bulfield et al., 1984; Sicinski et al., 1989; Pastoret and Sebille, 1995). All of these animals have X-linked mutations that abolish the expression of full-length dystrophin in skeletal muscle. They all faithfully mimic the phenotype of DMD in man but to various degrees. The *mdx* mouse model is the best studied of all. The *mdx* mouse is fertile and seems relatively healthy up to about one year of age. It has a normal life span of about two years but after about one and a half years, the limb muscles are severely affected and stiffen, resulting in difficulty in walking (Pastoret and Sebille, 1995).

The *mdx* mouse has a point mutation that introduces a premature translational stop codon into exon 23 of the dystrophin gene, which abolishes the expression of full-length dystrophin. The mutant arises from a spontaneous mutation in the highly inbred C57BL/10 Scn/Scn strain of mice (Bulfield et al., 1984; Hoffman et al., 1987; Sicinski et al., 1989). The mouse shows features characteristic of the early myopathic phase of the DMD disease. These include repeated cycles of skeletal muscle degeneration and regeneration as seen by the appearance of variable fibre size with centrally located nuclei, phagocytotic and fibrocollagenous infiltrations and elevated

serum creatine kinase (CK). The necrotic fibres are often seen in clusters (group necrosis) as in human.

The reason why the *mdx* mouse shows milder clinical features and is able to regenerate muscle fibres more successfully than human is not well understood. One of the reasons proposed is that the *mdx* mouse is able to redistribute calpain in the cytosol homogeneously. This redistribution enables the *mdx* mouse to suppress calpain-mediated proteolysis enabling the *mdx* muscle to arrest necrosis and regenerate successfully (Spencer and Tidball, 1992; Spencer et al., 1995; Spencer and Tidball, 1996). Muscle degeneration appears in the *mdx* mouse at approximately three weeks of age and by four weeks old necrosis and phagocytosis is widespread. At two weeks mitotic satellite cells can be seen but by four weeks they are seen in both normal and necrotic fibres (Cullen and Jaros, 1988). Regenerating fibres are common from three weeks onward and commonest at about seven weeks when about fifty percent of the soleus muscle is regenerating. At two months of age, more than half of the fibres show evidence of having been through a cycle of necrosis and regeneration. However, after the initial necrotic phase the mice recover well. Fatty and fibrous tissues are seen in older *mdx* mice as in DMD but not in younger ones.

One reason for arguing against the use of the *mdx* mouse as an animal model for the human disease, is the milder clinical feature observed. The mouse does not show the characteristic rapid progressive degeneration of the limb muscles, which forces the human patients to become wheelchair bound. However, the diaphragm muscle is more severely affected and with time, it becomes infiltrated with fibrous tissues and has severe functional deficit comparable to human DMD patients. This is probably a better indicator of the disease state (Stedman et al., 1991). The *mdx* mouse does not

show similar clinical progression to human DMD. However, a recent report provides evidence of progressive limb muscle deterioration in old *mdx* mice albeit at an attenuated rate (Pastoret and Sebille, 1995). In addition, there are several physiological abnormalities similar to those seen in man. These are as follows: increased fragility to hypo-osmotic shock and forced lengthening (Menke and Jockusch, 1991), abnormal opening time of stretch-sensitive calcium channels at rest resulting in raised resting intracellular calcium ion concentration (Turner et al., 1988; Franco and Lansman, 1990; Turner et al., 1993; for review see Gillis, 1996) and reduced normalised maximal tetanic force production in limb and diaphragm muscles (Coulton et al.; 1988; Stedman et al., 1991; Petrof et al., 1993; Deconinck et al., 1997). Many of these pathological and physiological changes are similar to those seen in human DMD patients and can be measured, allowing the dystrophic process in response to therapy to be monitored. Because of the above reasons, the *mdx* mouse has proved useful in the study of DMD despite its mild phenotype. (For a good review of the *mdx* skeletal muscle since 1987 see De la Porte et al., 1999).

Possibly, the utrophin-dystrophin-deficient mice (see Section 1.5.5) would be more appropriate as a model for DMD, because these mice show many signs typical of human DMD. They show severe progressive muscle dystrophy that results in premature death (Deconinck et al., 1997b; Grady et al., 1997b).

## 1.5 Utrophin

In 1989, our group, using probes from the dystrophin gene to screen skeletal muscle cDNA libraries, identified another large gene called the utrophin gene (Love et al., 1989). The utrophin protein exhibits over 80% homology to the N- and C-terminus domains of dystrophin. Sequence homology between utrophin and dystrophin extends

over the entire length (Tinsley et al., 1992). The protein structure consists of similar functional domains to the dystrophin protein. Similarities between the dystrophin and utrophin gene structure suggest that they arose from a common ancestral gene through a genomic duplication event (Pearce et al., 1993).

### **1.5.1 The utrophin gene**

The utrophin gene is localised to human chromosome 6q24 and mouse chromosome 10 (Buckle et al., 1990). The utrophin gene spans approximately 900 kb of genomic DNA and encodes a 13 kb mRNA transcript that gives rise to a protein of 395 kDa (Tinsley et al., 1992). Both dystrophin and utrophin genes contain multiple short exons but have a single long exon encoding the 3' UTR. There is a CpG island at the 5' end that contains a promoter driving the expression of the full-length utrophin (Dennis et al., 1996). This promoter has been shown to be synaptically regulated *in vivo* (Gramolini et al., 1999). A second promoter lying within the second intron of the utrophin gene was located recently (Burton et al., 1999). This promoter is tightly controlled and drives the transcription of a unique first exon that splices into a common full-length mRNA at exon 3. These two promoters are independently regulated.

Like dystrophin, there are also short utrophin isoforms with unique 5' exons, presumably driven by internal promoters at the 3' C-terminus region (Nguyen et al., 1995). Figure 2 shows the schematic representation of dystrophin and utrophin and their shorter isoforms. The shorter isoform, G-utrophin, is so named because it was found in the mouse dorsal root ganglion at embryonic day 11.5. It is the paralogue of Dp116, which is a 5.5 kb 3' utrophin transcript expressed in the sensory ganglia and

brain (Blake et al., 1995a). Up71 and Up140 are the homologues of Dp71 and Dp140 respectively (Wilson et al., 1999). The functions of these isoforms are unknown.

### **1.5.2 The utrophin protein**

The primary structure of utrophin is very similar to dystrophin. Homology between dystrophin and utrophin extends over their entire length and they share a surprisingly high number of identical amino acid residues. Sequence comparison of utrophin and dystrophin shows 88% amino acid identity to the  $\beta$ -dystroglycan binding domain of dystrophin and 80% amino acid identity to the C-terminus domain. (Love et al., 1989; Tinsley et al., 1992).

The N-terminus domain of utrophin, consisting of 260 amino acids, binds to F-actin and is regulated by calcium/calmodulin in a calcium-dependent manner (Winder et al., 1995a; Winder et al., 1995b; Winder et al., 1995c; Winder and Kendrick-Jones, 1995; for review see Morris et al., 1999). The cysteine-rich and C-terminus domains of utrophin bind to isoforms of dystrophin-associated proteins partners including  $\beta$ -sarcoglycan, syntrophins, and dystrobrevins in a similar way to dystrophin (James et al., 1995; Blake et al., 1996a; Peters et al., 1997a).

The amino acid conservation between the rod domains of utrophin and dystrophin is much weaker. Utrophin also has coiled-coil repeats of the central rod domain similar to dystrophin with proline-rich hinge domains, but lacks repeats 15 and 19 (Tinsley et al., 1992; Winder et al., 1995a). The rod domain of utrophin is less well conserved with respect to dystrophin. There are regions of the alignment of the rod domain showing less than 30% identity between the two proteins (Pearce et al., 1993). There

may be looser evolutionary constraints in this domain of the protein (Tinsley et al., 1992).

### **1.5.3 Expression pattern of utrophin**

Although utrophin and dystrophin proteins show a high degree of amino acid sequence identity, the two proteins have distinct patterns of expression and consequently this may reflect their different functions. Utrophin is ubiquitously expressed in a wide range of tissues (Khurana et al., 1991; Love et al., 1991; Nguyen et al., 1991; for review see Blake et al., 1996b). In contrast to dystrophin, which is expressed in cardiac, skeletal and smooth muscles and brain as discussed in Section 1.2.1.

#### **1.5.3.1 Utrophin in skeletal muscle**

In normal adult muscle, utrophin is exclusively localised to the cytoplasmic face of the MTJ and the post-synaptic membrane of NMJ. It is involved in the organisation and the maturation of NMJ (Ohlendieck et al., 1991; Khurana et al., 1995a; Blake et al., 1996b; Deconinck et al., 1997a; Grady et al., 1997a; Grady et al., 2000).

Utrophin is associated with a complex similar to the DPC (James et al., 1995; Winder et al., 1995c; Blake et al., 1996b; Peters et al., 1997a) as shown in the schematic representation in Figure 4. Recent work demonstrated that the actin-binding domain is localised in the first 240 amino acids sequence of utrophin and dystrophin. The functional actin-binding domain (ABD) of utrophin consists of three potential actin-binding sites, ABS1, ABS2 and ABS3, situated within the two calponin-homology

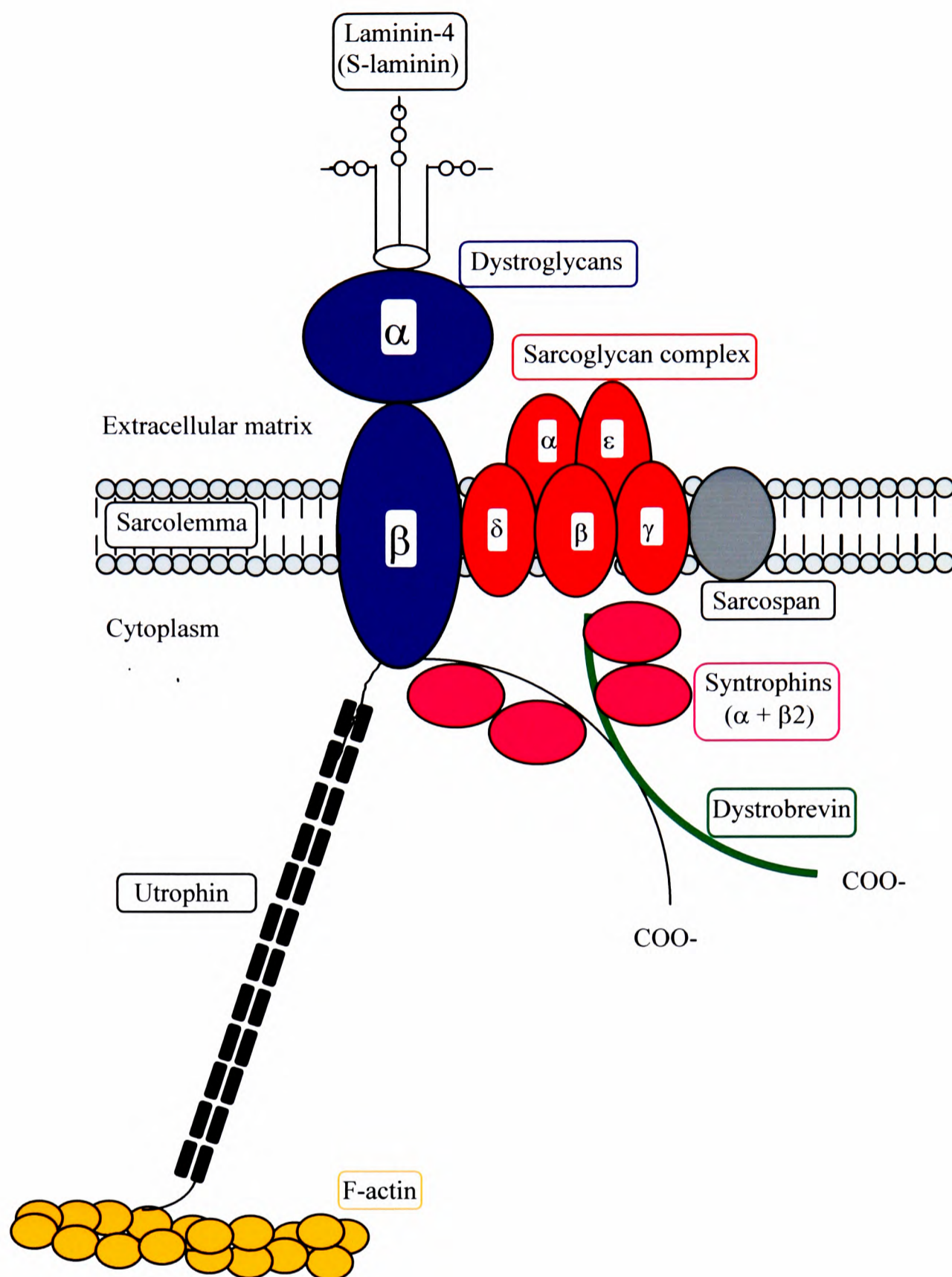


Figure 4 Schematic representation of the molecular interactions of utrophin at the neuromuscular junction.

In neuromuscular junction, utrophin, the homologue of dystrophin, has similar functional domains and has similar binding partners, the DPC. Here, the C-terminus of utrophin binds to  $\alpha$  and  $\beta 2$  syntrophin while dystrophin binds to  $\alpha$  and  $\beta 1$  syntrophin.

(CH1 and CH2) domains (Keep et al., 1999; Moores and Kendrick-Jones, 2000; Norwood et al., 2000). These sequences are highly conserved throughout the spectrin superfamily members, for example, dystrophin, utrophin,  $\alpha$ -actinin,  $\beta$ -spectrin and fimbrin. Dystrophin and utrophin ABDs share 70% sequence identity. This region is functionally important as mutation in this region of dystrophin leads to DMD (Roberts et al., 1994). Two dystrophin ABDs crystallise as anti-parallel dimer similar to utrophin (Norwood et al., 2000). The ABD of utrophin is more flexible than previously thought and this may allow domain reorganisation and play a physiological role in the actin binding mechanism not dissimilar to dystrophin (Keep et al., 1999).

Utrophin is precisely localised, together with acetylcholine receptors (AChRs), at the crests of the junctional folds whereas dystrophin is dispersed around the troughs of the folds. It is also found in the walls of blood vessels, both arterial, venous and endomysial capillaries, intramuscular nerves, the capsules of muscle spindles, the intrafusal fibres and at the surface of satellite cells (Khurana et al., 1991; Love et al., 1991; Nguyen et al., 1991; Ohlendieck et al., 1991; Zhao et al., 1992; Karpati et al., 1993b; Pons et al., 1993; Bewick et al., 1996; Blake et al., 1996b). This contrasts with the distribution of dystrophin in muscle, which is localised to the sarcolemma and enriched at the NMJs and MTJs (discussed in Section 1.2.2).

Utrophin levels are higher in developing muscle than in mature muscle. In man the levels peak during the second trimester of pregnancy and fall thereafter coinciding with the increase of dystrophin level, suggesting that the two proteins are coordinately regulated. By 26 weeks, the levels have fallen to a plateau showing that utrophin is down regulated before birth in man (Clerk et al., 1993). Clerk et al. (1993) also showed that the developmental profile of utrophin in dystrophin-deficient muscle

is similar to that of normal muscle (Clerk et al., 1993). In mouse, the developmentally regulated utrophin level is high until two weeks after birth (Khurana et al., 1991).

In *mdx* muscle, once muscle necrosis has started, utrophin is up-regulated by approximately twice that in normal mouse muscle and localised to the sarcolemma and associated with the components of DPC (Khurana et al., 1991; Karpati et al., 1993b; Mizuno et al., 1993; Law et al., 1994; Lin et al., 1998). This is consistent with the observations that regenerating muscle fibres recapitulate the normal developmental pattern of gene expression, possibly as part of an activated foetal programme during regeneration.

#### **1.5.3.2 Utrophin in heart muscle**

In the heart, utrophin and dystrophin have distinct patterns of expression (Pons et al., 1994). Utrophin is localised to the intercalated discs and cytoplasm of the Purkinje fibres but colocalised with dystrophin along the transverse-tubules in cardiomyocytes. In contrast, dystrophin is localised to the sarcolemma of the cardiomyocytes, the plasma membrane of the Purkinje fibres and transverse-tubules. In *mdx* heart, utrophin is over-expressed as in skeletal muscle sarcolemma, an area normally occupied by dystrophin but is not organised in the same network-like distribution (Pons et al., 1994).

#### **1.5.3.3 Utrophin in non-muscle tissues**

Utrophin is expressed in a wide range of non-muscle tissues, both in normal and DMD/*mdx* tissues. These are brain, kidney, spleen, liver, testis, intestine, stomach, heart and human placenta with the highest level in lung (Love et al., 1991). It is also

expressed in vascular smooth muscle, endothelium and Schwann cells of the peripheral nerves (Khurana et al., 1990; Love et al., 1991; Fabbri et al., 1995).

In the brain, utrophin is expressed in some neurons, choroid plexus, pia mater, intracerebral vasculatures, ependymal lining and end feet of perivascular astrocytes, which may play a role in the blood-brain barrier. In contrast, dystrophin is mainly localised within the neurons at the post-synaptic membrane (Lidov et al., 1990).

The expression pattern of utrophin and dystrophin genes during mouse embryogenesis is very different (Schofield et al., 1993). Whilst dystrophin is expressed in largely mesodermal derivatives, such as cardiac and striated muscle, utrophin shows a more wide spread distribution. It is expressed in neural tubes, neural crest derivatives and a variety of non-neural origin tissues.

#### **1.5.4 *Utrophin knockout mouse***

Utrophin null mutant mice are healthy and show no sign of muscle weakness (Deconinck et al., 1997a; Grady et al., 1997a). However, the NMJs have reduced numbers of AChRs and decreased numbers of post-synaptic folds although there are minimal physiological changes compared to normal mice. Despite these subtle changes, the NMJs and the DPC are normal. Thus utrophin is not essential for AChRs clustering at the NMJs but may contribute to the development and maintenance of the post-synaptic folds.

There is no known disease caused by the mutation of the utrophin gene. However, some cases of myasthenia gravis, caused by autoimmune end-plate destruction and congenital myasthenic syndromes show reduction in the number of AChRs and

junctional folds with a reduction of utrophin at the NMJs (Ito et al., 1996). Congenital myasthenic syndromes are a heterogeneous group of conditions in which muscle weakness resulting from impaired neuromuscular transmissions often present from infancy (Slater et al., 1997; Sieb et al., 1998). These patients have a defect in the development or maintenance of the post-synaptic clefts, and whether this defect results from or causes a reduced expression of utrophin and AChRs at the neuromuscular junctions is unclear.

### **1.5.5 Utrophin-dystrophin-deficient mutants**

If the utrophin knockout mouse is bred onto the dystrophin-deficient *mdx* mouse background, a severe phenotype characteristic of the human DMD is obtained (Deconinck et al., 1997b; Grady et al., 1997b). These utrophin-dystrophin-deficient mice show progressive muscular dystrophy that results in premature death. They have kyphosis, joint contractures, and ultra structural neuromuscular and myotendinous junctions abnormalities. Recent studies show that these mutants also have abnormal ECGs that are associated with decreased myocardial nNOS (neuronal nitric oxide synthase normally associated with dystrophin) and increased iNOS (inducible NOS) activities (Bia et al., 1999).

## **1.6 Therapeutic strategies for DMD**

There is currently no effective therapy for DMD and patients can only be given supportive management. Various therapeutic strategies have been considered in the past and present based on the knowledge of the molecular pathogenesis of the disease.

The identification of the DMD gene and the availability of the molecular diagnostic reagents made prenatal diagnosis possible, offering the choice of selective abortion of

affected fetuses (Ward et al., 1989). This strategy has not proved to be applicable in all cases because only 98% of the cases could be identified by DNA analysis (Beggs et al., 1990). The point mutations, micro-deletions or insertions are more difficult to detect. About 30% of the cases are spontaneous mutations (Emery, 1993). Therefore, only screening affected families will miss a third of affected fetuses. As the clinical diagnosis of DMD often occurs around the age of three, it is not uncommon for a second child to be born to parents harbouring a mutation (either a new mutation or asymptomatic female carrier) before the first child is diagnosed. For some families, due to religious or ethical reasons, termination of pregnancy is not an option. For these reasons, an effective therapy for DMD would be a more desirable option.

### **1.6.1 Dystrophin gene delivery**

For any delivery therapy to be successful it must be effective in the *in vivo* microenvironment and it must reach the target cells in optimal quantities. Dystrophin gene delivery is based on the fact that the DMD phenotype results from the absence of functional dystrophin. The over-expression of this protein corrects the dystrophic phenotype of the *mdx* mouse, without any toxic consequences (Wells et al., 1992; Cox et al., 1993; Wells et al., 1995). Full-length dystrophin is more effective than truncated dystrophin (Phelps et al., 1995). Effective prevention of dystrophic symptoms is dependent on at least 50% of the fibres in each muscle group expressing dystrophin (Hauser et al., 1997). The delivery of the dystrophin gene to 50% of the muscle cells of the body is a formidable challenge and dystrophin may provoke immune response in patients who has not seen dystrophin before. Therefore, the delivery of a non-immunogenic protein like utrophin might be a better proposition.

### **1.6.2 Myoblast transfer**

Early studies of myoblast transfer paved the way for several clinical trials (Partridge et al., 1978; Watt et al., 1982; Partridge et al., 1989). This involves the injection of normal donor myoblasts into DMD patients' muscles. Myoblasts would then fuse with regenerating myofibres and their nuclei would have a functional copy of the dystrophin gene capable of transcribing the dystrophin protein which is absent in the host fibres. However, clinical trials were disappointing (Gussoni et al., 1992; Mendell et al., 1995; Neumeyer et al., 1998; for review see Smythe et al., 2000). Generally normal dystrophin transcript was detected in the muscle of the patients for some months only, about one to eight months after myoblasts transfer and the efficiency of donor myoblasts fusing with the host fibres was low. Immune responses to the cells or dystrophin might be the cause (Ohtsuka et al., 1998). *Ex vivo* (autologous) dystrophin gene transfer using adenovirus-mediated delivery to the *mdx* muscle achieved a higher level of gene transfer to the dystrophic muscle (Floyd et al., 1998). However, this approach also triggered a cellular immune response both to the virus and myoblasts, which limited the duration of the transgene expression. Although immuno-suppressive drugs may be used, the poor fusion rate with the host myoblasts and the multisites of injection may not be ethically justified (Law et al., 1992, Karpati et al., 1993a; Miller et al., 1997).

### **1.6.3 Viral gene delivery**

Viral gene delivery involves inserting the dystrophin gene into a virus capable of delivering the gene to skeletal muscle. So, the choice of vector is important and is often dictated by the need for short or long-term expression of the transgene. In the case of DMD and other genetic diseases, sustained expression is needed. An

integrating vector without attendant immunological problems is most desirable. Patients often mount an immune response to the viral vectors and the dystrophin protein (Tremblay et al., 1993; Ferrer et al., 2000). Immuno-suppressants may prolong the expression of the transgene (Howell et al., 1998b).

An ideal vector for DMD therapy may have to be constructed from both viral and synthetic systems, and it should have the following properties:

1. High concentration of viral particles to allow many cells to be infected.
2. Convenience and reproducibility of viral production.
3. Ability to integrate in a site-specific location in the host chromosome, or to be successfully maintained as a stable episome.
4. There must be a transcriptional unit that can respond to the manipulation of its regulatory elements.
5. High transgene insert capacity.
6. Ability to target the desired cell types.
7. No accompanying components that will elicit an immune response.

Many different viruses have been used in dystrophin delivery to the *mdx* mouse muscle. Progress has been made in the design of suitable viral vectors but it has yet to prove its effectiveness as a therapeutic option. Adenovirus is less effective at infecting adult skeletal muscle than foetal muscle owing to reduced expression of the receptor for viral internalisation (Karpati et al., 1997). The limiting insert size that the many vectors can contain is small, about 6 kb (Hauser et al., 1997). This necessitates the generation of a dystrophin mini-gene that encodes a truncated form of the protein (Ragot et al., 1993). However, the new generation of gutted adenovirus, with the E1-E3 genes removed, has increased the insert size to 9 kb (Amalfitano et al., 1998). Retroviruses, which infect actively dividing cells, are suitable for skeletal muscle

satellite cell delivery and they also have been used after muscle damage (Dunckley et al., 1993).

Another problem with using a viral vector is that viruses tend to express a low level of viral genes in the host cells. This results in a virus-specific cellular immune response leading to the destruction of the genetically modified cells delivered (Hauser et al., 1997). Although this may be overcome by tolerating the recipients to viral antigens or subjecting them to chronic immuno-suppression. The gutted adenovirus reduced the immune response against the viral vector (Amalfitano et al., 1998). In *mdx* mouse that has never seen dystrophin in their cells before, therefore, regard the introduced dystrophin as foreign and produce an immune response to the introduced dystrophin protein (Ohtsuka et al., 1998). Immune responses to the transferred fibres are responsible for the failure of long-term expression of the transgene (Wells et al., 1997). AAV vectors which provoke less immune responses than adenovirus have also been use in several animal studies in mice and dogs (Fisher et al, 1997; Monahan et al., 1998; Li et al., 1999; for review see Hartigan-O'Connor and Chamberlain, 2000 and also Monahan and Samulski et al, 2000). Now the search is for less immunogenic viruses, such as the lenti-viruses (Kafri et al., 1997; Kim et al., 1998).

There are some successful gene delivery using viral vectors to dystrophic muscle but the long-term effectiveness is questionable (Ragot et al., 1993; Acsadi et al., 1996; Deconinck et al., 1996; Haecker et al., 1996). The direct injection into the muscle of  $\gamma$ -sarcoglycan knock out mice of a recombinant AAV with human  $\gamma$ -sarcoglycan shows improvement in muscle histology. However it needs to be injected early and before the development of significant muscle fibrosis (Cordier et al., 2000). The failure to obtain systemic delivery, getting the virus into the skeletal muscle and the

long-term expression of the transgene has so far, prevented successful application of viral vectors in gene delivery (Stedman et al., 2000).

#### **1.6.4 Non-viral gene delivery**

The injection of naked plasmid and plasmid-liposome complexes into mouse skeletal muscle has been attempted (Wolff et al., 1990; Wolff, 1997). However, the transfection of skeletal muscle by this technique is inefficient except in neonatal muscle of the *mdx* mouse (Acsadi et al., 1991; Danko et al., 1997; Howell et al. 1997; Howell et al. 1998a). The plasmid must be injected into a sufficiently high proportion of the muscle fibres to effect any phenotypic recovery (Acsadi et al., 1991). This technique does not seem like a viable method of gene delivery.

#### **1.6.5 Drug therapies**

Several drug therapies have been reported but most of the drugs used failed to lessen the progression of the disease with the exception of prednisone (Fenichel et al., 1991; Jacobs et al., 1996). Prednisone, given in appropriate amounts (1mg/kg), shows a 24% improvement in whole body strength. Prednisone, an immuno-suppressant, may suppress the inflammatory cascade involved in muscle damage and regeneration. This observation may explain the transient beneficial effect of prednisone in DMD patients (Jacobs et al., 1996; Granchelli et al., 2000). Several human trials using prednisone with or without other drugs are being carried out on DMD, BMD and sarcoglycanopathy patients (Report, 2000). Several controlled studies using steroids have shown an increase in muscle strength especially in the sarcoglycanopathies but the mechanism involved is not known (for review see Tawil, 1999).

### **1.6.6 Bone marrow-derived myogenic progenitor delivery**

Intravenous injection of genetically marked bone marrow into immuno-deficient mice revealed that marrow-derived cells migrate into areas of induced muscle degeneration, undergo myogenic differentiation, and participate partially in the regeneration of the damaged fibres. (Ferrari et al., 1998; for review see Chu et al., 1998; Gossoni et al., 1999; Cho et al., 2000). Thus systemic delivery of genetically modified marrow-derived myogenic progenitors or a novel population of muscle-derived stem cells could potentially be used to target therapeutic genes into muscle tissues, providing an alternative strategy for treatment of muscular dystrophy. This method of delivery stem cells that are able to disperse to sites of muscle damage via the blood vascular system and to participate in muscle repair may be a viable mean of treatment for muscular dystrophies.

### **1.6.7 Other approaches not based on gene delivery**

Experiments that are not based on gene delivery have been described. These have post-transcriptional effects on *mdx* mRNA re-establishing the open reading frame. The first uses the antibiotic, gentamicin, which interacts with ribosomes, such that they are able to read through stop codons and correct the expression of dystrophin post-transcriptionally (Barton-Davis et al., 1999a). Injection into *mdx* muscle results in functional improvement. Gentamicin is toxic to the inner ear hair cells and the renal tubules, and has neuromuscular blocking actions. The clinical use of this antibiotic in DMD therapy is doubtful in clinical trials.

The second approach to correct the dystrophin deficiency at the post-transcriptional level is by transfection of the primary *mdx* myoblasts with antisense RNA. A

chemically modified oligonucleotide complementary to the 3' splice site of murine dystrophin intron 22, is injected, so that precise splicing of exon 22 to exon 30 occurs, skipping the mutant exon and creating a novel in-frame dystrophin transcript (Dunckley et al., 1998). It is not known whether this will be effective *in vivo*.

The third approach is the use of anti-sense nucleotides to induce more revertant fibre production in dystrophin-deficient muscle (Fanin et al., 1992; Lu et al., 2000). A better understanding of the mechanism of how the reading frame has been corrected is needed.

The fourth approach that is not based on gene delivery is the therapeutic up-regulation of utrophin, the paralogue of dystrophin, which is discussed in more detail below.

## **1.7 Can utrophin replace dystrophin?**

Several observations lead to the hypothesis that utrophin can substitute for dystrophin in dystrophin-deficient muscle.

Firstly, there is a high degree of similarity in the functional domains of utrophin and dystrophin (Tinsley et al., 1992; Pearce et al., 1993; Dennis et al., 1996; Burton et al., 1999). Secondly, both utrophin and dystrophin bind to similar protein binding partners in muscle (Matsumura et al., 1992a; James et al., 1995; Winder et al., 1995c; for review see Blake et al., 1996b; Peters et al., 1997a). Thirdly, utrophin localises to the sarcolemma under certain circumstances, for example in regenerating and foetal muscle (Khurana et al., 1991; Clerk et al., 1993; Karpati et al., 1993b). Fourthly, the two proteins are evolutionarily conserved suggesting that they may play a fundamentally similar role in animal biology (Roberts and Bobrow, 1998; Wang et

al., 1998). Fifthly, in the *mdx* mouse and DMD patients, utrophin is up-regulated in muscles indicating that there is a natural attempt to compensate for dystrophin deficiency. This natural up-regulation is insufficient to protect dystrophin-deficient muscle from degenerating, but a utrophin therapy would provide adequate compensation.

Finally, In *Xenopus laevis*, utrophin instead of dystrophin is found in the heart naturally (Morris, 1997). Utrophin is associated with cardiac muscle membranes and its distribution is similar to that of  $\beta$ -dystroglycan, which is a transmembrane protein responsible for the localisation of both dystrophin and utrophin at cell membranes. The data suggest that utrophin in *Xenopus* heart can perform similar functions to dystrophin in mammalian heart, lending further support to the possibility of utrophin in therapy for DMD.

There may be some degree of common functions between the two proteins and thus enhanced expression of utrophin may be an effective way of compensating for the absence of dystrophin. Instances of this nature have been described in several diseases (see below).

## **1.8 Phenotypic rescue by functionally related proteins**

### **1.8.1 Haemoglobinopathies**

$\beta$ -Thalassaemia and sickle cell disease are caused by mutation in the gene encoding  $\beta$ -globin. There is a point mutation in the  $\beta$ -globin gene in sickle cell disease, resulting in a substitution of an amino acid that alters the biophysiological properties of circulating haemoglobin. Mutations in the gene either reduce or abolish its expression in  $\beta$ -thalassaemia. The foetal isoform of  $\gamma$ -globin is a close homologue of

$\beta$ -globin. After birth, a switch between the foetal ( $\alpha_2\gamma_2$ ) and adult ( $\alpha_2\beta_2$ ) forms of haemoglobin occurs (for review see Hoffbrand and Pettit, 1984).

One strategy to ameliorate the phenotype in the  $\beta$ -globinopathies is to reactivate transcription from the  $\gamma$ -globin locus in the red cell precursors, thus replacing the mutant or absent  $\beta$ -globin by its functional homologue,  $\gamma$ -globin (Faller and Perrine, 1995). Some small molecules, for example butyrate derivatives, are able to reactivate  $\gamma$ -globin transcriptionally by interacting with the 5' regulatory elements of the  $\gamma$ -globin promoter (Perrine et al., 1984; Hudgins et al., 1996). Early trials have some success, although clinical responses are variable (Perrine et al., 1993; Cappellini et al., 1998).

### **1.8.2 Adrenoleukodystrophy (ALD)**

This is an X-linked inherited disorder of peroxisomal  $\beta$ -oxidation of saturated very long chain fatty acids (VLCFAs), those containing over 20  $\text{CH}_2$  groups (Ligtenberg et al., 1995; Kemp et al., 1998; Smith et al., 1999). The genetic mutation abolishes the expression of a peroxisomal membrane transporter protein ALDP required for the uptake of the enzyme long-chain fatty acyl CoA synthase into the peroxisome from the cytosol, which is one of the family of related proteins (Kemp et al., 1998). Up-regulation of a related protein, ALDRP, by treatment with 4-phenylbutyrate reduces accumulation of VLCFAs, both in cultured fibroblast and in the brains of XALD mice was successfully carried out. This indicates that the homologue is able to replace the mutated protein functionally.

### **1.8.3 Utrophin complementing dystrophin**

The above studies demonstrate an important principle with respect to the present work. A closely related protein is up-regulated to compensate for the absent protein. In so doing, the patients improve clinically. An example of utrophin and dystrophin having complementary functions in the muscle can be seen from the *mdx* and the utrophin-null mutants. These two mutants have mild phenotypes, which could arise from functional redundancy allowing utrophin to compensate for dystrophin. In support of this, the utrophin-dystrophin-deficient mutants have a very severe myopathic phenotype that results in premature death. The mice develop contractures, kyphoscoliosis and muscle weakness characteristic of DMD patients (Rafael et al., 1994; Deconinck et al., 1997a; Grady et al., 1997a).

There are many challenges facing DMD therapy by up-regulating utrophin by small molecules. The hypothesis of whether utrophin can functionally replace dystrophin will be answered by generating a range of transgenic mice expressing truncated and full-length utrophin. The question of whether over-expressing utrophin in tissues other than the targeted tissues is toxic is addressed by the ubiquitous over-expression of utrophin experiments here. Other questions involving the screening of drugs that are able to target and up-regulate endogenous utrophin are being studied in the laboratory at the moment. Finally the toxicity of potential drugs will be screened in tissue culture and in mice.

## **1.9 Hypothesis of this thesis**

In this thesis, the main objective is to test the hypothesis whether utrophin could replace dystrophin functionally through the generation of transgenic mice. Here,

studies were carried out to demonstrate whether the over-expression of truncated and full-length utrophin could prevent muscle dystrophy and improve the mechanical properties of the *mdx* mouse, and to test whether over-expression of utrophin ubiquitously in all tissues is toxic in the *mdx* mouse. This raises the possibility of up-regulating the endogenous utrophin to compensate for dystrophin deficiency as a therapy for DMD. The defined plans are as follows:

- 1) The generation of transgenic mice for a utrophin minigene to study whether utrophin can localise to the sarcolemma of the *mdx* muscle membrane and improve the mechanical properties.
- 2) The generation of transgenic mice for a full-length utrophin to study whether the expression of full-length utrophin can rescue the dystrophic phenotype of the *mdx* mouse at a lower transgene level than that of the truncated utrophin minigene. This is based on the assumption that full-length utrophin has all the functional domains intact and therefore, might have improved function and in addition evidence from the dystrophin transgenic experiments showed that full-length dystrophin was better than truncated dystrophin (Phelps et al., 1995; Wells et al., 1995)
- 3) The generation of transgenic mice that over-express utrophin ubiquitously in the *mdx* mouse for toxicity studies with the aim of up-regulating utrophin by small molecules in the therapy of DMD, if the over-expression of utrophin is found to be not toxic.

4) The generation of tetracycline inducible expression of utrophin transgenic mice to determine the best disease progression stage to up-regulate utrophin in the treatment of dystrophin-deficient muscle degeneration in the *mdx* mouse.

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## Chapter 2

# Materials and methods

## 2.1 Materials

### 2.1.1 *Chemical reagents*

All the dry and liquid chemicals, enzymes, media and hormones were obtained from various commercial sources as noted.

### 2.1.2 *Solutions*

Solutions were made using standard formulae used in the laboratory.

### 2.1.3 *Truncated and full-length utrophin cDNA*

The truncated and full-length utrophin cDNA were constructed by Dr. Jon Tinsley and Mr. Steve Phelps using standard techniques and materials (Tinsley et al., 1996; Tinsley et al., 1998).

### 2.1.4 *Mouse strains*

CBA/CA and C57 BL/6J were purchased from Olac, UK. The *mdx* mice, C57BL/10 Scn/Scn, breeding pairs were kindly donated by Prof. George Radar, Biochemistry Department, Oxford, UK.

### **2.1.5 Micromanipulation apparatus**

A Leica DM 1RB inverted microscope with differential interference contrast and a Narishige manipulator were used for the pronuclear microinjection of the cDNA constructs. Other instruments used included a micropipette puller from Sutter, Model P97 and a microforge, from Micro-Instrument, Oxford, to prepare the microinjecting and holding pipettes respectively. A Nikon microscope for embryo manipulations and a stereoscope, model TS4, from Vision Engineering, UK, for embryo transfer.

### **2.1.6 Antibodies**

Antibodies were kindly provided by various people or obtained from various sources.

All monoclonals were mouse and polyclonals were rabbit unless specified otherwise.

The dilutions used were as following:

- URD40; polyclonal raised against G-utrophin but also picks up full-length utrophin, 1/500.
- 2166; polyclonal dystrophin, 1/1000. Detects full-length, Dp260, Dp140 Dp116 and Dp71.
- Alt C; polyclonal, 1/30, picks up the kidney isoform of Dp71.
- $\alpha$ -1CP-FP; polyclonal, 1/1000, picks up  $\alpha$ -1 dystrobrevin.

All the above-purified antibodies were kindly provided by Dr. D. Blake, Human Anatomy and Genetics, Oxford, UK.

- G3 and P6; polyclonal utrophin and dystrophin, 1/25, 1/400. Dr. C. Sewry, London.

- $\beta$ 1-syntrophin; syn35, polyclonal, 1/50. Dr. S. Froehner, USA.
- $\alpha$ -Sarcoglycan; polyclonal, 1/10, Dr K. Campbell, USA.
- $\alpha$ -,  $\beta$ -Dystroglycan; goat polyclonal, 1/10. Dr. K. Campbell, USA.
- Mancho 3; monoclonal utrophin, 1/200-1/400. Prof. G. Morris Wales.
- Anti-desmin monoclonal antibody, 1/400, DE-U-10, Sigma, UK.
- FITC conjugated secondary antibody to goat; 1/10. Sigma, UK.
- Cy3 conjugated secondary antibody to rabbit; 1/400. Jackson Laboratory, UK.

## **2.2 Methods**

All the animal work was carried out with careful planning and utmost care following the regulations of the Home Office of Great Britain. The embryos of the George line and sperms of all the transgenic lines used were frozen at MRC Mammalian Genetics Unit, Harwell, Didcot, Oxfordshire, UK, after the papers were published. The reasons were to insure against the loss of the unique transgenic lines and to minimise the maintenance cost. Initially 8-cells embryos of the George line were frozen but it was found to be too time consuming and expensive. With Dr. P. Glennister's collaboration, the freezing of sperms was tested and found to be successful. Thereafter, sperms from 3 to 5 fertile males of various ages of each line were frozen, usually after publication.

### **2.2.1 cDNA constructs**

Dr. Jon Tinsley and Mr. Steve Phelps constructed the truncated and full-length cDNA. The method was described in references (Tinsley et al., 1996; Tinsley et al., 1998).

## **2.2.2 Pronuclear microinjection**

The pronuclear microinjection technique was carried out essentially as in Hogan and Allen (Hogan, 1986; Allen et al., 1987). All the normal controls used were F1(C57BL6/J x CBA/CA) in our laboratory except when otherwise stated.

### **2.2.2.1 Mouse strains**

To obtain the largest number of fertilised eggs from the fewest females, F1 hybrids of (C57BL/6 x CBA/CA) were used. Fertilised eggs from F1 hybrid females also survive micromanipulation and *in vitro* culture better than those from inbred strains. With the help of our animal technicians, we did our own F1 stockbreeding starting with C57BL/6 females and CBA/CA males from Olac to ensure that we had the correct strains to start with.

### **2.2.2.2 Making pipettes**

Before microinjection, pipettes required for harvesting embryos, embryo transfer, holding and DNA transfer pipettes were prepared. The microinjecting pipettes were pulled on the morning of microinjection to avoid dust clotting the pores.

#### **2.2.2.2.1 Harvesting, embryo transfer and cDNA transfer pipettes**

These pipettes were made from blood collecting capillaries using a home made micro Bunsen burner, that is, using a large gauge steel hypodermic needle that was bent into a L-shape with the gas coming out of the shorter arm of the L. The capillary was held with both hands and a small region at the centre was heated, by rotating it in the microflame. When the glass became soft, it was pulled apart sharply to draw out a thin region of about 5-10cm long. A glasscutter was used to cut the capillary into 2 pipettes with a cleanly broken tip. The tips of the pipettes to be used for embryo

transfer were flame polished, so that it did not damage the oviduct during transfer. The cDNA transfer pipettes required a longer thin tapered tip so as to deliver DNA to as near the tip of an injection pipette as possible.

#### **2.2.2.2 Holding pipettes**

Holding pipettes were made from thin wall borosilicate glass capillaries without an inner filament from Clark Electromedical Instruments. The capillary was pulled under the microflame as for the transfer pipettes resulting in a tip with an external diameter of 80-100  $\mu\text{m}$  and an internal diameter of about 20  $\mu\text{m}$ . The tip was melted under the microforge to give a polished end, thus avoiding damaging the embryo while microinjecting.

#### **2.2.2.3 Injection pipettes**

Injection pipettes were made from thin wall borosilicate capillaries with an inner tube (Clark) to assist in the flow of cDNA. They were pulled by using a micropipette puller from Sutter. The best microinjecting pipettes were made by trial and error, to give the least lysis of injected embryos. The readings that gave the best pipettes were recorded for future use.

#### **2.2.2.3 Superovulation and time mating**

Three and a half to six weeks old F1 female weaners were used for superovulation.

- 1) On day 1: the mice were intraperitoneally (i.p) injected with pregnant mare serum which was used to mimic follicle-stimulating hormone (FSH, Folligon from Intervet at 5 international units/0.1 ml in 0.9% NaCl, stored at  $-20^{\circ}\text{C}$ ) and left for 2 days.

- 2) On day 3: the same mice were injected with 5 i.u. of the human chorionic gonadotrophin which was used to mimic luteinizing hormone (HCG, from Intervet) to mature and release the eggs and mated with F1 male studs overnight. Ovulation which should occur about 12 hours after HCG administration, which was timed to coincide with the mid-point of the dark cycle, when mating is most likely to occur. Our animal room light/dark cycle was from 8 p.m. to 5 a.m. At the same time, sufficient numbers of F1 females aged between 2-6 months were mated with vasectomised males, to be used as foster mothers. These pseudo-pregnant females display the hormonal profile of a pregnant female and their uterus was receptive to embryo implantation. F1 females were used as pseudo-pregnant recipients because they were robust, healthy, reproduce easily and they were good mothers. The vasectomised males were sterilised by tying off and ligated both the vas deferens at least two weeks before the start of microinjection and they could be used for about eight months. The best males to be vasectomised were those young stud males that have been mated with female weaners a few times before and they were likely to remain active after vasectomy.
- 3) On day 4: all super-ovulated females and pseudo-pregnant mice were plugged in the morning and those super-ovulated females with plugs were sacrificed by neck dislocation for embryo harvest. Plugs which were composed of dried up male secretion from the vesicular and coagulating glands, were taken as an indication of mating. The plugged foster mothers were left aside for embryo transfer later on. Table 1 shows my weekly schedule of superovulation and pronuclear microinjection sessions.

<b>Day</b>	<b>Job</b>
Monday	FSH
Tuesday	<b>FSH</b>
Wednesday	HCG + Mating and Pp mating
Thursday	Microinjection. <b>HCG + Mating</b> <b>and Pp mating</b>
Friday	<b>Microinjection.</b>

Table 1 Weekly time table for microinjection

Showing my weekly schedule of superovulation and microinjection. Microinjection was carried out twice a week. Superovulation started on Monday and timed mating started on Wednesday. Microinjection and embryo transfer were carried out on Thursday and Friday.

Key: FSH, follicle stimulating hormone; HCG, human chorionic gonadotrophin; Pp, pseudo-pregnant.

#### **2.2.2.4 Harvesting of fertilised embryos**

Oviducts from the mated weaners were removed into M2 medium which is a medium especially formulated for embryos and is buffered by air (Sigma, UK). The oviducts were teased open with a pair of watchmaker forceps and clumps of embryos surrounded by cumulus cells could be seen to float in the medium. 10 $\mu$ l of hyaluronidase at 10 $\mu$ g/ml was added into the medium for about one minute to remove the cumulus cells. The embryos were washed twice in M2, once in warm M16, which is a specially formulated medium for embryos use, buffered by carbon dioxide (Sigma, UK) and put into warm M16 microdrops under light paraffin oil (Sigma) and incubated at 37<sup>0</sup>C, buffered with 5% CO<sub>2</sub>. About 150-200 embryos would be ideal for a good microinjection session.

#### **2.2.3 The microinjection set-up**

##### **2.2.3.1 Microinjection microscope**

The microinjection was performed by using a Leica DM 1RB inverted microscope with differential interference contrast optics, sitting on an anti-vibration table from (Wentworth Laboratory, UK). The magnification used for microinjection was 600x.

##### **2.2.3.2 Micromanipulator**

A Narishige (Japan) micromanipulator was used for the mouse embryo micromanipulation. The syringes to control holding pipette and injection pipettes (Narishige) were attached to the micromanipulator and the movement was achieved by joystick control.

### **2.2.3.3 Injection chamber**

The cover of a 50mm Petri dish (Sterilin, UK) was used as the injection chamber. Several M2 microdrops were made on the dish and covered with light paraffin oil.

### **2.2.4 Injection of the pronuclei**

Five to six injection pipettes were filled with cDNA using a cDNA transfer pipette and left aside for the cDNA to settle to the tips, ensuring no air bubbles were trapped inside. About 20-30 embryos were transferred into a microdrop in the chamber. Using low power, 50x or 100x magnification, selection of abnormal looking embryos was carried out and these were rejected. A normal healthy embryo should not appear to be fragmented, it should have 2 polar bodies and the cytoplasm contains 2 pronuclei (Allen et al., 1987). During injection, under 600x magnification, the embryo was held by the holding pipette on the left hand side and the injection pipette attached to the micromanipulator on the right as shown in Figure 1. The holding pipette system was filled with light paraffin oil. After choosing the larger male pronucleus, using the manipulator, the injection pipette that was under positive air pressure, was pushed through the zona pellucida, plasma and pronuclear membranes firmly but gently. A small amount of cDNA (pl) was injected and the pronucleus could be seen to swell. It is not known that how much cDNA remains in the pronucleus. If an injection pipette caused too much embryo lysis or was unable to pierce through the zona pellucida and pronuclear membrane cleanly or kept pulling out the cytoplasmic contents, it was replaced by a new one. An injecting pipette could be used to inject approximately 10 embryos successfully. The injected embryos were put to one side until all the embryos in the chamber were injected. The injected embryos were then incubated at 37<sup>0</sup>C with 5% CO<sub>2</sub> for at least an hour before transferring into pseudo-pregnant females.

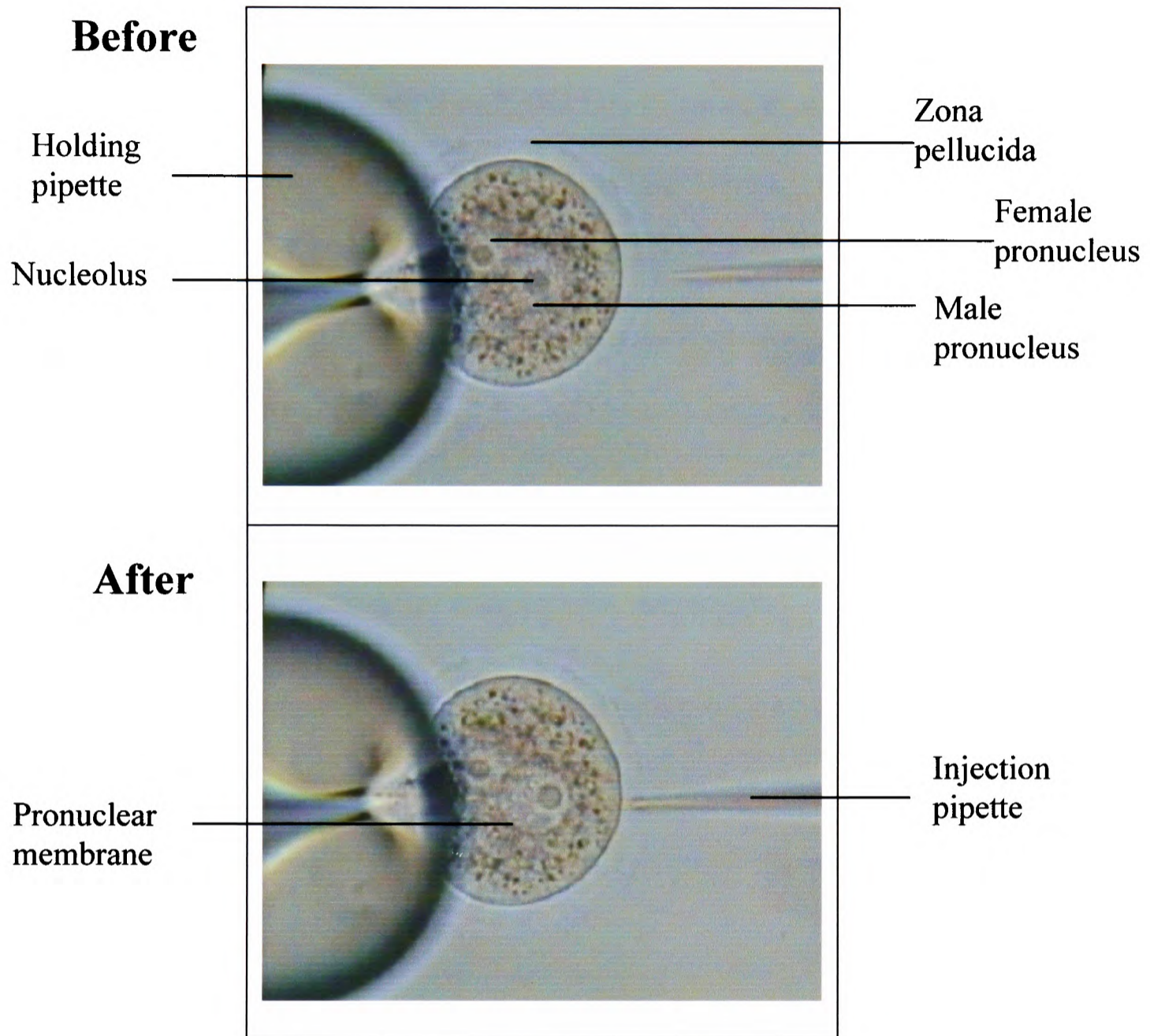


Figure 1 Pronuclear microinjection

Showing before and after the microinjection of the male pronucleus of a one day old fertilised mouse egg. After microinjection, the pronucleus can be seen to be swollen with cDNA and has a defined pronuclear membrane outline. A nucleolus can be seen in each of the pronuclei. Magnification: x600.

### **2.2.5 Oviduct transfer**

All the instruments used in surgery were washed in antibacterial soap and sterilised in 70% alcohol. The injected embryos, still in the one cell stage except a few in the two cells stage by late afternoon, were transferred into the oviduct of the pseudo-pregnant females as shown in Figure 2. The recipient was anaesthetised using 1.25 % Avertin anaesthetic at 0.2ml per 10 gm body weight. About 15-18 injected embryos were transferred into the opening (infundibulum) of the oviduct of each recipient. The mice were left in the cage with a lamp over them for warmth until they recovered.

### **2.2.6 Analysis of transgenic mice**

#### **2.2.6.1 Tail biopsy of potential transgenic founders**

After a gestation period of 19 –21 days, the pseudo-pregnant females gave birth to potential transgenic founders. Approximately 0.5cm tail biopsies were taken at weaning age of between 3-4 weeks. Mice were tagged by toe clipping and later changed to ear tagging.

#### **2.2.6.2 Extraction of tail DNA**

DNA was extracted by the phenol-chloroform method. DNA was extracted from either fresh or frozen tails. Tails were lysed over night at 55°C by proteinase K (30 µg) in tail buffer (10 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 0.1 M NaCl, 0.5% SDS). 1 µg RNA A was added to digest RNA at 37' C for 1 hour. DNA was precipitated in ethanol followed by 2x phenol-chloroform extractions (pH 8.0, Biorad), and 1x chloroform extraction. DNA was pelleted in a bench centrifuge at top speed, washed twice in 1.5 ml 70% ethanol, air dried for 5 min and resuspended in 400 µl distilled

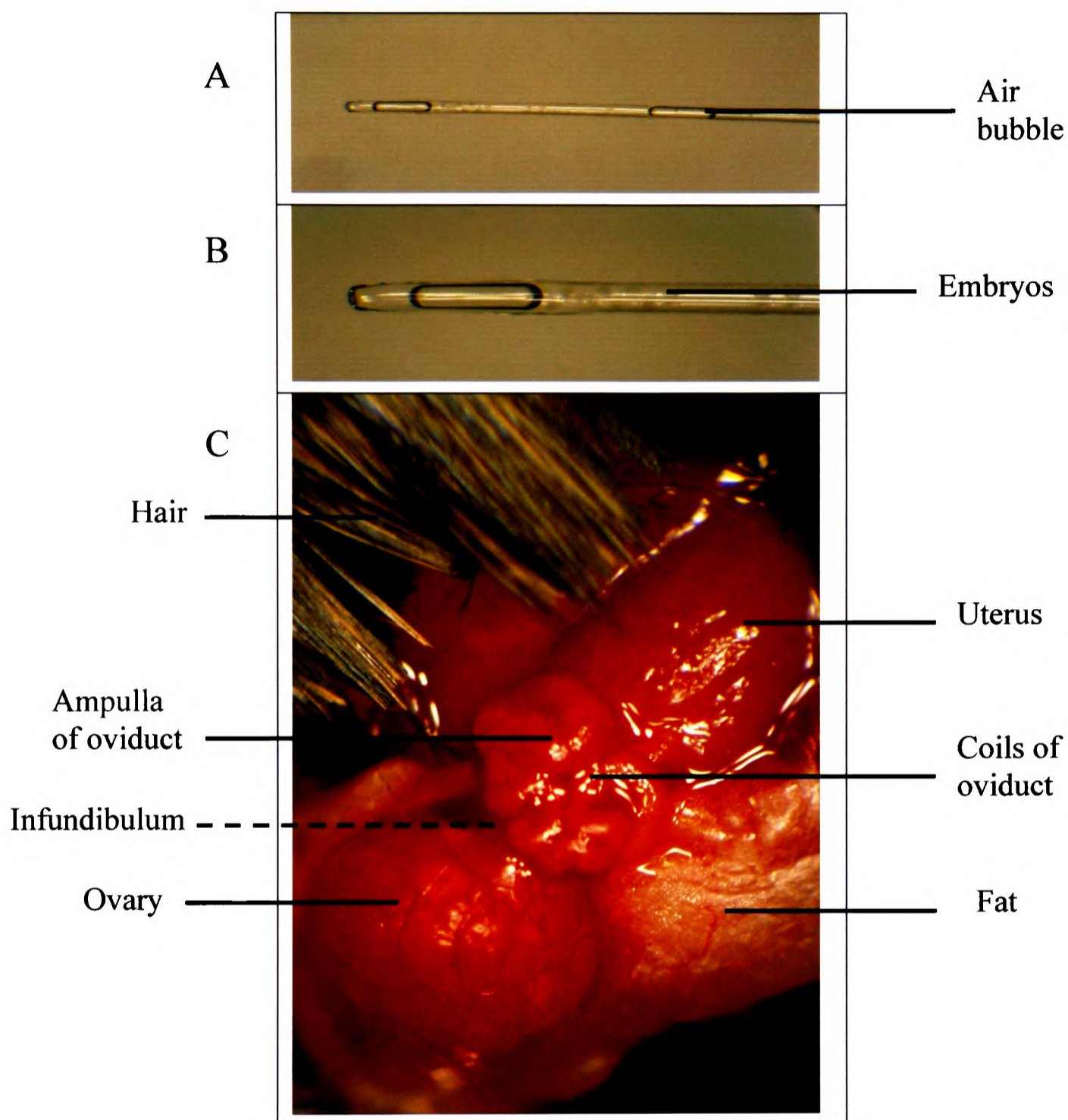


Figure 2 Oviduct transfer

A, Embryo transfer pipette showing arrangement of 2 air bubbles entrapping the embryos. This arrangement was to avoid the loss of the embryos through suction when the tip of the transfer pipette first came into contact with the infundibulum and to monitor the successful transfer of the embryos. Magnification: x8.

B, Higher magnification (x20) to show the embryos inside the pipette.

C, Detail for oviduct transfer. The pipette was inserted into the infundibulum which was usually hidden beneath the coils of oviduct. The ampulla (swollen portion of the oviduct) can be seen to contain 2 air bubbles after successful embryo transfer. Magnification: x50.

water over night on a roller in a cold room. *Bam*H1 digest was set up, using 10 µg of the extracted DNA and analysed by Southern blotting (Section 2.2.6.3).

### **2.2.6.3 Southern blotting**

Positive transgenics were identified by Southern blotting, using a probe to the central part of the transgene (a human fragment). This was the modified technique originally described by Southern (Southern, 1975). The *Bam*H1 digested DNA was separated by gel electrophoresis in 0.8% agarose in TAE buffer (Tris, 10.8 gm; Boric acid, 5.5 gm; EDTA buffer, 0.98 gm, 1 li distilled water). Ethidium bromide was added to the buffer, as well as the gel, so that DNA bands could be visualised under UV light. DNA was depurinated in 0.25M HCl (10 ml HCl /500 ml water) by gel immersion for 15 min, then denatured in 0.4M NaOH (50 ml NaOH in 500 ml water) for 15 min. Single stranded DNA was transferred onto Hybond TH-N membrane (Amersham). After fixing the DNA to the membrane by drying between Whatman filter paper, the membrane was used in hybridisation (Section 2.2.6.4).

### **2.2.6.4 Nucleic acid hybridisation**

Membranes were prehybridised at 65<sup>0</sup> C in 20 ml of Church and Gilbert hybridisation buffer (0.25 M NAHPO<sub>4</sub>, pH 7.2; 0.25 M NaCl; 5mM EDTA; 7% SDS; 5% dextran sulphate) for 2-3 hours. About 40 ng gel purified PCR fragment 4.0 DNA (central region of the microinjected cDNA) was labelled using <sup>32</sup>P dCTP (Amersham, UK) and separated from unincorporated label by a Pharmacia S-300 microspin column. Hybridisation of the membrane was done in a roller over night at 65<sup>0</sup> C. Membrane was washed in 3X SSC at 60<sup>0</sup> C for 30 min, prior to exposure to X-ray film at -70<sup>0</sup> C.

Depending on radiation monitored, washing stringency was increased by soaking membrane in 0.2X SSC at 65<sup>0</sup> C for another 30 min.

### **2.2.7 Transgenic breeding**

Positive transgenic offspring born from the microinjected embryos were the transgenic founders. Founders were kept apart and did not interbreed. Each founder was used to derive a transgenic line. The transgenic mice were bred with *mdx* partners to obtain transgenic-*mdx* mice for analysis. Tissues were taken for western blot analysis and cryosections for morphological analysis when the mice were at the appropriate age. The minimum time needed to breed transgenic-*mdx* offspring from microinjected embryos for analysis was approximately 15 weeks. Figure 3 shows the time scale of breeding of a male founder.

### **2.2.8 Western blot protein analysis**

Total protein extracts were prepared by homogenisation of the tissues in half or 1 ml extraction buffer (75mM Tris, pH 6.8; 3.8% SDS; 4 M Urea; 20% glycerol; 5%  $\beta$ -mercaptoethanol), then heated to 95<sup>0</sup> C for 5 min. The protein concentration was measured using a Bio-Rad DC Protein Assay kit or Bradford Protein Assay kit (BioRad, Richmond, California), in triplicate in a spectrophotometer. The protein extract was diluted into a constant volume of 20  $\mu$ l containing either 25  $\mu$ g or 50  $\mu$ g of total protein extract to minimise the loading variations. Coomassie blue staining and anti-desmin monoclonal antibody (Sigma, DE-U-10) were used to confirm equal loading of the protein samples on gel electrophoresis. 50  $\mu$ g of total protein was loaded onto 6% polyacrylamide gel and transferred to nitro-cellulose membrane. The utrophin transgene was detected using a primary antibody of 1/400 dilution of mouse

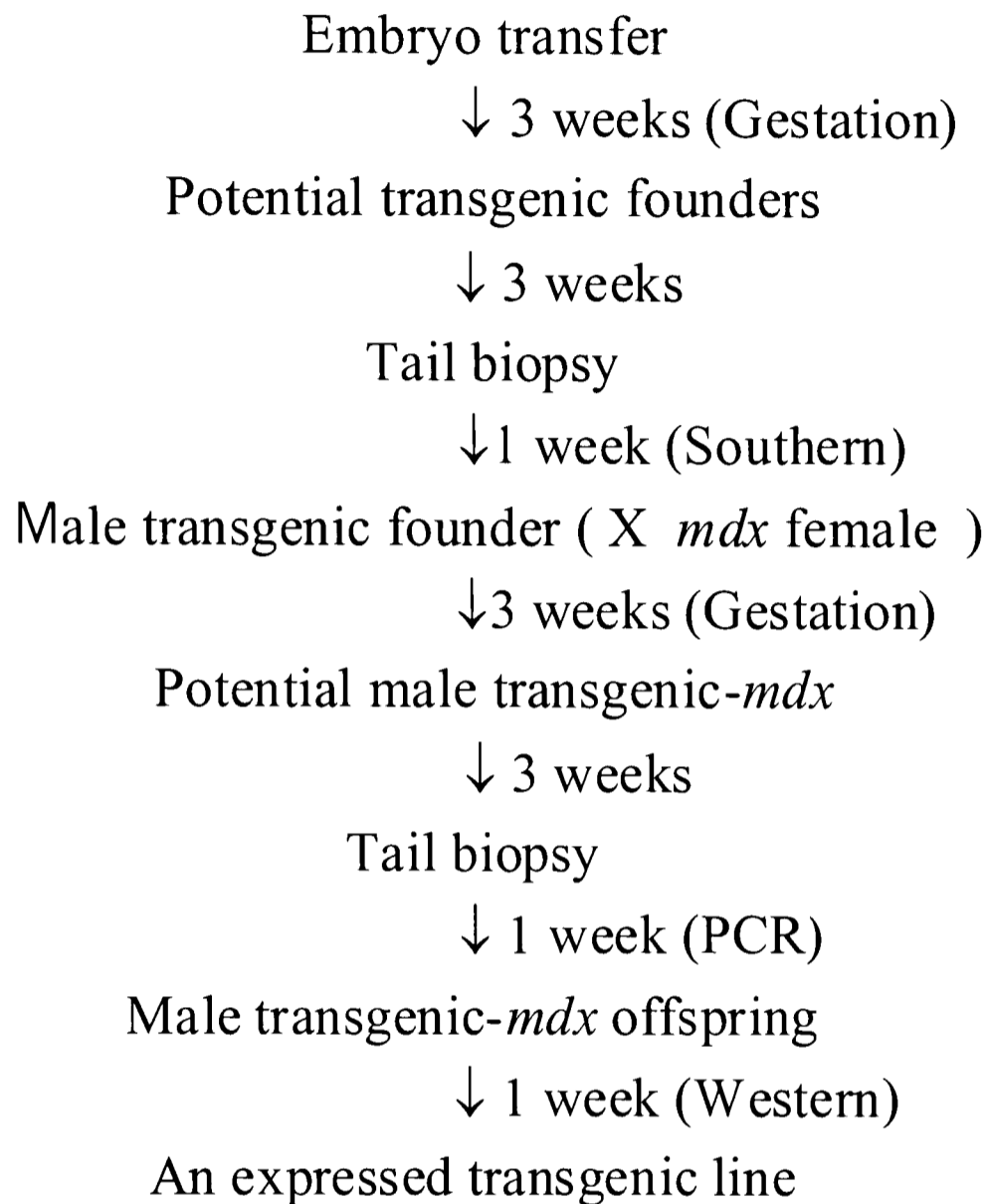


Figure 3 Outline of transgenic breeding

The minimum time scale needed to breed transgenic-*mdx* offspring for analysis from microinjected embryos was about 15 weeks from a male founder.

anti-utrophin monoclonal antibody (Mancho 3, (Nguyen et al., 1991)) and visualised using a secondary anti-mouse HRP (Jackson) and chemiluminiscence (Boehringer). Normally, at least two gels were run to confirm each result.

### **2.2.9 Creatine kinase analysis**

Blood from 4-5 week old mice were collected, allowed to clot, and serum was removed. Serum creatine kinase (CK) levels were measured using the Boehringer NAC-CK kit and 5  $\mu$ l of serum. The rate was averaged over 4 min and calculated as  $UL^{-1}$ .

### **2.2.10 Creatinine analysis**

The analysis of creatinine levels was done by Professor Jo Martin, Royal London School, London, UK. For full detail, please refer to (Martin, 1995). The urine samples from age-matched transgenic, normal and *mdx* mice were tested for the levels of creatinine using proton Nuclear Magnetic Resonance spectroscopy. Defrosted samples were mixed well and placed in a cuvette and analysed, using a 400MHz, 500MHz or 600MHz machine with a standard protocol. A representative spectrum was obtained and analysed with reference to control spectra obtained from the experimental controls and from the database comprising a range of urine samples from a range of mouse strains (Swr, C57BL/6, Balb/c, C3H). Known substance standards have previously been used to establish the spectral characteristics of a range of substances including trimethylamine, trimethylamine-N-oxide, lactate, creatinine, dimethylamine, oxoglutarate, creatine, succinate, acetate, taurine. Experimental samples (from Cleo and HCleo mice) were analysed with the operator blind to the

genetic status of the animals and interpreted with reference to the height of creatinine peaks in order to compensate for possible dilution factors.

### **2.2.11 Analysis of weight versus age**

At the start of the experiment, six mice per group of transgenic, *mdx* and normal mice were weighed at the beginning of each month, starting at three months old. After about one and a half years, some mice died or had to be sacrificed due to ill health. The graph of weight versus age was plotted and the standard error of the means (s.e.m.) calculated.

### **2.2.12 Histology**

Tissues were collected from age-matched mice in each experiment. For the HSA-truncated and HSA-full-length utrophin experiments, TA, EDL, soleus, diaphragm and heart were collected. For the ubiquitin C promoter full-length utrophin experiment, between 20-24 different tissues were collected. These were TA, EDL, heart, lung, liver, diaphragm, intestine, brain, kidney, adrenal gland, spleen, stomach, tongue, testis, ovary, skin, oesophagus, trachea, eye, thymus, bone, uterus, blood and urine.

#### **2.2.12.1 Haematoxylin and eosin staining (H&E)**

The tissues were collected into formal saline and wax embedded and sectioned. The sections were stained with haematoxylin and eosin for comparative histological analysis. Some cryosections were used as well.

### **2.2.12.2 Centrally nucleated myofibre count and total fibres**

H&E slides of TA, EDL, soleus and diaphragm were used to count the percentage centrally nucleated fibres (CNF). For the ubiquitin C utrophin experiments, all the fibres of four complete sections from each mouse were counted. Three to four mice per group were counted. Histogram of %CNF was plotted for each group of mice with  $\pm$  s.e.m. The total muscle fibres for 18 months old mice were obtained by adding together the normal and centrally nucleated fibres counted.

### **2.2.12.3 Masson staining**

Sections used to visualise the progressive infiltration of the fibrocollagenous tissues were stained progressively with Hansen's haematoxylin such that the collagen was green while the cytoplasm was still orange/red. The sections were stained in Harris's haematoxylin for 2-4 min. Washed in running tap water for 5-10 min. Stained in xylidine red and differentiated in dedeca-molybdo-phosphoric acid (BDH). Rinsed in distilled water and followed by Masson light green (Clin-Tech, UK.) for 1-5 min. Dehydrated through alcohol and mounted with Dpx.

### **2.2.12.4 Immunofluorescence staining**

Tissues for immunohistochemistry were dissected, immersed in OCT (BDH) and frozen in liquid nitrogen cooled isopentane. Frozen unfixed section of 10  $\mu$ m were cut and stored at  $-70^{\circ}\text{C}$ . Immunostaining was performed by air drying the cryosections at room temperature and blocking them in 10% normal donkey serum in 50 mM Tris, 150 mM NaCl, TBS at pH 7.5. The primary antibodies used were all rabbit polyclonal antibodies and the following dilutions (in TBS) were used: utrophin (URD40, 1/500; G3, 1/25), dystrophin (2166, 1/1000; P6 (Sherratt et al., 1992), 1/400),  $\alpha$ -dystrobrevin

( $\alpha$ 1-CT-FP, (Blake et al., 1998), 1/1000), Dp71 (Alt-C, 1/100),  $\alpha$ -sarcoglycan, (Roberds et al., 1993) (1/5),  $\beta$ 1-syntrophin (syn35, 1/50); goat polyclonal against  $\alpha/\beta$ -dystroglycan (FP-B,(Ibraghimov-Beskrovnaya et al., 1992), 1/10). FITC conjugated secondary antibody to goat (1/50, Sigma) and Cy-3 conjugated secondary antibody to rabbit (1:500 dilution, Jackson) were used. The primary antibodies were incubated for 2 hours while the secondary antibodies were incubated for an hour at room temperature. The slides were mounted with Vecta Shield (Vector) and photographed under identical conditions of illumination and exposure using a Leica microscope.

### **2.2.13 Mechanical performances**

Dr Jean-Marie Gillis and colleagues in Belgium did the mechanical force and calcium homeostasis experiments. For full details, please refer to (Deconinck et al., 1996; Deconinck et al., 1997c; Tinsley et al., 1998).

### **2.2.14 Statistics**

For the truncated minigene experiments, a series of independent tests were made comparing each pair of means, for example, Utr<sup>++</sup> versus C57, Utr<sup>++</sup> versus *mdx*, for each parameter tested. However, as in most cases, means to be tested were based on small and unequal numbers of data, an adapted version of the *t*-test (two tail) was used with a reduced number of degrees of freedom. Statistical significance,  $p < 0.01$  for Utr versus C57, Utr versus *mdx* for mechanical forces.

For the full-length utrophin experiments, all the data were first submitted to a variance analysis. The significance of the two groups was further evaluated by the Turkey test. The differences between all *mdx* results and all those of other groups (Fiona, Fergie and Freddie) were statistically significant ( $p < 0.05$ ).

For the ubiquitin C full-length experiments, the Kolmogorov-Smirnoff non-parametric test was used in the analysis and showed highly significant differences ( $p < 0.001$ ) in every case (month) except the 7<sup>th</sup> month ( $p < 0.007$ ) between all the age-matched pairs of utrophin-*mdx*, control and *mdx*. This analysis was done with help from Dr. F. Marriott, Statistics Department, Oxford.

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## Chapter 3

# Generation of mice transgenic for a utrophin minigene

### 3.1 Introduction

A truncated dystrophin minigene modelled on the Becker dystrophin mutation (discussed in Section 1.2.4.2) was shown to correct the dystrophic phenotype of *mdx* mice (Phelps et al., 1995; Wells et al., 1995). It was hypothesised that utrophin, a paralogue of dystrophin with similar functional domains, could replace dystrophin (Love et al., 1989; Tinsley et al., 1992; Tinsley and Davies, 1993; Hoffman, 1994; Blake et al., 1996b). Utrophin is localised at the neuromuscular junctions in adult muscle (Pons et al., 1991). However, variable amounts of utrophin are found in the sarcolemma of DMD patients and the *mdx* mouse (Matsumura et al., 1993; Mizumo et al., 1993).

The advantage of using utrophin in the therapy of DMD is to avoid immunological reactions as might be expected in the case of dystrophin, since most DMD patients have never expressed dystrophin before, expressed dystrophin at low levels or in an abnormal form. Therefore patients may mount immune responses to previously unseen epitopes (Huard et al., 1992; Tremblay et al., 1993; Bittner et al., 1995). This

may also be true of the Becker patients where specific epitopes will be missing in the truncated dystrophin that is present. DMD patients have a normal copy of the utrophin gene, therefore immune responses to utrophin transgene would not be expected.

As a first step to validate the use of utrophin up-regulation in the therapy of DMD, it was decided to show that utrophin could replace dystrophin functionally through the generation of transgenic-*mdx* mice using a truncated utrophin minigene driven by the human skeletal  $\alpha$ -actin (HSA) promoter (Muscat and Kedes, 1987; Brennan and Hardeman, 1993). We decided to use the truncated minigene, which was modelled on the Becker dystrophin minigene first for the following reasons. This was, firstly to test whether the N- and C-terminal domains of the minigene could function like the Becker dystrophin minigene; secondly to see if it could rescue the *mdx* phenotype as well as restoring muscle strength like dystrophin as shown by Wells and Phelps (Phelps et al., 1995; Wells et al., 1995); thirdly if it worked, the mini-utrophin gene would be a better candidate to be considered in the therapy of DMD. As a shorter utrophin would facilitate the incorporation into a viral vector (as discussed in Section 1.6.3). The full-length utrophin transgenic experiment will be discussed in the next chapter to compare the advantages and disadvantages of using truncated or full-length utrophin transgene in DMD therapy.

Here, experiments were carried out to test whether the truncated utrophin minigene localised to the sarcolemma of skeletal muscle in the absence of dystrophin and whether utrophin was able to re-establish the dystrophin-associated protein complex at the sarcolemma. Finally mechanical functions specifically the isometric force, peak force and percentage force drop of the skeletal muscle in the utrophin transgenic mice were evaluated in collaboration with Dr Gillis group in Belgium.

## 3.2 Results

The experiments in this chapter were carried out in collaboration with Dr. Jon Tinsley, Mrs Allyson Potter, Mr Steve Phelps and Mr Jeffrey Trickett in the laboratory. I generated the transgenic mice, performed all the transgenic breeding and took tissues for various analyses at the appropriate time. I also participated in the genotyping of the transgenic founders and offspring, was involved in protein analysis, creatine kinase assay and immunostaining.

### 3.2.1 *Transgene construction*

The HSA-truncated utrophin minigene (mini-G) used in this experiment was constructed by Dr. Jon Tinsley in the laboratory (Tinsley et al., 1996). The human skeletal  $\alpha$ -actin promoter was chosen because it promotes high expression of the transgene specifically in skeletal muscle, which is the main site of expression of dystrophin (Muscat and Kedes, 1987; Brennan and Hardeman, 1993). The skeletal muscle of DMD patients and the *mdx* mouse are severely affected (Bulfield et al., 1984; Emery, 1993). The truncated minigene was modelled on the Becker dystrophin transgene as shown in Figure 1A which has been shown to partially correct the dystrophic phenotype of the *mdx* mouse (Phelps et al., 1995; Wells et al., 1995). Figure 1B shows the utrophin transgene vector containing the human skeletal  $\alpha$ -actin promoter and regulatory regions and SV40 large T poly A site. The amino- and carboxy-terminal ends of utrophin were cloned as PCR product using overlapping cDNAs as template, then ligated in-frame to produce the truncated utrophin cDNA. The PCR product was then cloned into a vector containing the 2.2 kb human skeletal

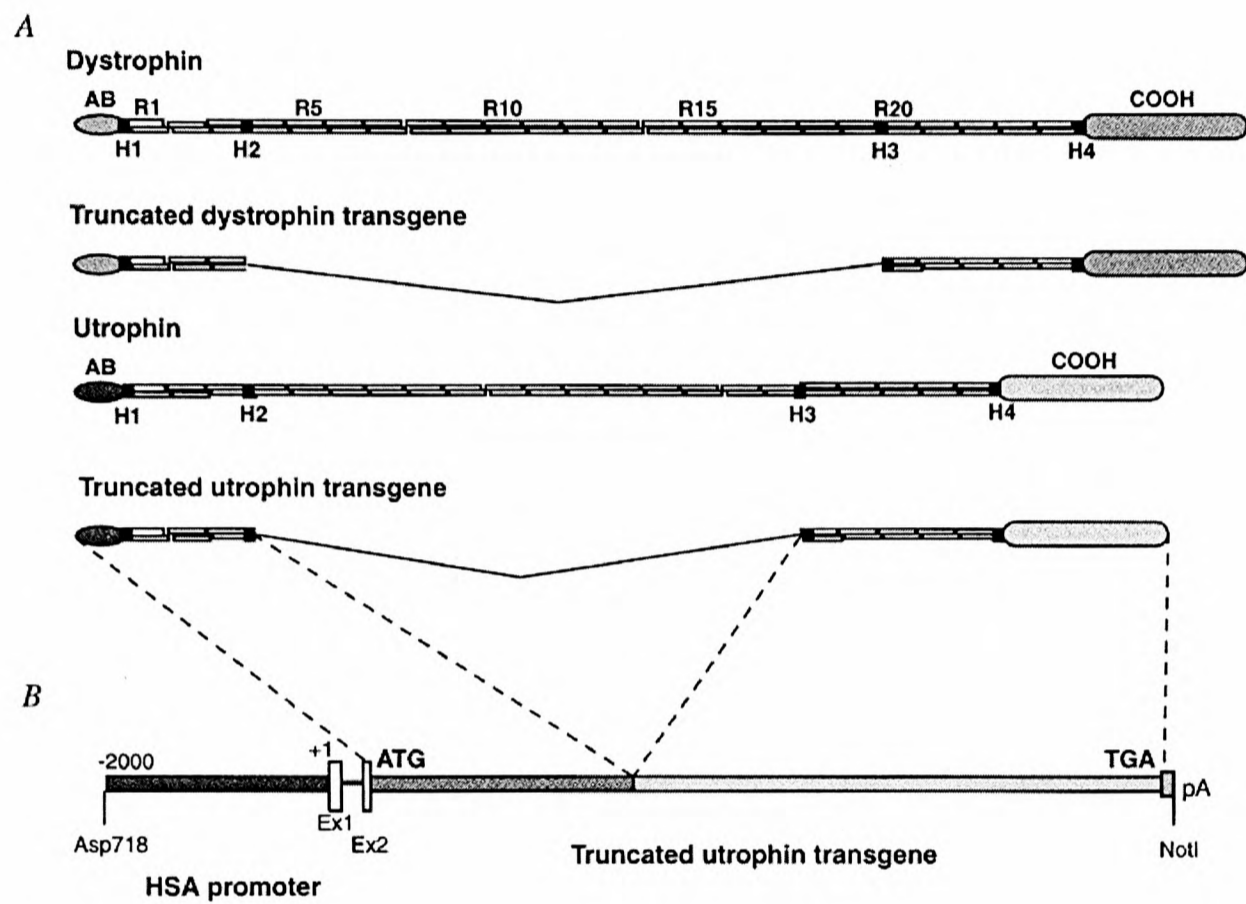


Figure 1 HSA-truncated utrophin transgene construction

A, Representation of dystrophin, utrophin and the two truncated transgenes modelled on the Becker dystrophin mutation.

B, Utrophin transgene vector containing the human skeletal  $\alpha$ -actin (HSA) promoter and regulatory regions and SV40 large T poly (A) site.

Key: Spectrin-like repeats (R, double rectangle), hinge sites (H), acting-binding (AB) and C-terminal-binding (OOH) domains. From (Tinsley et al., 1996).

$\alpha$ -actin promoter and regulatory regions and SV40 large T poly (A) site (Muscat and Kedes, 1987, Brennan, 1993 #1216). Asp 718i was used to liberate the complete fragment, which was purified and resuspended in double distilled water at 2-3 ng/ $\mu$ l for microinjection.

The 6 kb truncated utrophin minigene was a human-mouse hybrid with the rod region deleted between hinges 2 and 4 to produce a predicted protein of 200kDa. A human cDNA was generated but it was unstable while being constructed. The reason was not understood. As the human and mouse utrophin genes are very similar, approximately 87% amino acid identity using the NCBI Blast 2 sequences search results, version blastP 2.2.1. The human gene was expected to function in the *mdx* mouse.

### **3.2.2 Generation of transgenic mice**

The purified HSA-transgene was microinjected into the pronucleus of one day old fertilised F2 hybrid embryos from (C57BL/6J x CBA/CA) parents (Section 2.2.2). The concentration of cDNA used was between 2-3 ng/ $\mu$ l. Positive transgenic mice were identified by Southern blotting (Section 2.2.6) using a probe of 2.93kb (PCR 4.0 probe) to the central part of the utrophin transgene as shown in Figure 2. Twelve transgenic founders were generated, giving a transgenesis rate of about thirteen-percent. They were fertile and gave germ line transmission. Table 1 shows record of the pups born from microinjected embryos. 448 embryos were microinjected and 147 pups were born, giving a successful implantation rate of 33%. About 40% of pups died by weaning age of 3-4 weeks. The causes were not known, but it was observed

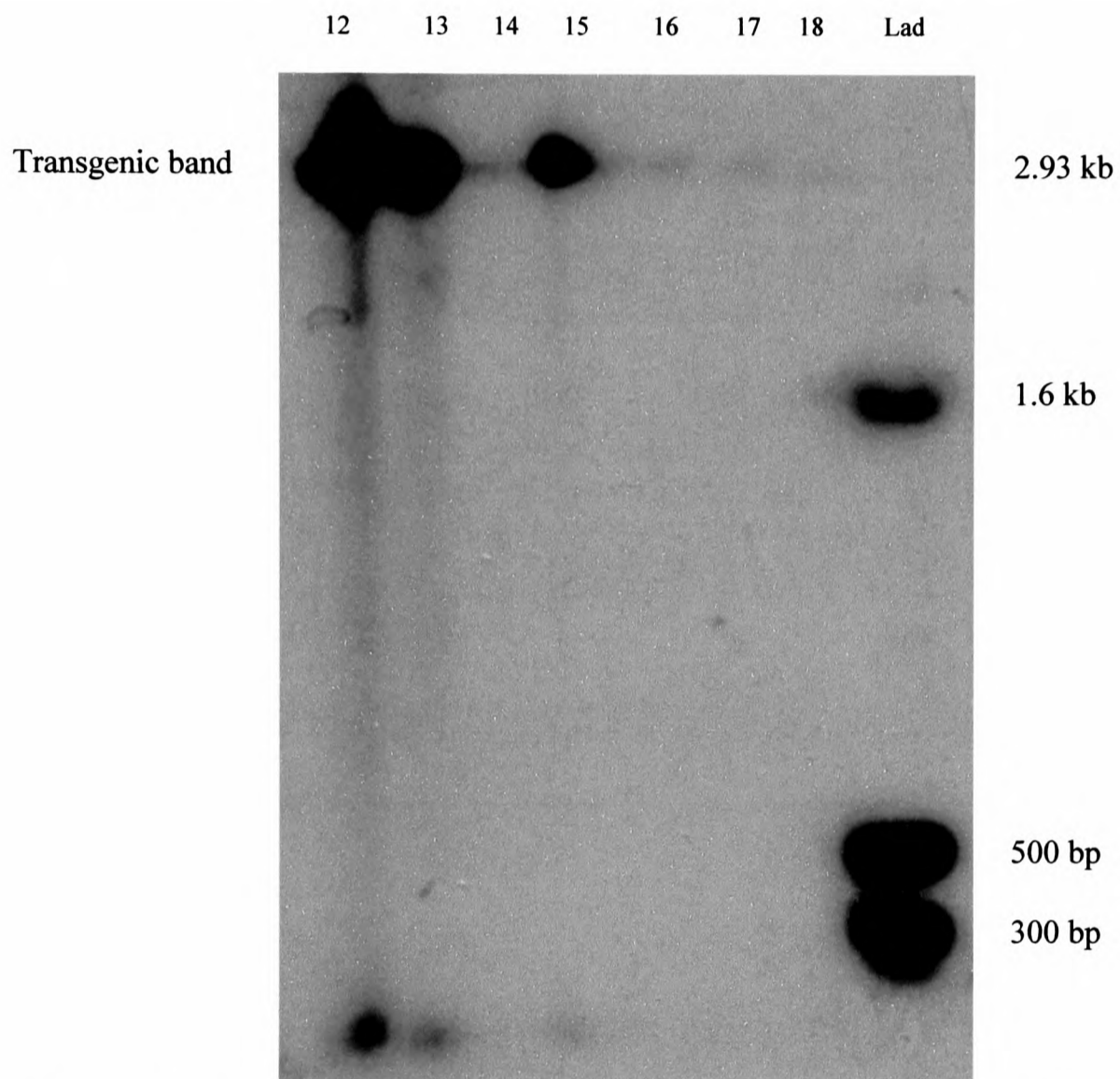


Figure 2 Southern results of the potential transgenic founders

Mice numbers 12, 13 and 15 were the founders of the George, Gareth and Gemma lines. The 2.93 kb band was the radiolabelled PCR 4.0 probe which picked up the transgene. The faint bands of numbers 14, 16, 17 and 18 were false positives as subsequent Southern blotting tested negatives.

Key: Lad, the 1 kb DNA ladder.

No. of microinjected embryos transferred.....	448
No. of pups born.....	147
% of successful implantation.....	33%
No. of pups survived up to 3 - 4 weeks.....	93
% of survival up to 3 - 4 weeks.....	63%
No. of transgenic lines.....	12
% Transgenesis.....	13%

Table 1 Record of potential transgenic founders

Record of pups born from microinjected embryos after oviduct transfer. Successful implantation of the injected embryos was 33%. The percentage of survival of the pups born up to 3-4 weeks was 63%. The percentage transgenesis rate was about 13%.

The percentage transgenesis rate was calculated from No. of transgenic lines/No. of pups of 3-4 weeks x 100. (12/93 x 100). This is because only the tails of the pups that survived up to 3-4 weeks were genotyped.

Key: No., number.

that the mothers tended to eat pups of small litters of one or two. The transgenic founders were used to establish transgenic lines by breeding with *mdx* partners. The offspring produced were on the *mdx* background and used for analysis. The transgenic mice had no overt phenotype and were healthy and fertile except the Gemma line, which was sub-fertile. Table 2 summarises the expression pattern of the transgene in the twelve lines generated. Eleven out of twelve lines expressed the transgene. The Gavin and George lines had the highest level of expression in muscle and diaphragm. The Grant line had the highest level in heart. Only four out of twelve lines expressed significant amounts of the transgene in the heart. These were Grant, Gordon, George and Gemma lines. The Graham line contained the transgene but did not express it.

### **3.2.3 Analysis of transgenic lines**

The human skeletal  $\alpha$ -actin promoter promotes high levels of transgene expression in skeletal muscles. Therefore skeletal muscles (a mixture of type 1 and 2 muscle fibres dissected from the thigh region of hind limbs), in addition to the diaphragm and heart from twelve age-matched transgenic lines were analysed for the expression of the utrophin transgene by Western blot analysis (Section 2.2.8). The tissues were homogenised in 1 ml Newcastle lysis buffer for protein analysis. 50  $\mu$ g of total protein extract from each tissue was loaded onto identical 6% polyacrylamide gels. One gel was stained with Coomassie blue and the other transferred to nitrocellulose membrane and stained with utrophin specific antibody, MANCHO 7.

No. of Tg. lines	Tg-Founders	Names	Germ-line Transmission	Expression	Muscle	Heart	Dia.
1	12	George	Yes	Yes	+++(+)	+	++
2	13	Gareth	Yes	Yes	++	-	++
3	15	Gail	Yes	Yes	+++	-	++
4	33	Gemma	Yes	Yes	++	+	+
5	40	Gene	Yes	Yes	++	-	+
6	44	Gavin	Yes	Yes	++++	-	+++
7	45	Graham	Yes	No	-	-	-
8	52	Gordon	Yes	Yes	++	+(+)	+
9	87	Grant	Yes	Yes	++	++	++
10	120	Greg	Yes	Yes	++	-	+
11	163	Gill	Yes	Yes	++	-	+
12	181	Gerald	Yes	Yes	++	-	++

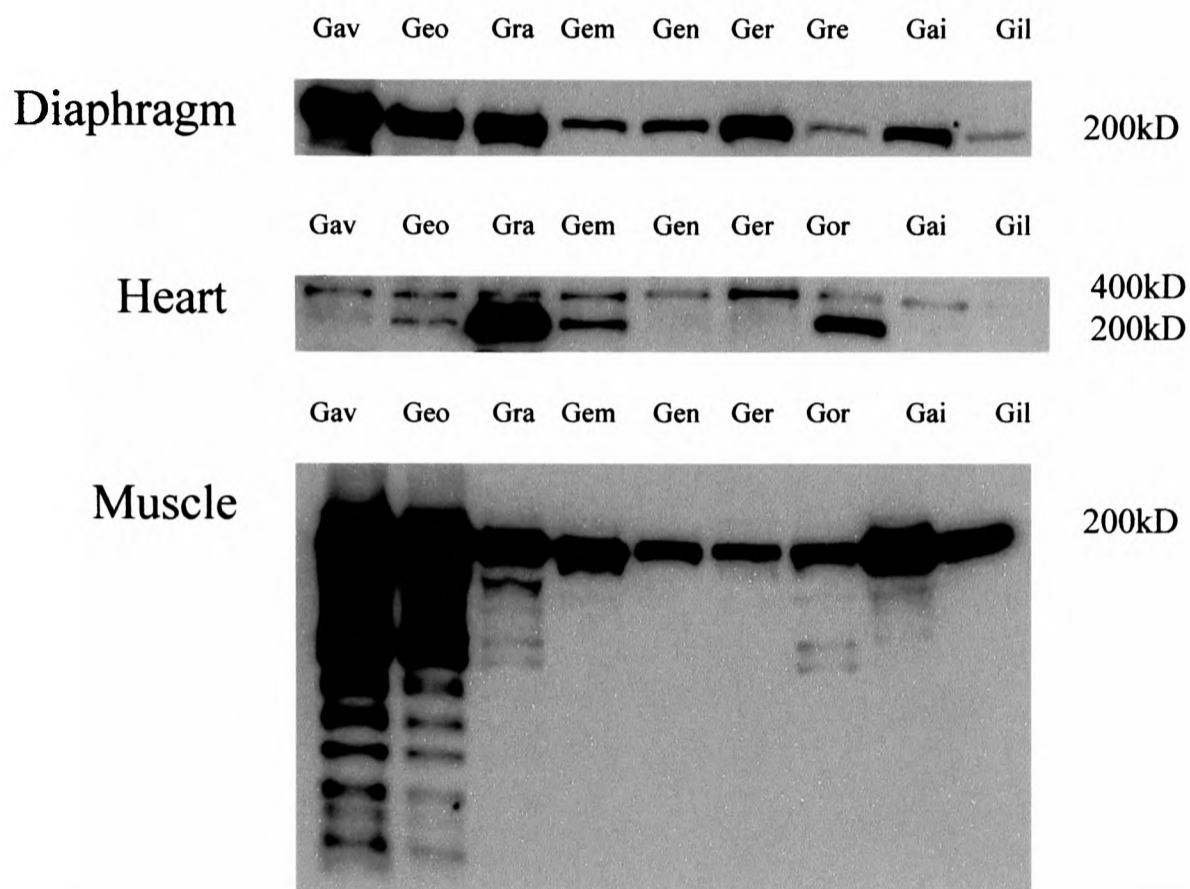
Table 2 Utrophin transgene expression pattern

Expression pattern of 12 HSA-truncated utrophin-*mdx* transgenic lines generated. The founders were bred with *mdx* partners and the offspring were analysed by Southern technique (Section 2.2.6). Tissues were taken from positive offspring for protein analysis by the Western blot technique.

Key: Tg, transgenic; + or ++ indicate approximate comparative levels of transgene expression by western assay; (+), slightly lower level than +; -, no expression.

A 200-kDa-transgene band was expected in the Western blotting. Figure 3 shows different lines expressed different levels of the transgene with Gavin and George lines expressing the highest levels in muscle and diaphragm among the eleven expressing lines. The multiple faint bands of George and Gavin muscles probably resulted from the proteolytic breakdown of the highly expressed transgene product (Cox et al., 1993). It was postulated that the binding sites for utrophin protein at the DPC were saturated in the very high expressing lines and that those unbound protein diffused into the cytoplasm and degraded eventually. This may provide an explanation for the degradation products observed.

The Grant line expressed the highest level in heart and the Gordon, Gemma and George lines expressed at a lower level. In the immunoblot of the heart, the upper bands were the endogenous utrophin bands (400 kDa) and the lower bands were those of the transgene (200kDa). Figure 4 shows the Coomassie-stained-gel as a control for loading accuracy. The approximately equally stained bands in the diaphragm, muscle and heart showed that equal amounts of the total protein extracts had been loaded. Figure 5a shows the expression of utrophin in TA, EDL, soleus and diaphragm in George and Gill lines, which were used as the high and low expressing lines in the mechanical force experiment. The EDL and Diaphragm muscles were used in the mechanical force measurement experiments. The difference between the levels of the transgene expression in EDL and diaphragm of George and Gill lines were more marked than that of TA. Figure 5b and c show the expression of the transgene in the TA and soleus muscles of the Gavin, Gordon, Gill, George, Gerald and Grant lines. The highest expressing lines of Gavin and George showed high levels of the transgene in TA (type 2 fast-twitch muscle) and soleus (type 1 slow-twitch muscle) which



**Figure 3 Expression of the truncated transgene in nine transgenic lines**

The truncated transgene gave a band at 200 kDa in the immunoblot of diaphragm, heart and skeletal muscle of the transgenic lines generated. The multiple faint bands of Gavin, George, Graham, Gordon and Gail muscles probably resulted from the proteolytic breakdown of the highly expressed transgene product (Cox et al., 1993).

Key: kD, kilo-Dalton; Gavin (Gav); George (Geo); Graham (Gra); Gemma (Gem); Gene (Gen); Gerald (Ger); Gail (Gai); Gill (Gil); Gordon (Gor).

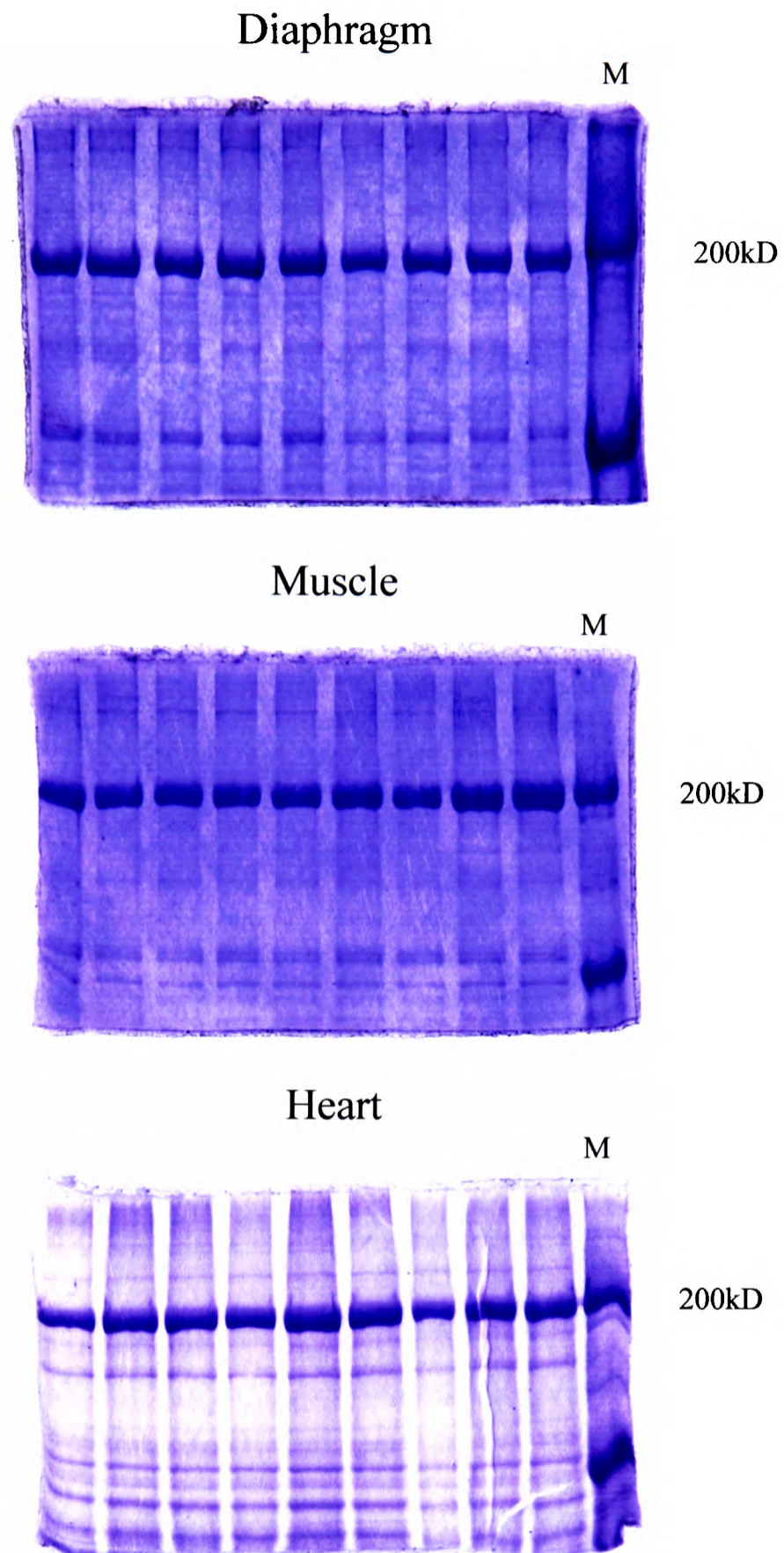
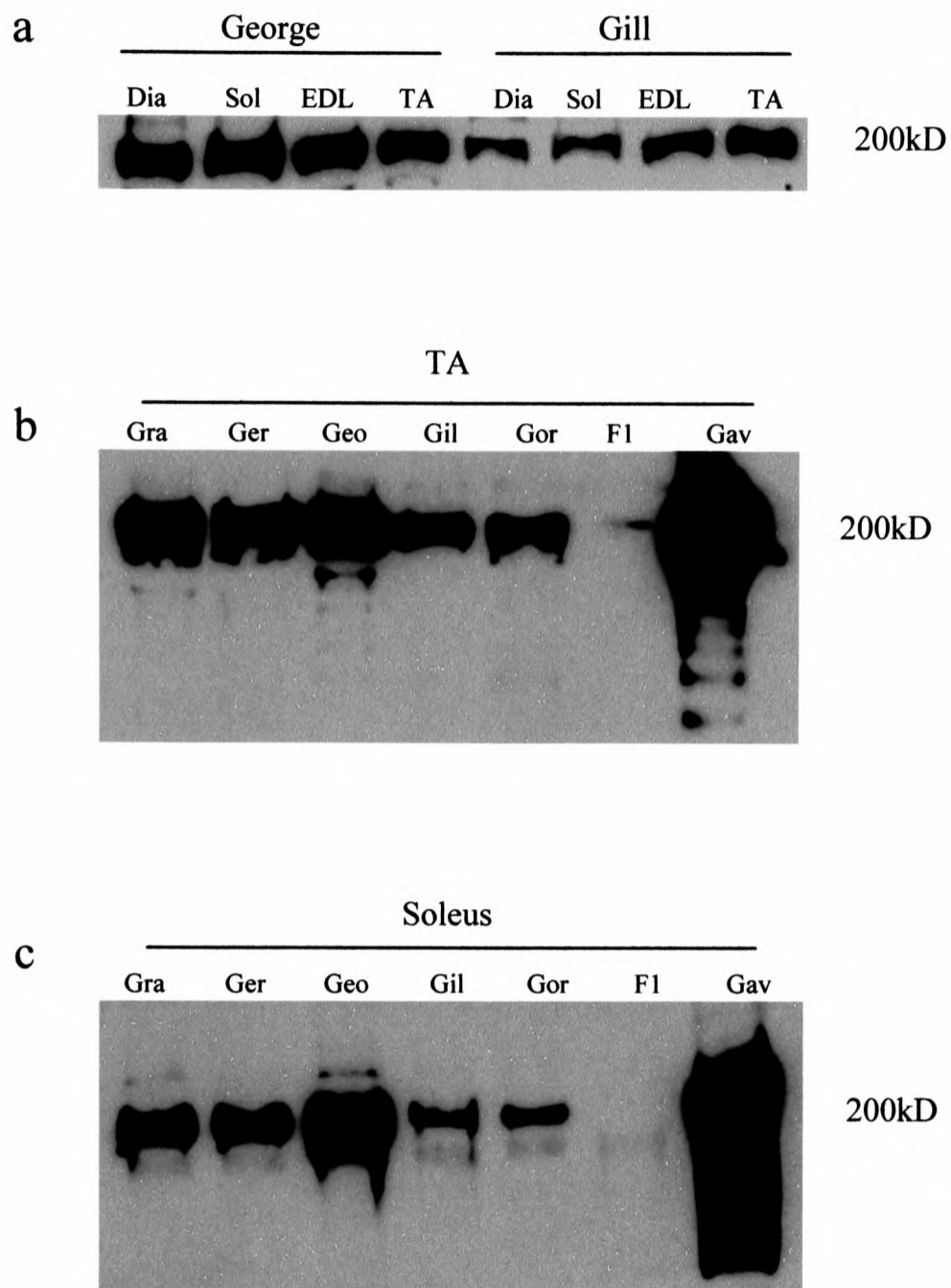


Figure 4 Coomassie-blue-stained gel

Coomassie-blue-stained 6% SDS-polyacrylamide gel of diaphragm, skeletal muscle and heart of the transgenic lines shown in Fig. 3 as a control for loading accuracy. The Coomassie-blue-stained bands look approximately equal.

Key: M, protein rainbow marker.



**Figure 5** Utrophin transgene expression in the high and low expressing lines.

a, Immunoblot of diaphragm (Dia), soleus (Sol), extensor digitorum longus (EDL) and Tibialis anterior (TA) showing the high expressing line, George, compared to the low expressing line, Gill.

b and c, Immunoblot of TA and soleus of Gavin (Gav), F1 (normal control), Gordon (Gor), Gill (Gil), George (Geo), Gerald (Ger), Grant (Gra) lines. The transgene bands were compared to that of the normal mice.

The multiple faint bands probably resulted from the proteolytic breakdown of the highly expressed transgene product.

agreed with the whole muscle (mixture of type 1 and 2) expression level shown in Figure 3. Considering the amount of the transgene expressed, the George, Gerald and Gordon lines were grouped together as the high expressing lines and the Gill line as the low expressing line for the mechanical force and calcium homeostasis experiments which will be discussed later on in this chapter. Further analysis showed that there was no evidence of the expression of the utrophin transgene in other non-muscle tissue like brain, kidney, lung, liver, intestine, skin or pancreas (data not shown).

### **3.2.4 Immunohistochemistry**

Using antibodies specific to utrophin (G3) and dystrophin (P6) as shown in Figure 6, the utrophin transgene localised to the sarcolemma of the TA just like dystrophin, demonstrating that utrophin could localise to the sarcolemma in the absence of dystrophin. However, some of the utrophin transgene was localised to the cytoplasm as a diffused signal (Figure 7). The immunostaining of the DPC components in TA showed that  $\beta$ 1-syntrophin, dystroglycan (DG) and  $\alpha$ -sarcoglycan re-localised to the sarcolemma and the intensities appeared brighter compared to normal controls (Figure 7). This suggested that the amount of DPC components at the sarcolemma was limited to some degree by the amount of dystrophin or utrophin present in the sarcolemma of myofibres. This phenomenon was similar to that observed in dystrophin-*mdx* transgenic mice (Cox et al., 1993).

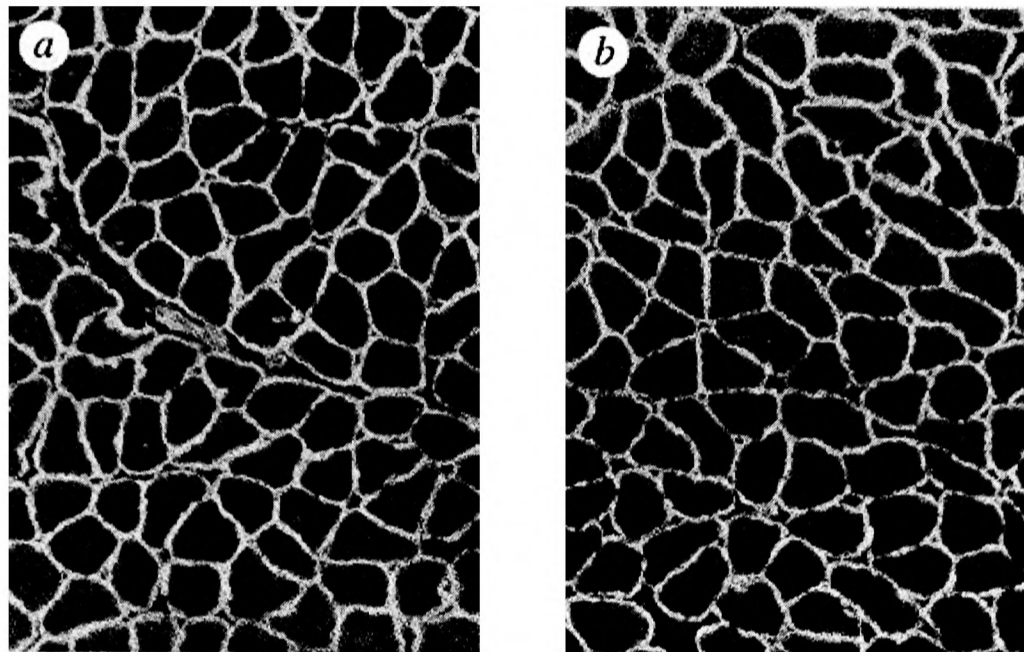


Figure 6 HSA-truncated utrophin transgene expression in TA

Immunohistochemistry of TA showing dystrophin in normal wild type (a) and utrophin in low expresser, F-3 line (b) localised to the sarcolemma using specific antibodies.

Cryosections of littermate control (a) and utrophin-*mdx* transgenic (b) stained with dystrophin, P6 at 1/400 (Sherratt et al., 1992) and utrophin, G3 at 1/25 dilutions. The results show that utrophin localised to the sarcolemma of the muscle in the absence of dystrophin (b). The photos were taken with a Leica microscope at the same exposure. Magnification, x100. From (Tinsley et al., 1996).

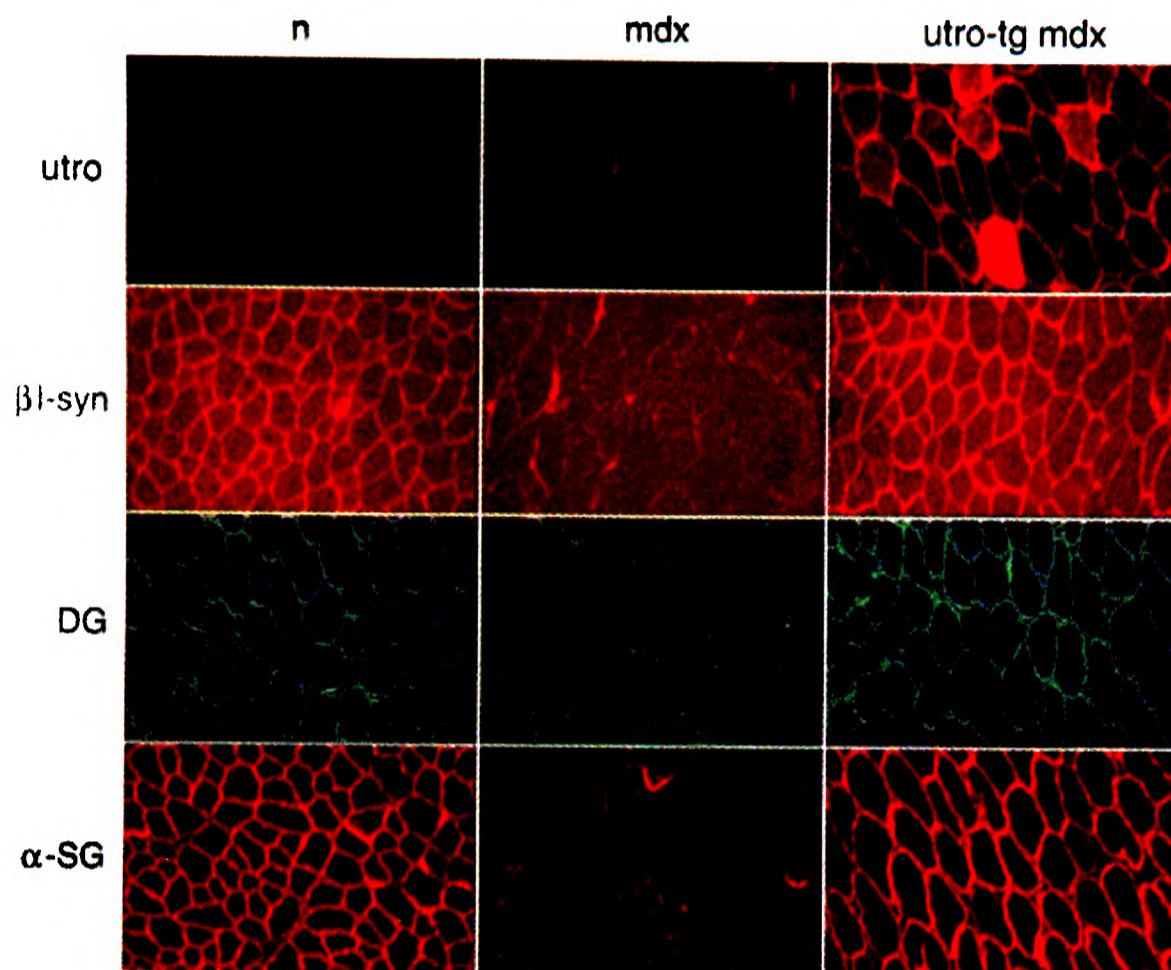


Figure 7 Immunostaining of the dystrophin-associated protein complex (DPC) components.

Immunohistochemistry of utrophin and components of the DPC in TA muscle of George transgenic-*mdx*, (*utro-tg mdx*), *mdx* and normal (n) mice using specific antibodies to utrophin (utro),  $\beta$ 1-syntrophin ( $\beta$ 1-syn),  $\alpha/\beta$ -dystroglycan and  $\alpha$ -sarcoglycan ( $\alpha$ -SG). Increased staining in DPC at the sarcolemma of transgenic-*mdx* mice was observed. Magnification, x100. From (Tinsley et al., 1996).

### **3.2.5 Haematoxylin and eosin staining**

The diaphragm of normal, *mdx* and transgenic mice was stained with haematoxylin and eosin for comparative morphological studies. The diaphragm in the transgenic mice appeared essentially normal (data not shown).

### **3.2.6 Creatine kinase and percentage of centrally nucleated fibres**

Creatine kinase (CK) levels are raised in DMD patients and the *mdx* mouse due to proteolytic breakdown of muscle proteins and the percentage of centrally nucleated fibres (CNF) is increased because of muscle degeneration and regeneration (Bulfield et al., 1984; Sicinski et al., 1989; Emery, 1993). In order to establish whether the utrophin minigene had improved the dystrophic morphology in the muscle of the *mdx* mice, blood sera of age-matched mice, four to five weeks old were measured from normal, *mdx* and utrophin-*mdx* transgenic mice for creatine kinase levels (see Section 2.2.9). The rate was averaged over four minutes and calculated as units per litre (UL<sup>-1</sup>). Figure 8 shows that the recovery score (explained in the next section) of creatine kinase levels of the high expressing lines returned to near normal levels indicating that muscle breakdown had decreased.

The number of centrally nucleated fibres was counted in individually dissected TA, soleus and diaphragm muscles and showed a marked decrease of muscle regeneration. The percentage of centrally nucleated fibres was markedly decreased in pooled data of TA, soleus and diaphragm muscles as shown in Figure 8 with a recovery score of 85%.

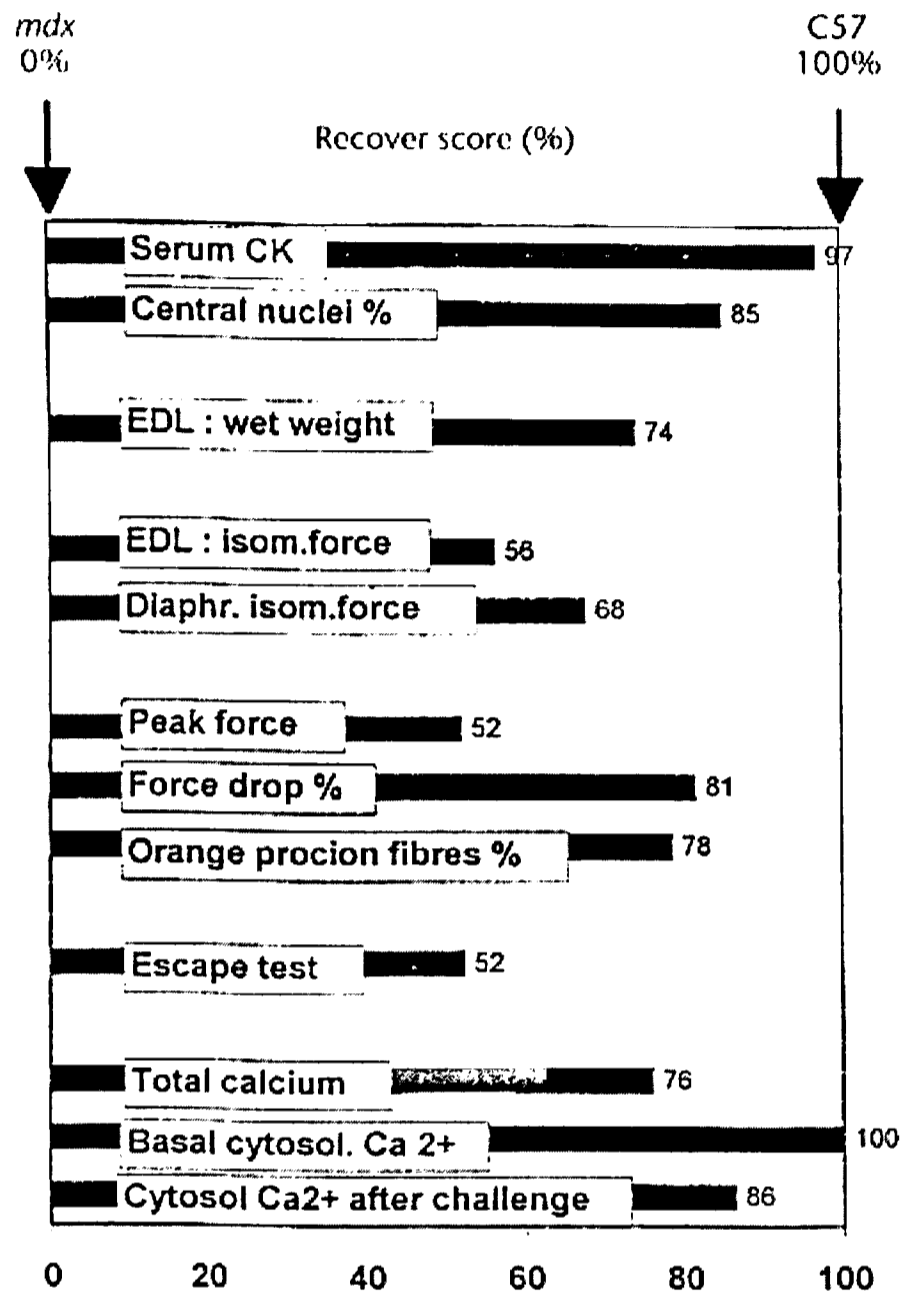


Figure 8 Recovery scores

Summary of the results obtained by Dr Jean-Marie Gillis and colleagues.

The recovery score is defined as  $(Utr++ - mdx) / (C57 - mdx) \times 100$  was calculated for each of the parameters studied to measure the severity of the dystrophic phenotype. Where an animal scores a 100% it would be performing equivalent to a normal wild type control and 0% to a *mdx* mouse.

There were some degrees of functional recovery in all the tests. Eccentric contractions i.e. resistance to stretch (% force drop) and the subsequent damage to muscle fibres (% orange procion fibres), total calcium content and calcium homeostasis, serum CK levels and the % CNF (the recovery score was calculated from an average value of pooled data of TA, EDL and diaphragm in George, Gerald and Gordon lines), the *Utr++* recovery scores reached 75-85% or more. The isometric force was less affected with a recovery score of about 50%.

From (Tinsley et al., 1996).

### 3.2.7 Mechanical performance and calcium homeostasis

DMD patients are wheel chair bound by the age of 12 due to progressive skeletal muscle degeneration (Emery, 1993). Although the *mdx* mouse does not seem to suffer to the same extent physically, the mechanical forces of EDL and diaphragm muscles and regulation of calcium homeostasis are reduced compared to normal mice (Gillis, 1996; Deconinck et al., 1997b). Experiments on mechanical force and calcium homeostasis were carried out to see the effect of the transgene on the mechanical performance of the utrophin-*mdx* transgenic mice.

Dr Jean-Marie Gillis and colleagues in Belgium did the mechanical performance and calcium homeostasis experiments. The results are summarised here for completeness in assessing the efficiency of the truncated utrophin minigene transgenic experiments. The transgenic mice used were divided into high expressing lines, Utr<sup>++</sup>, consisting of the George, Gerald and Gordon lines and low expressing lines, Utr<sup>+</sup>, consisting of the Gill line basing on similar characteristics e.g. (a) the levels of creatine kinase, (b) percentage of centrally nucleated fibres in TA and diaphragm, (c) protein levels and (d) intensities of immunostaining.

To summarise the benefits of the truncated utrophin minigene in the *mdx* mouse, a recovery score was tabulated as shown in Figure 8. A quantitative evaluation of recovery of normal characteristics can be obtained by comparing the difference between Utr<sup>++</sup> and *mdx* muscles, to the difference between normal C57 and *mdx* muscles. The recovery score is defined as  $(Utr^{++} - mdx) / (C57 - mdx) \times 100$ , such that 100% recovery score indicates that the animal performs identically to a normal

wild-type control and 0% indicates that the animals performs identically to a *mdx* mouse.

### 3.2.7.1 Maximal isometric force

The absolute isometric force production in the young *mdx* mouse was normal but the *mdx* mouse muscles were larger (hypertrophy) than normal mice muscles (Coulton et al., 1988). When the muscle size was accounted for, the *mdx* mice were weaker than normal controls. The normalised force of the *mdx* EDL muscle was approximately seventy percent of normal values (Deconinck et al., 1997).

Hypertrophy in the *mdx* EDL muscles was reduced significantly by the utrophin minigene in utrophin-*mdx* transgenic mice, which reached a recovery score of 74%. The normalised isometric force of both the EDL and diaphragm muscles was improved significantly, with the recovery scores reaching 56% and 68% respectively. The recovery scores of diaphragm for the low (Utr+) and high (Utr++) utrophin expressing lines were 37% and 68% respectively (data not shown). Phelps and colleagues expressed their results of the diaphragm in specific force (KN/m<sup>2</sup>) for their mini-dystrophin transgenic mice. Since the value of the normalised isometric forces for diaphragm of C57 and *mdx* mice were practically the same in both studies, for comparison, the specific force of the diaphragm of the high and low mini-dystrophin expressing lines were converted to recovery scores using the formula, (dystrophin - *mdx*)/(C57 - *mdx*) x 100, that was used here. If the values of normalised isometric forces of diaphragm of the mini-dystrophin transgenics (Table 1, column 7) of low, 0.2 times the normal dystrophin content, (0.2X(U)) and high, 0.9 times (0.9X(U)) expressing lines were calculated as recovery scores, values of 60% and 77% were obtained respectively. These values were very similar to the values obtained for the

mini-utrophin transgenic mice here. Therefore, both mini-dystrophin and mini-utrophin transgenes were able to improve the mechanical performance of the *mdx* mice.

### **3.2.7.2 Force drop during repetitive eccentric contractions**

The *mdx* muscles are particularly susceptible to forced lengthening contractions leading to muscle necrosis, a procedure known as “eccentric contraction” (Weller et al., 1990). This results in a steady drop of forces as such contractions are repeated (Moens et al., 1993; Petrof et al., 1993). After the EDL muscle was submitted to repetitive stimulations, muscle injury was assessed by monitoring the force signal after each contraction. Membrane damage was evaluated by bathing the muscle in orange procion after the forced lengthening experiments, a dye that does not penetrate healthy, intact muscle membrane and subsequent analysis by light microscopy. Histological analysis of the EDL muscle demonstrated that force drop was associated with muscle damage. The primary function of dystrophin has been postulated to provide mechanical reinforcement to muscle membrane and protect it from mechanical stresses (Ohlendieck and Campbell, 1991; Petrof et al., 1993; Pasternak et al., 1995; Gillis, 1996). Therefore, the resistance to eccentric contractions is a critical test for evaluating the success of a potential therapy in dystrophin-deficient muscle (Deconinck et al., 1996). The recovery scores of the percentage force drop and the percentage orange procion fibres of the mini-utrophin transgenic mice reached 81% and 78% respectively.

### **3.2.7.3 Muscle activity of the whole mouse**

The tension generated by the whole *mdx* mouse was significantly reduced during burst of activity compared to normal mice (Carlson and Markiejus, 1990). The whole body

tension (WBT), which is the overall force exerted by the mice when provoked to escape, was measured by a non-invasive procedure in response to gentle pinching of the tail. The transgenic mice reached a recovery score of 52% showing that the transgenic mice developed escape forces significantly higher than the *mdx* mice did.

#### **3.2.7.4 Calcium content and calcium homeostasis**

An increase of total calcium content in DMD and *mdx* muscles has been reported (Bodensteiner and Engel, 1978; Gailly et al., 1993). This was seen as a loss of intracellular calcium homeostasis. However, there are conflicting reports of the direct measurement of intracellular calcium ion concentration which was thought to be due to increased leak channel activity (Turner et al., 1991; Gailly et al., 1993; Head, 1993; Pressmar et al., 1994). Nevertheless, there was an increased permeability of the *mdx* myotubes to calcium ions under specific stress conditions (Leijendekker et al., 1996). When these fibres are challenged by the combination of increased external calcium ion concentration and a hypo-osmotic shock, this results in a rise of cytosolic calcium ion concentration of about two folds, showing that calcium homeostasis is definitely impaired in the *mdx* muscle fibres. Here, the effect of over-expression of utrophin minigene had upon the total calcium content and intracellular calcium homeostasis was examined.

##### **3.2.7.4.1 Total calcium content**

The recovery score of the total calcium content in gastrocnemius muscle of mini-utrophin transgenic mice was 76% as measured by atomic adsorption flame photometry.

#### 3.2.7.4.2 Challenge of $[Ca^{2+}]_i$ homeostasis

The *mdx* muscle had a consistently raised ratio of the intracellular calcium ion concentration  $[Ca^{2+}]_i$ . Therefore, the  $[Ca^{2+}]_i$  was measured before and after hyposmotic-hypercalcic challenge where results were expressed as the ratio of fura-2 fluorescence excited at 340 and 380 nm as reported previously (Turner et al., 1991; Gailly et al., 1993; Gillis, 1996). The flexor digitorum brevis (FDB) muscle of mini-utrophin transgenic mice had a recovery score of 86% of the cytosolic calcium content after challenge. Thus the presence of utrophin minigene restored the normal behaviour of the muscle membrane to maintain low values of intracellular calcium ion concentration. These data show that utrophin minigene was able to restore the *mdx* muscle property and maintain intracellular calcium homeostasis.

### 3.3 Discussion

The data presented here demonstrate that a truncated utrophin minigene under the control of the HSA-promoter localised to the sarcolemma of the skeletal muscle fibres when expressed in the *mdx* mouse. This specific localisation in the place of dystrophin significantly improved the dystrophic muscle phenotype, improved mechanical performances and maintained intracellular calcium homeostasis.

Twelve transgenic mouse lines were generated and expressed different levels of the transgene. They showed high levels of expression of the transgene in skeletal muscle and diaphragm. The Grant and Gordon lines also showed high levels of expression of the transgene in the heart. The different levels of utrophin transgene expressed here were similar to those obtained by Phelps and Wells in their Becker dystrophin minigene transgenic mice experiments (Phelps et al., 1995; Wells et al., 1995). They

too obtained different expression levels of the mini-dystrophin transgene in different transgenic lines. The reason for the different expression levels of the utrophin transgene in different lines could be due to the site of integration of the transgene into the chromosomes. If it integrated near an enhancer that could promote the expression of the transgene, as in the case of the George and Gavin lines, high expression levels of the transgene were obtained. And if the transgene integrated near a suppresser or silencer, low levels of the transgene were expressed in the transgenic mice, for example, the Gill line and the Graham line that did not express the transgene at all. It is not clear what factor controls tissue transgene expression and what controls the expression levels. The HSA promoter promoted the expression of the utrophin transgene in the skeletal muscle, but it was observed that TA, EDL and soleus muscle expressed different levels of utrophin transgene in different transgenic lines. This pattern of expression was observed throughout this thesis work.

Utrophin was able to localise to the sarcolemma correctly in the absence of dystrophin. In normal mice, utrophin is localised to the neuromuscular and myotendinous junctions. The utrophin minigene was also found to replace dystrophin functionally in the following tests. The serum creatine kinase level returned to near normal levels and the percentage of centrally nucleated fibres was significantly reduced, with recovery scores of 97% and 85% respectively, indicating that there was a marked decrease in muscle degeneration. These results were similar to those obtained by Phelps and Wells in their high mini-dystrophin expressing lines (Phelps et al., 1995; Wells et al., 1995).

Additional utrophin staining was observed in the cytoplasm and may have been due to saturation of membrane-binding sites. Similar observations for dystrophin were

recorded by Cox and colleagues (1993). This might imply that all the binding sites for utrophin at the sarcolemma were saturated in the very high expressing lines, George and Gavin, and the unbound utrophin diffused into the sarcoplasm. The reason why the expression level of the transgene varied from fibre to fibre in the same TA bundle was not well understood. Cox and colleagues (1993) also observed this mosaic expression pattern in their high expressing lines. It was established that uniformly expressed dystrophin transgene was better at rescuing the dystrophic morphology at a lower level than a non-uniformly expressed transgene at a high level. This mosaic utrophin expression pattern was observed here in two very high expressing lines only, Gavin and George. Both these lines contained the truncated utrophin minigene, discussed in this thesis. The cellular and molecular mechanisms regulating the expression of utrophin in normal fibres are not determined yet. In this case, possibly post-transcriptional regulatory mechanisms might be involved in the regulation of the utrophin transgene in individually over-expressed muscle fibre; some fibres might be more efficient in degrading the over-expressed transgene. However, this seemed to make no difference to the distribution of DPC components.

Over-expression of the utrophin minigene was also able to re-establish the DPC to the sarcolemma. Loss of dystrophin results in varying degrees of loss of the DPC (Campbell, 1995). It was vital that the transgene was able to re-establish the DPC to the sarcolemma as in the case of dystrophin-*mdx* transgenic mice to restore muscle strength (Cox et al., 1993; Rafael et al., 1994; Phelps et al., 1995; Wells et al., 1995). The experimental results here show that there was notable increased staining of  $\beta$ 1-syntrophin,  $\alpha/\beta$ -dystroglycan,  $\alpha$ - and  $\gamma$ -sarcoglycans at the sarcolemma of TA and

diaphragm of utrophin-*mdx* transgenic mice compared to the *mdx* controls, indicating an elevation of sarcolemmal DPC components.

As diaphragm was the most severely affected muscle in the *mdx* mouse, haematoxylin and eosin stained diaphragm sections of the utrophin-*mdx* transgenic mice were analysed. They showed great morphological improvement. There were greatly reduced areas of fibrosis, cellular infiltrations and centrally nucleated fibres and appeared essentially as normal. These results were comparable to those obtained by Phelps and Wells expressing mini-dystrophin transgene at similar levels in the *mdx* mice (Phelps et al., 1995; Wells et al., 1995).

The data here demonstrate importantly that the expression of a utrophin minigene leads to significant functional improvements in the *mdx* mouse muscle (Deconinck et al., 1997c). The normalised force of both the EDL and diaphragm muscles was increased significantly, reaching a recovery scores of 56% and 68% respectively for the transgenic mice. The low expressing line, Utr<sup>+</sup> showed significant increase in normalised force but less so than that of Utr<sup>++</sup>, thus showing that mechanical improvement depends on the amount of the transgene expression. The *mdx* muscles are particularly susceptible to forced lengthening contractions, a procedure known as “eccentric contraction”, resulting in a steady drop of forces as such contractions are repeated (Weller et al., 1990; Moens et al., 1993; Petrof et al., 1993). The resistance to eccentric contractions was a critical test for evaluating the success of potential therapy in the treatment of dystrophin-deficient muscle (Deconinck et al., 1996). The force drop of EDL muscle was significantly improved (81%) in the utrophin transgenic mice compared to that of the *mdx* mouse. The fibres were minimally damaged compared to normal controls as shown by the orange procion staining. The utrophin

transgenic mice generated higher escape forces, which reached a recovery score of 52%. Thus, we show here that the over-expression of utrophin improves the mechanical performances by (a) the generation of higher mechanical stress, (b) the lowering of percentage of force drop, (c) the increase of peak force and (d) the generation of higher whole-body tension escape force. All these mechanical improvements could be classed as “functional” recovery and they were in the region of 50-80%. How much extra force is needed to produce a significant improvement in life quality? Is 50% enough to alter the severe DMD phenotype to the mild BMD phenotype? If the whole-body tension escape force (50%) is considered as sufficient, then truncated utrophin minigene may be considered as a candidate for gene therapy.

The total calcium content in gastrocnemius muscle of Utr<sup>++</sup> mice decreased to approximately similar level of normal controls but that of Utr<sup>+</sup> approximated to that of the *mdx* mice (Data not shown). Here it is shown that the higher transgene levels conferred greater benefit in maintaining total calcium content. However, this was difficult to interpret as why there was an increase in the calcium content in DMD and *mdx* muscle is not well understood (Haws and Lansman, 1991; McArdle et al., 1994). The intracellular calcium ion concentration was measured before and after hyposmotic-hypercalcic challenge. This challenge revealed the poorer regulation of the *mdx* muscle as compared to normal and utrophin transgenic muscle. The ability of the utrophin transgenic muscles to regulate calcium ion concentration was as good as normal. Here the data showed that the Utr<sup>++</sup> fibres were able to handle all aspect of calcium regulation: total calcium content, basal intracellular calcium ion concentration level and intracellular calcium ion concentration reaction to hyposmotic-hypercalcic challenge, the average values obtained were not significantly different from that of the normal preparations. All these improvement described here,

including those of serum creatine kinase and %CNF could be classed as “structural” recovery and they were in the region of 80-100%.

Therefore the over-expression of a truncated utrophin should prevent the initiation of the muscle degenerative process as a loss of calcium homeostasis has been implicated in activating calcium-dependent proteolysis in dystrophin-deficient muscle (Turner et al., 1988; Franco and Lansman, 1990; Turner et al., 1993).

### 3.4 Conclusion

In conclusion, the utrophin minigene was able to localise to the sarcolemma of muscle fibres in the absence of dystrophin and re-establish the DPC to the sarcolemma resulting in a significant improvement of the dystrophic phenotype and partial restoration of muscle strength in the *mdx* mouse similar to those results obtained by Phelps and Cox for the truncated mini-dystrophin experiments. The high utrophin expressing lines had a greater degree of “structural” recovery (80-100%) than “functional” recovery (50-80%). This experiment showed that high level of truncated utrophin expression was better at inducing “structural” recovery than “functional” recovery. Could the missing rod-domains confer better recovery both “structurally” and “functionally”? (See next chapter). These experiments did not tell us that utrophin equals dystrophin but nevertheless it showed that utrophin was able to rescue the *mdx* phenotype as well as truncated dystrophin. These data show that a truncated utrophin minigene expressed at a high enough level was sufficient to improve the mechanical performance and maintain the intracellular calcium homeostasis. Thus, the utrophin minigene functioned very well in the *mdx* mouse. Possibly full-length utrophin is better but a smaller utrophin transgene would be easier to manipulate.

These results suggest strongly that utrophin can replace dystrophin and this has implications for the use of utrophin in gene therapy for DMD patients.

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## Chapter 4

# Generation of mice transgenic for full-length utrophin

### 4.1 Introduction

It was demonstrated that a truncated utrophin minigene driven by the human skeletal  $\alpha$ -actin (HSA) promoter improved partially the *mdx* phenotype in chapter 3. In this chapter, the hypothesis was to test whether full-length utrophin under the control of the human skeletal  $\alpha$ -actin promoter resulted in complete recovery of mechanical functions and prevented the development of muscular dystrophy in the *mdx* mouse and to determine the levels needed to effect full recovery measured.

### 4.2 Results

The experiment in this chapter was carried out in collaboration with Dr. Jon Tinsley, Dr. Nicolas Deconinck, Dr. David Kahn, Mr Steve Phelps and Dr. Jean-Marie Gillis. I carried out the generation of the transgenic mice, transgenic breeding, taking of tissues for various analyses at the appropriate times. I also carried out the protein analysis and immunostaining. I was involved in all the analysis at various times but did not do all of them. The mechanical performance experiments were carried out in

the laboratory of Dr. Jean-Marie Gillis in Belgium using age-matched (3.5 to 4 months old) mice sent from our laboratory.

#### **4.2.1 Transgene construct**

The full-length utrophin transgene used in this experiment was constructed by Dr. Jon Tinsley and Mr. Steve Phelps in the laboratory. The method was described in Tinsley et al. (1998). Briefly, the truncated utrophin transgene was linearised at a single HpaI restriction endonuclease site and the remainder of the rod domain was ligated in-frame to produce the full-length utrophin cDNA. The complete transgene was then cloned into an expression vector containing the 2.2-kb human skeletal  $\alpha$ -actin promoter (Brennan and Hardeman, 1993), regulatory regions and the SV40 large T poly-A site (Tinsley et al., 1996). The transgene was located downstream of the first human skeletal  $\alpha$ -actin untranslated exon, and flanking KpnI/NotI restriction endonuclease sites were used to liberate the complete expression fragment which was purified and resuspended in double distilled water at 2-3 ng/ $\mu$ l final concentration for microinjection.

The full-length utrophin transgene was a human-mouse hybrid, giving a predicted protein product size of approximately 400 kDa. Figure 1 shows the schematic representation of the full-length utrophin transgene compared to the truncated minigene previously described in chapter 3. Both constructs were human-mouse hybrids. The human full-length utrophin transgene was unstable during construction. Mouse utrophin shares 84% identity at the nucleotide level and 87% identity at the amino acid level with human utrophin. Sequences at the N-terminus and C-terminus

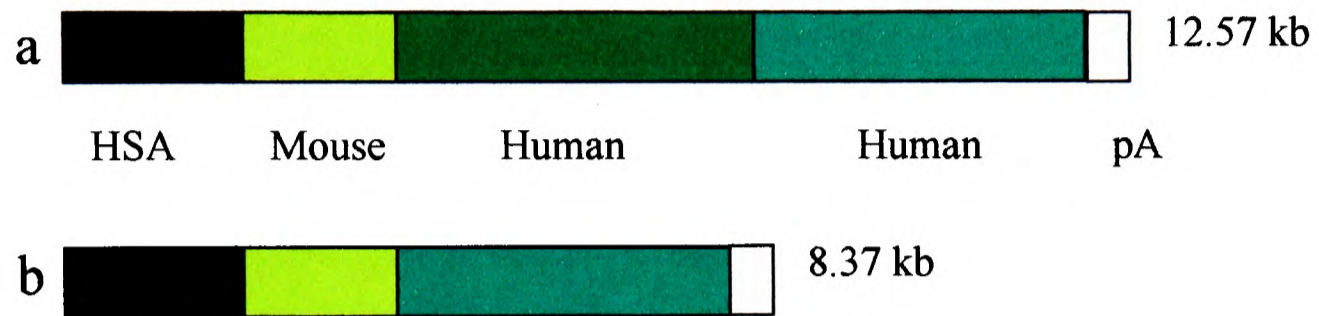


Figure 1 Schematic representation of the cDNA constructs

a, The HSA-full-length utrophin cDNA used for microinjection. It was approximately 12.57 kb. b, The HSA-truncated minigene (mini-G) used in the previous experiment (chapter 3) was approximately 8.37 kb. The diagram is not to scale.

are particularly well conserved between the two species with 92% and 97% identities for the amino acids (Guo et al., 1996). Therefore the full-length human-mouse hybrid utrophin cDNA was predicted to work in this experiment

#### **4.2.2 Generation of full-length utrophin transgenic mice**

The purified transgene was microinjected into the pronucleus of F2 hybrid oocytes from (C57BL/6J x CBA/CA) parents (Section 2.2.2). Positive transgenics were identified by Southern blotting (Section 2.2.6). Four transgenic founders were generated giving a transgenesis rate of five percent, which was rather low, compared to the truncated utrophin transgenesis rate of thirteen percent. This could be due to dirty cDNA containing some bacterial plasmid killing off some of the injected embryos. Table 1 shows the record of new-borns of the full-length (column F) compared to the truncated minigene (column G) derived from microinjected embryos. 396 injected embryos were transferred, giving a successful implantation rate of 27%. 78% of new-borns survived up to 3-4 weeks old. All three lines, Fiona, Fergie and Freddie were fertile. Fritz line was sub-fertile and the new-borns tended to die before weaning age. The reasons were not known. This could be due to the integration site of the transgene which caused premature death in the new-borns. As a result this line was rejected. All the lines gave germ line transmission. Table 2 shows the transgene expression levels in skeletal muscle of the four lines generated. A mixture of Type 1 and 2 muscle fibres from the thigh region of the hind limbs was taken. Fiona expressed the highest level of the transgene, followed by Fergie, Fritz and Freddie in the skeletal muscle tissues as shown in Figure 2. The founders were crossed with dystrophin-deficient *mdx* partners to derive offspring on *mdx* background for analysis. Age-matched mice of 10-12 weeks were analysed.

	G	F
No. of microinjected embryos transferred...	448	396
No. of pups born.....	147	105
% of successful implantation.....	33%	27%
No. of pups born.....	147	105
No. of pups survived up to 3 - 4 weeks.....	93	82
% of survival up to 3 - 4 weeks.....	63%	78%
No. of transgenic lines.....	12	4
% Transgenesis.....	13%	5%

Table 1 Record of potential transgenic founders

Record of pups born from microinjected embryos after oviduct transfer. F column: Successful implantation of the injected embryos was 27%. The percentage of survival of the pups born up to 3-4 weeks was 78%. The percentage transgenesis was approximately 5% compared to that of the truncated minigene of about 13% (G column).

The percentage transgenesis rate was calculated from No. of transgenic lines/No. of pups of 3-4 weeks x 100. (4/82 x 100). This was because only the tails of pups that survived up to 3-4 weeks were genotyped.

Key: G, the truncated mini-G series; F, the HSA-full-length utrophin series.

No. Tg. lines	Tg. Founders	Names	Germ-line transmission	Expression	Muscle
1	1119	Freddie	Yes	Yes	+
2	1122	Fergie	Yes	Yes	+++
3	1147	Fiona	Yes	Yes	++++
4	1171	Fritz	Yes	Yes	++

Table 2 Utrophin transgene expression pattern

Expression pattern of the 4 full-length utrophin transgenic lines generated. Fiona line had the highest expression level in muscle, followed by the Fergie, Fritz and Freddie lines. The Fritz line was difficult to breed. All gave germ line transmission. Muscle was a mixture of Type 1 and 2 skeletal muscle from the thigh region of the hind limbs.

Key: No., number; Tg., transgenic; +, level of protein as judged visually. The more +, the higher the level of transgene expression.

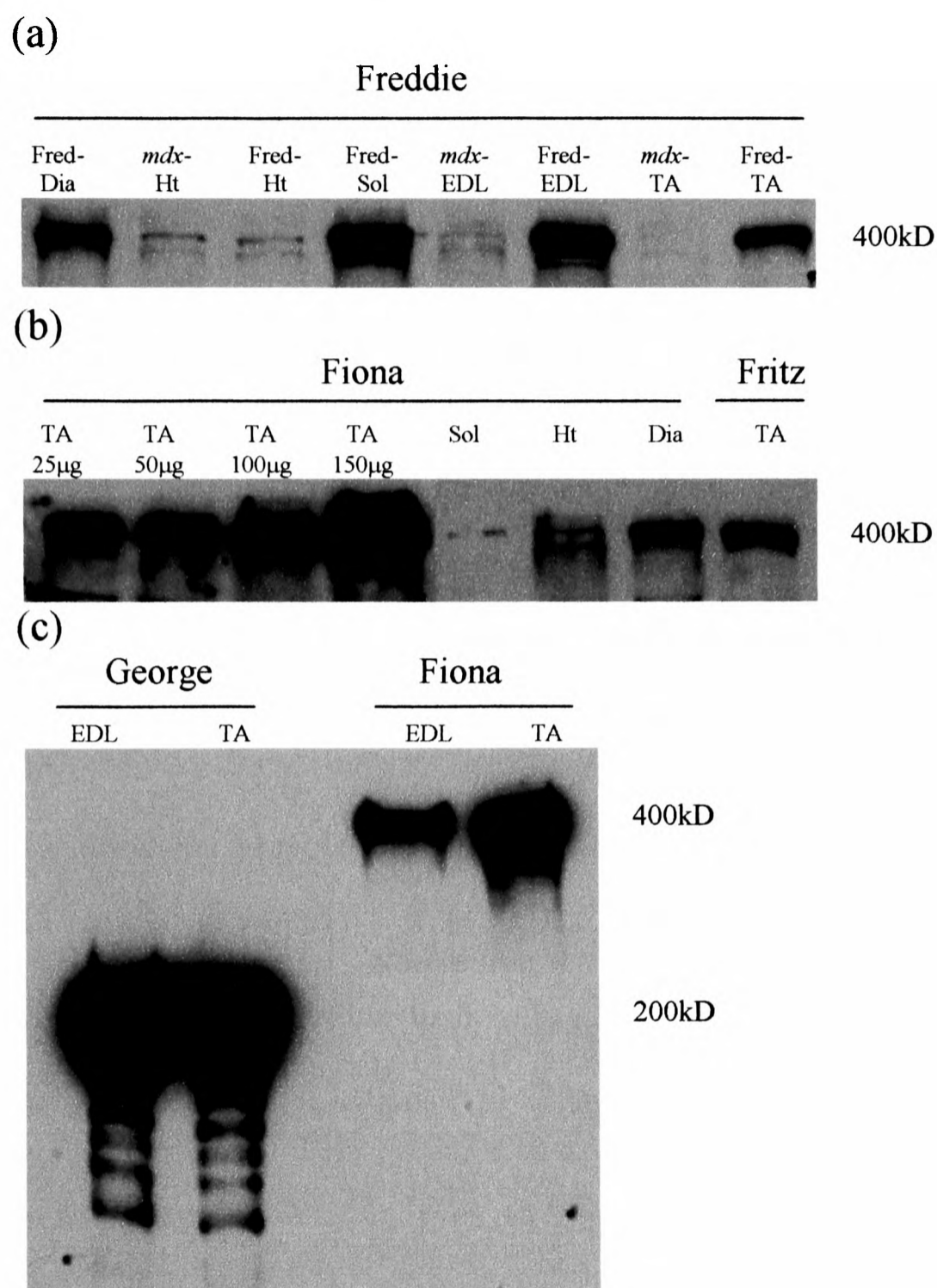


Figure 2 Expression pattern of utrophin in the muscle of Fiona, Freddie, Fritz and George lines

(a), The expression pattern of the utrophin transgene in TA, EDL and heart of Freddie line compared to that of the *mdx* mice. (b) Serial dilution of Fiona TA protein extracts to check the accuracy of the loading. Fiona TA showed the highest level of expression of the transgene compared to Fritz and Freddie (a). (c) The expression pattern of the TA and EDL of Fiona line compared to that of the George line of the truncated mini-G transgenic mice.

Protein extract loaded was 50µg except when specified otherwise. The results were repeated for the purpose of publication as shown in Figure 3.

The multiple faint bands probably resulted from the proteolytic breakdown of the highly expressed transgene product.

Key: Fred, Freddie; Dia diaphragm; Ht., heart.

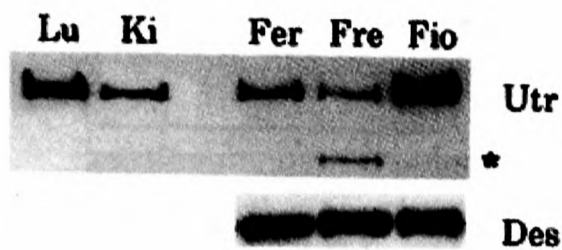
### **4.2.3 Western analysis of the transgenic lines**

The muscles chosen to be analysed were a group decision. Tibialis anterior (TA), extensor digitorum longus (EDL) and diaphragm were dissected from transgenic-*mdx*, normal and *mdx* mice. In addition, lung and kidney were collected from normal F1 controls for comparative study. They were homogenised in Newcastle extraction buffer and analysed for the expression of utrophin by the Western blot technique (Section 2.2.8). Desmin was used to control for equal loading of the total protein extract. Figure 3a-c shows the transgene expression levels of the Fiona, Fergie and Freddie lines in TA, EDL and diaphragm compared to normal kidney and lung which expressed high level of endogenous utrophin (Love et al., 1991). Fiona line expressed the highest level of the transgene in TA, EDL and diaphragm. Freddie line expressed the least. The transgene expression levels of the three lines were similar to or less than those of a normal utrophin promoter in kidney and lung (Figure 1a-c).

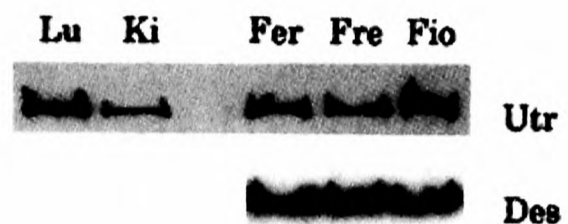
### **4.2.4 Immunohistochemistry**

The TA cryosections of Fiona, Fergie and Freddie were stained with utrophin and  $\alpha$ -dystrobrevin specific antibodies (Section 2.2.12). The results of utrophin staining of TA muscle correlated with the western blot results, showing increasing sarcolemmal staining of the lines from Freddie to Fergie to Fiona which was the highest expressing line as shown in Figure 3d. There was no intracellular staining of utrophin transgene observed in the Fiona line as for the minigene transgenic lines of George and Gavin. For successful amelioration of the dystrophic phenotype in muscle, re-establishment

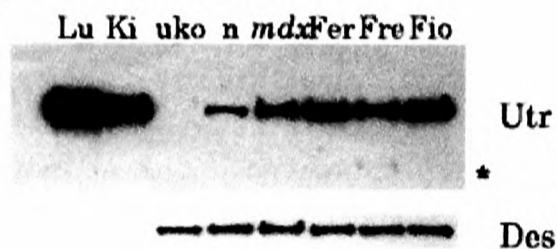
(a) TA



(b) EDL



(c) Diaphragm



(d) TA

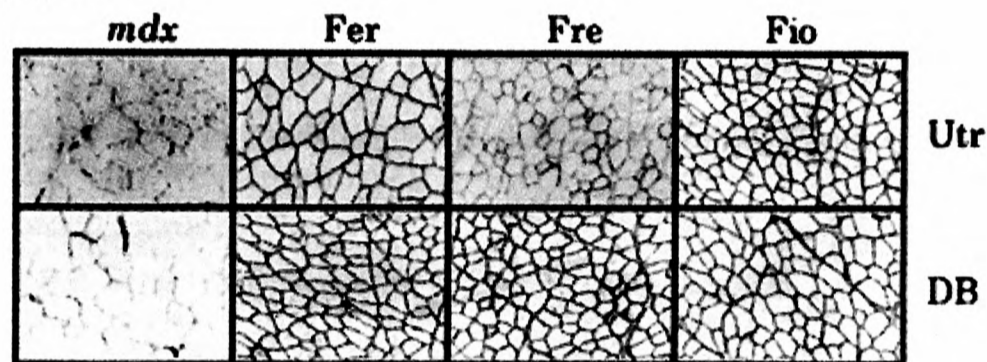


Figure 3 Expression pattern of full-length utrophin transgene and immunocytochemistry of cryosections of TA muscle.

a-c, Western blot of transgene total muscle extracts from Tibialis anterior (TA; a), extensor digitorum longus (EDL; b) and diaphragm (c) and two control tissues, lung (lu) and kidney (ki), which expressed high levels of endogenous utrophin. In c, diaphragm from a utrophin-deficient mouse (uko) was included to confirm the specificity of the utrophin antibody (Deconinck et al., 1997a). Asterisks denote probable proteolytic breakdown product of Freddie samples. Desmin (Des) staining was used to control for equal loading.

d, Immunocytochemistry of cryosections of TA muscle from *mdx* and Fergie (Fer), Freddie (Fre) and Fiona (Fio) lines, stained with utrophin (Utr) and a-dystrobrevin (DB). Exposure times were the same to allow for comparison of the intensity of staining of the samples. The image has been electronically reversed for easier interpretation. From (Tinsley et al., 1998).

of the dystrophin-associated protein complex is an important criterion, as failure to re-establish the sarcoglycans results in limb girdle muscular dystrophies (Straub and Campbell, 1997). Immunostaining of TA sections with  $\alpha$ -dystrobrevin, a cytoplasmic DPC component, showed that it had re-established to the sarcolemma in all the three lines compared to the *mdx* mice (Figure 3d) (Suzuki et al., 1995; Sadoulet-Puccio et al., 1997; Blake et al., 1998).

#### **4.2.5 Number of centrally nucleated fibres (CNF)**

To determine whether the transgene had decreased the rate of muscle degeneration and regeneration, the number of centrally nucleated fibres (CNF) were counted in EDL and diaphragm. Newly regenerated muscle fibres have centrally located nuclei, while normal fibres have peripheral nuclei. The sections were stained with haematoxylin and eosin (Section 2.2.12) and the number of centrally nucleated fibres counted in EDL and diaphragm and calculated as a percentage compared to normal controls. The recovery score (discussed in the following section) of the percentage of centrally nucleated fibres (%CNF) in the EDL and diaphragm muscles of the Fiona line was 100% and that of the Freddie line reached 100% in EDL and 70% in diaphragm as shown in Figure 4. This differential reduction of centrally nucleated myofibres correlated the transgene expression pattern in the high and low expressing lines studied, showing evidence of reduced regeneration depended on the amount of the utrophin transgene expressed. The %CNF recovery scores of EDL and diaphragm of the minigene transgenic mice were approximately 87%.

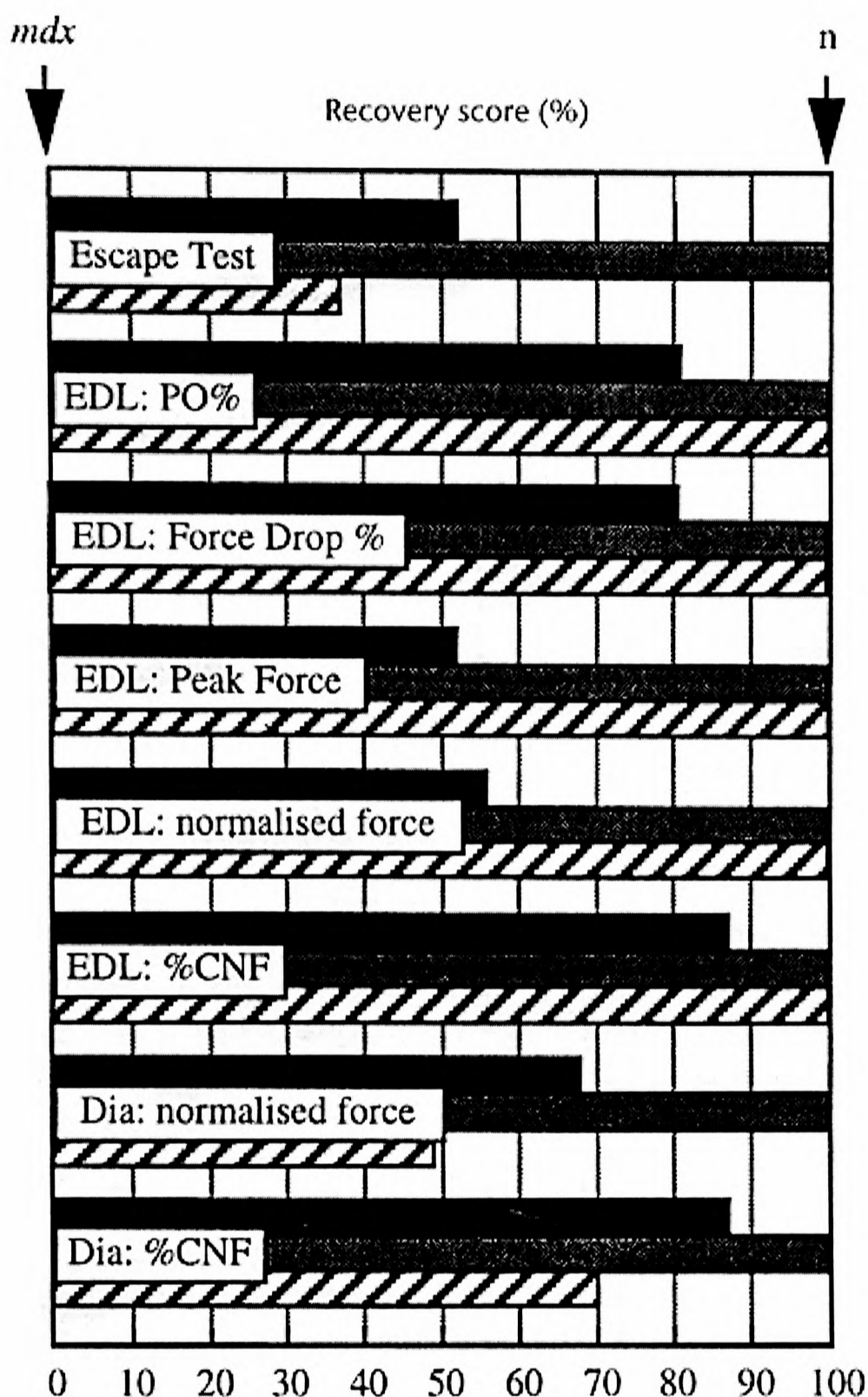


Figure 4 The comparative recovery scores of the truncated and full-length utrophin transgenic mice.

Recovery scores obtained from the high expressing lines (Utr++) of the truncated utrophin minigene compared to that of the full-length utrophin transgenic lines of Fiona and Freddie. The recovery scores of Fiona in all parameters tested reached 100%. The utrophin minigene did not ameliorate the *mdx* phenotype as well as the full-length utrophin transgene.

Key: ■ Truncated minigene, Utr++, ■ Fiona, ▨ Freddie. From (Tinsley et al., 1998).

#### 4.2.6 Mechanical performances

Dr Jean-Marie Gillis and colleagues carried out the mechanical performance experiments but the results are summarised here for completeness. The mechanical performance of muscle from Fiona, the high expressing line and Freddie, the low expressing line, was assessed and the damage to the membrane was studied. The muscles were isolated under anaesthesia which preserved the circulation (Deconinck et al., 1996; Tinsley et al., 1998).

To summarise the benefits of the full-length utrophin transgene in the *mdx* mouse, the recovery score of Fiona and Freddie lines was calculated and compared with that of the high expressing lines (Utr++) of the truncated utrophin minigene transgenic mice described in the last chapter. A quantitative evaluation of recovery of normal characteristics was obtained by comparing the difference between utrophin transgenic and *mdx* muscles, to the difference between normal C57 and *mdx* muscles as in the previous chapter. The recovery score is defined as  $(Utr - mdx) / (C57 - mdx) \times 100$  in percentage, such that 100% or 0% recovery scores indicate the performances identical to normal wild-type or the *mdx* mice. Figure 4 shows the comparative recovery scores of the truncated and full-length utrophin transgenic mice.

##### 4.2.6.1 Normalised force

The normalised force (the force per unit cross sectional area in milliNewton/millimetre square) was measured in isolated muscle preparations of diaphragm and EDL from the full-length utrophin-*mdx* transgenic, normal and *mdx* mice. Fiona and Freddie lines had normal force generation in EDL muscle with a recovery score of 100% while that of the mini-utrophin transgene reached 56%. The

normalised force of Fiona diaphragm reached 100% while that of Freddie was approximately similar to that of the minigene transgenic mice showing that a higher level of the transgene was needed to restore normalised force in diaphragm.

#### **4.2.6.2 Force drop in eccentric contractions**

Dystrophin-deficient muscles are susceptible to the drop in force during a series of repetitive eccentric contractions (Weller et al., 1990; Moens et al., 1993; Petrof et al., 1993; Deconinck et al., 1996). Isolated EDL muscle from normal, *mdx* and utrophin-*mdx* transgenic mice was forcibly stretched while it contracted. The percentage force drop in the EDL of Fiona and Freddie lines was 100% showing that the full-length utrophin transgene protected the muscle from repetitive contractions. The percentage force drop of minigene transgenic mice was approximately 82%. Both Fiona and Freddie lines developed 100% peak force, that was the highest value of tension developed during the imposed stretch, at the first tetanus in EDL muscle compared to that of the minigene transgenic mice of 52%.

#### **4.2.6.3 Membrane damage**

There was a direct correlation between eccentric contraction and membrane damage in normal and the *mdx* mice (Moens et al., 1993). To assess the damage in EDL muscle after forced eccentric contractions, the muscle was stained with orange procion which is a dye that does not penetrate intact membrane. The recovery scores of %CNF of EDL of both Fiona and Freddie lines was 100% while that of the minigene lines was approximately 80%. These results correlated well with the %CNF recovery. These results showed that full-length utrophin was more efficient in the protection of the muscle from repetitive contractions.

#### 4.2.6.4 Whole body tension

To determine the beneficial therapeutic benefits of the full-length utrophin transgene had on the muscles of the whole animal, the whole body tension (WBT) test was carried out in Fiona, Freddie, normal and *mdx* mice. This was a non-invasive test. The recovery score of the Fiona line was 100%. While that of Freddie line and minigene transgenic mice was approximately 35% and 52% respectively. This conflicting data could be due to the fact that Freddie line was analysed at the age of 2 months (to finish force analysis for publication quicker), instead of 4 months. This WBT test showed that younger transgenic-*mdx* mice exerted less total force at younger age. High level of full-length utrophin transgene had conferred a greater therapeutic value by reducing the stress and fatigue in the Fiona mice after several minutes of testing.

#### 4.2.6.5 Summary of results

The comparative results of the recovery scores of truncated and full-length utrophin experiments are summarised in Table 3. High level of full-length utrophin transgene gave complete recovery of the *mdx* phenotype while low level gave very variable rescue. Full-length utrophin was better than truncated in both structural and functional recovery. This shows that the rod domains of the full-length utrophin played an important role in the complete rescue of the dystrophic phenotype, both structurally and functionally. This is shown by the Freddie line which expressed low level of the utrophin transgene and yet obtained a significant recovery scores of between 70-100%.

	Truncated Utr <sup>++</sup>	Fiona	Freddie
“Functional” recovery	50-80%	100%	40-100%
“Structural” recovery	80-100%	100%	70-100%

Table 3 Summary of results of truncated and full-length utrophin experiments.

### 4.3 Discussion

The data presented here show that high level of full-length utrophin resulted in complete recovery of mechanical functions and prevented the development of muscular dystrophy in utrophin-*mdx* transgenic mice. Previous experiments (chapter 3) and the present ones showed that prevention of muscle necrosis depended on the amount of the utrophin transgene expressed at the sarcolemma of the muscle fibres. For all the parameters tested, these include the “functional” tests of escape test, percentage force drop and peak force of EDL, normalised force of EDL and diaphragm, and the “structural” tests of percentage procion orange, percentage of CNF in EDL and diaphragm muscle, obtained 100% recovery score in the Fiona line. Even the low expressing line Freddie, obtained “structural” recovery scores of between 70-100% which was comparable to the high expressing lines of the truncated utrophin minigene. This shows that only a small amount of full-length utrophin is needed to be up-regulated to do certain jobs like normal wild type mice (Figure 4).

The minigene transgenic mice obtained a “functional” recovery of 50-80% and “structural” recovery of 80-100%. These data show that the longer rod domain of the full-length utrophin transgene, that is 14 spectrin-like repeats more than the truncated minigene, which contains only 8 repeats, and is important in conferring full functional recovery in the *mdx* mice. Therefore, the full-length utrophin is needed to confer complete recovery, both structurally and functionally. This shows that an intact muscle sarcolemma is vital to maintain the intracellular calcium homeostasis and only intact muscle can provide 100% mechanical strength. Therefore, the rod domain of the full-length utrophin contributes to both “structural” and “functional” recovery in the Fiona transgenic-*mdx* mice. The rod domain might act as cushion to buffer the

shock of mechanical stresses and thus preserved sarcolemmal integrity. Low level of full-length utrophin (40-100%) was comparable to high level of truncated utrophin (50-80%) in effecting “functional” recovery. Thus, full-length utrophin transgene would be more suitable than the truncated transgene to be considered in DMD gene therapy.

The *mdx* diaphragm reproduces the degenerative changes of DMD and therefore is a good model to study the beneficial effect of the full-length utrophin transgene (Stedman et al., 1991). The western blots of Fiona and Freddie diaphragm samples were carefully quantified and compared the results with normal and *mdx* utrophin endogenous levels of lung and kidney (Figure 1c). The Fiona line expressed twice as much utrophin as the *mdx* diaphragm for complete recovery. However, this level was only 50% and 25% of the normal physiological utrophin expression levels seen in kidney and lung respectively (Figure 1c). Thus, in muscles, utrophin expression needs to be up-regulated to the normal endogenous levels found naturally in kidney and lung to prevent muscular dystrophy and restore mechanical performance. This means that the up-regulation of utrophin need not be higher than the physiological level naturally found in some tissues and this may avoid toxicity problems. The level of utrophin in Freddie diaphragm (low expressing line) was only slightly higher than that found in *mdx* diaphragm and yet all the recovery scores were substantially improved.

Most of the over-expression of utrophin in the *mdx* diaphragm and muscles is from the regenerating fibres which, when fully regenerated and developed decreased in their sarcolemmal staining. It is not fully understood at which stage dystrophin-deficient muscles start to degenerate. If it is at the stage when the sarcolemmal

utrophin starts to decrease, then it might be possible to prevent it by drug up-regulation of utrophin and thus slows or even halts the degeneration of the muscles.

The functional effects of the transgene would be expected to persist over time basing on the assumption that since the %CNF of the Fiona line at age 14 months was found to be the same as 3 months (5%) and 12% at 22 months (refer to Figure 8 in the next chapter). Other tests were not done for older transgenic-*mdx* mice.

#### **4.4 Conclusion**

The data from this experiment shows that expression of full-length utrophin is better than truncated utrophin in preventing the development of muscular dystrophy and results in complete recovery of mechanical functions in the *mdx* mouse when expressed at high level. The utrophin levels required in muscle were significantly less than the endogenous levels expressed in kidney and lung, and it was shown that the degree of “structural” and “functional” improvement in muscle depended on the amount of utrophin expression levels and that full-length was better than truncated. These data have important implications in the gene therapy of Duchenne muscular dystrophy. In the next chapter, it will be demonstrated whether the expression of utrophin ubiquitously is toxic in the *mdx* mouse.

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## Chapter 5

# Non-toxic ubiquitous over-expression of utrophin in the *mdx* mouse

### 5.1 Introduction

In the previous chapters, it was demonstrated that the continual presence of utrophin at the muscle fibre membrane prevents the development of muscular dystrophy in the HSA-truncated and HSA-full-length transgenic-*mdx* mice. If the utrophin level was high enough, the mechanical properties and the intracellular calcium homeostasis in the dystrophin-deficient *mdx* mouse muscle were completely normal showing that utrophin was able to replace dystrophin.

The strategy of replacing dystrophin with utrophin has the advantage of avoiding the immune problems surrounding gene delivery to muscle. Since DMD patients already have a functional copy of the utrophin gene, it has been proposed that the use of small molecules (screening for suitable small molecules are being carried out in the laboratory at the moment) capable of up-regulating utrophin expression, is a viable alternative approach to the therapy for DMD (Tinsley and Davies, 1993). There are two problems involving the use of a small molecule to up-regulate a protein for

therapy. Firstly, the toxicity of the small molecule unrelated to utrophin expression, for example, general toxicity plus effects on other gene promoters. It is important that the small molecule only acts on the utrophin promoter and promotes the expression of utrophin. Secondly, the toxicity of ectopic utrophin expression in tissues other than the target tissues. In the case of utrophin, the protein is expressed in all tissues but to a varying degree. It was therefore important to carry out experiments to study whether the over-expression of utrophin in a broad range of tissues was toxic to the *mdx* mouse, before embarking on a large-scale drug screen. The following experiments were carried out to study the toxicity effect in the *mdx* mouse when utrophin was ubiquitously over-expressed by a transgenic approach.

## **5.2 Results**

### **5.2.1 Transgene Construct**

In order to over-express utrophin in muscle and non-muscle tissues of the *mdx* mouse a truncated utrophin minigene (mini-A) driven by the SV40 promoter was constructed by Dr. Jon Tinsley modelled on the Becker dystrophin minigene previously described in chapter 3. The SV40 promoter was chosen because it had been shown to promote strong ubiquitous expression of transgenes but unfortunately we did not get expression in the ten transgenic lines generated (Brondyk, 1994). The results are summarised in Table 1 and 2, and Figure 1. Subsequently, a full-length utrophin cDNA driven by the human ubiquitin C promoter was kindly constructed by Dr. Jon Tinsley and Mr. Steve Phelps (Schorpp et al., 1996). The ubiquitin C promoter was chosen because it seemed to be a strong ubiquitous promoter driving the expression of the transgene in most tissues, both muscle and non-muscle tissues, compared to other

Transgene constructs.....	G	F	A	C
No. of microinjected embryos transferred...	448	396	695	423
No. of pups born.....	147	105	218	118
% of successful implantation.....	33%	27%	31%	28%
No. of pups survived up to 3 - 4 weeks.....	93	82	147	108
% of survival up to 3 - 4 weeks.....	63%	78%	67%	92%
No. of transgenic lines.....	12	4	10	11
% Transgenesis.....	13%	5%	7%	10%

Table 1 Record of potential transgenic founders.

Column A shows the pups born from microinjected embryos after oviduct transfer of the truncated SV40-utrophin mini-A and column C that of the ubiquitin C full-length utrophin transgene.

Successful implantation rate of the ubiquitin C utrophin embryos was 28%, approximately the same as that of the HSA-full-length (column F) utrophin transgenic mice. The percentage transgenesis rate of ubiquitin C utrophin was 10% and that of the G series was higher, 13%.

Key: No., number; G, truncated minigene-G; F, HSA-full-length; A, SV40 minigene-A; C, ubiquitin C full-length utrophin transgenic mice.

No. of Tg. lines	Tg. founder numbers	Names	Germ-line Transmission	Expression
1	5	Adam	Yes	No
2	73	Anthony	Yes	No
3	81	Anita	Yes	No
4	105	Arthur	Yes	No
5	154	Alice	Yes	No
6	182	Alfred	Yes	No
7	183	Albert	Yes	No
8	202	Alec	Yes	No
9	203	Andy	Yes	No
10	219	Alan	Yes	No

Table 2 Expression pattern of SV40 utrophin mini-A transgenic mice

All the ten transgenic lines generated did not express the transgene. They were sacrificed.

Key: No., number; Tg., transgenic.

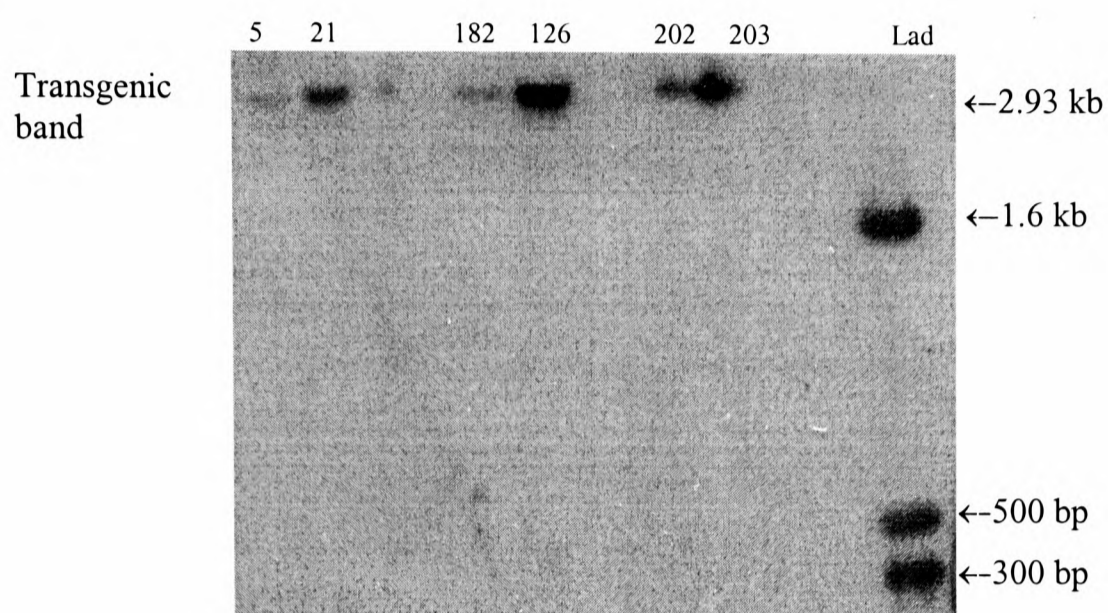


Figure 1 Southern results of the SV40 utrophin mini-A potential transgenic founders

Mice numbers 5, 182, 202 and 203 were the founders of the Adam, Alfred, Alec and Andy lines respectively. Mice 21 and 126 were false-positives; subsequent Southern tested negative. The 2.93-kb band was the band detected by the radiolabelled PCR 4.0 probe, which hybridised with the transgene.

Key: Lad, the 1-kb DNA ladder.

promoters (Schorpp et al., 1996). The full-length utrophin cDNA was identical to that previously described in chapter 4 in preventing muscular dystrophy in the *mdx* mouse. Briefly, the complete utrophin transgene was cloned into an expression vector containing the 1.2-kb human ubiquitin C promoter and regulatory regions (Schorpp et al., 1996). Flanking NotI / Asp718I restriction endonuclease sites were used to liberate the complete fragment for microinjection. The cDNA was purified and diluted in double distilled water with a concentration of 2-3 ng/ $\mu$ l for microinjection. This cDNA was a human-mouse hybrid. The human full-length cDNA was unstable during construction.

### **5.2.2 Generation of transgenic mice**

The purified transgene was microinjected into one pronucleus of F2 (C57BL/6 x CBA/CA) zygotes (Section 2.2.2). Eleven transgenic founders were generated. Table 1 shows the record of pups born from microinjected embryos. The number of microinjected embryos transferred were 423 and about 28% implanted successfully. The full-length utrophin transgene had a lower successful implantation rate compared to the truncated utrophin mini-G of 33%. The survival rate up to weaning age was very high, 92% compared to that obtained for the truncated and full-length transgenic mice. The percentage transgenesis rate was approximately 10%. Table 3 shows the expression pattern of the eleven lines generated. Nine founders were fertile and transmitted the transgene in a Mendelian fashion. Two lines, Cliff and Carole, did not transmit the transgene; they might have been mosaics, that is, only some of the germ cells contain the transgene. The Clark line was sub-fertile.

No. of Tg. lines	Tg.- founder numbers	Names	Germ-line Transmission	Expression
1	1253	Charlie	Yes	yes
2	1264	Colin	Yes	yes
3	1278	Cleo	Yes	yes
4	1279	Chelsea	Yes	yes
5	1345	Christine	Yes	yes
6	1374	Curtis	Yes	yes
7	1375	Clark	Yes	yes
8	1398	Conrad	Yes	yes
9	1400	Calvin	Yes	yes
10	1248	Cliff	No	ND
11	1371	Carole	No	ND

Table 3 Expression of full-length utrophin transgene in the 9 transgenic lines generated.

Expression pattern of the nine lines generated. 9 out of 11 lines expressed the transgene and gave germ-line transmission. The numbers were the numbers of the mice given when tailing. Cliff and Carole lines were sacrificed after about three litters of babies tested negatives.

Key: Tg, transgenic; No., number; ND, not done.

### **5.2.3 Heterozygote breeding**

The transgenic lines were established and maintained by breeding with *mdx* partners. The offspring produced were heterozygous for the transgene on the *mdx* background and used for analysis. Normal F1 (C57BL/6 x CBA/CA) of the same genetic background as the microinjected embryos was always used as normal wild type controls. The transgenic mice did not have any overt phenotype.

### **5.2.4 Homozygote breeding to increase the transgene levels**

It was hoped that by producing homozygous Cleo that the transgene levels would double. Therefore, heterozygous mice (Cleo) from the Cleo line, which had the highest transgene expression levels in most tissues analysed, were bred to homozygosity (HCleo) on the *mdx* background. Homozygous offspring were identified by western blotting using tail protein extracts, which showed double-intensity bands compared to that of the heterozygotes (data not shown). These suspected potential homozygotes were then bred with normal F1 non-transgenic mice to do a genetic assay for homozygosity. The first two litters born to each potential homozygote with pup numbers ranging from thirteen to fourteen in total were sacrificed and screened for transgenesis by PCR with assistance from Mrs Sarah Squire and Miss Liz Townsend. All the pups obtained from the cross between possible homozygotes and non-transgenic F1 mice were positive for the transgene. Therefore the possible homozygotes for the utrophin transgene were true homozygotes because they transmitted to all their offspring. Since at least thirteen offspring per homozygous parent tested positive it could be confident at the 0.0001 level that they were indeed homozygotes. There was a probability of 1/8192 that a heterozygous animal will produce thirteen positive offspring and no negative

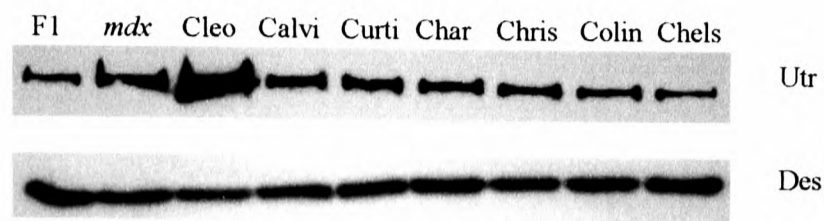
offspring. The HCleo line looked like normal wild type mice and was fertile. The females were good mothers.

### **5.2.5 Analysis of transgenic lines**

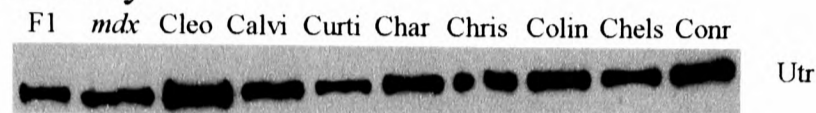
Initially, eight tissues: muscle, heart, diaphragm, lung, liver, intestine, brain and kidney were collected from each of the nine transgenic lines between four to six weeks old to screen for the expression of the transgene by western blot analysis. Desmin antibody staining was used to control for equal loading of the samples onto the gels. Desmin is an intermediate filament protein and is found in all tissues (Lodish et al., 1995). As an example of transgene expression, Figure 2 shows the utrophin transgene levels from several ubiquitin C utrophin transgenic lines compared to normal F1 and *mdx* derived from heart, kidney, muscle, diaphragm and brain tissues. The pattern of expression of the nine lines generated was summarised in Table 4. The differential expression of the transgene in the different lines was probably due to the different sites of integration of the transgene into the chromosomes. The Cleo line consistently showed the highest level of expression of the transgene in most tissues and was chosen for the transgene toxicity study. Some lines expressed at very low levels in most tissues, for example the Charlie and Curtis lines (Table 4).

Finally, twenty to twenty four tissues were collected from three to six age-matched (3 to 3.5 months old, after recovery from the initial degenerative foci by regeneration in the dystrophin-deficient muscle) mice from each group of the normal F1, *mdx*, Cleo and HCleo for detail analysis of H&E histology, including central nucleation count at three months old, immunohistochemistry and urine test. These tissues were, TA, EDL, heart, lung, liver, diaphragm, intestine (small and large), brain, trachea, eye, kidney,

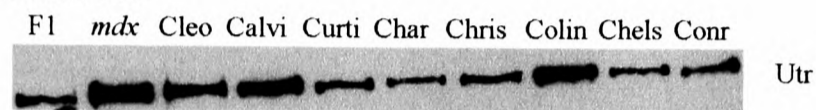
## Heart



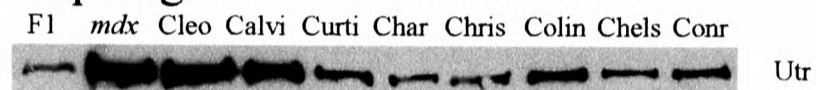
## Kidney



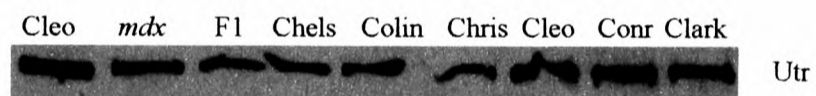
## Muscle



## Diaphragm



## Brain



**Figure 2** Western blot analysis of the full-length ubiquitin C utrophin transgenic lines generated

Showing the protein levels of the heart, kidney, muscle, diaphragm and brain of the lines generated. Cleo had the highest level of expression of the transgene in heart, kidney, diaphragm and brain. Desmin staining was used to control equal loading of the total protein extracts (data of kidney, muscle, diaphragm and brain not shown).

Key: F1, normal control mice; Calvi, Calvin; Curti, Curtis; Char, Charlie; Chris, Christine; Chels, Chelsea; Contr, Conrad; Utr, utrophin; Des, desmin.

	Mus/ TA*	Heart	Dia	Lung	Liver	Int.	Brain	Kid
<i>Mdx</i>	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
1 Chelsea	+	+	+	+	+	++	+	+
2 Colin	+(+)*	+(+)	++	+	+	++	+	++
3 Christine	-	+	+	+(+)	+	+	+	++
4 Cleo	+*	+++	++	+++	+++	(+)	++	+++
5 Conrad	-	+(+)	+(+)	++	+(+)	++	+(+)	++
6 Clark	++*	+++	++	+	+(+)	++	+(+)	++
7 Curtis	-	+	-	+	+	+	+	+
8 Charlie	+	+	-	+	+	+	+	+
9 Calvin	-	+	+	++	+	+++	+	++

Table 4 The expression pattern of 9 ubiquitin C full-length utrophin transgenic lines.

The differential expression of the transgene in the different lines was probably due to the different sites of integration of the transgene into the chromosomes. Ubiquitin C promoted the expression of the transgene in muscle as well as non-muscle tissues. Cleo line consistently showed high level of expression of the utrophin transgene in most tissues analysed.

Key: + indicated there was an over-expression of the transgene. It was accorded visually comparing with the endogenous level of the control *mdx* mice. The more + indicated the greater the over-expression of the transgene level by Western blot analysis. (+), endogenous level; TA\* means TA muscle. Mus means mixture of type 1 and 2 muscle fibre from the thigh region of the hind limbs. Dia, diaphragm; Int., intestine; Kid, kidney.

adrenal gland, pituitary gland, spleen, stomach, tongue, testis, ovary, skin, oesophagus, thymus, bone and uterus. Figure 3 shows the levels of utrophin in heart, kidney, liver, lung, oesophagus, spleen, eye, adrenal gland and tracheae from Cleo compared to *mdx* mice. Utrophin transgene levels were increased in all tissues ranging from a slight increase to one and a half times for diaphragm, two and a half times for lung and kidney and three times for heart. Figure 4 shows the serial dilution of the transgene over-expression levels in Cleo kidney, diaphragm, lung and heart compared to the *mdx* mice. The TA, EDL and diaphragm protein levels of Cleo line were compared to that of the Freddie line (discussed in chapter 4), the low expressing line of the HSA-full-length utrophin transgenic mice as shown in Figure 3. Cleo TA and EDL transgene expression levels were very low compared to that of Freddie. The expression in diaphragm was approximately similar to that of Freddie and the *mdx* mice. The utrophin level in lung, kidney, tongue and stomach of homozygous Cleo was approximately twice that in heterozygous HCleo, while in other tissues like TA, diaphragm, brain, testis, and bladder the levels increased only slightly compared to the *mdx* mice as shown in Figure 5. Table 5 shows the densitometry readings of TA of Cleo and HCleo compared to that of the *mdx* mice. These results show that there was an average increase of the utrophin transgene level of approximately 25% and 60% of the endogenous *mdx* utrophin level in TA muscle of Cleo and HCleo respectively over that of the *mdx* mice. The transgene expression in HCleo was more than doubled that of Cleo.

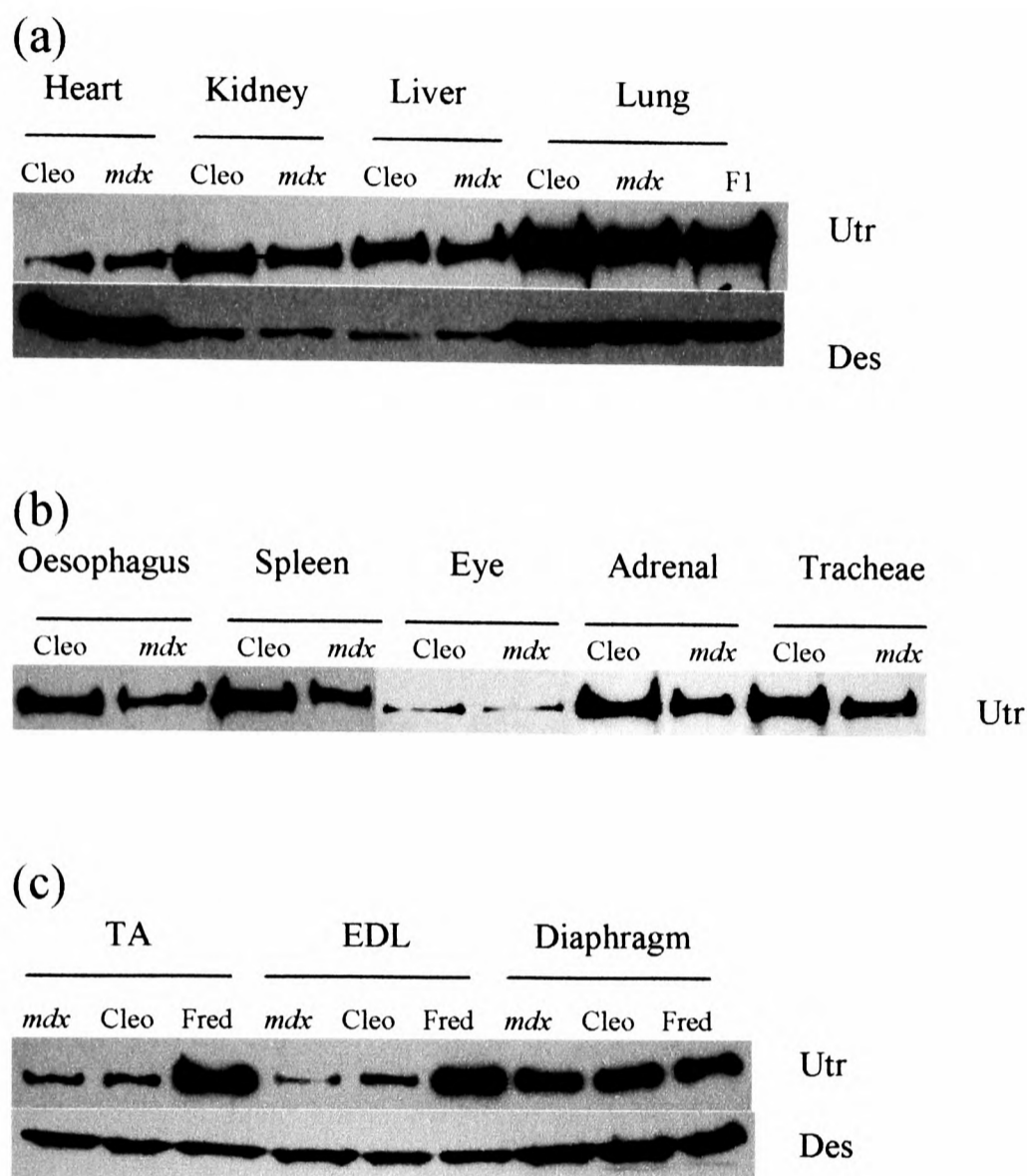


Figure 3 Expression pattern of the transgene in Cleo line

(a) and (b), Over-expression of the transgene in heart, kidney, liver, lung, oesophagus, spleen, eye, adrenal gland and tracheae of Cleo line compared to the *mdx* mice.

(c) Transgene protein level of TA, EDL and diaphragm of the Cleo line compared to that of the Freddie line, which was the lowest expressing line of the HSA-full-length utrophin transgenic mice, described previously in chapter 4. Desmin staining was used to control equal loading of the protein extracts (desmin data of b not shown).

Key: Utr, utrophin; Des, desmin.

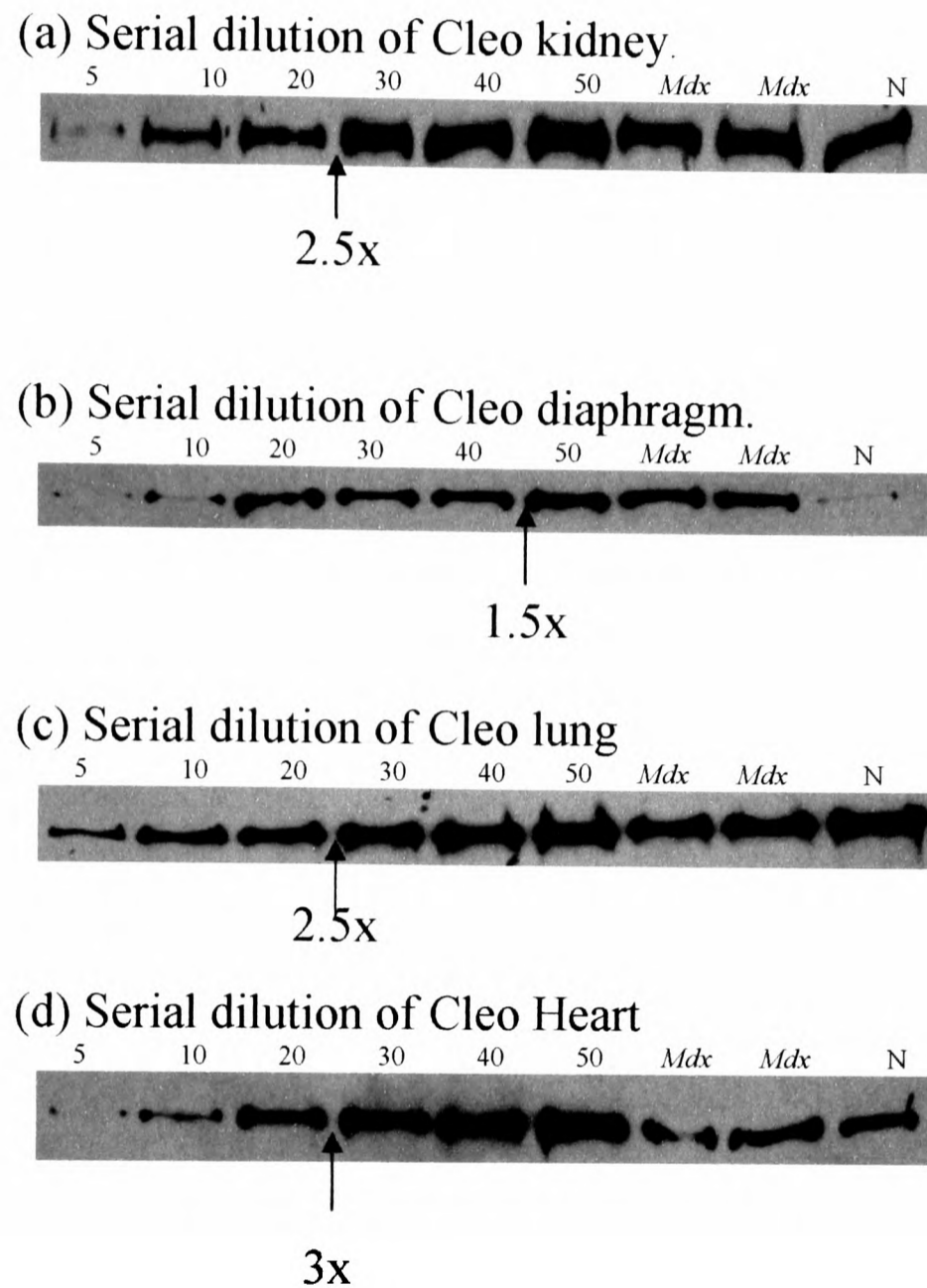


Figure 4 Serial dilutions of kidney, diaphragm, lung and heart.

Western blots of kidney, diaphragm, lung and heart of Cleo transgenic-*mdx* mice compared to Normal F1 (N) and *Mdx* mice. Protein loaded for N and *Mdx* mice was 50 $\mu$ g with serial dilution of 5,10,20,30,40 and 50 $\mu$ g for Cleo, to determine the level of over-expression of the transgene. The serial dilution of the protein was done using the original 50 $\mu$ g total protein extract as described in Materials and Methods. Serial dilutions were not done for those tissues whose levels of protein were only marginally increased compared to those of *mdx*, for example TA, EDL, eye etc..

There was a 2.5x, 1.5x, 2.5x and 3x over-expression of the transgene in Cleo kidney, diaphragm lung and heart respectively compared to the *mdx* mice.

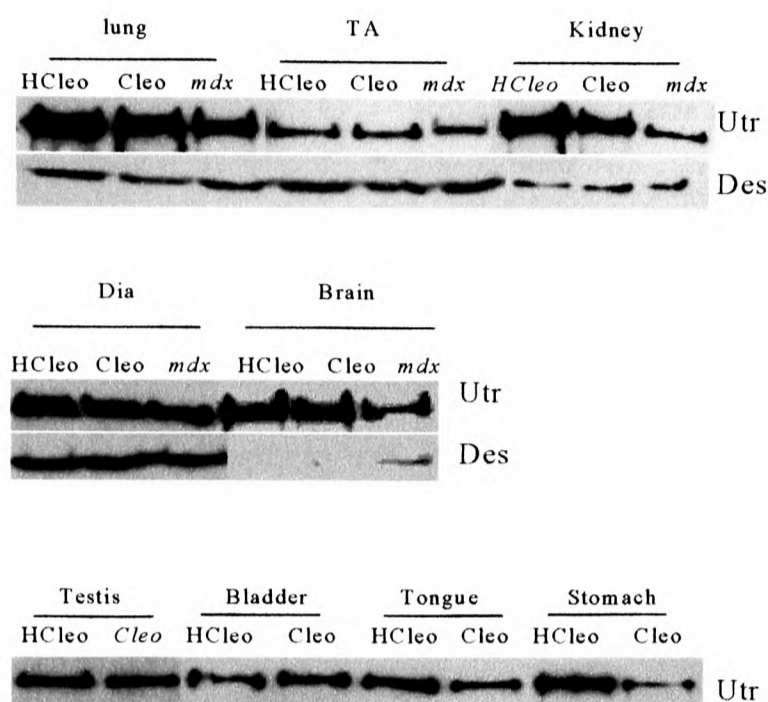


Figure 5 Expression pattern of Cleo and HCleo lines.

The level of utrophin transgene expression in various tissues from Cleo and HCleo lines compared to that of the *mdx* mice. The utrophin levels in lung, kidney, tongue and stomach of HCleo were approximately double that of Cleo, while that of TA, diaphragm, brain, testis and bladder remained approximately the same as that of Cleo. Ubiquitin C promoter seemed to promote very low levels of transgene expression in TA, EDL and diaphragm and higher levels in smooth muscle and tubular/glandular tissues.

Desmin staining was used to control for equal loading of the protein extracts.

Densitometry readings of utrophin transgene in TA of HCleo = 1.45  
 Cleo = 1.21  
*Mdx* = 1

Key: Utr, utrophin; Des, desmin. From (Fisher et al., 2001).

	Densitometry readings			Average
<i>mdx</i>	23.04	4.80	37.80	
Cleo	25.83	6.98	45.90	
HCleo	ND	9.20	53.86	
Ratio of Cleo/ <i>mdx</i>	1.12	1.45	1.21	1.26
Ratio of HCleo/ <i>mdx</i>	ND	1.92	1.42	1.67

Table 5 Densitometry of TA of Cleo and HCleo compared to *mdx* mice

The densitometry of TA of Cleo and HCleo compared to *mdx* mice was measured using Bio-Rad, Gel Doc 2000. These results show that there was a slight increase of the utrophin transgene of approximately 25% of the endogenous *mdx* utrophin level. The increase in HCleo was more than doubled. This increase resulted in a significant rescue of the dystrophic morphology of the *mdx* phenotype. Readings of 3 mice each of *mdx* and Cleo were taken and 2 of HCleo.

## **5.2.6 Effect of utrophin transgene expression in Cleo-mdx and HCleo-mdx skeletal muscle**

### **5.2.6.1 Percentage centrally nucleated fibres ( %CNF)**

To determine whether the levels of utrophin expression in Cleo and HCleo lines were sufficient for therapeutic benefit, the skeletal and diaphragm muscles from both lines were examined. The centrally nucleated fibres which were the result of continual muscle degeneration and regeneration were counted in the TA, EDL and diaphragm muscles and compared to dystrophin-deficient *mdx* and normal F1 muscles. Figure 6 shows a reduction from around 80% CNF down to approximately 30% in TA and EDL from Cleo line and down to approximately 13% and 5% respectively from HCleo line. The diaphragm also shows a significant improvement from 65% down to 50% CNF from Cleo line and down to approximately 25% from HCleo line. The percentage CNF in Figure 6 was calculated based on the number of centrally nucleated fibres in the *mdx* mice, which was of the C57/BL10 genetic background. In order to verify that the rescue in the utrophin transgenic mice was not influenced by its genetic background (C57BL/6 x CBA/CA), the percentage CNF in the negative littermates (F1-*mdx*) was counted as shown in Figure 7. There was no significant difference between the percentage CNF in the negative littermates and that of the *mdx* mice. Therefore the rescue of the muscle of the transgenic mice was not due to the effect of the genetic background of the *mdx* mouse but was due to the therapeutic effect of the utrophin transgene. By eighteen months of age TA, EDL and diaphragm of the Cleo line had approximately the same amount of central nucleation as the *mdx* mouse showing substantial muscular dystrophy (Pastoret and Sebille, 1993) as shown in Figure 8. Table 6 shows the total number of fibres in TA, EDL and diaphragm at 18

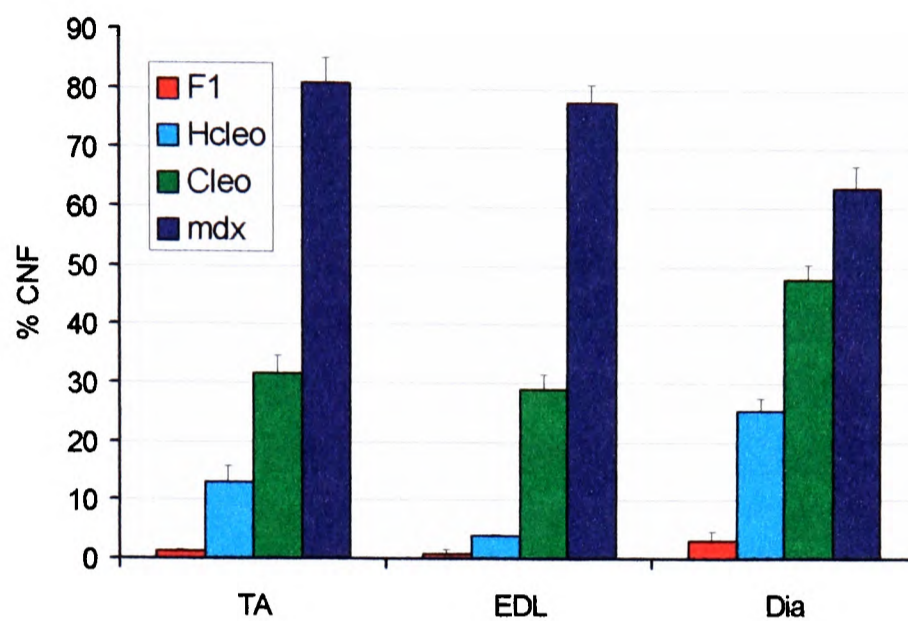


Figure 6 Percentage centrally nucleated fibres (%CNF) of Cleo and HCleo lines.

The %CNF in TA, EDL and diaphragm (Dia) of age-matched Cleo, 3 to 3.5 months old, HCleo, F1 and *mdx* mice. The %CNF of HCleo was reduced to 5% in EDL and 13% in TA respectively and that of diaphragm was reduced to 25%. The %CNF in Cleo was significantly reduced too. The bars represent the mean of 3 to 5 muscles  $\pm$  s.e.m. From (Fisher et al., 2001).

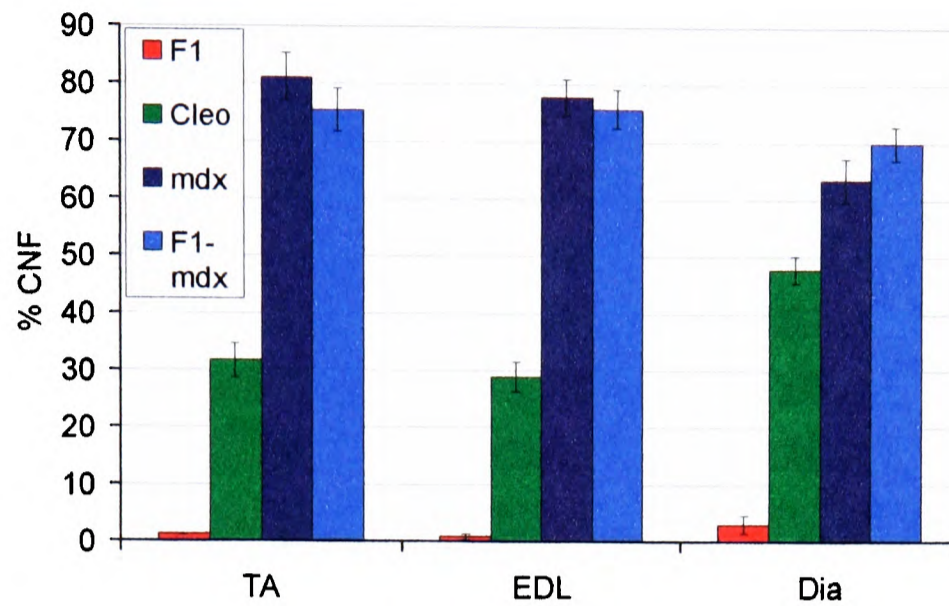


Figure 7 Percentage nucleated fibres of Cleo plotted against that of *mdx* and F1-*mdx* mice (3-3.5 months)

There was no significant difference between the %CNF of the *mdx* and F1-*mdx* (negative littermate) mice. Therefore the rescue of the muscle of the transgenic mice was not due to the effect of the genetic background of the *mdx* mouse but was due to the therapeutic effect of the utrophin transgene.

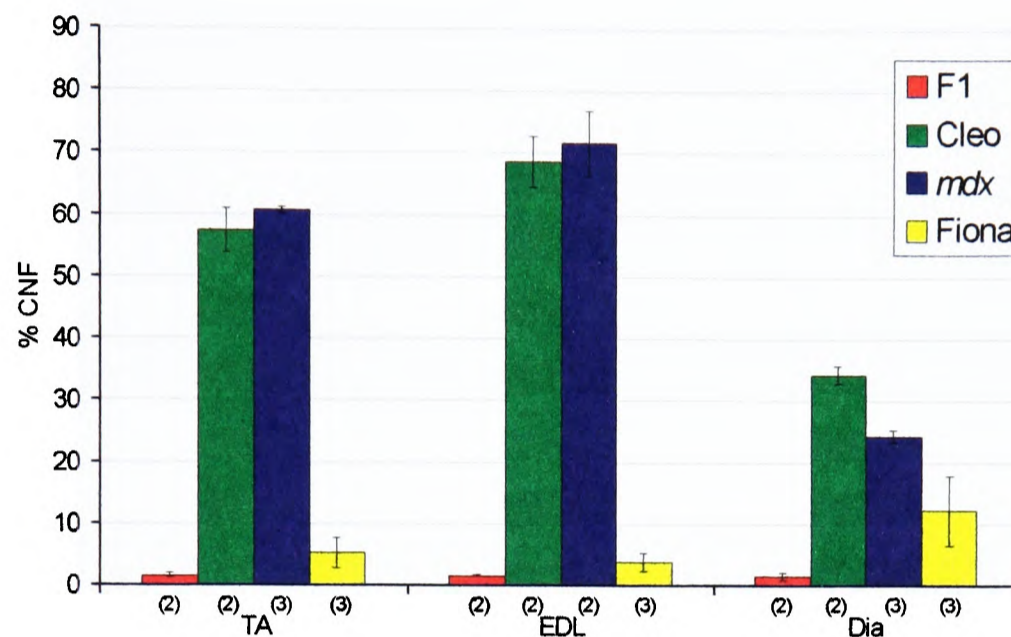


Figure 8 The % CNF of 18 months Cleo versus *mdx* and normal mice.

By 18 months of age, the TA, EDL and diaphragm (Dia) of Cleo and the *mdx* mice had the same amount of central nucleation. HCleo mice were not old enough to be analysed. The results of Fiona line, which contained the HSA-full-length utrophin, aged between 14 and 22 months, were included for comparison. The number of mice counted shown in parentheses. The %CNF of Freddie line was not done.

The bars represent the mean of 2-3 mice  $\pm$  s.e.m. From (Fisher et al., 2001).

	TA		EDL		Dia	
	Cleo	<i>mdx</i>	Cleo	<i>mdx</i>	Cleo	<i>mdx</i>
	1196	1518	545	487	1133	739
	2068	905	429	461	1037	667
Average	1632	1212	487	474	1228	703

Table 6 Total number of fibres in TA, EDL and Dia of age-matched 18 months old mice

Total number of fibres (normal peripherally and centrally nucleated fibres) in the sections analysed in TA, EDL and diaphragm (Dia) of Cleo and *mdx* mice were counted in complete transverse sections as described in Section 2.2.12.2. The total number of fibres in TA, EDL and diaphragm of Cleo were more than *mdx* mice but the majority of them were abnormal with central nuclei like those of the *mdx* fibres as shown in Figure 8.

The loss of total *mdx* muscle fibres in 18 months old *mdx* mice indicated that there was a block on regeneration. The utrophin transgene was able to effect some regeneration in the Cleo line at this late stage of dystrophy.

months old of age. There was some loss of total muscle fibres in the *mdx* tissues. The utrophin transgene levels in the Cleo line were very low and therefore the muscle rescue that was seen at age 3 months was not maintained at 18 months. It is important that the transgene level is up-regulated to high enough level in DMD therapy to maintain muscle regeneration at older age. The results of Fiona line, which contained the HSA-full-length utrophin that aged between 14 and 22 months, were included for comparison (discussed in chapter 4). Here, it is shown that the low level of expression of the ubiquitin C full-length utrophin did not ameliorate the dystrophic morphology as well as the HSA-full-length utrophin transgene did. Fiona was the high expressing line compared with Freddie, which was the low expressing line. The transgene level of the Cleo line compared to that of the Freddie line is shown in Figure 3c.

#### **5.2.6.2 Masson staining**

Histological analysis of Masson stained sections of TA and diaphragm of Cleo show a significant improvement of the dystrophic pathology as shown in Figure 9. The TA and diaphragm muscles of the *mdx* mice showed irregular muscle fibres with central nuclei, cellular infiltrations, fibrosis and increased fibrocollagenous tissues in common with DMD patients (Sicinski et al., 1989). The diaphragm of the *mdx* mice showed more severe symptoms than that of the TA with very extensive invasion of the fibrocollagenous tissues (Stedman et al., 1991). Both the TA and diaphragm of Cleo showed a lesser amount of fibrocollagenous and cellular infiltrations, lesser amount of degeneration and regeneration as seen by the fibre size and presence of centralised nuclei compared to the *mdx* mice. The levels of transgene expression in the muscles and diaphragm of Cleo resulted in a significant improvement in the early pathology of the *mdx* mice.

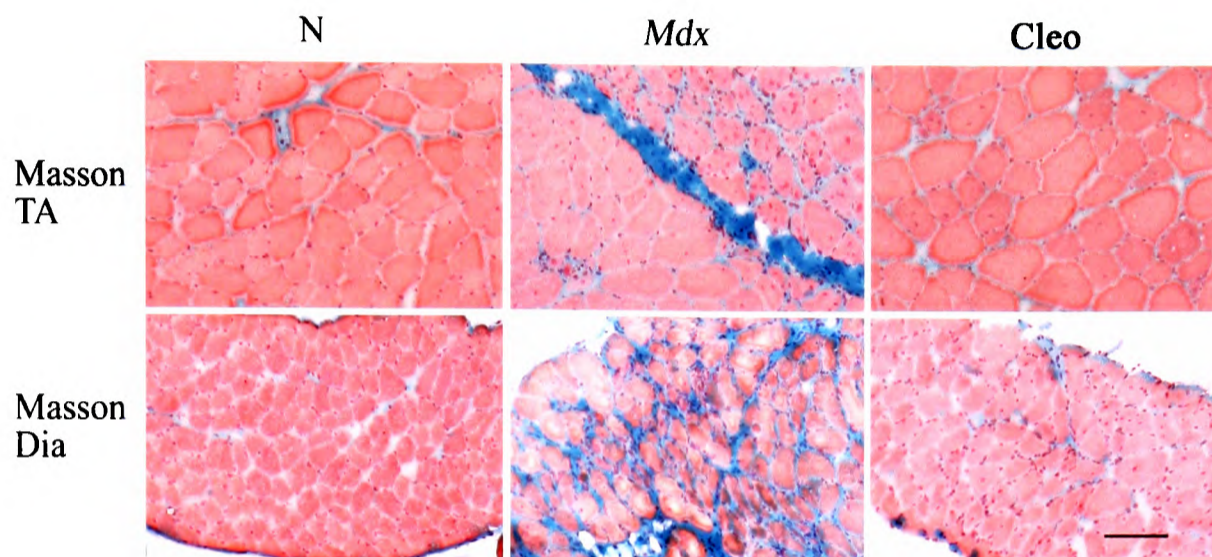


Figure 9 Masson stain of Cleo TA and Diaphragm

Histological analysis of Cleo, normal F1 (N) and *mdx* TA and diaphragm (Dia) from age-matched mice of 3 to 3.5 months old. Masson staining to show the infiltration of the fibrocollagenous tissues (blue/green). Both the TA and Diaphragm of Cleo showed lower amount of fibrocollagenous and cellular infiltrations and also decreased regeneration as seen by the uniformity of fibre size and lower numbers of centralised nuclei (stained dark reddish/brown) compared to the *mdx* mice. Scale bar =50 $\mu$ m. From (Fisher et al., 2001).

### 5.2.6.3 Immunohistochemistry

The TA and diaphragm of Cleo, normal and *mdx* mice were stained with specific antibodies for utrophin, dystrophin,  $\alpha 1$  dystrobrevin and  $\alpha$ -sarcoglycan. Figure 10 shows the immunohistochemistry of utrophin at TA and Figure 11 that of diaphragm. Utrophin and the DPC components,  $\alpha 1$  dystrobrevin and  $\alpha$ -sarcoglycan were shown to localise to the sarcolemma of both the TA and diaphragm muscles. In the TA and diaphragm sections of Cleo line, the utrophin staining was brighter and more uniform under the same staining and photographic conditions, than that of the normal and *mdx* controls showing that utrophin was over-expressed. In the normal TA and diaphragm muscles, utrophin stained brightly only at the neuromuscular junctions and cell/cell contacts. Utrophin was slightly up-regulated in the *mdx* muscles and the absence of dystrophin resulting in some loss of the DPC components. Immunostaining of 18 months old tissues were not done.

### 5.2.7 *Effect of utrophin transgene expression in Cleo- and HCleo-mdx non-muscle tissues*

#### 5.2.7.1 Haematoxylin and eosin (H&E) comparative morphology

The 20 to 24 tissues collected from each age-matched mouse were fixed in formalin and sent to independent veterinary pathologists for histopathological analysis. No abnormality could be seen in any of the tissues studied (data not shown). Skeletal muscle and heart of the *mdx* mice showed the usual dystrophic morphology but in the Cleo and HCleo sections a significant morphological improvement was observed. These data showed that the over-expression of the utrophin transgene did not result in any obvious morphological abnormality in the tissues tested.

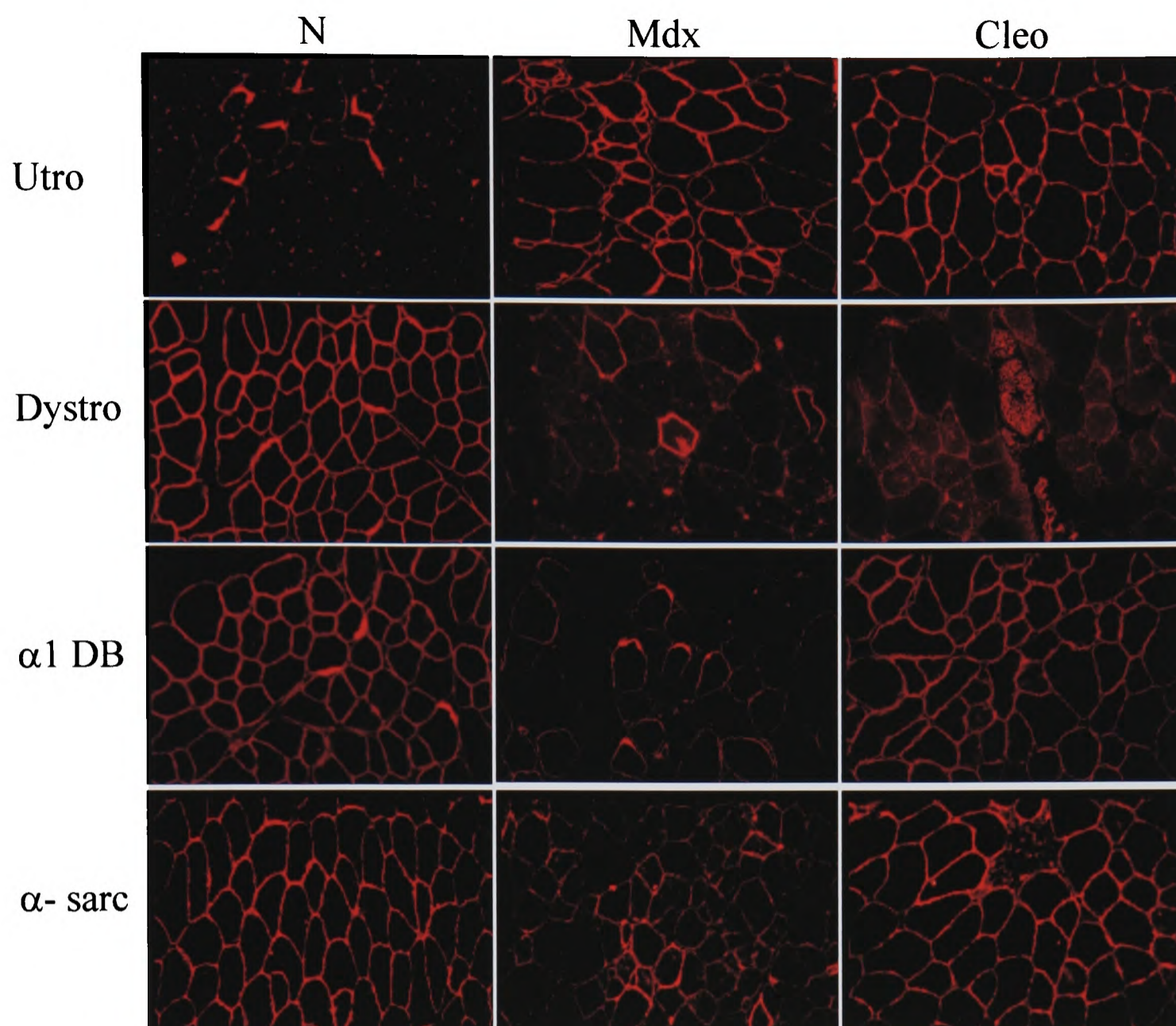


Figure 10 Immunohistochemistry of utrophin at TA muscle

Immunohistochemistry of utrophin, dystrophin,  $\alpha$ 1-dystrobrevin ( $\alpha$ 1-DB) and  $\alpha$ -sarcoglycan ( $\alpha$ -sarc) in TA muscle of normal F1 (N), *Mdx* and transgenic-*mdx* (Cleo) from age-matched mice of 3-3.5 months old, using specific antibodies.

Top row: Utrophin at TA: NMJ, blood vessels and cell/cell contacts were brightly stained in normal tissues. The sarcolemma of newly regenerated fibres of the *mdx* mice was stained strongly. Increased staining in sarcolemma of Cleo with some areas of cell/cell contacts resemble that of normal mouse tissue. 2<sup>nd</sup> row: Dystrophin at TA: The sarcolemma of N TA stained brightly especially the NMJ. Only 3 or 4 revertant (back mutation) fibres of *Mdx* were stained with dystrophin, the rest is not stained. Cleo sarcolemma is not stained. 3<sup>rd</sup> row:  $\alpha$ 1-dystrobrevin at TA: Sarcolemma brightly stained especially the NMJ of N tissues. NMJ and regenerated fibres stained in *Mdx*. NMJ and sarcolemma of Cleo brightly stained. 4<sup>th</sup> row:  $\alpha$ -sarcoglycan: Sarcolemma of N and Cleo brightly stained. Only small calibre regenerated fibres stained in *Mdx* tissues. Magnification: 200x. (Scale bar could not be calibrated because the microscope was stolen).

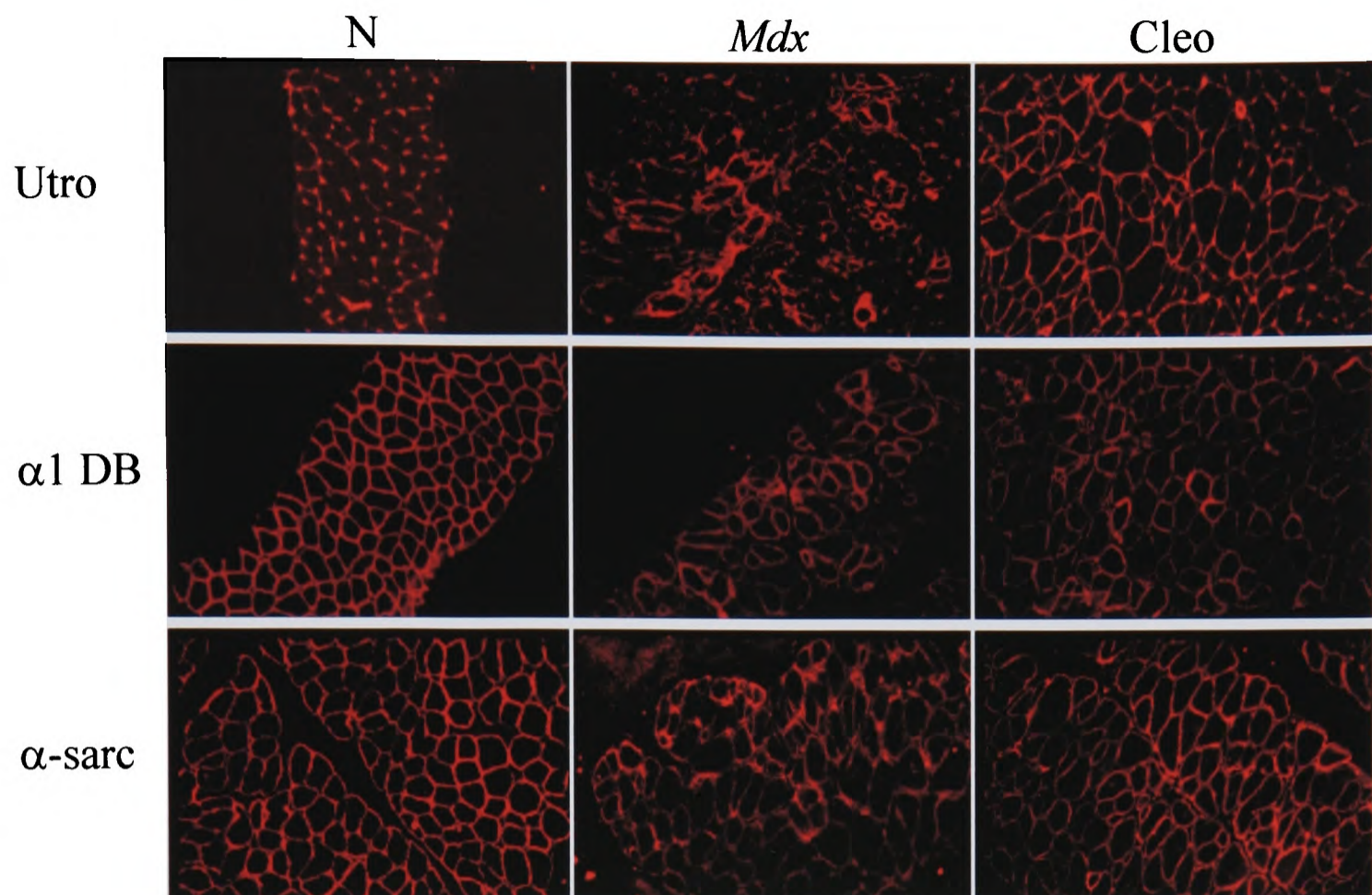


Figure 11 Immunohistochemistry of utrophin in diaphragm

Immunohistochemistry of utrophin (Utrophin),  $\alpha$ 1-dystrobrevin ( $\alpha$ 1 DB) and  $\alpha$ -sarcoglycan ( $\alpha$ -sarc) in diaphragm of normal F1 (N), *Mdx* and Cleo diaphragm of age-matched mice of 3-3.5 months old.

Top row: Utrophin at diaphragm: NMJ, blood vessels and cell/cell contacts were brightly stained in N Dia. Sarcolemma of *Mdx* was brightly stained. Some sarcolemma and cell/cell contacts were brightly stained in Cleo with some areas resembling that of N Dia. 2<sup>nd</sup> row:  $\alpha$ 1-dystrobrevin: Sarcolemma of N was brightly stained. In *Mdx*, cluster of regenerated fibres stained. NMJ and most sarcolemma stained but not as bright as N in Cleo. 3<sup>rd</sup> row:  $\alpha$ -sarcoglycan: Sarcolemma was brightly stained in N. Only parts of sarcolemma of small calibre fibres were stained in *Mdx*. All of the sarcolemma of Cleo stained but not as bright as N. Magnification: 200x. (Scale bar could not be calibrated because the microscope was stolen).

### 5.2.7.2 Urine analysis

The level of creatinine was examined in the urine to test for any potential deficit in kidney function that could have resulted from the over-expression of the transgene. Urine samples of Cleo, HCleo, normal F1, *mdx* and littermate-*mdx* mice were analysed for the levels of creatinine using proton nuclear magnetic resonance (NMR) by Professor Jo Martin (Section 2.2.10). No abnormalities were seen in Cleo and HCleo samples analysed compared with experimental (normal F1 and *mdx*) or historical controls, and no additional peaks were noted. (Data not shown).

### 5.2.7.3 Immunofluorescent staining

The cryosections of all the non-muscle tissues collected were stained with the utrophin antibody as described (Section 2.2.12). The results were summarised in Table 7. Utrophin staining was increased in all tissues analysed from Cleo and HCleo lines compared to the *mdx* mice confirming that utrophin was over-expressed. It was also found to localise to the sarcolemma in all tissues. In some tissues the staining of utrophin was observed to be slightly brighter in HCleo than the Cleo tissues under same conditions of staining run and photographing. The exposure times for photography were exactly the same for each set of tissues and the images had not been differentially processed in Photoshop etc. Figure 12 shows the immunofluorescence localisation of utrophin in bladder, heart, liver, intestine, eye and stomach where the utrophin intensity was slightly brighter in the HCleo line than that of the Cleo line. No change was observed in utrophin distribution in Cleo and HCleo tissues compared to normal F1 and *mdx* mice except in kidney where proximal

	<b>N</b>	<b>Mdx</b>	<b>Cleo</b>	<b>HCleo</b>
<b>KIDNEY</b> Utrophin	Glomerulus, distal tubules, medulla brightly stained.	Same as N.	Glomerulus, distal and in addition proximal tubules, medulla brightly stained	Same pattern as Cleo but slightly brighter stained.
$\alpha$ 1-dystrobrevin	Medulla, distal tubules, glomerulus brightly stained.	Same as N but medulla less brightly stained.	Same as N.	Same as N.
Kidney Dp71	Membranes of tubules in medulla (packing cells/tissues not stained like in utrophin), distal and proximal tubules brightly stained. Glomerulus not stained.	Same as N.	Same as N.	Same as N.
<b>HEART</b> Utrophin	Sarcolemma of myocytes especially at cell/cell contacts stained brightly. Intercalated discs stained faintly.	Sarcolemma and intercalated discs stained brightly.	Same as N but brighter.	Same as N but intercalated discs brighter still ( than Cleo).
<b>TONGUE</b> Utrophin	NMJ, cell/cell contacts, nerves and blood vessels brightly stained.	Same as N but sarcolemma stained brighter.	Same as N but sarcolemma stained brighter.	Same as N but sarcolemma stained brighter.
<b>OESO-PHAGUS</b> Utrophin	Basal layer of epithelium, blood vessels and nerves in lamina propria connective tissues and striated muscle stained.	Same as N.	Same as N.	Same as N.
<b>STOMACH</b> (body mucosa) Utrophin	Smooth muscle especially the squamous epithelium that lines the nonglandular stomach, blood vessels, nerves and internal membrane of all the glands in the glandular portion of the stomach stained brightly.	Same as N.	Same as N.	Same as N but brighter.
<b>INTESTINE</b> (T.S. small) Utrophin	Smooth muscle, columnar epithelial membrane of the villi and blood vessels stained brightly.	Same as N.	Same as N but slightly brighter stained.	Same as N but brighter still (than Cleo)
<b>LIVER</b> Utrophin	Hepatic arteries, hepatic portal veins and sinusoids are stained brightly. Hepatocytes not stained.	Same as N but brighter.	Same as N but brighter.	Same as N but very much brighter.

Table 7 Results of immunostaining of non-muscle tissues

Table 7 - continued

	<b>N</b>	<b>Mdx</b>	<b>Cleo</b>	<b>HCleo</b>
<b>BLADDER</b> Utrophin	Smooth muscle membrane, blood vessels in lamina propria stained brightly. Transitional epithelium (urothelium) not stained.	Same as N.	Same as N but brighter.	Same as N but brighter.
<b>TESTIS</b> Utrophin	Basement membrane of seminiferous tubules, stromal cells between tubules stained. Spermatogonia not stained.	Same as N but brighter.	Same as N but brighter.	Same as N but brighter.
<b>TRACHEA</b> Utrophin	Base of columnar epithelium, blood vessels and nerves in submucosa, some subset of hyaline cartilage cells stained brightly.	Same as N.	Same as N.	Same as N.
<b>LUNG</b> Utrophin	Blood vessels and alveolar endothelium stained brightly	Same as N but brighter.	Same as N but brighter.	Same as N but brighter still.
<b>EYE</b> (TS of cornea) Utrophin	Squamous corneal epithelium membrane, Bowman's membrane, Descemet's basement membrane stained. Stroma not stained.	Same as N.	Same as N.	Same as N but slightly brighter.
<b>BRAIN</b> (Cerebellum) Utrophin	Blood vessels stained.	Same as N.	Same as N.	Same as N.
<b>SKIN</b> Utrophin	Basal layer cells, blood vessels, nerves, premedullary epithelium and vascular basement membrane stained very brightly. Smooth muscles less.	Same as N.	Same as N.	Same as N.

Table 7 Results of immunostaining of non-muscle tissues.

From (Fisher et al., 2001).

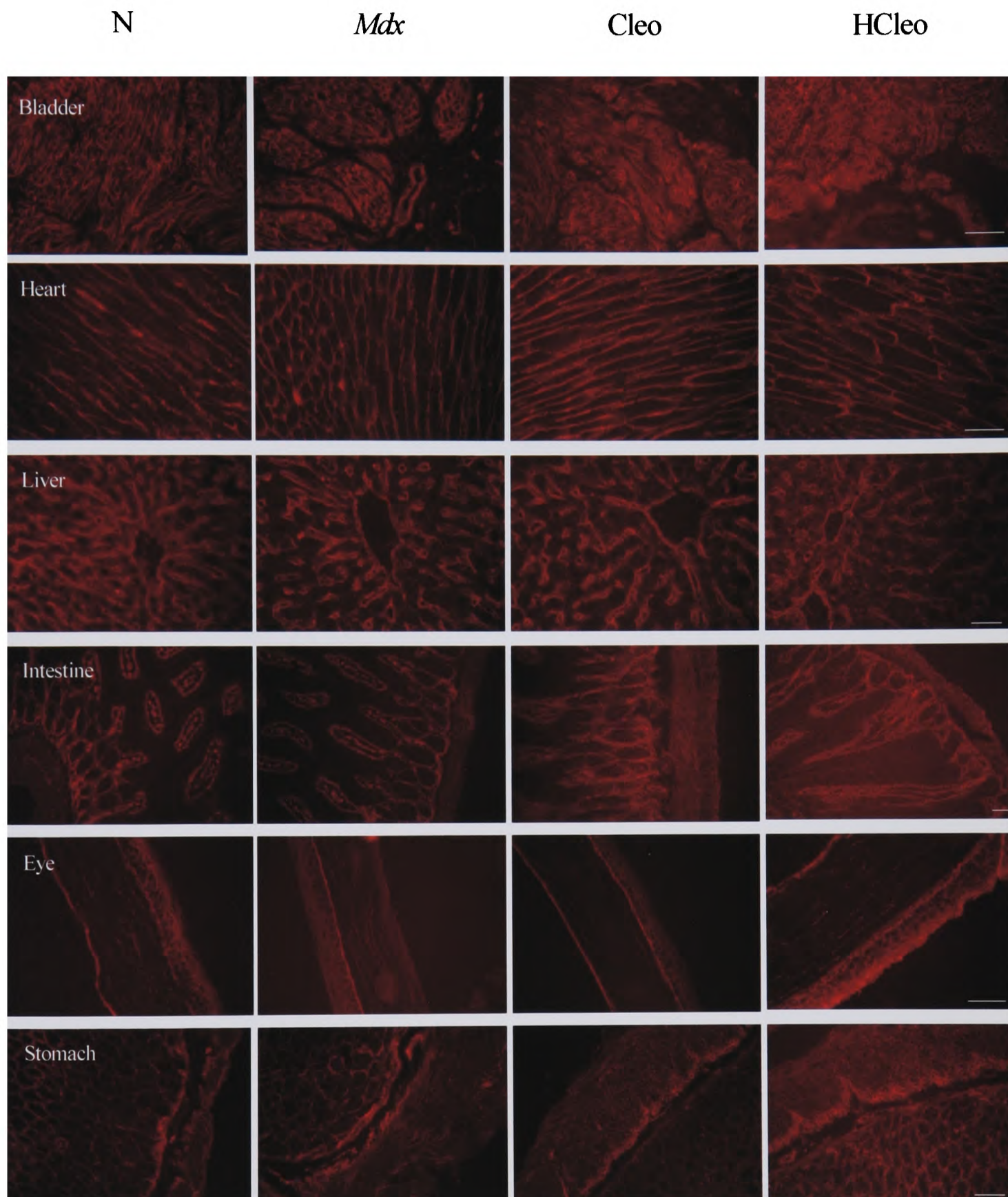


Figure 12 Immunohistochemistry of utrophin in non-muscle tissues

Immunohistochemistry of utrophin in bladder, heart, liver, intestine, eye and stomach of normal F1 (N), *mdx*, Cleo and HCleo. Increase in utrophin staining of Cleo and HCleo tissues was observed. Exposure times were the same for each set of antibody used to allow for comparison of the intensity of staining of the samples. Scale bar = 50 $\mu$ m. From (Fisher et al., 2001).

tubules were now stained in addition to distal tubules, glomerulus and medulla as shown in Figure 13. The localisation of  $\alpha$ -dystrobrevin and kidney Dp71 in Cleo were similar to that of the normal control mice.

### **5.2.8 Weight as a function of age**

At the start of the weighing experiment, six mice of normal F1, *mdx* and Cleo were weighed at the beginning of each month. At three months old the weight of normal *mdx* and Cleo mice was approximately the same, 32 gm, and their weight increased in parallel up to approximately six months as shown in Figure 14. The bars represent the mean of the weight of six mice  $\pm$  s.e.m. Thereafter the weight of the *mdx* mice dropped by approximately 25% ( $p < 0.001$ ) up to age 23 months and most of them died or had to be sacrificed due to ill health. While that of Cleo was the same as normal and still living healthily after two years of age. This showed that the utrophin transgene was still conferring its therapeutic benefit in muscle even after two years of age and the over-expression of utrophin in non-muscle tissues had not resulted in premature death.

## **5.3 Discussion**

The failure of the SV40 mini-A transgenic mice to express the transgene was not investigated further. This could be due to the very short SV40 promoter (1.05 kb) used. The ubiquitin C promoter drove the expression of the utrophin transgene weakly in most tissues, as could be seen by the comparison of the level of expression of the transgenes in TA and EDL of the Cleo and Freddie lines (Figure 3c). Only one line out of the nine lines of the ubiquitin C full-length utrophin transgenic mice generated

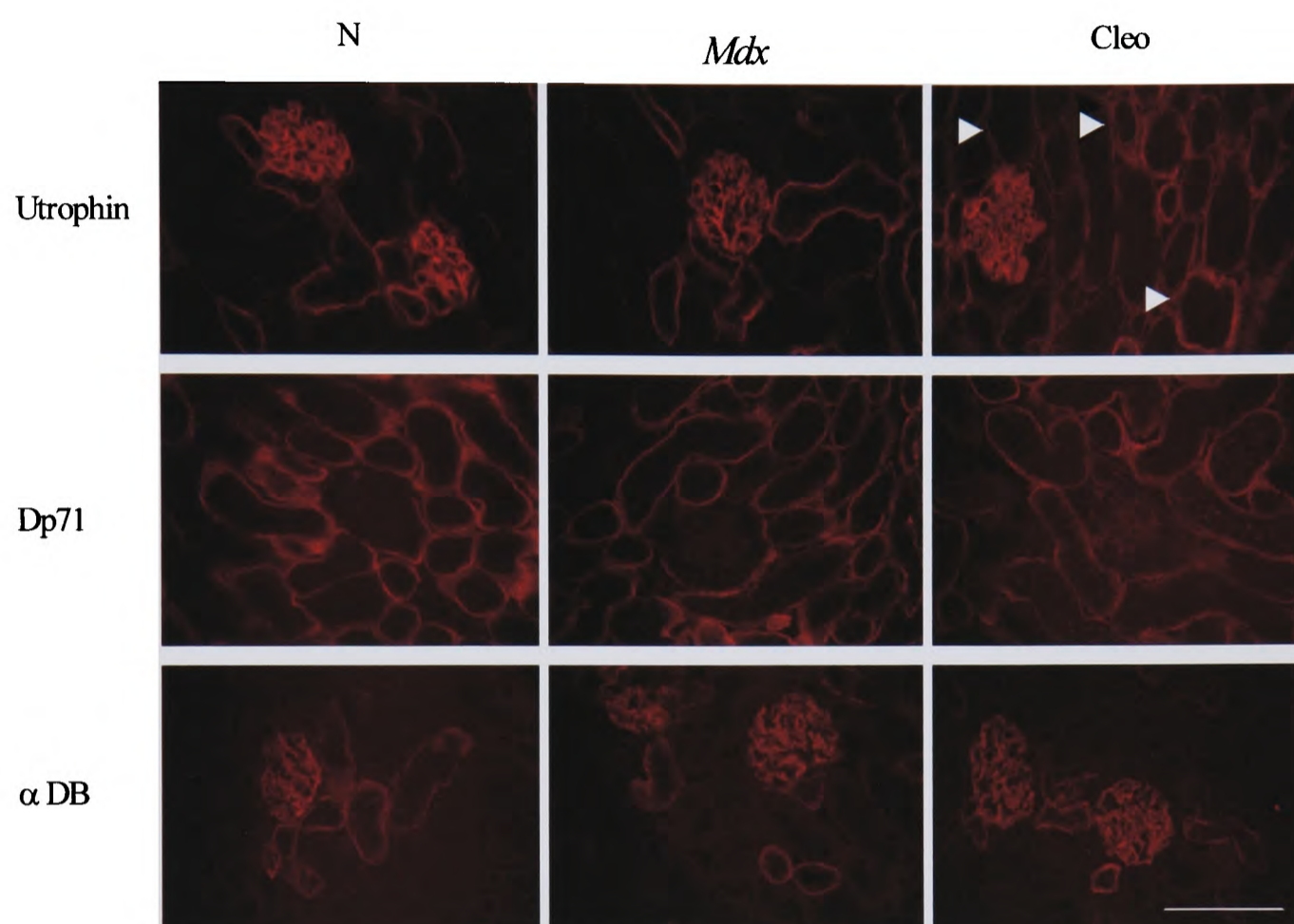


Figure 13 Immunohistochemistry of utrophin in Kidney

Kidney cryosections of normal F1 (N), *mdx* and Cleo mice were stained with utrophin, kidney dystrophin isoform Dp71 and  $\alpha$ -dystrobrevin specific antibodies. There was no increased distribution sites of Dp71 and  $\alpha$ -dystrobrevin but there was an increase distribution site of utrophin in Cleo, that is, the proximal convoluted tubules (arrowheads) were now stained in addition to the distal convoluted tubules and glomerulus. Scale bar = 100 $\mu$ m. From (Fisher et al., 2001).

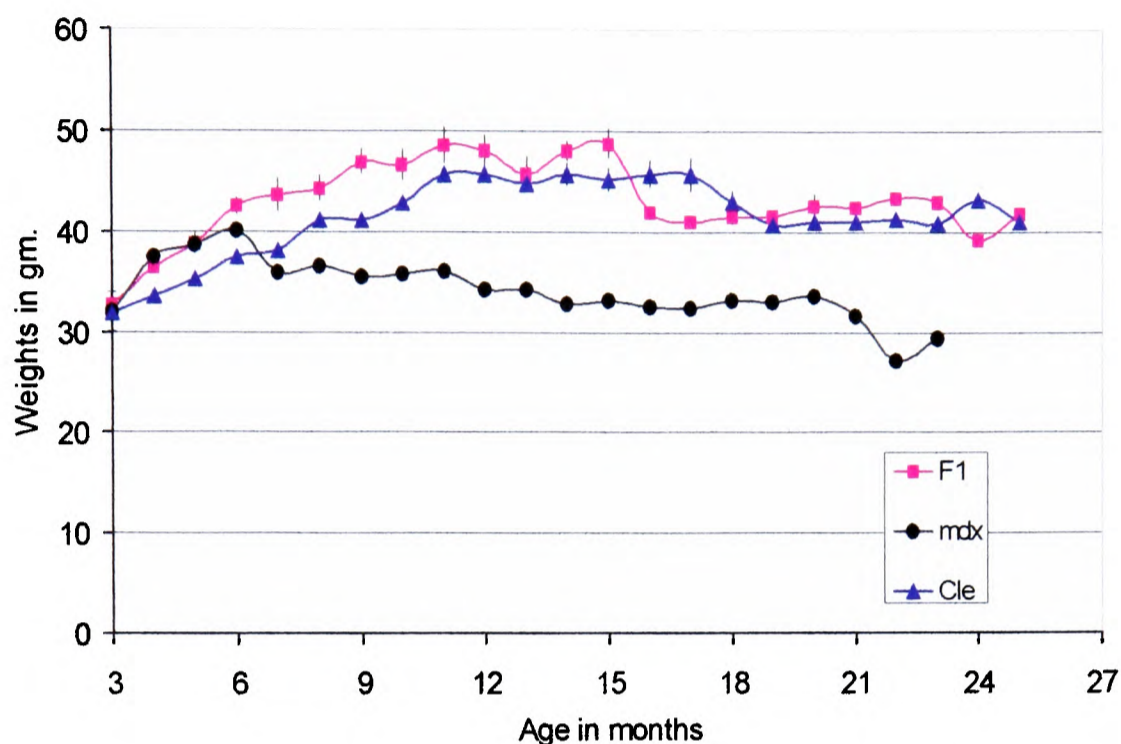


Figure 14 Weight as a function of age

At three months old, the weight of normal F1, *mdx* and Cleo was the same, 32g. The weight of the *mdx* mice dropped by approximately 25% up to age 23 months compared to normal F1 and Cleo. The graph was plotted as the mean weight of 6 mice (except for the *mdx* mice where 4 mice were used after 18 months due to the death of 2 *mdx* mice) as a function of age for normal F1, *mdx* and Cleo.

The Kolmogorov-Smirnoff non-parametric test was used in the analysis and showed highly significant differences ( $p < 0.001$ ) in every case (month) except the 7<sup>th</sup> month ( $p = 0.007$ ) between all the age-matched pairs of utrophin-*mdx*, control and *mdx*.

The bars show the  $\pm$  s.e.m. From (Fisher et al., 2001).

expressed high levels of the utrophin transgene in almost all the tissues analysed by the Western blot analysis. In the Cleo line the utrophin levels in all tissues were increased, ranging from a slight increase to approximately three folds in heart compared to the *mdx* mice. The reason why if ubiquitin C promoter is a ubiquitous promoter is there differential expression between tissues of the same mouse line is unclear. It might depend on the activity of the utrophin promoter in each particular tissue type, the number of active cells, the expression may be transient and the degradation rate of the transgene may be different in different tissues. As can be seen in Figures 3 and 4, some changes in protein levels between Cleo and *mdx* mice were marginal. With the biggest changes seen in non-muscle tissues. TA for example, the transgene was over-expressed by approximately 25% of that of the *mdx* utrophin endogenous level.

Therefore, it was decided to concentrate on that line, Cleo, to answer the question of whether the over-expression of utrophin in a broad range of tissues was toxic to the *mdx* mouse. To further increase the level of expression of the transgene, the Cleo line was bred to homozygosity. The increase in transgene level in HCleo line was approximately twice that of Cleo line in some tissues only like lung, kidney, tongue and stomach while in other tissues the level increased only slightly. The transgene levels did not double in all the tissues analysed in this experiment as expected. The effect of marginal increase of approximately 25% in utrophin over-expression in skeletal muscle of Cleo and approximately 60% in HCleo lines over *mdx* mice was dramatic, resulting in a reduction of around 80% CNF down to approximately 30% and 13% from Cleo and HCleo in TA and 30% and 5% in EDL respectively compared to *mdx* mice. The diaphragm also shows a significant improvement from 65% down to

approximately 50% and 25% from Cleo and HCleo respectively. These results showed a positive correlation between the transgene levels and the rescue benefits. Both the TA and diaphragm muscle of Cleo showed a great improvement in the morphology judging from the Masson stained sections. However, by 18 months of age TA, EDL and diaphragm had approximately the same amount of central nucleation as the *mdx* mouse. The total number of fibres in TA, EDL and diaphragm of Cleo were more than that of the *mdx* mice but the majority of them were abnormal, that is, with central nucleation. The loss of total *mdx* muscle fibres in 18 months old *mdx* mice indicated that there was a block on regeneration. The utrophin transgene was able to effect some regeneration in the Cleo line at this late stage of dystrophy. In both Cleo and *mdx* mice, the remaining muscle fibres were supported by intensive fibrocollagenous tissues (photos not shown). The lower level of the transgene expression compared to that of Fiona was not able to reduce the %CNF significantly at old age. This observation has implication in using utrophin in therapy. The amount of utrophin must be up-regulated to high enough level to effect continual beneficial benefits into old age.

The transgene level conferred greater beneficial effect at the early stage of the disease, that is, at three months of age. At two years of age all the *mdx* mice died or had to be sacrificed due to dehydration and extreme poor health, mainly stiffening of the limb muscles. It was not known whether the hearts of old Cleo mice suffered from arrhythmia or the kidney function deteriorated. These functional tests were not carried out due to the constrain of time but it would be informative to carry out in the future. But the Cleo mice were still living like the normal mice, showing that the utrophin transgene was still conferring its therapeutic benefits after two years of age, in spite of

the failure to regenerate any new muscle fibres. However, the utrophin levels at 18 months old were not assayed.

It was further confirmed that the over-expression of utrophin did not perturb the localisation of utrophin and the dystrophin-associated protein complex. Analysis of the TA, EDL and diaphragm of Cleo line showed that utrophin and components of the DPC were localised to the sarcolemma similar to previous findings in chapter 3 and showed a major recovery of the utrophin transgenic muscle tissues from a dystrophic phenotype. No difference was observed in the distribution of utrophin in all tissues of Cleo and HCleo lines studied compared to normal and the *mdx* mice except in kidney where proximal tubules were now stained, as well as the expected staining of the glomerulus, distal tubules and medulla. The localisation of dystrobrevin, a component of the DPC and kidney Dp71 was similar to normal mice, confirming that their expression was not affected by the over-expression of the utrophin transgene. In many tissues, for example heart, stomach, intestine, bladder, lung and eye the intensity of utrophin staining was slightly increased in Cleo and HCleo lines, showing that the utrophin transgene was over-expressed but did not lead to perturbation of the localisation of utrophin.

All the age-matched tissues examined by the independent specialist veterinary pathologists were normal except the skeletal muscle and heart of the dystrophin-deficient lines, which showed the usual muscular dystrophic symptoms in the *mdx* mice but the morphology of Cleo and HCleo lines was greatly improved. This study showed that the over-expression of the utrophin transgene did not result in any abnormal morphology. Study of urine samples showed that the levels of creatinine

were normal, indicating that the over-expression of utrophin did not lead to kidney or organ/tissue damage.

The mechanical performance of the Cleo and HCleo lines was not carried out. This decision was taken in view of the fact that the mechanical performance of the full-length transgenic mice, Fiona, Fergie and Freddie lines were already carried out previously (Chapter 4). It was shown that the full-length utrophin was able to improve the mechanical performance of the transgenic mice.

## 5.4 Conclusion

In summary, the data presented here showed that over-expression of full length utrophin in a broad range of tissues did not lead to toxic effects in the *mdx* mice. This suggested that tight control over utrophin expression in specific tissues might not be necessary for the correction of muscular dystrophy. Thus the search for small molecules to up-regulate utrophin expression represents a promising approach to improving therapy for DMD patients. The amount of utrophin up-regulated must be sufficiently high enough to effect continual benefits into old age.

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## Chapter 6

# An inducible expression system

### 6.1 Introduction

It was demonstrated in the previous chapters that the over-expression of either truncated or full-length utrophin in the *mdx* mouse could protect the muscle fibres from dystrophic degeneration and improve the mechanical properties of the muscle (Tinsley et al., 1996; Deconinck et al., 1997c; Tinsley et al., 1998). In these studies, the transgene was initiated *in utero* and not somatically. Therefore, some questions regarding the efficacy of the use of utrophin in gene therapy for DMD patients remain to be answered. For example: what is the best disease progression stage to up-regulate utrophin? Would up-regulation of utrophin in muscle fibres be as effective at later stages as at earlier stages? Would the up-regulation of utrophin at later stages be of any benefit to the patients, and if so, for how long? To answer these questions, transgenic mice containing a murine full-length utrophin transgene under the control of tetracycline were generated.

The system used here is the tet-off system, that is, when tetracycline (tet) is administered, the expression of the utrophin transgene is switched off. This tet-

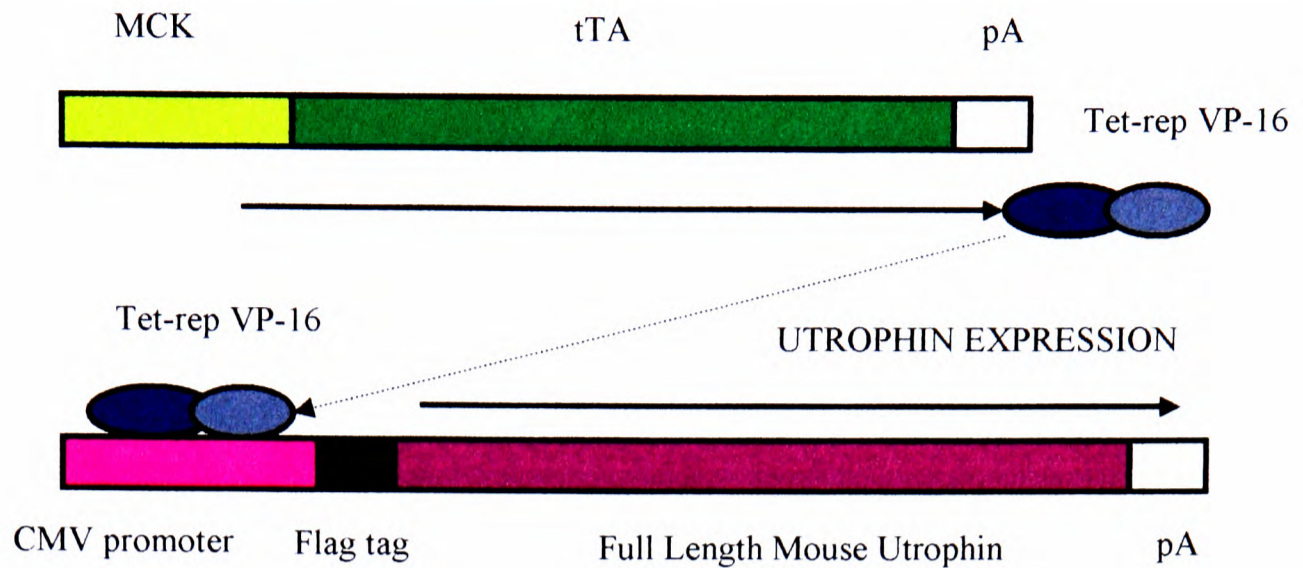
regulatable expression is reversible, that is, when the tetracycline is removed, the expression of the transgene is switched on again. This inducible expression of the transgene is facilitated by the use of the tetracycline responsive-transactivating system originally described by Gossen *et al.* (Gossen and Bujard, 1992). Figure 1 shows schematic representation of the tet-off inducible expression system. Both the transgenes, MCK-tTA/*mdx* and CMV-full-length mouse utrophin/*mdx* are bred in the double transgenic lines; Anna/Tex and Abe/Tex as explained in Section 6.2.3. In the absence of tetracycline, the transgene is switched on and in the presence of tetracycline the transgene is switched off. The suppression of the transgene expression is brought about by the tetracycline inhibiting the binding of the tTA transactivator to the tet-operator motif flanking the utrophin transgene. The tetracycline is administered in the drinking water at the concentration of 1 mg/ml.

## 6.2 Results

### 6.2.1 Tet-inducer mice

The tet-inducer mice, Anna and Abe transgenic lines, were a kind gift from Dr. R. Hooft, Glaxo-Wellcome, Geneva, Switzerland. These mice contain the muscle-specific creatine kinase (MCK) promoter driving the tetracycline-controlled transactivator (tTA). This transactivator stimulates transcription from a minimal promoter sequence derived from the human cytomegalovirus (CMV) promoter 1E combined with tet-operator sequences. For the construction of this transgene MCK-tTA cDNA, see (Ghersa *et al.*, 1998). Anna and Abe were bred onto the *mdx* background to produce MCK-tTA/*mdx* lines to facilitate the future breeding of double transgenic mice for analysis.

## No Tetracycline



## Addition of Tetracycline

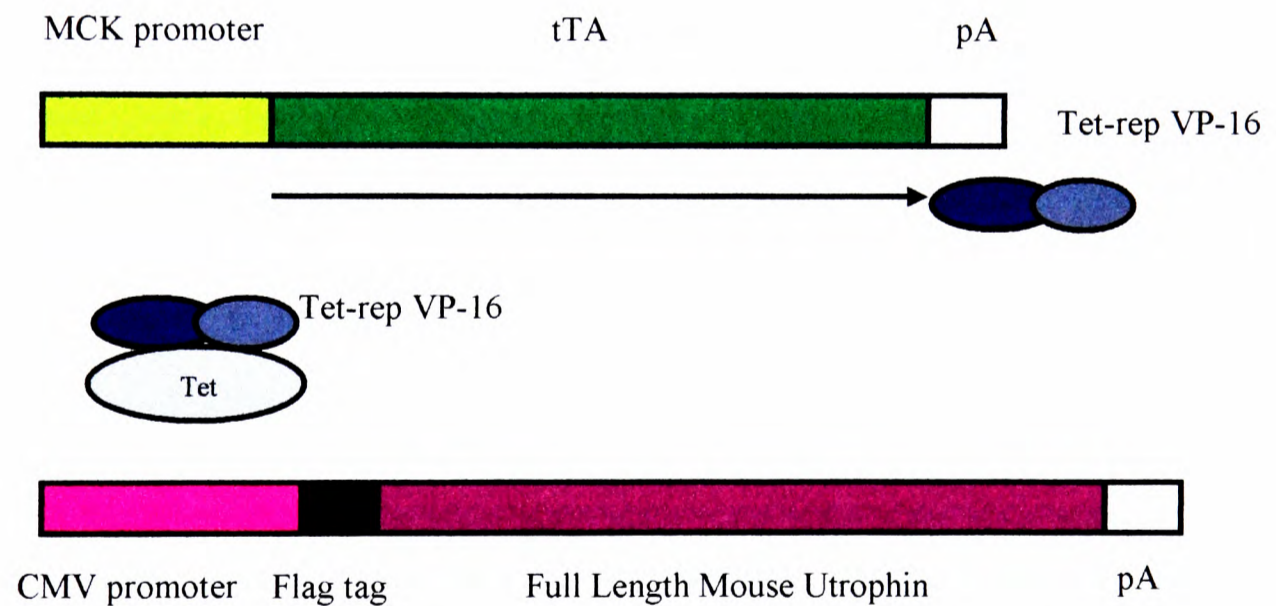


Figure 1 Schematic representation of the tet-off inducible gene expression system

The double-transgenic mice, Anna/Tex and Abe/Tex, contained both the transgenes MCK-tTA and CMV-full-length mouse utrophin. The tetracycline (Tet) is administered in the drinking water. In the absence of Tet, the transgene is expressed. In the presence of Tet, the expression of the transgene is switched off.

Key: MCK, Muscle-specific creatine kinase; tTA, tet-transactivator; pA, SV40 large poly (A); CMV, cytomegaloma virus; Tet-rep VP16, Tet-repressive transactivator VP-16 protein.

### **6.2.2 Generation of the tet-inducible utrophin transgenic mice**

Several attempts were carried out to generate the tet-inducible utrophin transgenic mice. Initially, six transgenic founders were generated from the Mouse Phase Truncated Utrophin (MPTU) cDNA constructed by Dr. Jon Tinsley. Unfortunately, the construct was found to contain a mutation and the founders were sacrificed.

An inducible transgenic mouse was made using the murine full-length utrophin (MU) cDNA with a Flag epitope tag at the 5'-end driven by the CMV promoter construct, which was a kind gift from Dr. Athena Guo, Department of Molecular Biology and Pharmacology, School of Medicine, St. Louis, USA (Guo et al., 1996). It was very difficult to generate this transgenic mouse line. Several different batches of this construct, prepared by Dr. Jon Tinsley and Mr Steve Phelps, were microinjected at various periods and only one transgenic founder, Tex, was generated, giving a transgenesis rate of 1% at this second attempt. Recently, a third attempt using the same cDNA to generate more tet-inducible utrophin transgenic mice failed to generate any more transgenic founders. The reason for the difficulty of generating this particular transgenic mouse was not known. Table 1 shows the record of microinjection of the different constructs. 1883 microinjected embryos of the MU construct were transferred into the pseudo-pregnant females and only one transgenic line, Tex, was generated. Tex was bred onto the *mdx* background to produce CMV-full-length utrophin/*mdx* mice. This was to facilitate the future breeding of the double-transgenic/*mdx* mice for analysis

Transgene construct	MPTU	MU (1)	MU (2)
No. of microinjected embryos transferred...	398	1038	845
No. of pups born.....	114	117	169
% of successful implantation.....	28%	11%	20%
No. of pups survived up to 3 - 4 weeks.....	103	102	139
% of survival up to 3 - 4 weeks.....	90%	87%	82%
No. of transgenic lines.....	6	1	0
% Transgenesis.....	6%	1%	0%

Table 1 Record of generating tet-inducible utrophin transgenic mice

1883 microinjected embryos with MU construct were transferred into pseudo-pregnant females and 286 pups were born. Only one transgenic founder, Tex, was obtained.

Key: No., number; MPTU, Mouse Phase Truncated Utrophin construct; MU, mouse full-length utrophin construct.

### **6.2.3 Interbreeding of two transgenic mouse lines**

The tet-inducer mice, MCK-tTA/*mdx* was inter-bred with the CMV-full-length utrophin/*mdx* mice to generate a double-transgenic/*mdx* mouse line. These double transgenic lines are known as Anna/Tex and Abe/Tex and currently they are under investigation in the laboratory

## **6.3 Discussion**

Preliminary results show that the expression of the utrophin transgene can be induced in Abe/Tex and Anna/Tex lines. The immunofluorescent localisation of utrophin in EDL of Abe/Tex and the *mdx* mice was carried out using the rod-domain utrophin specific antibody, URD40, as described in chapter 2. The Abe/Tex line showed the expression of the utrophin transgene could be induced (see figure 2). The sarcolemma was brightly stained compared to that of the *mdx* EDL where only the newly regenerated fibres were stained showing that the transgene correctly localised to the sarcolemma. These experiments are ongoing. Further discussion of this experiment is given in chapter 7.

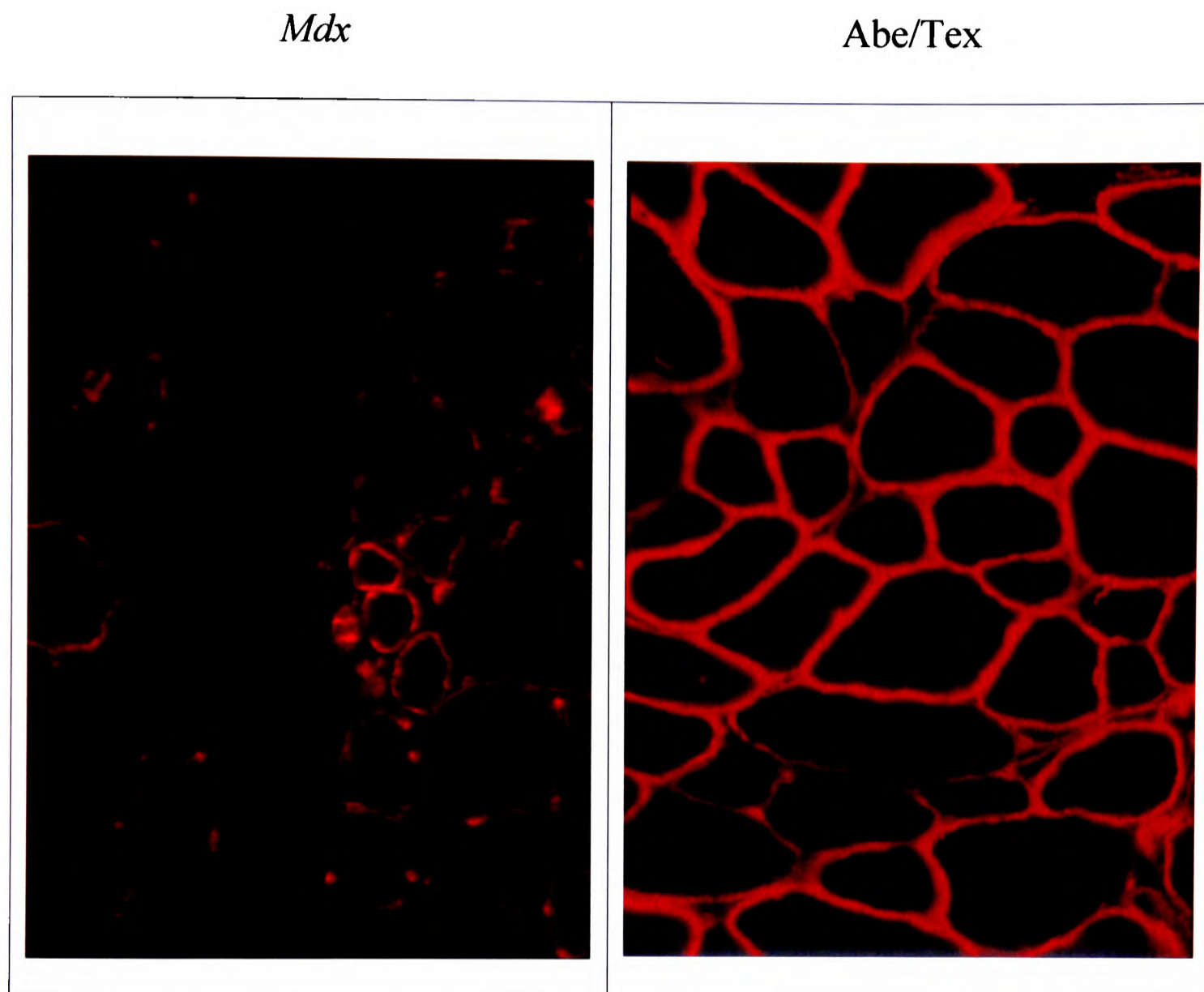


Figure 2 Induction of utrophin expression in *Abe/Tex* line

Immunofluorescent localisation of utrophin in EDL of the *mdx* mouse and *Abe/Tex* line. The *Abe/Tex* line showed the expression of the utrophin transgene could be induced. The sarcolemma was brightly stained compared to that of the *mdx* EDL. Magnification: 200x; done in collaboration with Mrs. S. Squire and Mrs. A. Potter. The mice used in this experiment were between 2 to 4 months old.

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## Chapter 7

# Summary, discussion and future work

### 7.1 Introduction

The transgenic work described in this thesis contributes significantly to the realisation of replacing dystrophin with utrophin in the gene therapy of Duchenne muscular dystrophy. The data presented here indicate that utrophin can replace dystrophin structurally and functionally in the *mdx* mouse.

### 7.2 Discussion

The points of detailed discussion concerning the experimental data have been covered in each individual chapter. This section discusses how this work as a whole relates to other work in the field.

#### **7.2.1 Comparison of truncated utrophin to truncated dystrophin minigenes**

Previous work on truncated dystrophin up-regulation in the *mdx* mouse demonstrated that over-expression of truncated dystrophin was able to partially prevent the development of dystrophic morphology and effect partial functional improvement in

the skeletal muscle (Cox et al., 1993; Rafael et al., 1994; Phelps et al., 1995; Wells et al., 1995; Rafael et al., 1996). The present work showed that over-expression of truncated utrophin in the *mdx* mouse was able to localise to the sarcolemma of the skeletal muscle and to re-establish the dystrophin-associated protein complex (DPC) to the sarcolemma of the skeletal muscle fibres. This resulted in a significant improvement of the dystrophic muscle morphology similar to that shown by truncated dystrophin, that is, the percentage of centrally nucleated fibres (%CNF) was decreased and the H&E histological analysis showed decreased fibrosis and cellular infiltrations.

Here, it was demonstrated importantly that the over-expression of a truncated utrophin minigene led to major functional improvement in the *mdx* muscle. Both the normalised force and the eccentric contraction improved significantly. The resistance to eccentric contraction is a critical test for evaluating the success of a potential therapy in the treatment of dystrophin-deficient muscle. Here, it was shown that over-expression of a utrophin minigene was able to resist eccentric contraction as seen by minimal muscle damage from the orange procion staining. If the specific force of the diaphragm of the high and low dystrophin minigene expressing lines are converted to recovery scores, 60% and 77% are obtained respectively (Phelps et al., 1995). These values are very similar to the values obtained for the utrophin minigene transgenic mice. Therefore, the utrophin minigene is able to improve the mechanical performance of the *mdx* mouse as well as the dystrophin minigene. Therefore, utrophin minigene can functionally replace dystrophin in the *mdx* mouse.

The total calcium content in the muscle of the utrophin minigene transgenic mice decreased to approximately similar levels to normal controls. However, this is

difficult to interpret as the increase in the calcium content in the *mdx* and DMD muscles is not well understood (Haws and Lansman, 1991; McArdle et al., 1994). The intracellular calcium ion concentration was measured before and after hyposmotic-hypercalcic challenge. This challenge revealed the poorer regulation of the *mdx* muscle as compared to normal and utrophin transgenic mouse muscle. The ability of the utrophin transgenic muscles to regulate intracellular calcium ion concentration was close to normal. Here, it is shown that the over-expression of a utrophin minigene was able to handle all aspects of calcium regulation: total calcium content, basal intracellular calcium ion concentration level and intracellular calcium ion concentration. The average values obtained were not significantly different from those of the normal muscle preparations. Therefore the over-expression of a truncated utrophin minigene should prevent the initiation of the muscle degenerative process, as a loss of calcium homeostasis has been implicated in activating calcium-dependent proteolysis in dystrophin-deficient muscle (Turner et al., 1988; Franco and Lansman, 1990; Turner et al., 1993).

It was observed that in the very high expressing lines, George and Gavin, the transgene diffused into the cytoplasm in some fibres of TA muscle giving a mosaic pattern. It was postulated that the binding sites at the sarcolemma were saturated and the excess utrophin transgene diffused into the cytoplasm. Why only some fibres transcribed/translated more utrophin transgene than others in the same muscle tissue was not clear. It could be due to different muscle fibre types, for example fast and slow twitch fibres that responded differentially to the HSA promoter. The levels of transgene expression could depend on how active the different cell types were, expression might be transient and the degradation rate of the transgene might be different in different fibre types. It would be interesting to find out the different

muscle fibre types that expressed very high levels of the transgene in TA of George and Gavin mice. This would provide the vital information as to which promoter to use in what muscle fibres in the up-regulation of utrophin.

### **7.2.2 Comparison of full-length utrophin to full-length dystrophin**

Previous work on the over-expression of full-length dystrophin in the *mdx* mouse showed that full-length dystrophin was better at improving the dystrophic phenotype and mechanical performances than the truncated dystrophin minigene (Cox et al., 1993; Phelps et al., 1995; Wells et al., 1995). Here, comparable results were obtained for the over-expression of full-length utrophin in the *mdx* mouse. The full-length utrophin was shown to localise to the sarcolemma of the muscle fibres and re-establish the DPC to the sarcolemma as well as the full-length dystrophin. The prevention of the dystrophic muscle phenotype depended on the amount of the utrophin transgene expression. The recovery scores of all the parameters tested, the escape test, percentage procion orange, percentage force drop, peak force, normalised force and percentage CNF in skeletal muscle, was 100% in the high expressing line. The recovery scores of the truncated utrophin transgenic mice were between 50% and 85%. Western blot analysis showed that expression levels in the full-length high expressing line was 30% to 50% of the truncated utrophin expression level. Thus, the full-length utrophin was better than the truncated utrophin in ameliorating the dystrophic morphology. The expression levels needed to effect full recovery of the dystrophic phenotype were determined at only 25% and 50% of the normal utrophin expression levels seen in lung and kidney of normal mice respectively. Thus, in skeletal muscle, the utrophin expression level needs to be up-regulated to the normal levels found naturally in lung and kidney to prevent muscle dystrophy and restore muscle strength. In the full-length dystrophin experiment, the investigators had shown

that expression levels of 20% to 50% of the endogenous control levels in different groups of muscles was sufficient to prevent essentially all the dystrophic symptoms (Phelps et al., 1995). Therefore, the therapeutic effects of full-length utrophin relative to full-length dystrophin are very similar. However, these two experiments could not be compared directly, as the promoters used were different. The timing and contribution of the two promoters were different. The MCK promoter was used in the dystrophin experiment (Jaynes et al., 1986; Johnson et al., 1989; Amacher et al., 1993). The HSA promoter was used in the utrophin experiment (Muscat and Kedes, 1987; Brennan and Hardeman, 1993). The MCK promoter contributed differentially to muscle creatine kinase activity in skeletal and cardiac muscles. The use of MCK promoter resulted in up to 50-fold greater than normal control levels but significant amount of mosaic dystrophin expression was also observed (Cox et al., 1993; Phelps et al., 1995). This mosaic expression of the transgene made it difficult to interpret the experimental results. The HSA promoter promotes strong specific transgene expression uniformly in the skeletal muscle. The uniformity of the transgene expression is an important criterion for the success of a therapy. Previous studies demonstrated that uniformly expressed dystrophin was more effective than the overall level of the transgene expression (Phelps et al., 1995). Low levels of uniform dystrophin expression resulted in a better rescue of the dystrophic pathology than in animals expressing high levels of dystrophin in variable and non-uniform pattern. However, in transgenic experiments, the non-uniformity of the transgene expression is not unusual and the reasons for it is not fully understood, but it could be due to the integration site of the transgene.

On the whole, the full-length utrophin transgene is as effective in the rescue of the dystrophic phenotype and in the restoration of the muscle strength as the full-length

dystrophin. Therefore, the full-length transgenes would be more suitable than the truncated transgenes to be considered in DMD gene therapy but the minigene may be easier to be packaged into viral vectors.

### **7.2.3 Ubiquitous over-expression of utrophin is non-toxic**

The over-expression of the full-length utrophin under the control of the ubiquitin C promoter in a broad range of tissues was not deleterious to the *mdx* mouse. Homozygous Cleo was bred to increase the transgene dose for the toxicity study. However, it was observed that in this experiment, the transgene dose did not always double in all tissues. The transgene dose in lung, kidney, tongue and stomach was double that of the heterozygous Cleo, while in other tissues the levels were increased slightly, but that of the heart was three times the Cleo level in the age-matched mice tested. The H&E data showed that, the over-expression of utrophin transgene at levels, which prevented the muscle pathology, was not toxic in the 20 to 24 tissues investigated. The %CNF in TA, EDL and diaphragm was greatly reduced. The densitometry readings of TA showed that there was an average of 25% and 60% increase of the transgene expression over the *mdx* endogenous utrophin expression in the Cleo and HCleo lines respectively. These increase resulted in improved dystrophic morphology and reduced %CNF. These results showed that there was a positive correlation between the transgene dose and morphological rescue.

Masson staining analysis showed that both the TA and diaphragm of Cleo showed a lesser amount of fibrocollagenous and cellular infiltrations, lesser amount of degeneration and regeneration as seen by the fibre size and presence of centralised nuclei compared to the *mdx* mice. The levels of transgene expression in the muscles

and diaphragm of Cleo resulted in a significant improvement in the pathology of the *mdx* mouse.

Immunostaining using utrophin specific antibody confirmed that the transgene localised to the sarcolemma of the skeletal muscle fibres and re-established the DPC to the sarcolemma. In all the tissues looked at, the transgenic mouse tissues were brighter than the normal control tissues under the same immunostaining conditions, confirming that the level of utrophin was over-expressed. The distribution of utrophin was similar to that of the normal controls except in kidney; the proximal tubules were now stained, in addition to the distal tubules, glomerulus and medulla. The creatinine levels were measured in the urine samples and found to be normal, thus showing that the over-expression of utrophin did not damage the kidney.

The weight of the utrophin transgenic-*mdx* mice was maintained like the normal controls for two and a half years. While the weight of the *mdx* mice dropped by approximately 25% up to age 23 months and most of them died or had to be sacrificed due to ill health. This showed that the utrophin transgene was still conferring its therapeutic benefit in muscle even after two years of age and the over-expression of utrophin in non-muscle tissues had not resulted in premature death.

The data here suggest that tight control over utrophin expression in specific tissues might not be necessary for the correction of muscular dystrophy. As no experiment on ubiquitous expression has been carried out with dystrophin, a comparison can only be made with those experiments, which demonstrated that 50-fold over-expression of full-length dystrophin in the skeletal muscle of the transgenic-*mdx* mice was not toxic

(Cox et al., 1993; Phelps et al., 1995). It is impossible to extrapolate the muscle results to other non-muscle tissues.

One of the problems in using drugs to up-regulate a gene for therapy is the toxicity of ectopic expression in tissues other than the target tissues. Data here suggest that it might be safe to up-regulate utrophin at levels which would be of therapeutic benefit in tissues other than the targeted tissues.

#### **7.2.4 Immune response to dystrophin and utrophin**

There is considerable evidence that cell-mediated immunity can be directed against a therapeutic transgene product, particularly in the case of genetic null mutations in which the therapeutic protein is foreign to the host (Tripathy et al., 1996; Yang et al., 1996; Morral et al., 1997). Many DMD patients will not have been exposed to some or all of the epitopes of dystrophin, and therefore, mount an immune response to it. DMD patients receiving donor myoblast transplantation (Huard et al., 1992; Tremblay et al., 1993) and BMD patients after cardiac transplantation (Bittner et al., 1995) produced antibodies to dystrophin protein. Similarly, anti-dystrophin antibodies had been reported in animal studies following congenic normal myoblast transplantation (Bittner et al., 1994; Vilquin et al., 1995) or *in vivo* gene delivery using recombinant adenovirus (Yang et al., 1994; Petrof et al., 1995; Acsadi et al., 1996; Lochmuller et al., 1996; Petrof et al., 1996; Howell et al., 1998b). Up to 50% of DMD patients show between 0.2 and 4% dystrophin positive revertant fibres. These are presumed to be somatic cell back mutations (reverting to the expression of dystrophin) of the endogenous dystrophin gene and are found in DMD patients and the *mdx* mouse (Qui Lu et al., 1998; Lu et al., 2000). It was originally thought that with careful patient selection, those with revertant fibres might benefit from dystrophin replacement.

However, the presence of revertant fibres did not confer immunologic tolerance of exogenous dystrophin in *mdx* mouse (Ebihara et al., 2000). This is explained in part by the missing epitope within the back-mutated dystrophin gene product (Vilquin et al., 1995). Also, along these same lines, a BMD patient produced humoral response against a missing dystrophin epitope after cardiac transplantation with a normal dystrophin-expressing heart (Bittner et al., 1995). The immune response to dystrophin could place an important limitation on the therapeutic efficacy of dystrophin gene transfer with or without viral vectors.

More recently, naked plasmid DNA was injected into the skeletal muscle of the *mdx* mouse showed a strong cytotoxic response to human dystrophin but not to the mouse dystrophin (Ferrer et al., 2000). In another study, direct comparison of dystrophin and utrophin gene transfer effects into the *mdx* mouse using E1/E3 deleted adenovirus vectors containing either a dystrophin or a utrophin transgene was carried out (Ebihara et al., 2000). In the above study, the investigators concluded that dystrophin and utrophin were largely equivalent in their intrinsic abilities to prevent the development of dystrophic symptoms in the neonatal *mdx* mouse with an immature immune system. However, in mature mice, there was significantly greater transgene persistence and reduced inflammation with utrophin compared to dystrophin gene transfer. Therefore, the use of utrophin as an alternative gene transfer appears to offer a significant therapeutic advantage.

### **7.2.5 Timing of transgene expression**

It is important to determine when to administer therapeutic intervention in the treatment of DMD patients. Here, the timing experiments will be carried out using the tet-inducible system (Section 7.2.5.2).

### **7.2.5.1 Adenovirus-mediated utrophin/dystrophin transfer**

A timing experiment of utrophin administration has been carried out (Wakefield et al., 2000). In this study, an E1+E3 deleted adenovirus containing a utrophin minigene was injected into the hind limb muscle of utrophin-dystrophin-deficient neonatal mutant mice. Up to 95% of the fibres around the injected site were transduced and they continued to express the minigene 30 days after injection. The above study demonstrated that a utrophin therapeutic intervention in two-week-old neonatal mice afforded substantial protection against muscle necrosis. The above results correlate well with the direct injection of adenoviral mediated mini-dystrophin transgene into neonatal mice muscle (Deconinck et al., 1996).

### **7.2.5.2 Tetracycline inducible expression of utrophin and dystrophin**

The use of this inducible expression system is to allow for the expression of utrophin to be inducibly controlled, at any point throughout the mouse's lifetime, simply by the oral administration of tetracycline to the animals. The aim is to determine when is the best disease progression stage to up-regulate utrophin in future gene therapy.

Preliminary results show that the *Abe/Tex* double transgenic mice express utrophin in a tTA-dependant manner. When these double transgenic mice are fed tetracycline, the expression of utrophin is inhibited by the binding of tetracycline to the tTA transactivator. When tetracycline is removed, expression of the transgene is induced, please refer to Figure 1 in chapter 6. Therefore, this tetracycline inducible utrophin expression system is reversible.

Immunostaining of TA muscle shows that the utrophin transgene localised uniformly to the sarcolemma and the expression was stable. Now, it is established that the

tetracycline inducible expression of utrophin works in the Abe/Tex double transgenic line. When tetracycline is removed from drinking water, utrophin expression is still observed at the sarcolemma but for how long it is still being studied. This will eventually give the half-life of utrophin and will tell how long the beneficial effects of each administration would last and how often tetracycline might need to be administered.

When is the best time to induce the expression of utrophin? Experiments can be carried out to establish the induction of the expression of utrophin in the double transgenic mice at various times after birth and the results can be compared to those of the dystrophin double transgenic mice (Ahmad et al., 2000). The above study shown that *in utero* expression resulted in more dramatic muscle morphological improvement and that the early induction of the expression of dystrophin gave better rescue of several muscle groups by the counting of %CNF. Induction at 2-4 days after birth resulted in a better rescue of the dystrophic phenotype than that of more advanced age of 4-5 weeks or after 30 weeks of age. These last two groups did not significantly prevent, nor reverse, the morphological evidence of dystrophy.

How long could utrophin maintain normal muscle morphology after termination of gene transcription? Ahmad et al. (2000) gave tetracycline at different ages after birth to inhibit somatically tTA dependent dystrophin gene transcription, and found that the dystrophin mRNA transcript decreased confirming that the absence of tet-operator derived dystrophin RNA transcription during tetracycline treatments. However, *in situ* immunofluorescent staining showed that dystrophin protein persisted at the sarcolemma six months after the expression of the transgene was switched off. The persistent protein may allow for a “long-term” functional protection of muscle fibres,

once significant amounts of dystrophin protein expression have been successfully initiated. Here, in a similar tetracycline treatment regime, the “long-term” benefits of utrophin protein have yet to be established.

There were several drawbacks in the experiments of Ahmad et al. (2000). The level of expression of dystrophin was low and only one line provided adequate levels of dystrophin expression in this study. Despite the use of MCK enhancer/promoter, the investigators were not able to isolate a double transgenic line that consistently achieved 100% rescue of the dystrophic morphology. The tet-inducible system is believed to be slightly “leaky”, that is, a very low level of the transgene is being transcribed all the time. Thus extremely low levels of the tet-operator-derived transgene transcription, coupled with a relatively low turnover rate, may influence the observed results of Ahmad and colleagues. The utrophin tet-inducible system may suffer from the “leaky” system as well. It was also noted that a significant amount of mosaic dystrophin expression in the various muscle groups of the double transgenic mice, both within the same muscle groups of individual mice as well as between transgenic littermates. These drawbacks diminished the statistical significance of the experimental observations. However, this study provided information with regard to the appropriate timing for up-regulation of dystrophin. That is, *in utero* expression of the transgene resulted in more dramatic improvement in the muscle morphology, in contrast to induction of dystrophin expression within the first few days after birth.

### **7.2.6 Up-regulation of utrophin isoforms in DMD**

Recent work demonstrates that there are four different 5' utrophin promoters driving the expression of at least two if not four full-length utrophin isoforms (Dennis et al., 1996; Gramolini et al., 1997; Burton, 1999; Burton et al., 1999). Promoter A contains

an N-box (sequence found in many genes that are expressed in synapses) and an E-box (sequence binds to MRFs like myoD, myogenin and MRF-4) and is synaptically regulated (Gramolini et al., 1997). Promoter B does not contain a N-box or an E-box (Burton, 1999). Promoters C and D are being characterised in the laboratory at the moment (Perkins, 2001). Promoters A, B, C and D are independently regulated and have distinct expression patterns. Promoters A and B are independently regulated in different types of cells and each has its own pattern of expression. Any attempt to up-regulate utrophin expression, which might involve the up-regulation of other components of the NMJs that are involved in signalling cascades, could disrupt the structure and function of NMJs. To avoid the disruption of NMJs, B, C or D isoforms of utrophin may prove a more attractive target for therapeutic up-regulation. Alternatively, up-regulation of A-utrophin by a different pathway, via the E-box, may be considered. *In vivo* analysis of promoter A and B driving the expression of Lac-Z in transgenic mice to find out whether the promoters contain all the important regulatory fragments, is being carried out in the laboratory at the moment (Weir, 2001).

One strategy for the up-regulation of utrophin is by small pharmacological molecules. Promoter A has been used in high throughput systematic screening by OSI Pharmaceutical Inc. to identify these small molecules (Dennis et al., 1996). In these assays, cis-acting elements for the utrophin gene are linked to a reporter gene, for example, luciferase. The compounds are tested for their ability to increase reporter gene expression in tissue culture using the dystrophin deficient H2K cell line. A small number of drugs were found to increase the expression of utrophin and these drugs are being tested in our laboratory in tissue culture at the moment. The inclusion of other

utrophin promoters (B, C and D) in the screening programme would increase the yield of small molecules.

Which promoter is responsible for the expression of utrophin in the heart? Absence of dystrophin from the heart is associated with cardiomyopathy (de Kermadec et al., 1994; Boland et al., 1996; Melacini et al., 1996a; Sasaki et al., 1998; Ferlini et al., 1999). Both dystrophin and utrophin are expressed in the normal heart muscle (Byers et al., 1991; Bies et al., 1992; Chevron et al., 1994; Pons et al., 1994). Therefore, the search for utrophin cardiac promoter would be beneficial for the therapy of cardiomyopathy in DMD.

### **7.3 Gene therapy versus small molecule therapy**

At the moment there is no cure for DMD, only supportive management. The isolation of the gene contributed to the development of current screening methods. However, one third of the mutations are due to new spontaneous mutations. Therefore, the development of post-natal therapeutic strategies is crucial. The central argument in favour of gene therapy is that it can treat desperately ill patients, or prevent the onset of horrible illness, or convert the DMD phenotype to that of BMD. Conventional treatment has failed for these patients and gene therapy may be the only hope for a future.

Effective prevention of dystrophic symptoms is dependent on at least 50% of the fibres in each muscle group expressing dystrophin (Hauser et al., 1997). The delivery of the dystrophin gene to 50% of the muscle cells of the body is a formidable challenge and dystrophin may provoke immune response in patients who has not seen

dystrophin before. Therefore, the delivery of a non-immunogenic protein like utrophin might be a better proposition.

Chamberlain and colleagues have shown that about 20% of the wild type dystrophin protein levels will be needed for therapeutic benefit by introducing dystrophin minigenes into the *mdx* mouse (Phelps et al., 1995). However, 50 times over-expression does not seem to be detrimental (Cox et al., 1994). The data here show that only 50% and 25% of the normal physiological utrophin expression levels seen in kidney and lung respectively are needed to have therapeutic effect in the *mdx* mouse. The ubiquitous over-expression of utrophin in *mdx* mouse is not toxic. Thus, a precise control of the levels of the gene might not be necessary for the correction of muscular dystrophy. Thus the search for small molecules to up-regulate utrophin expression represents a promising approach to improving therapy for DMD patients. The amount of utrophin up-regulated must be sufficiently high enough to effect continual benefits into old age.

Early studies for the delivery of dystrophin using direct injection of dystrophin plasmids has been attempted (Wolff et al., 1990; Wolff, 1997), However, the transfection of skeletal muscle by this technique is inefficient except in neonatal muscle of the *mdx* mouse (Acsadi et al., 1991; Danko et al., 1997; Howell et al. 1997; Howell et al. 1998a). The plasmid must be injected into a sufficiently high proportion of the muscle fibres to effect any phenotypic recovery (Acsadi et al., 1991). This technique does not seem like a viable method of gene delivery and also ethically unacceptable to inject the plasmid into the skeletal muscle of the DMD patients at multiple sites. The use of adenovirus and AAV viral vectors to introduce the dystrophin transgenes into the skeletal muscle did not prolong the expression of the

transgene significantly, due to the immune responses to the transgenes and the viral vectors (Tremblay et al., 1993; Acsadi et al., 1996; Howell et al., 1998b; Ferrer et al., 2000). Transplantation of myoblasts appeared to be inefficient and short lived in DMD boys, either with or without immuno-suppressants like cyclosporin A, prednisone and cyclophosphamide (Gussoni et al., 1992; Law et al., 1992; Karpati et al., 1993a; Tremblay et al., 1993; Miller et al., 1997). The short-term dystrophin expression was due to immune responses to dystrophin and a range of other proteins after myoblast transfer (Tremblay et al., 1993). The efficiency of delivery of the above techniques was low and the resultant immune response resulted in short lived expression of the dystrophin transgene. An improvement in both gene delivery and levels of expression are needed before any of these techniques can be used clinically. Subcutaneous injection of gentamicin resulted in 10-20% of normal level increase in dystrophin protein in the skeletal muscle (Barton-Davis et al., 1999a). This treatment could benefit up to 15% of DMD patients with premature stop codon mutations. However, gentamicin is very toxic to the inner ear hair cells and kidney, so, the clinical use of this drug is doubtful.

However, the use of small molecules or drugs to increase endogenous utrophin gene expression could be a more likely method in the therapy of DMD. Results in this thesis show that utrophin can replace dystrophin structurally and functionally and that full-length is better than truncated utrophin and also the over-expression of utrophin is not toxic in the *mdx* mouse. A drug may promote the over-expression of endogenous full-length utrophin in all tissues that expressed utrophin but it will not lead to toxicity problems.

There are two problems involving the use of a small drug to up-regulate utrophin for therapy. Firstly, the drug may be toxic due to general toxicity including effects on other gene promoters. Secondly, the toxicity of ectopic utrophin expression in tissues other than the target tissues. In the case of utrophin, the protein is expressed in all normal tissues but to a varying degree. Data here show that the over-expression of utrophin in tissues other than muscle is not toxic in the *mdx* mouse; therefore, the general up-regulation of utrophin in all tissues may not be deleterious.

## 7.4 Conclusion

The data in this thesis shows that utrophin can replace dystrophin structurally and functionally and also the ubiquitous over-expression of utrophin in the *mdx* mouse is not toxic. Therefore, the choice of utrophin for gene therapy of DMD patients is a better strategy than dystrophin. The use of utrophin would circumvent the immune response that accompanied the administration of dystrophin because the utrophin gene is intact in DMD patients and that its transcript generates the utrophin protein already known to the immune system. Thus the search for small molecules to up-regulate utrophin expression represents a promising approach to improving therapy for DMD patients.

The work described here proved the hypothesis that utrophin could replace dystrophin structurally and functionally and it has been adequately tested through the generation of a range of transgenic mice containing truncated and full-length utrophin as confirmed by the data presented here.

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## Chapter 8

# Future work

The work described here strongly supports the hypothesis that utrophin could replace dystrophin. Therefore, up-regulation of utrophin may indeed be a plausible therapeutic target for Duchenne muscular dystrophy, but more detailed information would be helpful on several aspects:

1. TA, EDL and diaphragm muscle from 18-month old transgenic mice of the HSA-truncated, HSA-full-length and Ubiquitin C full-length experiments need to be analysed, in order to learn whether aged transgenic mice still express the utrophin transgene and that it is localised at the sarcolemma correctly.
2. The percentage of centrally nucleated fibres of TA, EDL and diaphragm of the 18-month old transgenic mice must be assessed to confirm that the utrophin transgene still exerts its therapeutic effects at old age.
3. In order to find out whether the transgene could maintain the functional benefits in old transgenic mice, 18-month old mice could be subjected to the mechanical force experiment.

4. As shown in Figure 14, Chapter 5, the weight of *mdx* mice remains the same as that of the normal controls for the first six months of life, but thereafter decreases by approximately 25%, despite hypertrophy. Transgenic-*mdx* muscles also show hypertrophy, suggesting that utrophin up-regulation may not alter this feature of the dystrophic process. It would be interesting to examine this discrepancy by directly comparing the weights of individual muscle groups (e.g. TA, EDL and soleus) as the muscle of *mdx* mice are for long period of their lives bigger than normal mice (Coulton et al., 1989).
  
5. Individual TA muscle fibres of the high expressing lines, George and Gavin, expressed different levels of the transgene. Knowing whether these differences are unique to the fast or slow twitch fibres may hold implications regarding the therapeutic success of utrophin up-regulation. Several methods exist to differentiate between the slow and fast twitch fibres. These include the myosin ATP-ase method (Brooke and Kaiser, 1970a, b, c) and the combined method, in which the myosin ATP-ase reaction is used to differentiate between fast and slow twitch muscle fibres, NADH-tetrazolium reductase activity can further identify the subgroups of fast twitch fibres (Ashmore and Doerr, 1970).

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## Appendix

### In Press and Published Work:

- Fisher, R., Tinsley, J. M., Phelps, S. R., Squire, S. E., Townsend, E. R., Martin, J. E., and Davies, K. E. (2001). "Non-toxic ubiquitous over-expression of utrophin in the mdx mouse." *Neuromuscular Disorders*. 11(8):713-721.
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## Non-toxic ubiquitous over-expression of utrophin in the *mdx* mouse

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### Abstract

Duchenne muscular dystrophy (DMD) is an inherited, severe muscle wasting disease caused by the loss of the cytoskeletal protein, dystrophin. Patients usually die in their late teens or early twenties of cardiac or respiratory failure. We have previously demonstrated that the dystrophin related protein, utrophin is able to compensate for the loss of dystrophin in the *mdx* mouse, the mouse model of the disease. Expression of a utrophin transgene under the control of an HSA promoter results in localization of utrophin to the sarcolemma and prevents the muscle pathology. Here we show that the over-expression of full-length utrophin in a broad range of tissues is not detrimental in the *mdx* mouse. These findings have important implications for the feasibility of the up-regulation of utrophin in therapy for DMD since they suggest that tissue specific up-regulation may not be necessary. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Dystrophin; Utrophin; Duchenne muscular dystrophy; *mdx* mouse

### 1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive progressive muscle disorder affecting 1 in 3000 new-born males. Patients are generally wheelchair bound by about the age of 10 and death ensues in late teens or early twenties [1]. At present, there is no effective treatment for this devastating disease. Although prenatal diagnosis is available, the disease has a very high new mutation rate resulting in the occurrence of the disease in families with no previous history of DMD. Thus the development of therapeutic protocols is essential.

The DMD gene, encoding the protein product dystrophin, is located at Xp21 and spans 2.4 Mb of genomic sequence and contains at least 83 exons including seven independent promoters (for review see [2]). The full-length gene is expressed in muscle with smaller amounts in brain [3]. Defects in this gene give rise to DMD and the milder form of the disease, Becker muscular dystrophy (BMD). In DMD patients, dystrophin is much reduced or completely absent, while in BMD patients a partially functional dystrophin molecule is expressed. Dystrophin is localized to the cytoplasmic surface of the sarcolemma of adult skeletal

muscle and is associated with a large oligomeric protein complex known as the dystrophin-associated protein complex (DPC) [4–6]. Dystrophin forms a link between the myofiber cytoskeleton and the extracellular matrix. The DPC consists of transmembrane proteins, the dystroglycans, sarcoglycans and the cytoplasmic proteins, syntrophins and dystrobrevins [7–10] for reviews [11,12]. Immunostaining of muscles from DMD patients or the *mdx* mouse model of the disease, shows that loss of dystrophin leads a selective reduction or absence in the staining of the DPC [4].

The dystrophin related protein, utrophin, is a ubiquitously expressed transcript encoded by a gene on the long arm of chromosome 6 [13]. There is approximately 80% similarity in amino acid sequence between the dystrophin and utrophin actin and DPC binding domains and they have similar binding partners [14,15]. In mature muscle fibres, utrophin is localized to the cytoplasmic face of the neuromuscular and myotendinous junctions, nerves and capillaries [16–18] but in developing and regenerating muscle it is found at the sarcolemma [16,19].

The *mdx* mouse is an X-linked myopathic mutant, an animal model for human DMD. There is a mutation in exon 23, which abolishes the production of full-length dystrophin [20]. These mice display a range of dystrophic symptoms similar to DMD including fibrosis and phagocytic infiltration of skeletal muscle tissue, variation in myofi-

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ber size, increased numbers of centrally nucleated fibres which are indicators of muscle degeneration and regeneration [21,22] and elevated serum creatine kinase (CK) levels [23]. We have previously demonstrated that the continual presence of utrophin at the muscle fibre membrane prevents the development of muscular dystrophy in *mdx* mice transgenic for truncated and full-length utrophin under the control of the human skeletal actin promoter [24–26]. If the utrophin level is high enough, the mechanical properties and the intracellular calcium homeostasis in the transgenic dystrophin-deficient *mdx* mouse muscle are completely normal showing that utrophin is able to replace dystrophin.

Since DMD patients already have a functional utrophin gene, we have proposed the use of small molecules capable of up-regulating utrophin expression, as a viable alternative approach to the therapy for DMD, (for reviews, see [27,28]). The strategy of replacing dystrophin with utrophin has the advantage of avoiding the problems surrounding gene delivery to the muscle. The data presented in this paper show that over-expression of utrophin in non-muscle tissues has no deleterious effects in the *mdx* mouse. This suggests that a small drug therapy may be a safe approach to the treatment of DMD even if utrophin is upregulated in a non-tissue specific manner.

## 2. Materials and methods

### 2.1. Transgene constructs and transgenesis

The complete utrophin transgene [26] was cloned into an expression vector containing the 1.2 kb human ubiquitin C promoter and regulatory regions [29]. Flanking *NotI*/*Asp718I* restriction endonuclease sites were used to liberate the complete fragment for microinjection. The transgene was purified and microinjected into one pronucleus of F2 (C57BL/6 × CBA/CA) zygotes, using standard techniques [30,31]. Nine transgenic founders were generated: one founder was sub-fertile and eight founders were fertile and transmitted the transgene in a Mendelian fashion. Transgenics were identified by Southern blotting, using a probe to the central part of the utrophin transgene. Transgenic lines were initially established and maintained by breeding with *mdx* partners. The normal transgenic founders do not have any phenotype.

To increase the transgene expression levels, heterozygous mice from the Cleo line (Cleo) were bred to homozygosity (HCleo). Homozygote offspring were identified by Western blotting. Potential homozygotes were then bred with normal F1 non-transgenics to confirm homozygosity.

Three to six age-matched (3–3.5 months old) mice from each group of the normal (N) F1 (C57BL/6 × CBA/CA), *mdx*, Cleo and HCleo were used for histology, including the central nucleation count at this time point, immunohistochemistry and urine test. All the normal (N) mice used here was of the F1 (C57BL/6 × CBA/CA) genetic background.

### 2.2. Protein analysis

Tissues were collected from two 4-week-old mice per group of N, *mdx*, Cleo and HCleo for protein analysis. Total protein extracts were prepared by homogenisation of the tissues in half or 1 ml extraction buffer (75 mM Tris, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol, 5% 2-mercaptoethanol). The protein concentration was measured using a Biorad Bradford protein assay kit (from BioRad, Richmond, California), in triplicate in a spectrophotometer and diluted into a constant volume of 20 µl containing either 25 or 50 µg of protein extract to minimize the loading variation. Serial dilutions of the protein extracts of heart, lung, diaphragm and kidney were made to estimate the amount of the over-expression of the utrophin transgene. Usually 50 µg of total protein was loaded onto 6% polyacrylamide gels and transferred to nitrocellulose membrane. The utrophin transgene was detected using a 1/400 dilution of mouse anti-utrophin monoclonal antibody (Mancho 3 [17]) and visualized using anti-mouse HRP (Jackson) and chemiluminescence (Boehringer). Desmin monoclonal antibody (Sigma, DE-U-10) was used to confirm equal loading of the protein sample. At the manufacturer's recommended concentration, anti-desmin bands were also observed in non-muscle tissues, for example, lung and kidney.

### 2.3. Histology

Tissues were collected from age-matched mice and sent to independent veterinary pathologists for a comparative morphological study to detect any morphological changes potentially caused by the over-expression of the utrophin transgene. Between 20 and 24 different tissues were collected from each mouse. The following tissues were collected: tibialis anterior (TA), extensor digitorum longus (EDL), muscle, heart, lung, liver, diaphragm, intestine, brain, kidney, adrenal gland, spleen, stomach, tongue, testis, skin, oesophagus, trachea, eye, thymus, bone, uterus, blood and urine. The sections of the tissues were stained with haematoxylin and eosin (H&E) for examination. Tissues for immunohistochemistry were also collected, immersed in OCT compound (BDH) and frozen in liquid nitrogen cooled isopentane. Frozen, unfixed cryosections of 10 µm were cut and stored at  $-80^{\circ}\text{C}$ . For assessing the percentage of centrally nucleated fibres (%CNF), sections were stained with haematoxylin and eosin and four complete sections were counted per muscle per mouse. Sections used to visualize the progressive infiltration of fibrocollagenous tissues were stained progressively with Hansen's haematoxylin such that the collagen was green while the cytoplasm was still orange/red.

### 2.4. Immunohistochemistry

Immunostaining was performed by air drying the cryosections at room temperature and blocking them in 10% normal donkey serum in TBS (50 mM Tris, 150 mM

NaCl, pH 7.5). The primary antibodies used were all rabbit polyclonal and the following dilutions in TBS were used: utrophin (URD40, 1:500), dystrophin (2166,1:1000),  $\alpha$ -dystrobrevin ( $\alpha$ 1-CT-FP, 1:1000 [32]), Dp71 (Alt-C, 1:100), and  $\alpha$ -sarcoglycan (1:200). The primary antibodies were incubated for 2 h then washed in TBS. Cy-3 conjugated rabbit secondary antibody (1:500 dilution, Jackson) was incubated for an hour at room temperature. The slides were mounted with Vecta Shield (Vector) and photographed under identical conditions of illumination and exposure as shown in Fig. 4.

### 2.5. Urine test

The urine samples were tested for the levels of creatinine using proton nuclear magnetic resonance spectroscopy. Defrosted samples were mixed well and placed in a cuvette and analyzed, using a 400, 500 or 600 MHz machine with a standard protocol. A representative spectrum was obtained and analyzed with reference to control spectra obtained from the experimental controls and from the database comprising a range of urine samples from several mouse strains (Swr, C57BL/6, balb/c, C3H). Known substance standards have previously been used to establish the spectral characteristics of a set of substances including trimethylamine, trimethylamine-*N*-oxide, lactate, creatinine, dimethylamine, oxoglutarate, creatine, succinate, acetate, taurine. Experimental samples (from Cleo mice) were analyzed with the operator blind to the genetic status of the animals and interpreted with reference to the height of creatinine peaks in order to compensate for possible dilution factors.

### 2.6. Weight measurement

At the start of the experiment, six mice of Cleo, *mdx* and normal were weighed at the beginning of each month, starting at 3 months old. After about 1.5 years, one *mdx* mouse died and one had to be sacrificed due to ill health.

## 3. Results

### 3.1. Analysis of transgenic lines

In order to over-express utrophin in muscle and non-muscle tissues a transgenic approach was taken using a full-length utrophin cDNA driven by the human ubiquitin C promoter to generate ubiquitous expression of the transgene in *mdx* mouse. The utrophin cDNA was identical to that previously described in preventing muscular dystrophy in the *mdx* mouse [26]. Initially, eight tissues (skeletal muscle, heart, diaphragm, lung, liver, intestine, brain and kidney) were collected from each of the nine transgenic lines between 4 and 6 weeks old to screen for high expression by Western blot analysis. Desmin antibody staining was used to control for equal loading of samples on the gels. Desmin is an intermediate filament protein and is

found predominantly in skeletal, cardiac and smooth muscle [33]. As an example of transgene expression Fig. 1a shows the total utrophin levels in heart from seven ubiquitin C utrophin transgenic lines compared to normal F1 and *mdx* tissues. The Cleo line had the highest level of expression in the heart tissue. Cleo line also had the highest level of expression in lung, liver and kidney (data not shown) and was chosen for further analysis. Fig. 1b shows the level of utrophin in liver, oesophagus, spleen, eye, adrenal gland and tracheae from Cleo compared to *mdx* mice. Utrophin levels were increased in all tissues compared to *mdx* mice ranging from a slight increase to one and a half times for diaphragm, two and a half times for lung and kidney and three times for heart.

### 3.2. Breeding to homozygosity to increase utrophin levels

Mice homozygous for the transgene (HCleo) were bred from heterozygous (Cleo) animals. The utrophin levels in lung, kidney, tongue and stomach of HCleo were approximately twice that in heterozygous Cleo (Cleo), while in other tissues like TA, diaphragm, brain, testis, and bladder the levels were increased only slightly compared to *mdx* mice as shown in Fig. 2.

### 3.3. Effect of utrophin transgene expression in Cleo-*mdx* and HCleo-*mdx* skeletal muscle

To determine whether the levels of utrophin expression in Cleo and HCleo were sufficient for therapeutic benefit, the skeletal muscle from Cleo was examined. The centrally nucleated fibres (CNF) which are the result of muscle degeneration and regeneration were counted in the TA, EDL and diaphragm muscles and compared to dystrophin-

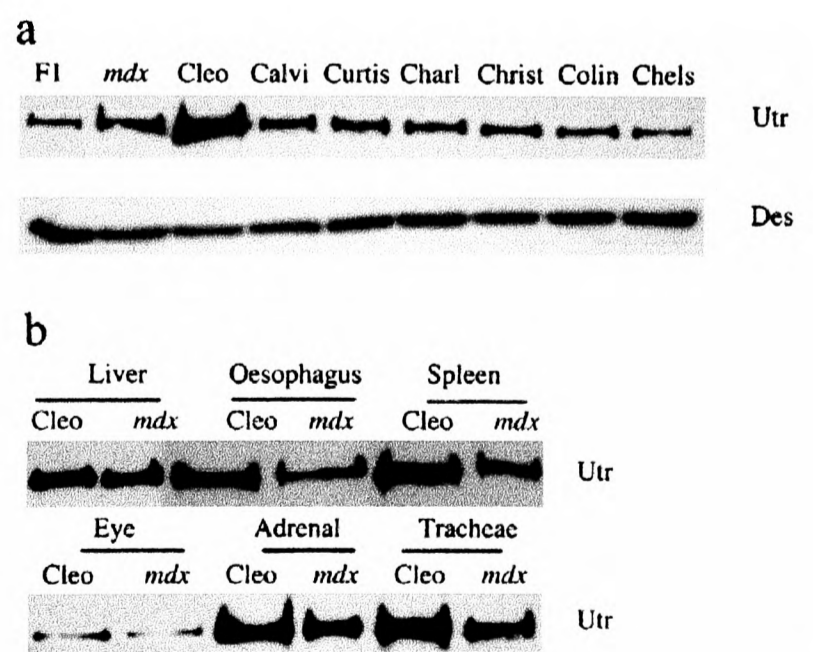


Fig. 1. Western blot analysis of utrophin in the transgenic lines generated compared to normal (F1) and *mdx* mice. (a) Protein levels in the heart of seven transgenic lines. Cleo line expressed the highest level of the utrophin transgene in the heart. Desmin was used to control for equal loading of the protein extracts. (b) Over-expression of utrophin in liver, oesophagus, spleen, eye, adrenal gland and tracheae of Cleo compared to *mdx* mice.

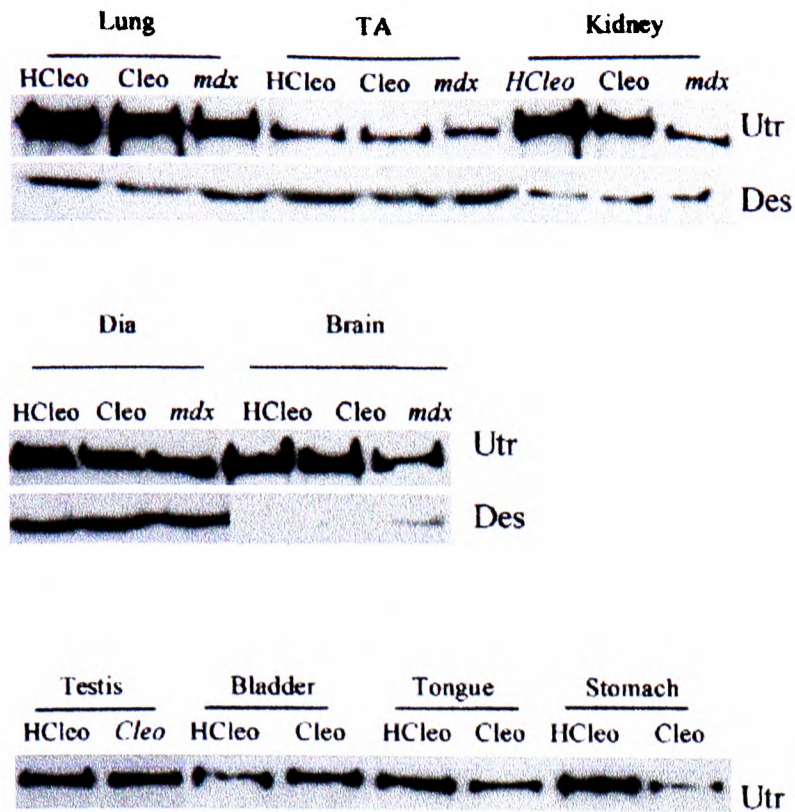


Fig. 2. The level of transgene expression in various tissues from heterozygous (Cleo) and homozygous Cleo (Hcleo) compared to *mdx* mice. The utrophin level in lung, kidney, tongue and stomach of Hcleo were approximately double that of Cleo, while TA, diaphragm, brain, testis and bladder remained the same as that of heterozygous Cleo. Desmin was used to control for equal loading of the protein extracts.

deficient *mdx* and normal muscles. Fig. 3a shows a reduction from around 80% CNF down to approximately 30% in TA and EDL from Cleo and down to approximately 13 and 5%, respectively from Hcleo. The diaphragm also shows a significant improvement from 65 down to 50% CNF from Cleo and down to approximately 25% from Hcleo. By 18 months of age TA, EDL and diaphragm of Cleo came to approximately the same amount of central nucleation as the *mdx* mouse showing substantial muscular dystrophy as shown in Fig. 3b [34]. These results in a transgenic mouse line with levels of expression that completely correct the phenotype (Fiona [26]), aged between 14 and 22 months, are included for comparison. It is worth noting that the ubiquitin C utrophin transgene was not expressed at high enough levels to maintain a rescue in the older mice as compared to the Fiona line. No data on this issue in relatively old mice are available for dystrophin transgenes. However, it is not surprising that therapy needs to administered as early as possible with maximal levels of expression for optimal benefit.

Histological analysis of Masson stained sections of TA and diaphragm of Cleo show a significant improvement of the dystrophic pathology as shown in Fig. 3c. The TA and diaphragm muscles of *mdx* mice showed irregular muscle fibres with central nuclei, cellular infiltration, fibrosis and increased fibrocollagenous tissues in common with DMD patients. The diaphragm of *mdx* mice showed more severe symptoms than that of the TA with very extensive deposition of the fibrocollagenous tissue [35]. Both the TA and

diaphragm of Cleo showed a lesser amount of fibrocollagenous and cellular infiltrations, lesser amount of degeneration and regeneration as seen by the fibre size and presence of centralized nuclei compared to *mdx* mice. The levels of transgene expression in the muscles and diaphragm of

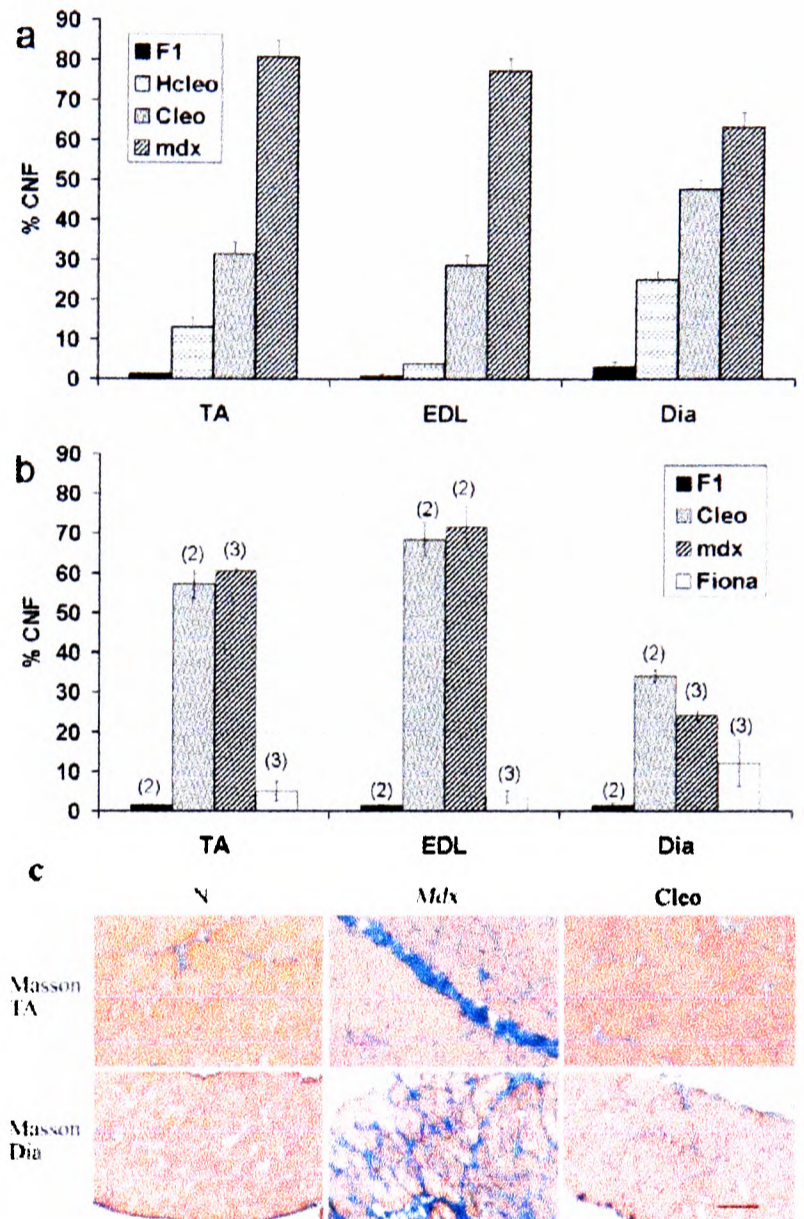


Fig. 3. Prevention of pathology by the utrophin transgene expression in muscle. (a) Percentage centrally nucleated fibres (% CNF) in TA, EDL and diaphragm (Dia) of age matched (3–3.5 months) Cleo, Hcleo, F1 and *mdx* mice. Therapeutic benefit of transgene expression resulted in a reduction from around 80% down to approximately 30% in Cleo TA and EDL, and down to approximately 13 and 5%, respectively from Hcleo. The diaphragm also shows a significant improvement from 65% down to approximately 25% from Hcleo. The bars represent the mean of 3 to 5 muscles  $\pm$  SEM. (b) The % CNF of 18 months old age-matched F1, *mdx* and Cleo. By 18 months of age the TA, EDL and diaphragm had the same amount of central nucleation. The results of Fiona which contained the HSA-full-length utrophin [26] aged between 14 and 22 months were included for comparison. The number of mice counted shown in parentheses. The bars represent the mean of two to three mice  $\pm$  SEM. (c) Histological analysis of normal F1 (N), *mdx* and Cleo mice. Sections of TA and diaphragm from age matched mice of 3–3.5 months old were stained with Masson stain to show the infiltration of the fibrocollagenous tissues (blue/green). Both the TA and diaphragm of Cleo show lower amounts of fibrocollagenous and cellular infiltrations, also decreased regeneration as seen by the uniformity of fibre size and lower numbers of centralized nuclei compared to *mdx* mice. Scale bar, 50  $\mu$ m.

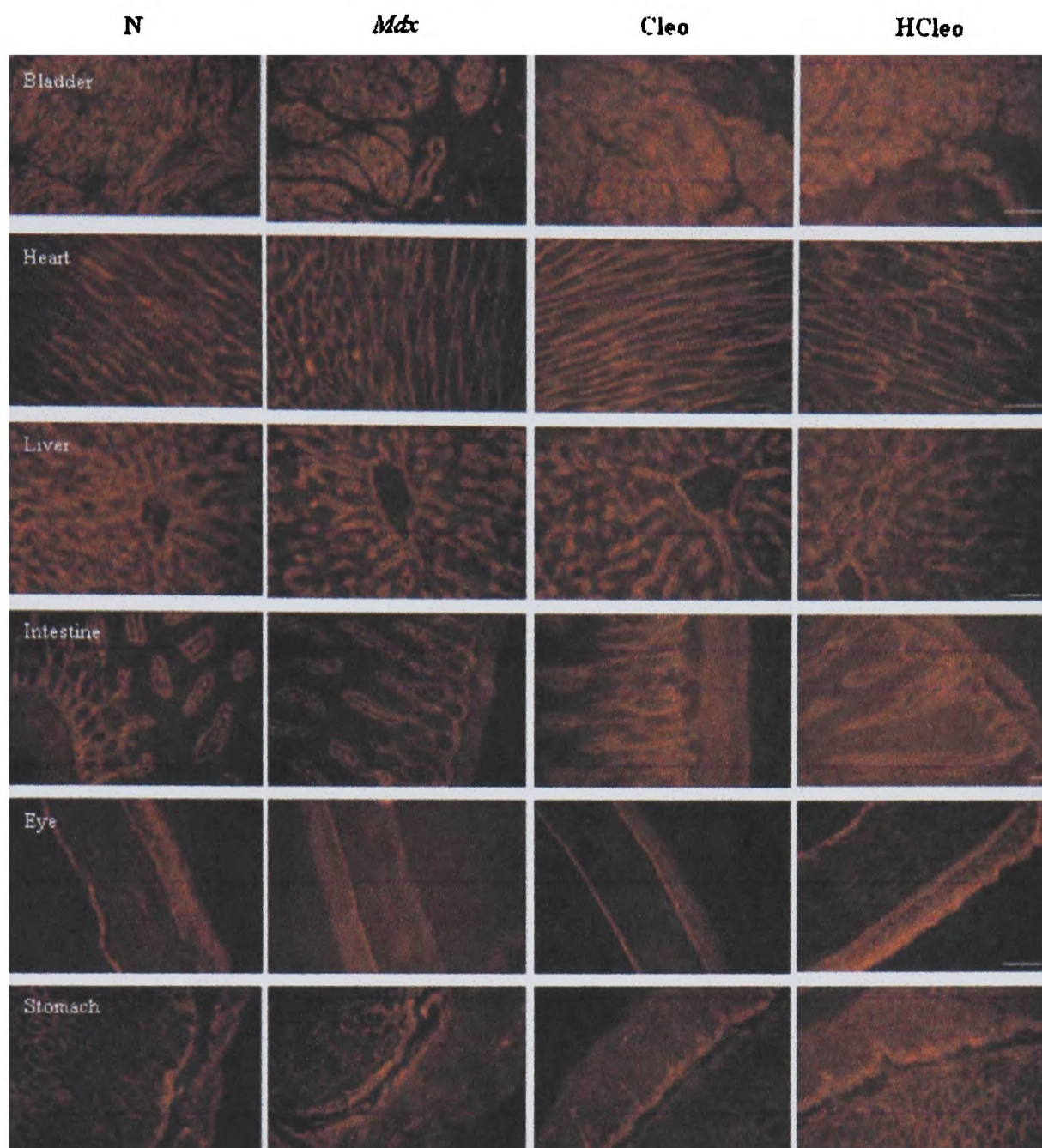


Fig. 4. Immunohistochemistry of utrophin in non-muscle tissues. Exposure times were the same for each set of antibody used to allow comparison of the intensity of staining of the samples. Immunohistochemistry of utrophin in bladder, heart, liver, intestine, eye and stomach of normal F1 (N), *mdx*, Cleo and HCleo. Increased in utrophin staining in Cleo and HCleo. Scale bar, 50  $\mu$ m.

Cleo, result in a significant improvement in the pathology of the *mdx* mice.

### 3.4. Effect of utrophin transgene expression in Cleo-*mdx* and HCleo-*mdx* non-muscle tissues

#### 3.4.1. Haematoxylin and eosin (H&E) comparative morphology

Reports from the independent veterinary pathologists did not detect any pathological changes that may have been caused by the over-expression of the utrophin transgene. Skeletal muscle and diaphragm of the *mdx* tissues showed the usual dystrophic symptoms but in the Cleo and HCleo sections the dystrophic symptoms showed a quantitative improvement. Both the TA and diaphragm of Cleo show lower amounts of fibrocollagenous and cellular infiltrations, decreased regeneration as seen by the uniformity of the fibre size and low numbers of centralized nuclei compared to *mdx*

mice (see Fig. 3c). These data show that the over-expression of the utrophin transgene does not result in any obvious morphological abnormality in the tissues tested.

#### 3.4.2. Urine analysis

The level of creatinine was examined in the urine to test for any abnormalities in kidney function that could have resulted from the over-expression of the transgene. Urine samples were tested for the levels of creatinine using proton NMR spectroscopy as described in methods. No abnormalities were seen in Cleo and HCleo samples analyzed compared with experimental (normal F1 and *mdx*) or historical controls, and no additional peaks were noted.

#### 3.4.3. Immunofluorescent staining

The cryosections of all the non-muscle tissues collected were stained with the utrophin antibody as described. The results are summarized in Table 1. Utrophin staining was

Table 1  
Results of immunostaining of non-muscle tissues

	N	Mdx	Cleo	HCleo
<i>Kidney</i> Utrophin	Glomerulus, distal tubules, medulla brightly stained	Same as N	Glomerulus, distal and in addition proximal tubules, medulla brighter stained than N	Same as Cleo but slightly brighter stained
$\alpha 1$ -dystrobrevin	Medulla, distal tubules, glomerulus brightly stained	Same as N but medulla less brightly stained	Same as N	Same as Cleo
Kidney Dp71	Membranes of tubules in medulla (packing cells/tissues not stained like in utrophin), distal and proximal tubules brightly stained. Glomerulus not stained	Same as N	Same as N	Same as Cleo
<i>Heart</i> Utrophin	Sarcolemma of myocytes especially at cell/cell contacts stained brightly. Intercalated discs stained faintly	Sarcolemma and intercalated discs stained brightly	Same pattern as mdx but brighter	Same as Cleo but intercalated disc brighter stained
<i>Tongue</i> Utrophin	NMJ. cell/cell contacts, nerves and blood vessels brightly stained	All sarcolemma stained brightly	Same as N	Same as mdx
<i>Oesophagus</i> Utrophin	Basal layer of epithelium, blood vessels and nerves in lamina propria connective tissues and striated muscle stained	Same as N	Same as N	Same as N
<i>Stomach</i> (body mucosa) utrophin	Smooth muscle especially the squamous epithelium that lines the nonglandular stomach, blood vessels, nerves and internal membrane of all the glands in the glandular portion of the stomach stained brightly	Same as N	Same as mdx	Same as Cleo but brighter
<i>Intestine</i> (T.S. small) utrophin	Smooth muscle, columnar epithelial membrane of the villi and blood vessels stained brightly	Same as N	Same as N but slightly brighter stained	Same as Cleo but brighter still
<i>Liver</i> Utrophin	Hepatic arteries, hepatic portal veins and sinusoids are stained brightly. Hepatocytes not stained.	Same as N but brighter	Same as mdx but brighter	Same as Cleo
<i>Bladder</i> Utrophin	Smooth muscle membrane, blood vessels in lamina propria stained brightly. Transitional epithelium (urothelium) not stained	Same as N	Same as N but brighter stained	Same pattern as Cleo but brighter stained
<i>Testis</i> Utrophin	Basement membrane of seminiferous tubules, stromal cells between tubules stained. Spermatozoa not stained.	Same as N but brighter stained.	Same as N but brighter stained than Mdx.	Same as Cleo.
<i>Trachea</i> Utrophin	Base of columnar epithelium, blood vessels and nerves in submucosa, some subset of hyaline cartilage cells stained brightly	Same as N	Same as N	Same as N
<i>Lung</i> Utrophin	Blood vessels and alveolar endothelium stained brightly	Same as N but brighter	Same as mdx	Same as Cleo but brighter still
<i>Eye</i> (TS of cornea) Utrophin	Squamous corneal epithelium membrane, Bowman's membrane, Descemet's basement membrane stained	Same as N	Same as N but slightly brighter stained	
Stroma not stained.	Same as N			
<i>Brain</i> (Cerebellum) Utrophin	Blood vessels stained	Same as N	Same as N	Same as N
<i>Skin</i> Utrophin	Basal layer cells, blood vessels, nerves, premedullary epithelium and vascular basement membrane stained very brightly. Smooth muscles less	Same as N	Same as N	Same as N

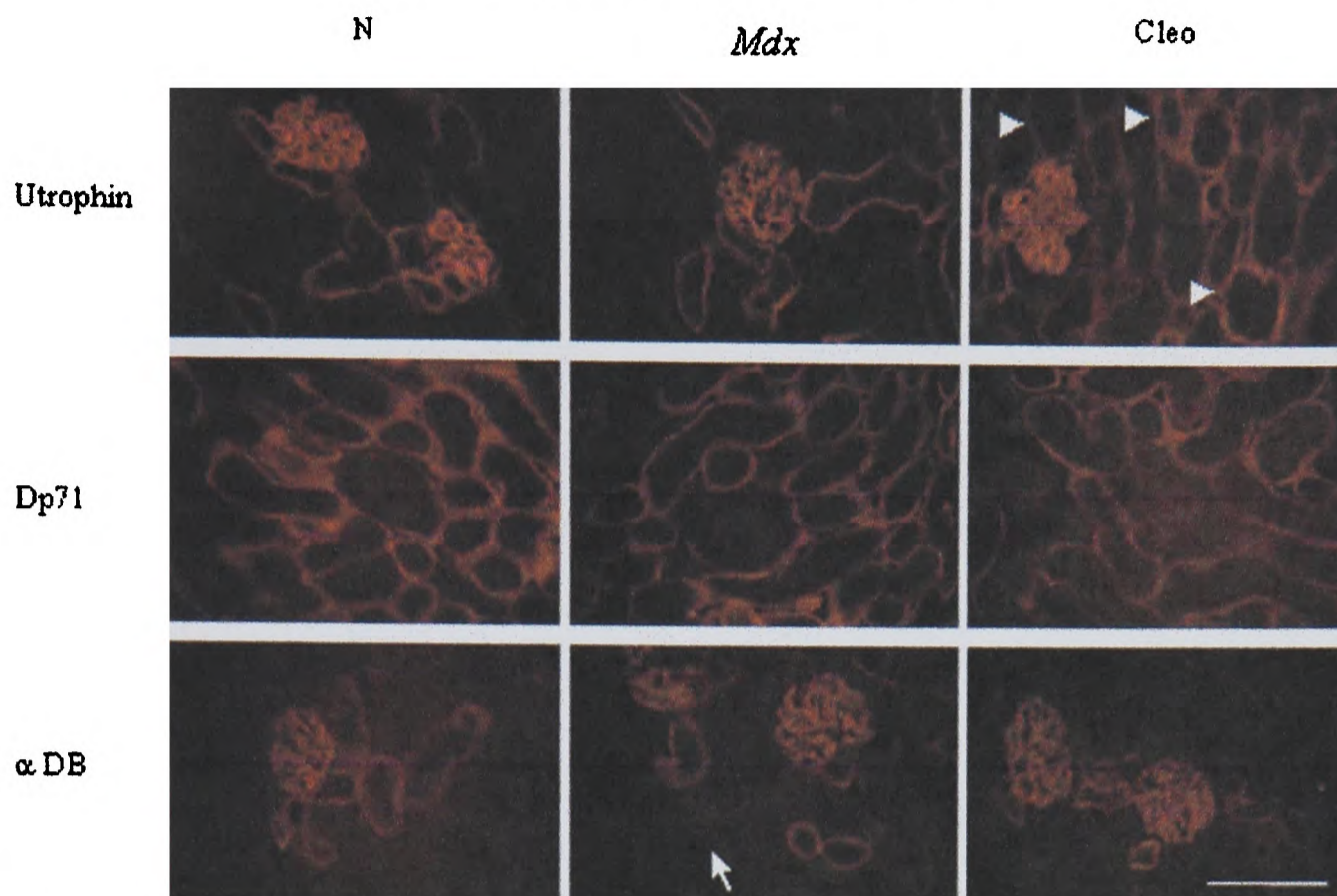


Fig. 5. Immunohistochemistry of the kidney cryosections of Cleo, *mdx* and normal F1 (N) mice stained with antibodies specific to utrophin, Dp71 and  $\alpha$ -dystrobrevin. There was a slight increase of utrophin staining. There was no increased distribution of Dp71 and  $\alpha$ -dystrobrevin but there was an increased distribution of utrophin in Cleo, that is, the proximal convoluted tubules (arrowheads) were now stained in addition to the distal convoluted tubules and glomerulus. Scale bar, 100 $\mu$ m.

increased in all tissues tested from Cleo and HCleo compared to *mdx* confirming that utrophin was overexpressed. It was also found to localize correctly to the sarcolemma in all tissues. In some tissues the staining of utrophin was observed to be slightly increased in HCleo than the Cleo tissues. Fig. 4 shows the immunofluorescence localization of utrophin in bladder, heart, liver, intestine, eye and stomach (image electronically reversed) where the utrophin intensity was slightly brighter in HCleo than that of Cleo. No change was observed in utrophin localisation in Cleo and HCleo

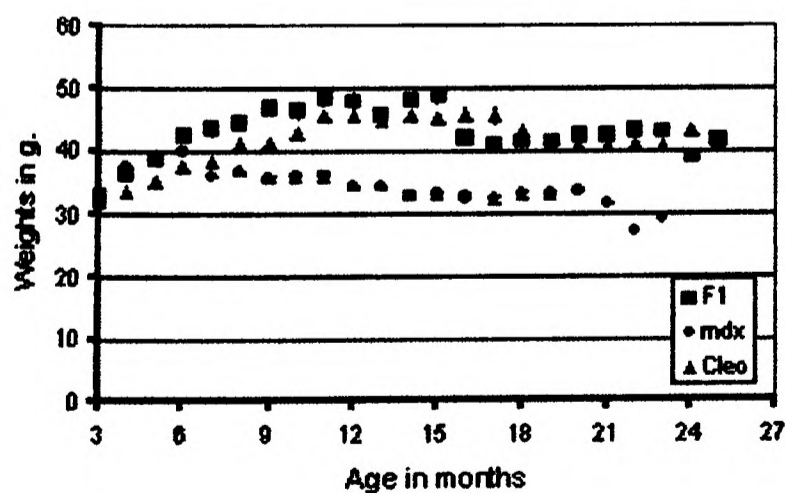


Fig. 6. Weight as a function of age of normal F1 (N), *mdx* and Cleo mice. The graph was plotted as the mean weight of six mice (except for the *mdx* curve where four mice data were used after 18 months due to death of two *mdx* mice) as a function of age for N, *mdx* and Cleo. The bars show the  $\pm 1$  SEM.

tissues compared to normal and *mdx* mice except in kidney where proximal tubules were now stained in addition to distal tubules, glomerulus and medulla as shown in Fig. 5. The localization of the dystrophin related protein,  $\alpha$ -dystrobrevin and kidney Dp 71 were similar to that of the control mice.

### 3.5. Weight as a function of age

At 3 months old the weight of normal, *mdx* and Cleo was approximately the same (32 g) and their weight increased in parallel up to approximately 6 months. Thereafter the weight of *mdx* mice dropped by approximately 25% up to age 23 months as shown in Fig.6 and most of them died or had to be culled due to ill health. The weight of Cleo mice was the same as normal and they were still living healthily after 2 years of age. This showed that the utrophin transgene was still conferring its therapeutic benefit in muscle even after 2 years of age and the over-expression in non-muscle tissues had not resulted in premature death.

## 4. Discussion

One of the major problems in using a small drug to up-regulate a protein for therapy is the toxicity of ectopic expression in tissues other than the target tissue. In the case of utrophin, the protein is expressed in all normal tissues but to a varying degree. It was therefore important

to address this issue before embarking on a large drug screen. The data presented here suggest that there is unlikely to be a detrimental effect of up-regulating utrophin at levels, which are therapeutic in the muscle.

One transgenic line (Cleo) expressed high levels of the utrophin transgene in almost all the tissues analyzed. To further increase the level of expression of the transgene, the Cleo line was bred to homozygosity. All the Cleo and Hcleo mice analyzed were on dystrophin-deficient *mdx* background and expressed the transgene at sufficient levels in muscle to prevent significant pathology. In Cleo the utrophin levels in all tissues were increased, ranging from a slight increase to approximately three folds in heart and kidney. The utrophin level in lung, kidney, tongue and stomach of HCleo was approximately twice that of Cleo while in other tissues the level increased only slightly. No pathology was observed in any tissue. At 2 years of age all the *mdx* mice died or had to be sacrificed due to dehydration and extreme poor health, mainly stiffening of the limb muscles. But the normal and Cleo mice were still healthy, showing that the utrophin transgene was still conferring its therapeutic benefits after 2 years of age.

In summary, the data presented here show that over-expression of full-length utrophin in a broad range of tissues does not lead to toxic effects in *mdx* mice. This suggests that tight control over utrophin expression may not be necessary for the correction of muscular dystrophy. Thus the search for small molecules to up-regulate utrophin expression represents a promising approach to therapy for DMD patients. This is a particularly encouraging strategy in view of the recent identification of a second promoter at the 5' end of the utrophin gene which is not responsive to the synaptic regulation reported for the first promoter, which makes this pharmacologically more feasible [36,37].

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# Expression of full-length utrophin prevents muscular dystrophy in *mdx* mice

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Duchenne muscular dystrophy (DMD) is a lethal, progressive muscle wasting disease caused by a loss of sarcolemmal bound dystrophin, which results in the death of the muscle fiber leading to the gradual depletion of skeletal muscle<sup>1</sup>. The molecular structure of dystrophin is very similar to that of the related protein utrophin<sup>2</sup>. Utrophin is found in all tissues<sup>3</sup> and is confined to the neuromuscular and myotendinous junctions in mature muscle<sup>4</sup>. Sarcolemmal localization of a truncated utrophin transgene in the dystrophin-deficient *mdx* mouse significantly improves the dystrophic muscle phenotype<sup>5,6</sup>. Therefore, up-regulation of utrophin by drug therapy is a plausible therapeutic approach in the treatment of DMD. Here we demonstrate that expression of full-length utrophin in *mdx* mice prevents the development of muscular dystrophy. We assessed muscle morphology, fiber regeneration and mechanical properties (force development and resistance to stretch) of *mdx* and transgenic *mdx* skeletal and diaphragm muscle. The utrophin levels required in muscle are significantly less than the normal endogenous utrophin levels seen in lung and kidney, and we provide evidence that the pathology depends on the amount of utrophin expression. These results also have important implications for DMD therapies in which utrophin replacement is achieved by delivery using exogenous vectors.

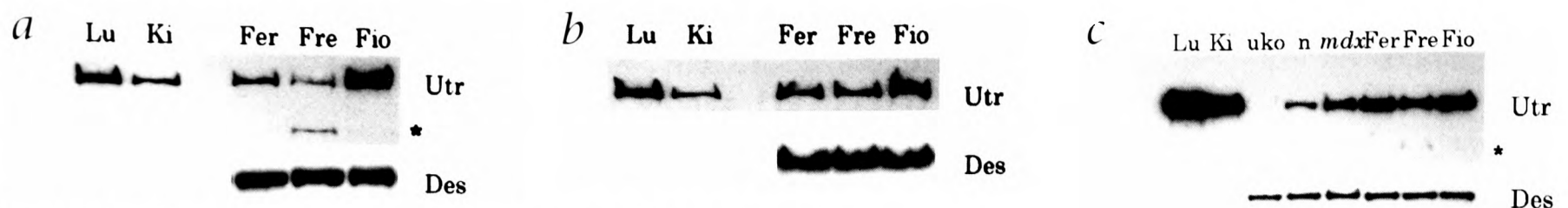
We generated lines of transgenic mice expressing the full-length utrophin protein in skeletal muscle, using the human skeletal  $\alpha$ -actin (HSA) promoter<sup>7</sup>. These lines were then crossed with dystrophin-deficient *mdx* mice<sup>8</sup> to produce mice that lack dystrophin and have utrophin localized at the sarcolemma. Three transgenic *mdx* lines, Fio, Fer and Fre, were chosen for analysis; each expresses a different amount of utrophin in muscle. We did western blot analysis of the tibialis anterior (TA; Fig. 1a), extensor digitorum longus (EDL; Fig. 1b) and diaphragm (Fig. 1c) muscles, staining for utrophin and desmin. For the TA and diaphragm, the highest utrophin transgene expression was in the Fio line and the lowest was in the Fre line (Fig. 1a and c, respectively). However, in the EDL, the difference between the Fio and Fre lines was less salient (Fig. 1b). We also compared the level of transgene expression with the highly expressed, endogenous utrophin levels in lung<sup>9</sup> and kidney<sup>3</sup>, and found that transgene expression levels are similar to or less than those of a normal utrophin promoter (Fig. 1a–c). Desmin staining was used to confirm that similar amounts of total muscle extract was loaded for all samples (Fig. 1a–c).

Immunostaining of TA sections with utrophin antibody correlates with the western blot results, showing decreasing sarcolemmal staining in the lines, from Fio (most) to Fer to Fre (least) (Fig.

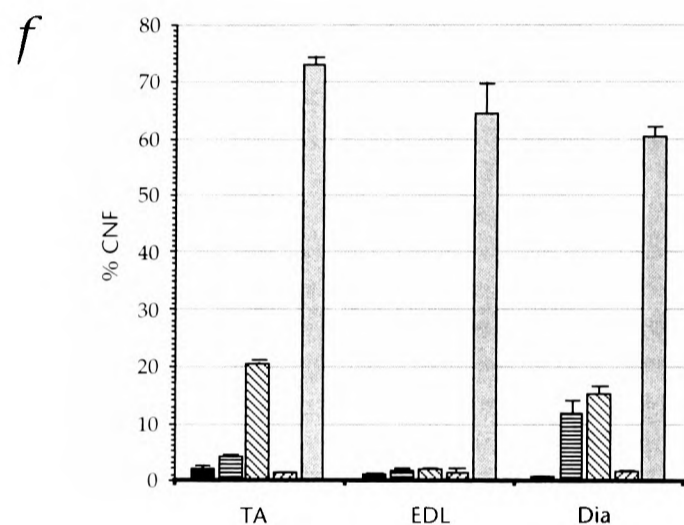
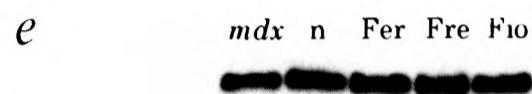
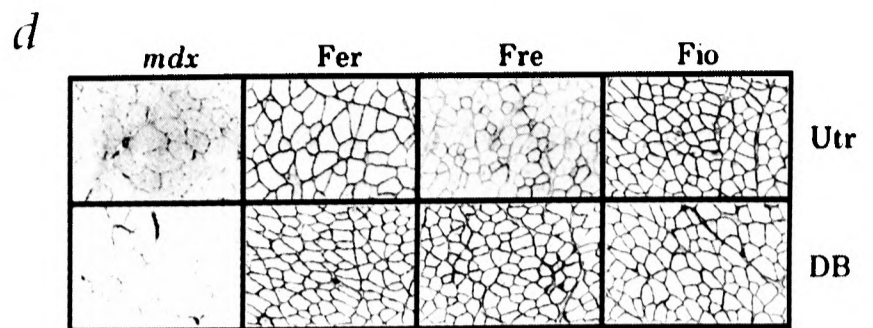
1d, Utr). Relocalization of the dystrophin protein complex is a prerequisite for successful amelioration of the dystrophic phenotype, as failure to relocalize the sarcoglycans results in limb-girdle muscular dystrophies<sup>10</sup>. Staining of TA sections with  $\alpha$ -dystrobrevin, a component of the dystrophin protein complex<sup>11–13</sup>, shows it is relocalized in all three lines compared with its location in the *mdx* line (Fig. 1d). Western blotting of total muscle extract demonstrated that the amount of  $\alpha$ -dystrobrevin was essentially the same in normal, *mdx* and utrophin transgenic lines (Fig. 1e). This confirmed that expression of  $\alpha$ -dystrobrevin was unaffected and that the observed increase in sarcolemmal immunostaining was due to relocalization. Similar results were also obtained with EDL and diaphragm muscle stained with antibodies to  $\beta$ -dystroglycan,  $\alpha$ -sarcoglycan and syntrophin (data not shown).

Muscle fibers that have recently regenerated as a result of fiber degeneration are easily identified by the presence of nuclei located centrally in the fiber rather than in the normal peripheral location. TA, EDL and diaphragm sections from normal C57, *mdx* and utrophin transgenic lines were stained with hematoxylin and eosin, and the percentage of centrally nucleated fibers was determined for each (Fig. 1f). The percentage of centrally nucleated fibers in the Fio line was the same as the percentage for the normal line for all muscles tested. The Fer and Fre lines had normal levels of centrally nucleated myofibers in the EDL, with significant reductions in these levels in the TA and diaphragm. This differential reduction of centrally nucleated myofibers reflects the expression pattern of the utrophin transgene (Fig. 1a–c), demonstrating that reduction of regeneration depends on the amount of utrophin present. Morphologically, no abnormal features could be identified in Fio muscle sections (data not shown).

We also assessed the mechanical performance of muscle from the transgenic lines. We measured the normalized force (the force per unit cross-sectional area) that could be exerted by the diaphragm and EDL from the normal C57, *mdx* and utrophin transgenic lines. In diaphragm, only the Fio line had normal force generation (Fig. 2a). The Fer and Fre lines showed substantial improvement compared with the *mdx* line, reaching about 78% of the normal value, whereas diaphragm muscle from the *mdx* produced only 54% of the normal value (Fig. 2a). However, EDL muscles from all three transgenic lines were able to exert a force that was the same as that of normal EDL muscle (Fig. 2b). A procedure that emphasizes specifically the susceptibility of dystrophin deficient muscle to stress is the drop in force during a series of eccentric contractions<sup>5,14–16</sup>. EDL muscle excised from the normal, *mdx* and transgenic lines was stretched forcibly while it contracted. We determined the percentage drop in force between the fifth eccen-



**Fig. 1** Utrophin transgene expression in the Fio, Fer and Fre lines. **a–c**, Western blot of transgene muscle extracts from tibialis anterior (TA; **a**), extensor digitorum longus (EDL; **b**) and diaphragm (**c**) and two control tissues expressing high levels of utrophin, lung (Lu) and kidney (Ki). In **c**, diaphragm from a utrophin-deficient mouse<sup>20</sup> (uko) was included to confirm the specificity of the utrophin antibody. For each sample, 20  $\mu$ g of total protein extract was used. Asterisks denote probable proteolytic breakdown product of Fre samples. Desmin (Des) staining was used to control for equal loading. **d**, Immunocytochemistry of cryosections of TA muscle from *mdx* and transgenic (Fer, Fre and Fio) lines, stained with antibodies that recognize utrophin (Utr) and  $\alpha$ -dystrobrevin (DB). Exposure times were the same to allow comparison of the intensity of staining of the samples. The image has been electronically reversed for easier interpretation. **e**, Levels of  $\alpha$ -dystrobrevin in total TA extracts from normal C57 (n), *mdx* and transgenic (Fer, Fre and Fio) lines. **f**, Percentage of centrally nucleated fibers (%CNF) in TA, EDL and diaphragm (Dia) muscle from normal C57 (■), *mdx* (□) and transgenic (Fio, ▨; Fer, ▩; and Fre, ▪) lines. The bars represent the mean of six muscles  $\pm$  s.e.m. The difference between *mdx* results and those of each other line was statistically significant ( $P < 0.05$ ). There was no statistically significant difference between the Fio and normal lines for TA, EDL or Dia. For the EDL, there were no statistically significant differences between the Fer, Fre and normal mice.



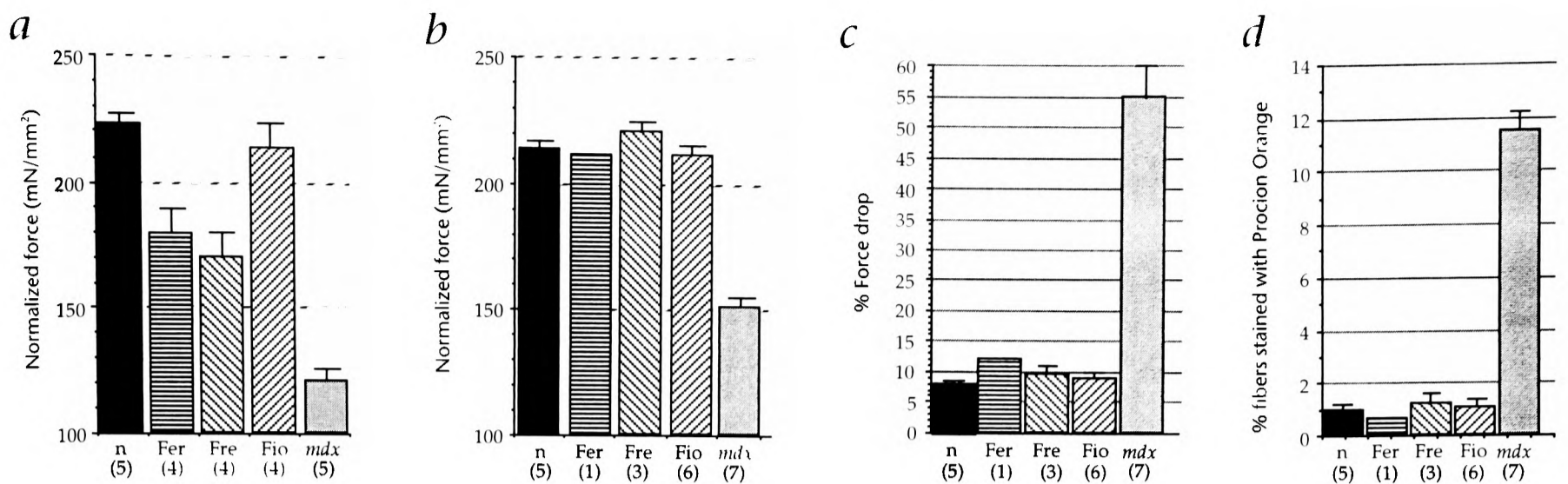
tric contraction and the first (Fig. 2c). Utrophin transgene expression in all three transgenic lines protected the EDL from any force drop compared with normal. However, repetitive eccentric contractions on the *mdx* EDL muscle resulted in a 55% drop in force. To assess the membrane integrity of the test EDL muscle after forced eccentric contractions, the muscle was stained with Orange Procion. Uptake of this low-molecular-weight dye into individual fibers demonstrates membrane damage. The number of fibers containing Orange Procion in all three transgenic EDL muscles was the same as that in normal EDL muscles (1%; Fig. 2d).

To demonstrate the overall beneficial effect of utrophin expression on all skeletal muscle, we used a non-invasive escape test, measuring the ability of a mouse to exert force (whole body tension, WBT). Mice of the Fio line, at 4 months of age, demonstrated the ability to exert a maximal force (WBT<sub>1</sub>) the same as that of normal mice (Fig. 3a). The maximum force exerted by the *mdx* mice was approximately 20% less than that of normal and Fio mice. There was no statistical difference between the maximum force exerted (WBT<sub>1</sub>) and the average of the five highest pulls (WBT<sub>1-5</sub>) for normal and Fio mice, indicating that there was little muscle fatigue during the several minutes of testing. However, *mdx* mice seemed to suffer from fatigue, as the average of their five highest pulls was approximately 20% less than their maximum (Fig. 3a, *mdx*). Mice 2 months of age of the Fre line, which has the least transgene expression, were able to exert a whole body tension (WBT<sub>1</sub> and WBT<sub>1-5</sub>) significantly greater than that of *mdx* mice of a similar age (Fig. 3b).

These results demonstrate that full-length utrophin, when localized to the sarcolemma of mouse muscle, results in a complete recovery of normal mechanical functions and prevents the occurrence of muscular dystrophy in the absence of dystrophin. However, prevention of muscle necrosis depends on the amount of full-length utrophin available. To compare the data obtained from the Fio and Fre lines with those from truncated utrophin

lines<sup>5</sup> (Utr<sup>++</sup>), we calculated the recovery score<sup>5</sup>, for which results equivalent to normal or *mdx* are plotted as 100% and 0%, respectively (Fig. 4). For all eight parameters tested, the recovery score of the Fio line (full-length utrophin) was 100%. It exceeded that obtained from the Utr<sup>++</sup> lines<sup>5</sup> (truncated utrophin), even though western blots showed that Fio<sup>++</sup> expression was 30–50% Utr expression (data not shown). In the line expressing the lowest amount of full-length utrophin, Fre, the recovery score was substantially better than that of the *mdx* line, varying from 40% to 100% for different parameters. Thus, if utrophin transgenes are to be considered for gene therapy, use of full-length utrophin rather than truncated utrophin would be prudent.

The *mdx* diaphragm is a good muscle model for DMD (ref. 17). To assess how much full-length utrophin is beneficial, we carefully quantified western blots of Fio and Fer diaphragm samples and compared the results with normal and *mdx* endogenous levels (Fig. 1c). For complete recovery, the Fio line expressed approximately twice as much utrophin as *mdx* diaphragm. However, this level was only 50% and 25% of the normal expression levels of kidney and lung, respectively (data not shown). Thus, in muscle, utrophin expression needs to be induced only to the normal levels found naturally in some tissues to prevent muscular dystrophy. The level of utrophin in the Fer diaphragm sample was only slightly increased compared with that in the *mdx* sample (Fig. 1c), yet the percentage of centrally nucleated fibers and mechanical



**Fig. 2** Mechanics of isolated muscle. **a** and **b**, Evaluation of normalized force (millinewton (mN)/mm<sup>2</sup>) in diaphragm (**a**) and EDL (**b**). **a**, For the diaphragm, Fer and Fre results were lower than those of normal C57 (n) and Fio mice. **b**, For the EDL, there was no statistically significant difference between the transgenic lines and C57 (n). **c**, % Force Drop measures the sensitivity of the EDL to eccentric contractions. **d**, % Procion Orange is a

measure of membrane damage. The same EDL muscle samples were used in **c** and **d**. Data are the mean  $\pm$  s.e.m. The number of animals tested is shown in parentheses. The differences between all *mdx* results and all those of other groups were statistically significant ( $P < 0.05$ ). The data were first submitted to a variance analysis, and the significance of the difference between two groups was further evaluated by the Tuckey test.

performance in Fre diaphragm muscle were substantially improved (Fig. 1*f*, Dia, and Fig. 2*a*). In the *mdx* diaphragm, most of the utrophin is from the regenerating fibers, which, once fully developed, lose their sarcolemmal utrophin staining. It is probably at this point that the newly developed fibers start to degenerate because of the lack of dystrophin. It is plausible that even after muscle degeneration has occurred, if the loss of sarcolemmal utrophin is prevented by drug upregulation, these newly formed myotubes will thrive and the cycles of degeneration will slow or even halt.

## Methods

**Full-length utrophin transgenic *mdx* lines.** The truncated utrophin transgene<sup>6</sup> was linearized at a single *HpaI* restriction endonuclease site, and the remainder of the rod domain was ligated in-frame to produce the full-length utrophin cDNA. The complete transgene was then cloned into an expression vector containing the 2.2-kb human skeletal  $\alpha$ -actin promoter, regulatory regions and the SV40 large T poly-A site. The cloning sites were such that the transgene was located downstream of the first human skeletal  $\alpha$ -actin untranslated exon, and flanking *KpnI/NotI* restriction endonuclease sites were used to 'liberate' the complete expression fragment. Transgenic mice were generated by microinjection of the purified human skeletal  $\alpha$ -actin transgene insert into the pronucleus of F2 hybrid oocytes from C57BL/6x CBA/CA parents. Positive transgenic mice were identified by Southern blotting. Mice 10–12 weeks of age were analyzed.

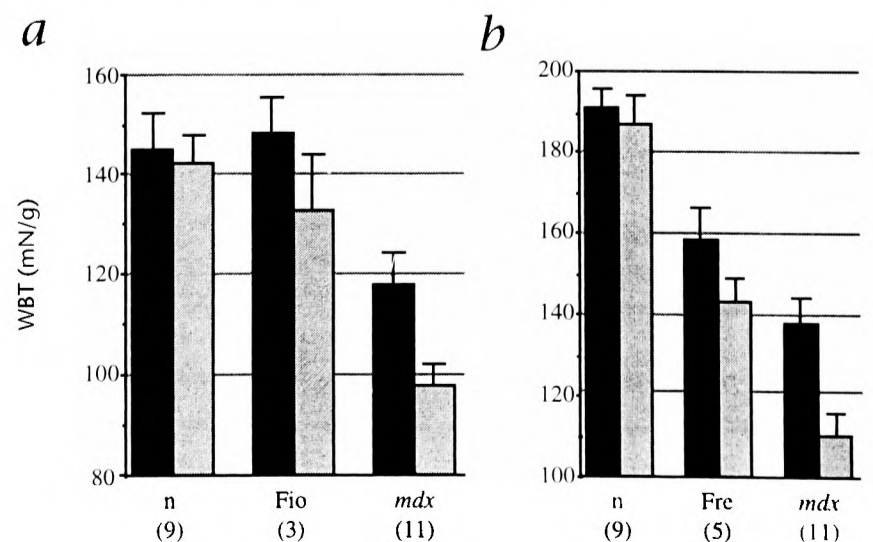
**Utrophin transgene expression.** Total muscle extracts and control lung and kidney extracts were prepared by homogenization in extraction buffer (75 mM Tris, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol, 5%  $\beta$ -mercaptoethanol), then heating at 95 °C for 5 min. Generally, 20  $\mu$ g of total protein (quantitated in triplicate using the Biorad DC protein assay kit; BioRad,

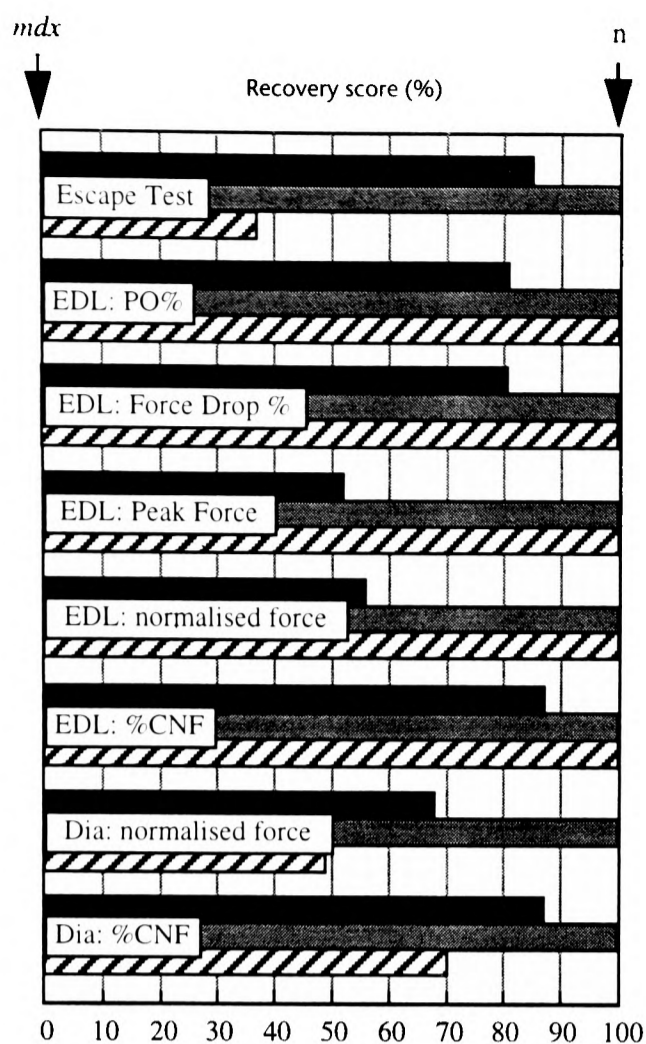
Richmond, California) was loaded onto 6% polyacrylamide gels and transferred to nitrocellulose. Utrophin transgene expression was detected using a 1:200 dilution of MANCHO3 (ref. 18) and made visible with anti-mouse IgG linked to horseradish peroxidase, and chemiluminescence (Boehringer). Desmin was detected using monoclonal antibody DE-U-10 (Sigma).

**Histology.** For sectioning, skeletal muscle samples were removed and immersed in OCT compound (Merck Ltd, Leics, UK) and frozen in liquid-nitrogen-cooled isopentane. Diaphragms were removed, cut in half, rolled longitudinally and frozen to facilitate orientation and easier sectioning. Frozen, unfixed cryosections 8  $\mu$ m in thickness were cut and stored at -80 °C. For calculations of percentage of centrally nucleated fibers, sections were stained with hematoxylin and eosin. Immunostaining was done by blocking the sections in 10% normal donkey serum in 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS). Primary rabbit antibody against utrophin (G3; 1:25 dilution) and  $\alpha$ -dystrobrevin ( $\alpha$ 1CT-FP (ref. 13); 1:1000 dilution) and Cy3-conjugated rabbit secondary antibody (1:400 dilution; Jackson Laboratories, Bar Harbor, Maine) were diluted in TBS. To evaluate membrane damage, EDL muscle was soaked for 1 h in Krebs solution containing 1% Orange Procion. After cryosectioning, the percent of fibers stained with Orange Procion contained in the entire cross-section was calculated.

**Muscle mechanics.** Isometric force production and eccentric contractions were assessed on isolated EDL muscles using a published procedure<sup>5</sup>. During tetanic stimulation, muscles were forcibly stretched by 7.7% (usually 1 mm), at 6.6 mm/sec. The isometric force drop (in %) was calculated as  $(F_1 - F_5)/F_1$ ,

**Fig. 3** Whole body tension (WBT) monitored in the 'escape' test. ■, WBT<sub>1</sub>; ▨, WBT<sub>1-5</sub>. **a**, Mice of the Fio line were tested at 4 months of age. The differences for WBT<sub>1</sub> and WBT<sub>1-5</sub> between normal and Fio mice were not statistically significant, whereas those between normal and *mdx* mice were ( $P < 0.05$ ). **b**, Mice from the Fre line, which express less utrophin than the other utrophin transgenic mice, were assessed at 2 months of age. Data are the mean  $\pm$  s.e.m. The number of animals tested is shown in parentheses. The data were first submitted to a variance analysis, and the significance of the difference between two groups was further evaluated by the Tuckey test.





**Fig. 4** Recovery scores obtained in muscles from the Utr++ lines (truncated utrophin; ■) and the Fio (■) and Fre (▨) lines (full-length utrophin). The scores were calculated as percentages of (transgenic result–mdx result)/(normal C57 result–mdx result).

where  $F_1$  and  $F_5$  are the isometric forces developed at the first and fifth tetanus, respectively. For isolated diaphragm analysis, narrow strips were removed by cutting radially from the central aponeurosis to a short segment of rib, then were attached at both ends to the force transducer. The muscle length of each preparation was adjusted to obtain the maximal isometric force. Forces were normalized ( $\text{mN}/\text{mm}^2$ ) by dividing the fiber length by the wet weight of the muscle.

Whole body tension (WBT), which is the overall force exerted by the mice when provoked to 'escape', has been described<sup>5,19</sup>. The force exerted by a mouse when it escapes after a gentle pinch of the tail was recorded by connecting the tail to a force transducer. The highest force peak (WBT<sub>1</sub>) and the average of the five highest peaks after repeating this several times over a period of several minutes (WBT<sub>1-5</sub>) were calculated. The results were normalized to the body weight of the animal ( $\text{mN}/\text{g}$ ) and this ratio is described as the WBT. Mice of the Fio and Fre lines (4 months of age and 2 months of age, respectively) were used for these analyses.

**Statistics.** Data (Figs. 2 and 3) were first submitted to a variance analysis. The significance of the difference between two groups was further evaluated by the Tuckey test.

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# Expression of truncated utrophin leads to major functional improvements in dystrophin-deficient muscles of mice

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**Dystrophin-deficient mice (*mdx*) expressing a truncated (*trc*) utrophin transgene show amelioration of the dystrophic phenotype. Here we report a multifunctional study demonstrating that *trc*-utrophin expression leads to major improvements of the mechanical performance of muscle (that is, force development, mechanical resistance to forced lengthenings and maximal spontaneous activity) and of the maintenance of the intracellular calcium homeostasis. These are two essential functions of muscle fibers, known to be impaired in *mdx* mouse muscles and Duchenne muscular dystrophy (DMD) patients. Our results bring strong support to the hypothesis that muscle wasting in dystrophin-deficient DMD patients could be prevented by upregulation of utrophin.**

Duchenne muscular dystrophy (DMD) patients suffer from an X-linked genetic defect resulting in the absence of a cytoskeletal protein, dystrophin. This causes a severe, progressive wasting of the muscle, resulting in fatal cardiac and respiratory failure during the second decade of life. Dystrophin belongs to a family of structural proteins (recently reviewed in ref. 1). One such protein, utrophin, encoded on chromosome 6, shows 80% homology with dystrophin<sup>2</sup>. In adult muscles, utrophin is normally localized at the neuromuscular junction<sup>3</sup>. However, variable amounts of utrophin are found at the sarcolemma of muscles of the dystrophin-deficient *mdx* mouse<sup>4</sup> and DMD patients<sup>5</sup>. This and the protein similarity led to the hypothesis that utrophin upregulation might compensate for dystrophin deficiency<sup>6,7</sup>.

Previous studies have shown that expression of a truncated form of dystrophin in *mdx* muscles results in a return to near-normal histology and function<sup>8,10</sup>. Recently, we reported<sup>11</sup> *mdx* mice expressing a truncated (*trc*) utrophin transgene encoding a 200-kDa protein consisting of the amino and carboxy domains, but lacking the majority of the spectrin-like repeat. Under the regulation of the human skeletal muscle  $\alpha$ -actin promoter, high levels of transgene expression were obtained in muscles, with the *trc*-utrophin uniformly localized at the sarcolemma, as is the case for dystrophin. This resulted in a dramatic decrease of the dystrophic histological phenotype, a significant drop in the creatine kinase (CK) levels in serum and relocalization of the dystrophin-associated proteins at the sarcolemma.

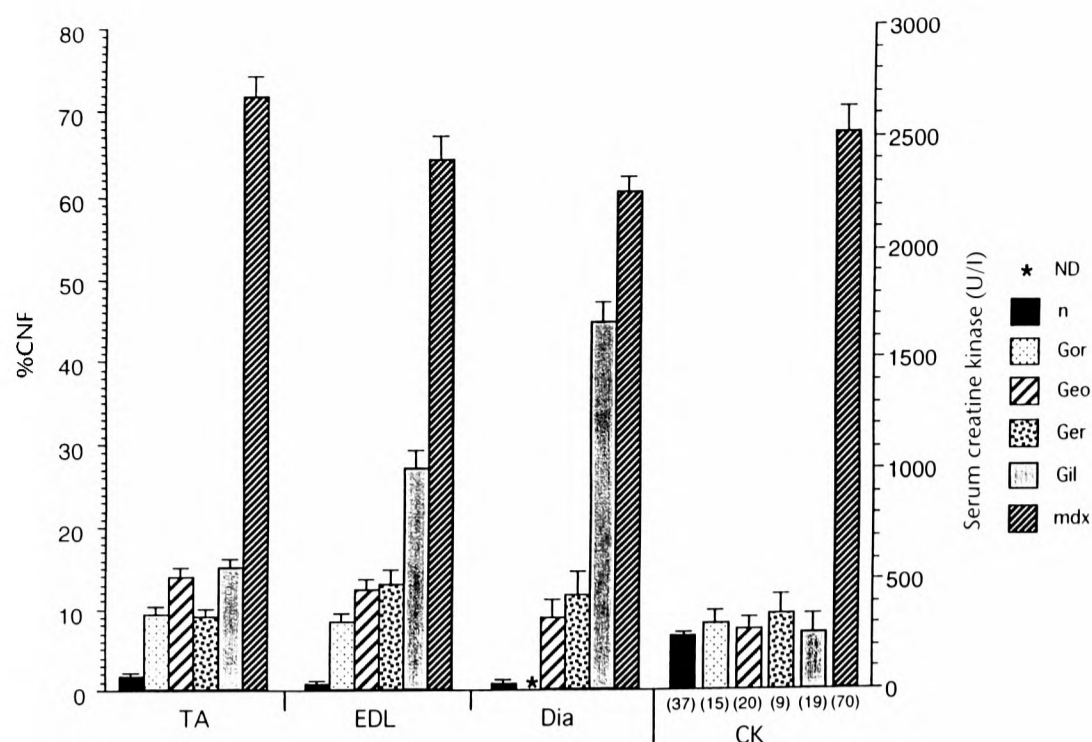
As recently discussed<sup>1</sup>, recovery of muscle function will be the ultimate criteria for judging the success of therapy of DMD by utrophin upregulation. Here we provide clear evidence that expression of truncated utrophin leads to a major recovery of two key functions in *mdx* muscle: the intracellular calcium homeostasis and mechanical performances, both in isolated muscles and, importantly, in the whole animal. Furthermore, we provide evidence that high recovery is critically dependent on the amount of the transgene expression.

## Characteristics of muscles from *trc*-utrophin transgenic *mdx* mice

Four lines of transgenic *trc*-utrophin *mdx* mice were obtained as described<sup>11</sup>. They were examined at 3–4 months for two classical markers of the dystrophic process: the serum creatine kinase (CK) level and the presence of centrally nucleated fibers (CNF). As shown in Fig. 1, the CK level, which is considerably increased in *mdx* mice, was practically normal in each transgenic line. The %CNF was determined in two limb muscles and in the diaphragm. For lines Geo, Ger and Gor, this percentage fluctuated around 10–15%, compared with normal (<2%) and *mdx* (60–70%), with little difference between muscles or between lines. In contrast, with Gil, the %CNF showed large differences between muscles: slightly above the level seen in other lines for tibialis anterior (TA), about twice as much for extensor digitorum longus (EDL), and nearly four times as much in the diaphragm. The results from all lines, and most obviously from the Gil line, demonstrate that a systemic marker, like the CK level, is much less sensitive to the dystrophic process than a local marker like the %CNF, which shows how the situation changes from muscle to muscle.

Immunostaining of *trc*-utrophin in muscle cryosections demonstrated the similarity in expression between the Geo, Ger and Gor lines and the difference compared with the Gil line. As illustrated in Fig. 2a, for TA, the Geo, Ger and Gor lines, the transgene is abundantly located at the sarcolemma of every fiber, along its complete periphery. In the Gill line, staining is weaker and more or less uniform in TA, whereas in some fibers of the EDL, *trc*-utrophin shows patchy staining around the fiber membrane (not shown). High and very similar levels of *trc*-utrophin expression, analyzed by western blots, were observed in muscles of the transgenic lines Geo, Ger and Gor. In Fig. 2b, transgene expression for three muscles of Geo and Gil lines are compared. Densitometry of the spots of Fig. 2b showed that expression in Geo muscles was 2.5 times that in Gil muscles, on the average, with variations from muscle to muscle. Because of their similar characteristics, the Geo, Ger and Gor lines will be abbreviated as

**Fig. 1** Percentage of centrally nucleated fibers (%CNF) for tibialis anterior (TA), extensor digitorum longus (EDL) and diaphragm (DIA) muscles (each bar represents the mean of eight muscles  $\pm$  s.e.m.) and serum creatine kinase (CK, units per liter, number of mice tested shown in parentheses) for four transgenic lines of *mdx* mouse expressing *trc*-utrophin (Gor, Geo, Ger, Gil) and for normal (n) C57 and *mdx* mouse. ND, not determined.



Utr++ (this pooling will be further justified in discussing the functional aspects below), and the Gil line, which expresses less, as Utr+.

### Mechanical performances

**Maximal isometric force.** Absolute force production in *mdx* limb muscles is about normal<sup>14</sup>. However, in spite of a hypertrophic phenotype, the normalized force (the force per unit cross sectional area) is usually around 70% of normal values. The first column of Table 1 illustrates that hypertrophy in *mdx* EDL muscles was largely reduced in *trc*-utrophin upregulated muscles, the reduction being statistically larger in (Utr++) muscles. The normalized force (Table 1) was significantly increased in EDL from Utr++, reaching 87% of the normal value. In the line expressing less transgene, Utr+, the increase of normalized force was lower than in the Utr++ samples, but still significantly higher than in *mdx* mouse muscle. In the diaphragm (Table 1, last column), the most affected muscle in the *mdx* mouse<sup>14</sup>, the normalized force also presented a marked increase in Utr++ muscle, reaching 84% of the normal value, whereas muscle from *mdx* mice produced only 51%.

**Force drop during repetitive eccentric contractions.** Dystrophin-deficient muscles are particularly susceptible to forced lengthenings applied during maximal muscle activation<sup>15</sup>, a procedure known as "eccentric contraction". This produces a steady drop of force as such contractions are repeated<sup>16,17</sup>. As already shown, the resistance to eccentric contractions is a highly critical test for evaluating the success of any potential therapy of dystrophin-deficient muscles<sup>10</sup>. Results obtained in EDL muscles are summarized in Table 2, column 1, gives the normalized peak force produced during the first forced lengthening. It is a measure of the stress generated within the preparation. The drop of isometric force at the 5th eccentric contraction is given in column 2 of Table 2 as the percentage of the isometric force before the first imposed stretch [calculated as  $(F_1 - F_5)/F_1$ , see the Methods section]. Damage to individual fibers has been demonstrated by the diffusion of the extracellular dye orange procion and consequential cytoplasmic staining. The percentage of damaged fibers after the fifth eccentric contraction is shown in the last column of Table 2. In confirmation of previous studies<sup>10,16</sup>, this percentage is a linear function of the force drop.

In comparison with the situation in *mdx* muscles, the major improvement of muscle resistance to mechanical injury conferred by *trc*-utrophin expression is clearly demonstrated by (1) the generation of higher mechanical stress (2) the much lower drop of force and (3) the lower percentage of identified damaged fibers. Individual results are given in Fig. 3, which shows that results from C57 and (Utr++) muscles form two close but separated sets, whereas those from the lowest transgene expression (Utr+) and *mdx* muscle slightly overlap. As already reported<sup>10,16</sup>, force drops for *mdx* muscles are variable (from 37 to 82%); by contrast, results from Utr++ muscles show the same variations as normal muscles.

**Muscle activity in the whole animal.** The force developed during bursts of activity by the whole animal was reported to be weaker in *mdx* mice<sup>18</sup>. This can be quantified by measuring the force produced when a mouse tries to escape in response to gentle pinching of the tail (adapted from ref. 18, see Methods). The

**Table 1** EDL and diaphragm maximal tetanic force

	EDL		normalized force		Diaphragm normalized force	
	Weight (mg)	N	(mN/mm <sup>2</sup> )	N	(mN/mm <sup>2</sup> )	N
C57	12.2 $\pm$ 0.1	7	213.0 $\pm$ 2.1	7	236.0 $\pm$ 5.2	5
Utr++	14.3 $\pm$ 0.41 <sup>††</sup>	10	186.2 $\pm$ 3.5 <sup>††</sup>	10	199.0 $\pm$ 5.3 <sup>††</sup>	7
Utr+	16.7 $\pm$ 0.6 <sup>††</sup>	6	177.7 $\pm$ 3.2 <sup>††</sup>	6	163.5 $\pm$ 8.5 <sup>††</sup>	4
<i>mdx</i>	20.3 $\pm$ 0.2	7	150.7 $\pm$ 4.1	7	121.0 $\pm$ 5.0	5

Values are means  $\pm$  s.e.m. For each mouse line, the same EDL muscles contributed to entries of Table 1 (tetanic contractions) and Table 2 (eccentric contractions). Statistical significance,  $P < 0.01$  for \*Utr versus C57; †Utr versus *mdx*.

**Table 2** Eccentric contractions and damage in EDL muscle

	Normalized peak force		Force drop		Orange procion fibers (%)	
	(mN/mm <sup>2</sup> )	N	(%)	N	(%)	N
C57	354 $\pm$ 6.8	7	9.1 $\pm$ 1.4	7	1.5 $\pm$ 0.16	7
Utr++	296 $\pm$ 7.5 <sup>††</sup>	10	17.6 $\pm$ 2.1 <sup>††</sup>	10	3.5 $\pm$ 0.15 <sup>††</sup>	10
Utr+	258 $\pm$ 6.5 <sup>††</sup>	6	37 $\pm$ 2.8 <sup>††</sup>	6	7.5 $\pm$ 0.8 <sup>††</sup>	5
<i>mdx</i>	233 $\pm$ 4.7	7	55 $\pm$ 5.1	7	11.9 $\pm$ 0.7	7

Values are means  $\pm$  s.e.m. For each mouse line, the same EDL muscles contributed to entries of Table 1 (tetanic contractions) and Table 2 (eccentric contractions). Statistical significance,  $P < 0.01$  for \*Utr versus C57; †Utr versus *mdx*.

**Table 3** Whole-body tension (WBT, escape test)

	Weight (g)	WBT (mN/g)	N
C57	33.5 $\pm$ 0.18	142.1 $\pm$ 5.2	9
Utr++	35.6 $\pm$ 0.43 <sup>†</sup>	121.0 $\pm$ 3.8 <sup>††</sup>	15
Utr+	34.1 $\pm$ 0.65	98.1 $\pm$ 5.5	11

Values are means  $\pm$  s.e.m. Statistical significance,  $P < 0.01$  for \*Utr versus C57; †Utr versus *mdx*.

means of the five highest peaks of muscle activity during a session of such "escape tests" are given in Table 3. To avoid bias, only mice of matched age and weight were used. Utr++ mice developed forces significantly higher than *mdx* mice, which reached 85% of the normal values, confirming the results obtained on isolated muscles. This simple test provided a noninvasive, physical demonstration of the benefits of *trc*-utrophin expression on a complex muscle activity in the living animal.

#### Calcium content and calcium homeostasis

Muscles from DMD patients and from *mdx* mice present an increase of their total calcium content<sup>19,20</sup>, which has been seen as a sign of the loss of the intracellular calcium homeostasis. However, direct measurements of cytosolic  $[Ca^{2+}]$  in fibers from *mdx* mice have provided conflicting results concerning a chronic intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) overload<sup>20,21</sup>. Nevertheless, when these fibers are challenged by the combination of an increased external  $Ca^{2+}$  concentration and a hyposmotic shock,  $Ca^{2+}$  homeostasis is definitely impaired in dystrophin-deficient fibers<sup>21</sup>. Accordingly, we examined how *trc*-utrophin expression affected the total calcium content and the effect of the hyposmotic and hypercalcic challenge on the value of  $[Ca^{2+}]_i$ .

**Total calcium content** (gastrocnemius muscle). As shown in Fig. 4a, *mdx* muscles contain about twice as much total calcium as normal ones. This level was reduced to values not significantly different from normal ones in muscles from Utr++ mice. For the Utr+ line, the Ca content was definitely higher than normal and close to the *mdx* level.

**Challenge of  $[Ca^{2+}]_i$  homeostasis.** The intracellular  $[Ca^{2+}]_i$  concentration was measured in isolated fibers from the flexor digitorum brevis (FDB) muscles, before the hyposmotic and hypercalcic challenge, then 30 minutes after its application. Results are expressed as the ratio of fura-2 fluorescence excited at 340 and 380 nm (ref. 25). As previously reported, this ratio is slightly but consistently increased in fibers from *mdx* mice<sup>20,21</sup>, whereas in fibers from transgenic mice (Utr++ and Utr+), it is equivalent to that in C57 mice (no significant difference was observed between the transgenic lines). The fluorescence ratio increased by about the

same amount (by the addition of 17 to 22%) for challenged fibers from C57 and from (Utr++) and (Utr+) mice, whereas it nearly doubled in fibers from *mdx* mice (Fig. 4b). Thus the presence of *trc*-utrophin restored normal behavior in conditions meant to challenge the ability of muscles fibers to maintain low values of  $[Ca^{2+}]_i$ .

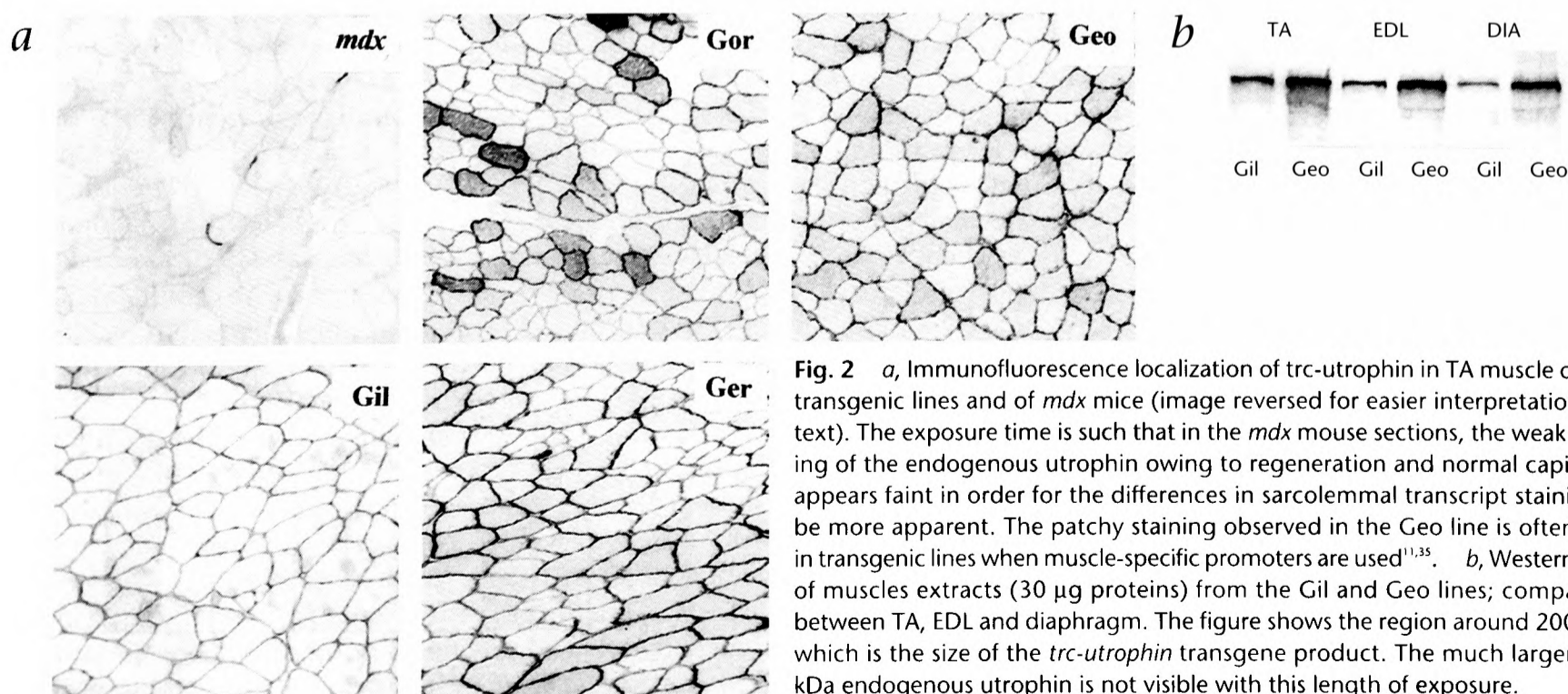
#### Discussion

Expressing a truncated form of utrophin (preserving the N and C terminal domains) in dystrophin-deficient muscles of the *mdx* mouse led to major improvements of the mechanical performances and of the intracellular calcium homeostasis.

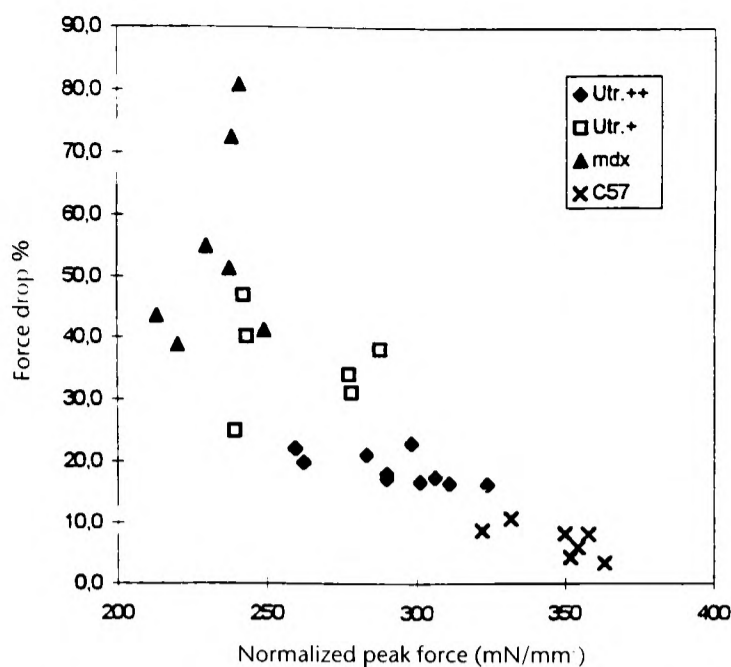
#### Overall evaluation of the benefit of *trc*-utrophin expression.

A quantitative evaluation of the extent of recovery of normal characteristics can be obtained by comparing the difference between Utr++ muscle and *mdx* ones, to the difference between normal (C57) muscles and *mdx* ones. The "recovery score," defined as  $(Utr++ - mdx)/(C57 - mdx) \times 100$ , was calculated for each of the parameters studied; where 100% would be equivalent to normal C57 mice and 0% to *mdx*. As seen in Fig. 5, for parameters that are most affected by the lack of dystrophin, namely, resistance to stretch, calcium content and homeostasis, serum CK levels, and the proportion of centrally nucleated fibers, the Utr++ recovery scores reached 75–85% or more. For the isometric force, which is less affected, recovery was around 50%.

**Muscle mechanics.** One current hypothesis<sup>26</sup> about the function of dystrophin is that it forms a mechanical link between the sub-membranous network of cytoskeletal actin through its N-terminal actin-binding domain and a complex of membrane-bound glycoproteins linked at its C-terminal domain; in turn, this complex is bound to the extracellular matrix through  $\alpha$ -2 laminin. In patients, mutations in the C-terminal domain result in a DMD phenotype, whereas mutations in the actin-binding domain result in a phenotype ranging from the typical severe form of DMD to the mild type of Becker dystrophy, depending on the mutation<sup>27</sup>. Utrophin and dystrophin display extensive sequence homology<sup>2</sup> covering the  $\beta$ -dystroglycan and the syntrophin binding domains and, *in vitro*, both have the ability to bind these



**Fig. 2** *a*, Immunofluorescence localization of *trc*-utrophin in TA muscle of four transgenic lines and of *mdx* mice (image reversed for easier interpretation, see text). The exposure time is such that in the *mdx* mouse sections, the weak staining of the endogenous utrophin owing to regeneration and normal capillaries appears faint in order for the differences in sarcolemmal transcript staining to be more apparent. The patchy staining observed in the Geo line is often seen in transgenic lines when muscle-specific promoters are used<sup>11,35</sup>. *b*, Western blots of muscles extracts (30  $\mu$ g proteins) from the Gil and Geo lines; comparison between TA, EDL and diaphragm. The figure shows the region around 200 kDa, which is the size of the *trc*-utrophin transgene product. The much larger 400-kDa endogenous utrophin is not visible with this length of exposure.



**Fig. 3** Individual drop of the isometric force at the fifth tetanus, as a percentage of the first tetanus, in eccentric contractions of mouse EDL, as a function of the peak force (highest value of tension developed during the imposed stretch, at the first tetanus). Normalized forces, milliNewton per millimeter squared.

sarcolemmal proteins. *In vitro* assays demonstrated that the binding of the N-terminal domain to actin is regulated by calmodulin in a  $Ca^{2+}$ -dependent way, which is much more pronounced for utrophin<sup>28</sup>. This suggested that, in a muscle fiber at rest, that is, for  $[Ca^{2+}]_i < 10^{-7}$  M, utrophin may be dissociated from actin. As discussed above, an uninterrupted mechanical link between cytoskeletal actin and the extracellular matrix is essential for muscle integrity, probably to sustain the stress generated by contraction. Thus utrophin has to remain associated with actin upon stimulation to substitute functionally for dystrophin. Hence, experiments on *mdx* contracting muscles containing the utrophin transgene are critical for evaluating the effectiveness of the substitution.

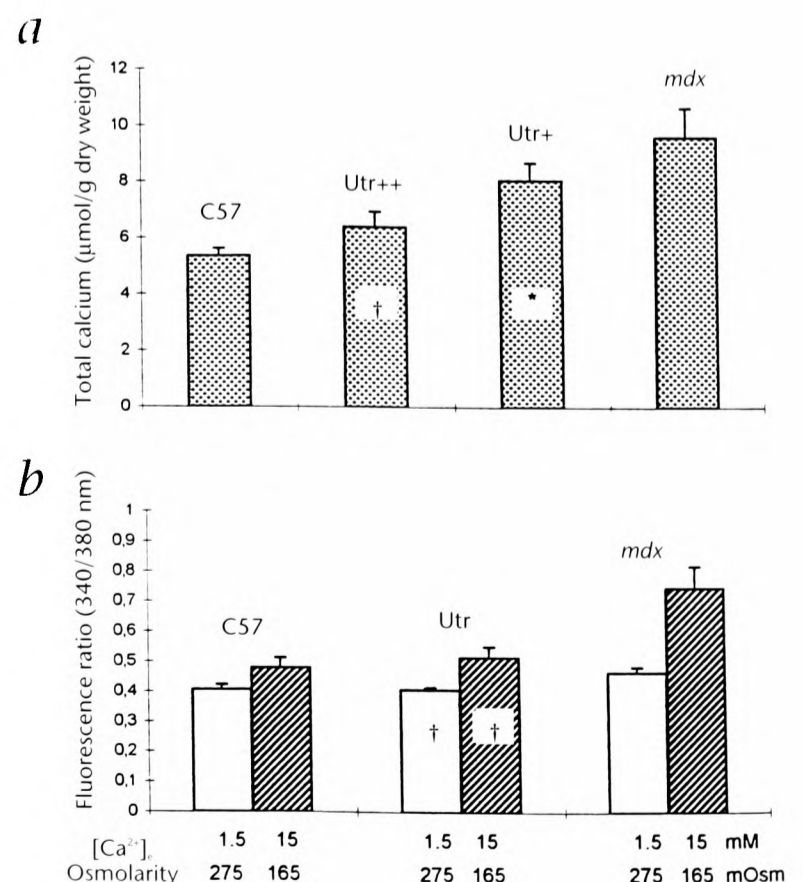
When trc-utrophin expression was high, recoveries were similar in the three Utr++ lines. However, the Utr+ line, which had a lower level of transgene expression in EDL and diaphragm, definitely showed poorer recovery for all mechanical parameters studied, and most obviously for the resistance to forced lengthenings during activation. This suggests that a critical level of expression is required, above which recovery is "saturated," and below which muscle pathology deteriorates. This might explain why the very moderate upregulation of utrophin occurring spontaneously in *mdx* diaphragm ( $2.5 \times$  normal) is insufficient to prevent the dystrophic process<sup>9</sup>. However, one can demonstrate possible beneficial levels of utrophin. In *mdx* small caliber muscles such as the extraocular and toe muscles there is sarcolemmal utrophin (not seen in C57) and retention of  $\alpha$ -sarco-

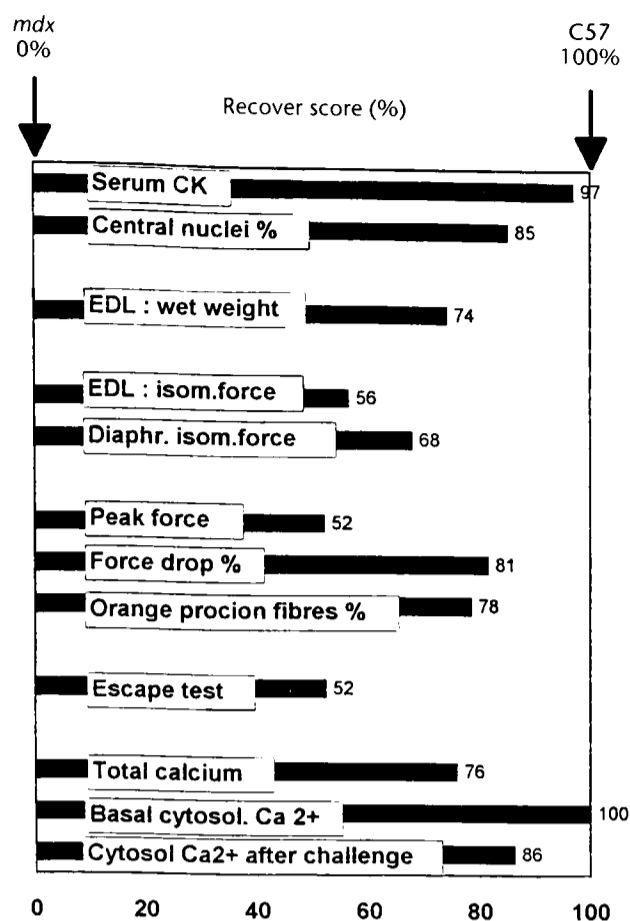
**Fig. 4** *a*, Total calcium content in gastrocnemius muscles of normal ( $n = 9$ ), *mdx* ( $n = 12$ ) and transgenic mice (Utr++:  $n = 15$ ; Utr+:  $n = 9$ ) in micromoles of calcium per gram of muscle dry weight ( $\pm$  s.e.m.). *b*, Measurement of cytosolic  $[Ca^{2+}]_i$  of isolated fibers, expressed as the intensity ratio of fura-2 fluorescence. open columns, Normal Krebs conditions; hatched columns, 30 min after incubation in the hyposmotic + hypercalcic solution (the Ca homeostasis challenge). Normal fibers,  $n = 13$ ; *mdx* fibers,  $n = 16$ ; Utr fibers  $n = 33$ . Error bars, s.e.m. As no differences were observed between Utr++ and Utr+ fibers, results were pooled. Significant ( $P < 0.01$ ) differences, marked \* or †, refer to the (Utr - C57) or the (Utr - *mdx*) comparisons, respectively.

glycan<sup>1</sup>. In *mdx* cardiac muscle, which is less affected than skeletal muscle, there is a fourfold increase in utrophin level (western blotting) with no appreciable decrease in  $\beta$ -sarcoglycan levels. In *mdx* quadriceps where the utrophin increase is only 1.3-fold, there is a drastic reduction of  $\beta$ -sarcoglycan. Most likely, maximal recovery was obtained when enough trc-utrophin was produced to saturate all possible binding sites at the membrane. Indeed, for all fibers of the Utr++ lines, the immunofluorescence reaction revealed the presence of trc-utrophin along the whole periphery of the fibers, and its intensity was about the same for all preparations; on the contrary, the reaction was definitely weaker for fibers of the Utr+ line (Fig. 2a), and the membrane localization was sometimes incomplete.

**Calcium content and homeostasis.** In muscles from the Utr++ lines, the calcium content did not differ significantly from normal muscles. However, in the Utr+ line, this content did not differ significantly from that of *mdx* muscles. The effect of trc-utrophin expression is dramatic, but difficult to interpret, as the origin of the excess of total calcium content in dystrophin-lacking muscles (DMD patients and *mdx* mice) is still not understood. It may reflect a larger filling of the intracellular calcium stores, in response to increase of calcium influxes through leaky channels<sup>30</sup>. Possibly, this calcium may also come from inert precipitates at the sites of fiber degeneration and necrosis<sup>31</sup>, and its amount could be an "integrated index of the dystrophic history" of the preparation, implying that measurements for comparative purposes should be made on muscles from age-matched mice, as we did here.

Intracellular calcium homeostasis was challenged by the combination of a shock leading to membrane distention and a high external calcium concentration<sup>32</sup>. This challenge revealed the poorer  $[Ca^{2+}]_i$  regulation of *mdx* fibers as compared with that of normal fibers and showed that trc-utrophin fibers had a recovery score of 86%. No differences were observed between the Utr++ and Utr+ line, possibly because the conditions of the challenge were mild in order to avoid contraction and irreversible damages, which develop for lower osmolarity<sup>32</sup>. Finally, it is noteworthy





**Fig. 5** Recovery scores obtained in muscles of *Utr<sup>+/+</sup>/mdx* transgenic mice, calculated as  $(Utr - mdx)/(C57 - mdx) \times 100$  (see text). The recovery score for the %CNF was calculated from an average value of pooled data of TA, EDL and diaphragm, in Geo, Ger and Gor lines, given in Fig. 1.

thy that, in the case of *Utr<sup>+/+</sup>* fibers, the average values of all aspects of Ca handling (total content, basal  $[Ca^{2+}]_i$  level and  $[Ca^{2+}]_i$  reaction to challenge) were not significantly different from those of normal preparations. As a loss of calcium homeostasis has been implicated in activating Ca-dependent proteolysis in dystrophin-lacking fibers, the dramatic recovery obtained with truncated utrophin should prevent this initiation of the degenerative process.

**Which strategic choice for gene therapy of DMD: dystrophin or utrophin?** When expressed in such amounts that they are present along the whole periphery of the fibres, truncated forms of either dystrophin or utrophin produce dramatic recovery, both structural and functional, toward the normal phenotype. Published data<sup>9</sup> on force recovery in diaphragm from transgenic *mdx* mice expressing truncated dystrophin and the present data allow a direct quantitative comparison of the relative advantage of truncated dystrophin versus truncated utrophin. The comparison is particularly sound as the values of normalized isometric forces for diaphragm of C57 and of *mdx* mice are practically identical in both studies. In the case of high expression of truncated dystrophin (Table 1 of ref. 9) the level of expression was 0.2 or 0.9 times the normal dystrophin content. From the corresponding values of normalized force, the calculated recovery score was 60% and 77%, respectively, whereas, as reported here, for truncated utrophin, it was 37% for *Utr<sup>+</sup>* diaphragm and 68% for *Utr<sup>+/+</sup>* diaphragm. From these elements of comparison, one could estimate that the level of utrophin expression attained in our *Utr<sup>+</sup>* and *Utr<sup>+/+</sup>* diaphragms would be functionally equivalent to 0.1 and 0.4–0.5 times the normal dystrophin level, respectively. Thus, if utrophin and dystrophin are one-for-one, functionally interchangeable molecules, the expression of the former should be

upregulated to reach at least one-half of the normal dystrophin content in order to confer major recovery to dystrophin-deficient muscles. (This preliminary conclusion should await a direct quantitative comparison between the amount of truncated utrophin and dystrophin.) For the future, even if better recovery could not be obtained with the full-length utrophin molecule, the improvements already achieved with the truncated form would radically change the clinical phenotype of the disease (provided that the results obtained on *mdx* mice could be extrapolated to the DMD patients). In addition, utrophin offers the advantage that its gene is intact in DMD and that its transcript generates a protein already known to the immune system. Its upregulation should therefore not produce adverse immune response, a possibility that cannot be excluded for dystrophin. These results also suggest that a truncated utrophin transgene used in gene delivery protocols, such as adenovirus, would be just as successful as truncated dystrophin in alleviating the dystrophic phenotype in DMD muscles. In conclusion, we presented three major results addressing the feasibility of using utrophin to replace dystrophin for DMD therapy. The utrophin minigene has a significant effect on dystrophin-deficient muscle strength, especially in fast glycolytic muscles (for example, EDL), which are preferentially affected in DMD patients<sup>33</sup>. The calcium content and homeostasis is stabilized. Finally, the amount of utrophin present in muscles directly relates to the degree of muscle recovery both functionally and structurally.

## Methods

**Transgenic lines of *mdx* mouse.** Four new lines of *mdx* mice expressing a truncated utrophin transgene in skeletal muscles were obtained as described<sup>11</sup>. Mice were killed between 3½ and 4 months.

**Mechanics on isolated muscles.** Isometric force production and eccentric contractions were studied on isolated EDL muscles following the procedure described previously<sup>9</sup>. During tetanic stimulation, muscles were forcibly stretched by 7.7% (usually 1 mm), at 6.6 mm/s. The isometric force drop (as a percentage) was calculated as  $(F_1 - F_5)/F_1$ , where  $F_1$  and  $F_5$  are the isometric forces developed at the first and fifth tetanus, respectively. Narrow strips of diaphragm were dissected out by cutting radially from the central aponeurosis to a short segment of a rib, and attachments were made at these two ends. Lengths of both preparations were adjusted to get maximal isometric force. Forces were normalized (milliNewton per millimeter<sup>2</sup>) by dividing the fiber length by the wet weight of the muscle.

**Histology.** All EDL muscles studied for mechanical experiments were soaked for 3 h in Krebs solution containing 1% orange procion to label damaged fibers. Then, they were frozen in isopentane-liquid N<sub>2</sub>. Cryosections were cut at midbelly, 0.5 mm apart; orange procion-positive fibers were counted over the entire cross section. Cryosections of TA were used for truncated utrophin detection (using an affinity-purified rabbit polyclonal antibody, 1/25, followed by FITC-conjugated goat anti-rabbit antibody, 1/100); for comparison, muscle sections were photographed under strictly identical conditions of illumination and exposure. The images were reversed for easier interpretation. For assessing the %CNF, each pair of muscles were removed from four mice in each line; the diaphragm was cut in half and both halves used (thus for each bar of Fig. 1,  $n = 8$ ). Between 300 and 400 hematoxylin and eosin-stained fibers were counted, and the number with centrally nucleated fibers was determined.

**Bursts of muscle activity monitored by the "escape test"**<sup>18</sup>. Mice were placed on a platform facing the entrance of a tube. A cuff was wrapped around the tail and connected to a fixed force transducer. In response to gentle pinching of the tail, mice tried to escape within the tube. This was prevented by the attachment of the tail to the force transducer, and a short peak of force was recorded. The test was repeated several times over a period of several minutes. The five highest peaks were averaged and normal-

ized to the body weight of the animal (milliNewton per gram). Animals of matched age and weight were selected for this test.

**Total Ca determination.** Isolated gastrocnemius muscles were rinsed for 15 min in a Ca-free Krebs solution containing 2 mM EGTA to remove extracellular calcium. They were freeze-dried. Each muscle was dissolved in 0.5 ml concentrated HCl. The concentration of calcium was measured by atomic absorption flame photometry, after adequate dilution in the presence of 1% LaCl<sub>3</sub>. Results are expressed in micromoles Ca/gram dry weight.

**Measurement of cytosolic [Ca<sup>2+</sup>].** Single fibers from FDB muscles were isolated after collagenase digestion and loaded with the diffusible fura-2AM (0.2 μM, for 30 min, at 20 °C). [Ca<sup>2+</sup>]<sub>i</sub> changes were monitored by the ratio-metric measurements of fluorescence intensity (510 nm) excited at 340 and 380 nm<sup>25</sup>. After determination of the ratio in normal Krebs solution, fibers were bathed in (160 mosM, 0.6× normal) Krebs solution containing 15 mM CaCl<sub>2</sub> (10× normal) for 30 min before a new determination of the fluorescence ratio. For all results reported in Fig. 4b, ratio determinations were made on each fiber, before and after the hyposmotic-hypercalcic challenge. During this test, fiber diameter increased, whereas length decreased, but all fibers were affected similarly; they all remained at rest with regular and similar sarcomere spacing (1.5–1.6 μm) along the entire fiber length.

**Statistics.** To evaluate significant differences between muscles with utr and muscles from both *mdx* and normal mice, a series of independent tests were made comparing each pair of means, for example, Utr++ versus C57, Utr++ versus *mdx*, for each parameter studied. However, as in most cases, means to be compared were based on small and unequal numbers of data, an adapted version of the *t*-test (two tails) was used<sup>34</sup> with a reduced number of degrees of freedom. Significant (*P* < 0.01) differences were marked \* or † when they refer to the (*Utr* vs C57) or the (*Utr* vs *mdx*) comparisons, respectively.

#### Acknowledgments

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## Amelioration of the dystrophic phenotype of *mdx* mice using a truncated utrophin transgene

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**DUCHENNE** muscular dystrophy (DMD) is a severe, progressive muscle-wasting disease that causes cardiac or respiratory failure<sup>1,2</sup> and results in death at about 20 years of age. Replacement of the missing protein, dystrophin, using myoblast transfer in humans or viral/liposomal delivery in the mouse DMD model is inefficient and short-lived<sup>3,4</sup>. One alternative approach to treatment would be to upregulate the closely related protein, utrophin<sup>5,6</sup>, which might be able to compensate for the dystrophin deficiency in all relevant muscles<sup>7,8</sup>. As a first step to this approach, we have expressed a utrophin transgene at high levels in the dystrophin-deficient *mdx* mouse. Our results indicate that high expression of the utrophin transgene in skeletal and diaphragm muscle can markedly reduce the dystrophic pathology. These data suggest that systemic upregulation of utrophin in DMD patients may lead to the development of an effective treatment for this devastating disorder.

A truncated utrophin transgene was modelled on the Becker dystrophin transgene, which has been shown to correct the dystrophic phenotype of *mdx* mice<sup>9,10</sup> (Fig. 1A). To generate high levels of muscle expression, the utrophin transgene was driven by the human skeletal  $\alpha$ -actin (HSA) promoter (Fig. 1B). Several transgenic lines expressing the utrophin transgene were generated with differing levels of transgene expression. Immunoblot analysis of muscle samples from transgenic lines demonstrating high-level expression are shown in Fig. 1C. The multiple fainter bands probably result from the proteolytic breakdown of the highly expressed transgene product<sup>11</sup>. Line 347 also shows weak expression of the transgene in the heart. Line 261 contains, but does not express, the transgene. Further analysis of the F-3 line shows no evidence of transgene expression in heart, brain, kidney, lung, liver, intestine, skin or pancreas. To demonstrate that the utrophin transgene localized to the sarcolemma, immunofluorescence of skeletal-muscle sections from the F-3 line was performed using antibodies specific to utrophin and dystrophin. The sarcolemmal localization pattern of dystrophin (Fig. 1D, a) and the utrophin transgene (Fig. 1D, b) in tibialis anterior (TA) muscle sections demonstrate that they can colocalize to the sarcolemma *in vivo*. In contrast with the transgenics, the normal localization of utrophin in adult skeletal muscle is exclusively at the neuromuscular and myotendinous junctions and in the capillaries and nerves<sup>12,13</sup>.

The limb muscles of the dystrophin-deficient *mdx* mouse only develop atrophy and weakness late in life. But histological and physiological analysis reveals several muscle defects in common with DMD patients, including muscle-fibre degeneration, giving rise to a dramatic elevation of serum creatine kinase (CK) level and evidence of massive myofibre regeneration, with most fibres having centrally located nuclei<sup>14</sup>. Thus changes in the levels of serum CK and numbers of centralized nuclei have been used to monitor the pathology of the muscle in several transgenic lines expressing dystrophin transgenes in *mdx* mice<sup>9,10,11,15</sup>. Male transgenic F-3 mice carrying the utrophin transgene were crossed with dystrophin-deficient female *mdx* mice. The resultant offspring were analysed (Fig. 2a) at approximately 5 weeks of age when the skeletal muscle is still undergoing rounds of degeneration and regeneration. The CK levels of male transgenic *mdx* mice (utro-tg

mdx) had fallen to approximately a quarter of that of non-transgenic *mdx* male littermates (*mdx*). Females, whether transgenic or not, have roughly normal levels of serum CK. The reduction in the serum levels of CK in the male utrophin transgenic *mdx* littermates signifies a change in the muscle pathology of these mice, and implies that a marked decrease in muscle degeneration has occurred. The contrast in numbers of

centralized nuclei in frozen sections from the soleus and TA muscle of transgenic (utro-tg *mdx*) and non-transgenic male *mdx* mice is shown in Fig. 2*b*. The numbers of centrally nucleated myofibres is markedly reduced in the two muscle types examined, showing that the amount of fibre regeneration is decreased. The difference in numbers of central nuclei between the utrophin transgenic *mdx* TA (~10%) and soleus (~30%) is probably

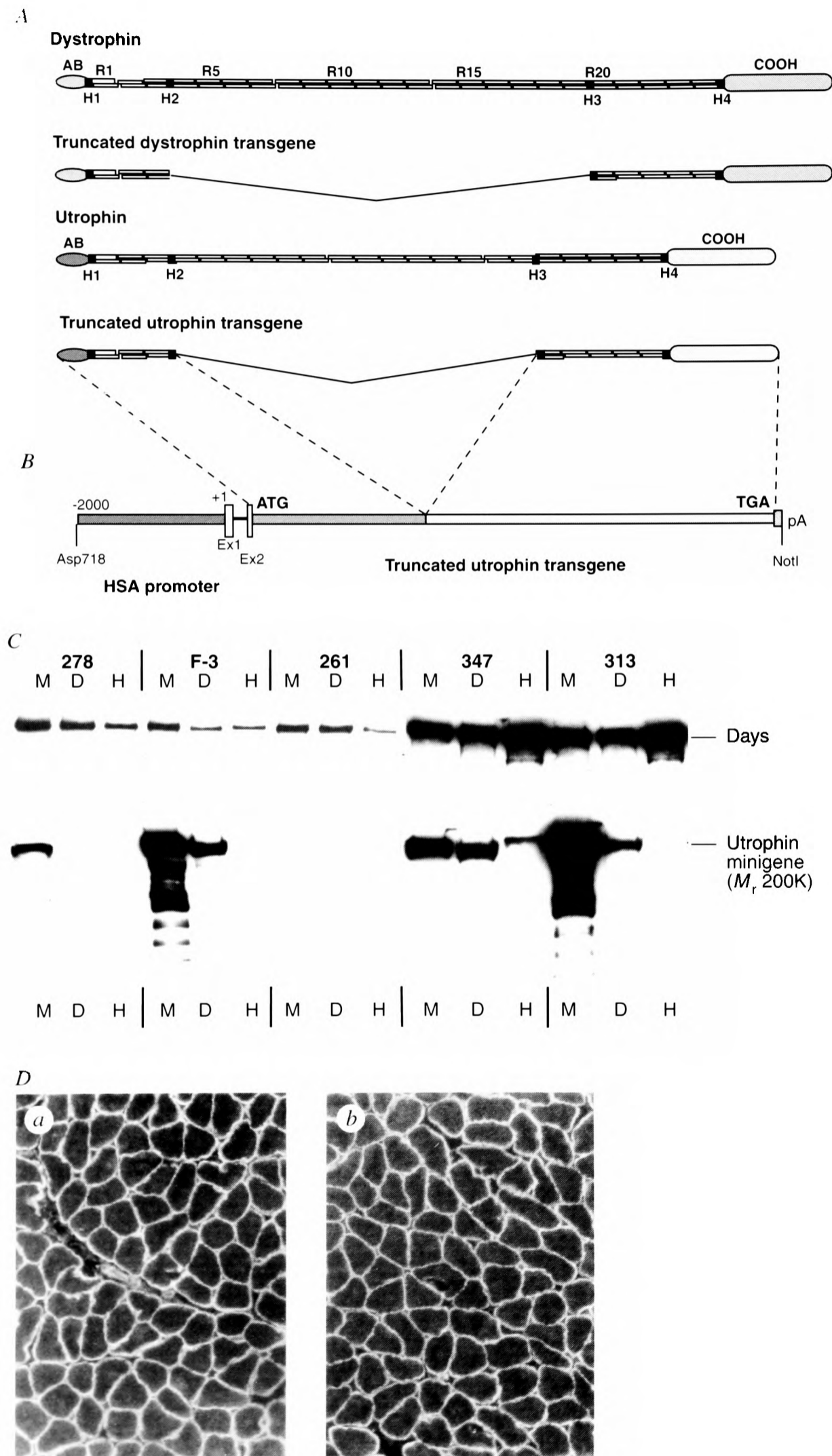


FIG. 1 Utrophin transgene construction and expression. **A**, Representation of dystrophin, utrophin and the two truncated transgenes. The spectrin-like repeats (R, each repeat depicted by the double rectangle), hinge sites (H), actin-binding (AB) and C-terminal-binding (COOH) domains. **B**, Utrophin transgene vector. For details of construction, see 7Methods. **C**, Immunoblot of muscle from utrophin-transgenic lines (278, F-3, 347, 313) and non-expressing line (261). M, skeletal muscle; H, heart; D, diaphragm. **D**, Immunohistochemistry of tibialis anterior muscle sections from the F-3 transgenic line showing dystrophin (*a*) and utrophin (*b*) sarcolemmal immunostaining. Magnification,  $\times 100$ .

explained by the fact that the human skeletal  $\alpha$ -actin promoter is expressed at lower levels in the slow-twitch fibres which essentially populate the soleus muscle than in the fast-twitch fibres of the TA (L. Levitt and E. Hardeman, personal communication). This observation implies that the levels of utrophin transgene are important for amelioration of the muscle phenotype.

Dystrophin is normally associated with a large oligomeric protein complex, the dystrophin protein complex (DPC),

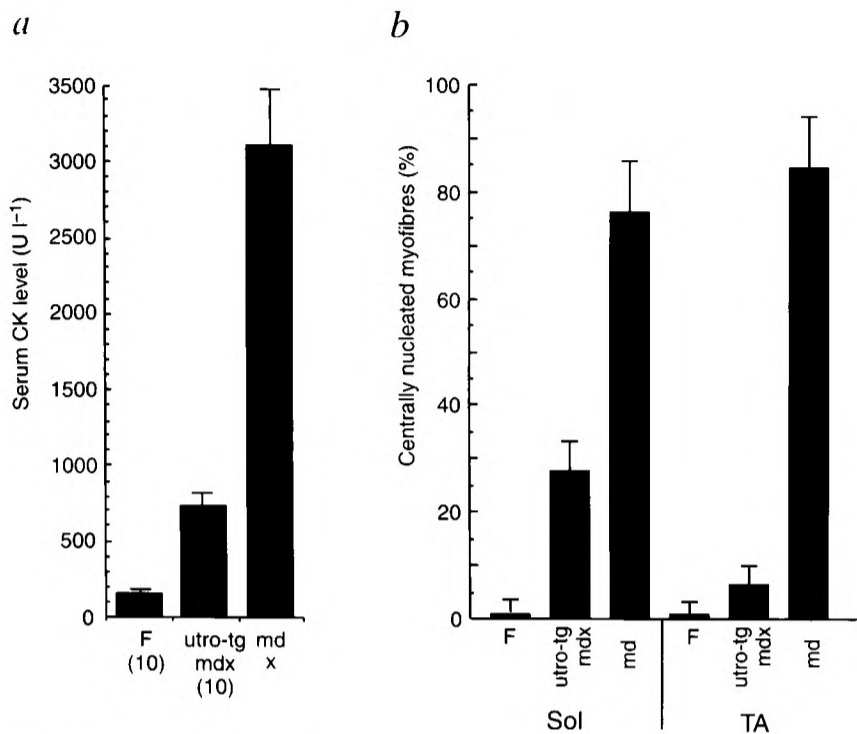


FIG. 2 Decrease in serum CK levels and centralized myofibres in transgenic *mdx* mice. *a*, Serum CK levels from male *mdx* (*mdx*), male utrophin transgenic *mdx* (*utro-tg mdx*) and heterozygous females (F). Female heterozygotes are not significantly different from wild type, and so can be used as normal controls. The number of mice in each group is given in parentheses and the s.e.m. is shown. *b*, Proportion of myofibres containing centralized nuclei. The s.e.m. is shown.

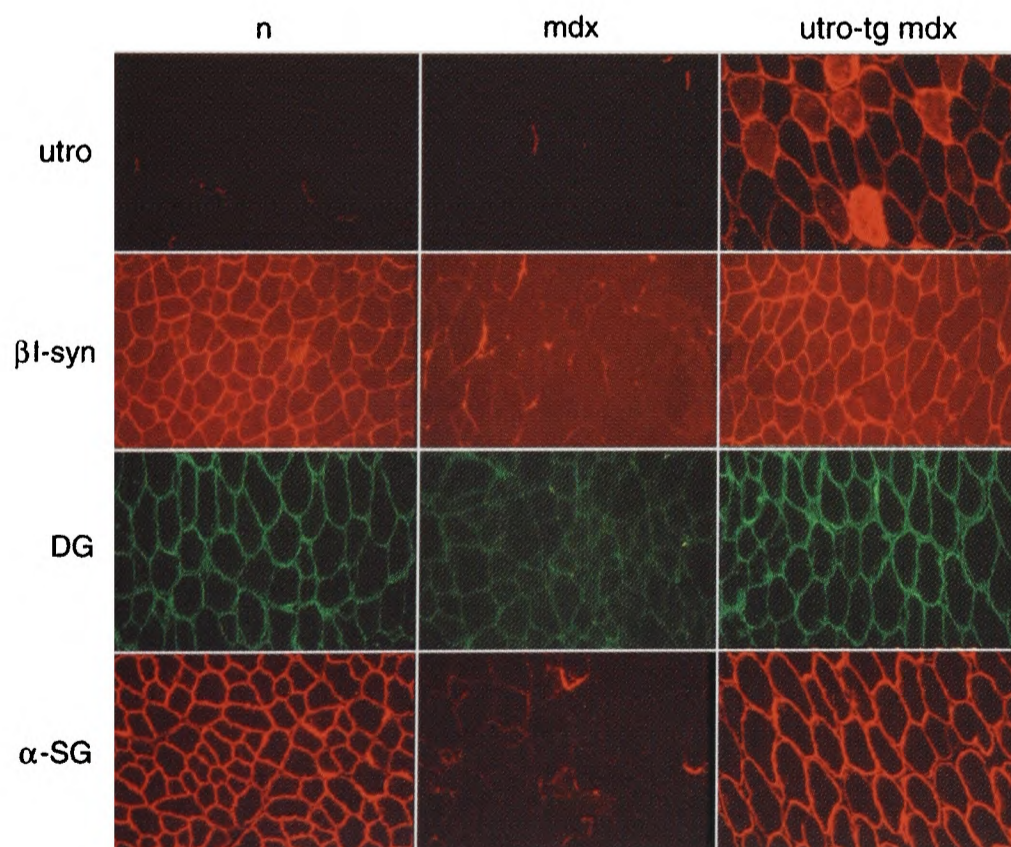


FIG. 3 Increase in DPC staining at the sarcolemma of transgenic *mdx* mice. Immunohistochemistry of utrophin and components of the DPC in TA muscle or normal (n), male *mdx* (*mdx*) and male transgenic *mdx* (*utro-tg-mdx*) using antibodies specific to utrophin (utro),  $\beta$ 1-syntrophin ( $\beta$ 1-syn),  $\alpha/\beta$ -dystroglycan (DG) and  $\alpha$ -sarcoglycan ( $\alpha$ -SG). Magnification,  $\times 100$ .

embedded in the sarcolemma<sup>12,16</sup>. Loss of dystrophin in DMD patients and *mdx* mice also results in a dramatic loss of sarcolemmal DPC<sup>17</sup>. In transgenic *mdx* mice expressing the full-length and truncated dystrophin transgenes, re-establishment of components of the DPC at the sarcolemma is an important marker for the restoration of muscle strength by dystrophin transgenes<sup>9,10,11,15</sup>. The results of immunostaining for components in the DPC in TA muscle from normal (n) mice, male *mdx* mice (*mdx*) or *mdx* mice expressing the utrophin transgene (*utro-tg-mdx*) are shown in Fig. 3. Sarcolemmal staining of all myofibres by utrophin-specific antibody was seen in utrophin transgenic *mdx* muscle. But in the normal and non-transgenic *mdx* mice there is virtually no sarcolemmal staining apart from neuromuscular junctions and regions likely to contain regenerating fibres. In all cases using polyclonal antibodies specific to  $\beta$ 1-syntrophin,  $\alpha/\beta$ -dystroglycan,  $\alpha$ -sarcoglycan and  $\gamma$ -sarcoglycan (data not shown), there was a notable increase in the staining at the sarcolemma of utrophin transgenic *mdx* TA muscle, indicating an elevation in sarcolemmal bound components of the DPC. The level of sarcolemmal staining of the utrophin transgenic *mdx* mice appears to be greater than that seen in the normal TA, suggesting that the number of dystrophin protein complexes at the sarcolemma is limited to some degree by the amount of dystrophin (or utrophin) present in the myofibres. This is a similar phenomenon to that observed in *mdx* mice expressing very high levels of a dystrophin transgene<sup>11</sup>. The increase in sarcolemmal staining of these components in the soleus muscle of transgenic *mdx* mice is greater than the non-transgenic *mdx* males but not as elevated as in the TA (data not shown). This result again implies that increased utrophin transgene expression correlates with an increase in sarcolemmal bound DPC.

Analysis of the *mdx* diaphragm has shown that this muscle exhibits a continued pattern of degeneration, fibrosis and functional deficit throughout the lifespan of the *mdx* mouse comparable to that of DMD skeletal muscle<sup>18</sup>. Thus, for utrophin to be capable of replacing dystrophin, overexpression of utrophin in this muscle has to alter the pathology in a similar way to that demonstrated for the dystrophin transgenic *mdx* mice<sup>9,10,11,15</sup>. Immuno-

staining of diaphragm sections using a utrophin antibody demonstrates the sarcolemmal localization of the utrophin transgene expressed in the transgenic *mdx* mouse (*utro-tg mdx*) compared to normal and *mdx* mice (Fig. 4*a*). The re-establishment of  $\alpha$ -sarcoglycan at the sarcolemma of the transgenic *mdx* diaphragm at levels similar to the normal diaphragm is shown in Fig. 4*a*. Sarcolemmal staining of the transgenic *mdx* diaphragm is similar to normal using antibodies specific to  $\alpha/\beta$ -dystroglycan and  $\gamma$ -sarcoglycan (data not shown). Thus, as in skeletal muscle, expression of the utrophin transgene in diaphragm relocalizes the DPC to the sarcolemma. Histological analysis of haematoxylin and eosin-stained sections of *mdx* diaphragm shows extensive regions of fibrosis, cellular infiltration and variable myofibre size containing centralized nuclei (Fig. 4*b*, *mdx*). But the utrophin transgenic diaphragm looks essentially the same as normal (Fig. 4*b*, 'n') with no necrosis, regular myofibre size and virtually no centralized nuclei (Fig. 4*b*, *utro-tg mdx*). In the *mdx* diaphragm, even in regions which have no necrosis and so appear more histologically normal, on higher magnification the myofibres are still of variable size, often containing centralized nuclei, which is indicative of continual regeneration (Fig. 4*c*, *mdx*). In the utrophin transgenic diaphragm, even at higher magnification, the whole muscle appears normal (Fig. 4*c*, *utro-tg mdx*). A return to normal histology and establishment of the DPC are two important observations, as seen with the dystrophin-transgenic

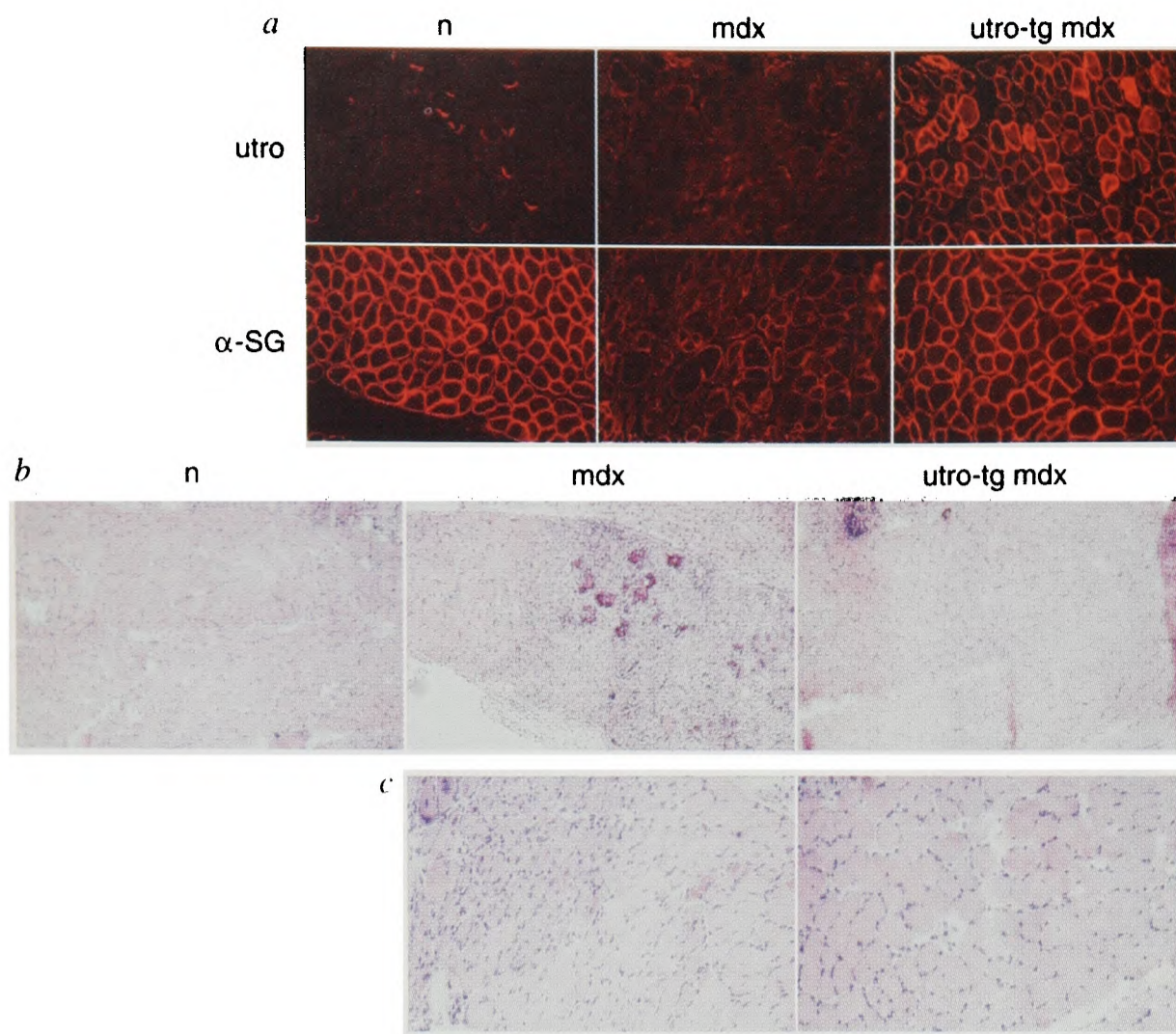


FIG. 4 Immunostaining and histological analysis of normal, *mdx* and utrophin transgenic *mdx* diaphragm. *a*, Immunostaining of diaphragm sections from normal (*n*), *mdx* (*mdx*) and transgenic *mdx* (*utro-tg-mdx*) stained with antibodies against utrophin (*utro*) and  $\alpha$ -sarcoglycan ( $\alpha$ -SG). Magnification,  $\times 100$ . *b*, Haematoxylin and eosin staining of normal (*n*), *mdx* (*mdx*) and transgenic *mdx* (*utro-tg-mdx*) diaphragm sections. Magnification,  $\times 100$ . *c*, Higher magnification ( $\times 200$ ) of *mdx* diaphragm (*mdx*) in areas with no obvious fibrosis/cellular infiltration still show evidence of continual regeneration, as seen by the variable fibre size and presence of centralized nuclei.

mice<sup>9,10,11,15</sup>, which predicts a major recovery of the utrophin-transgenic diaphragm from a dystrophic phenotype.

We have demonstrated a significant decrease in the dystrophic muscle phenotype of *mdx* mice by expressing a utrophin transgene at high levels in the skeletal muscle and the diaphragm. These results strongly suggest that utrophin can replace dystrophin *in vivo*. This implies that the use of small molecules which increase the normal utrophin muscle expression to compensate and therefore alleviate the consequences of a lack of dystrophin, is a promising area for DMD therapy. It remains to be seen if such compounds can be identified. However this approach would potentially target all muscles and thus prolong life by conserving the respiratory and cardiac muscles. Utrophin is expressed in many tissues so a generalized upregulation may not have detrimental side effects. The normal mice expressing the utrophin transgene at high levels seem to suffer no deleterious affects in their skeletal and diaphragm muscles. The side effects, if any, have yet to be demonstrated in other tissues. A precedent for such a gene-therapy approach using butyrate to upregulate fetal haemoglobin is having success in clinical trials of sickle-cell disease<sup>19,20</sup>. Only 20–30% of the wild-type levels of dystrophin are required to reduce significantly the dystrophic phenotype in *mdx* mice<sup>9,10</sup>. It will be interesting to determine whether similar level of utrophin will be adequate to compensate for dystrophin loss. Furthermore, as utrophin is normally expressed in all tissues including muscle, the use of this utrophin transgene rather than a dystrophin transgene in conventional gene-therapy approaches using viruses or liposomes may avert any potential immunological responses against the transgene. □

## Methods

**Transgene construction and microinjection.** The amino- and carboxy-terminal portions of utrophin were cloned as polymerase chain reaction (PCR) products using overlapping complementary DNAs as template, then ligated together in-frame to produce the truncated utrophin cDNA. The PCR product was then cloned into a vector containing the 2.2-kilobase human skeletal  $\alpha$ -

actin (HSA) promoter and regulatory regions<sup>21,22</sup> and SV40 large T poly (A) site. The cloning sites were such that the transgene was located near the beginning of the second HSA untranslated exon and the Asp 718/NotI sites were used to liberate the complete fragment. Transgenic mice were generated by microinjection of the purified HSA transgene insert into the pronucleus of F<sub>2</sub> hybrid oocytes from C57BL/6x CBA/CA parents<sup>23</sup>. Positive transgenic mice were identified by Southern blotting using a probe to the central part of the utrophin transgene. Several founder F<sub>0</sub> males were bred to generate more offspring for analysis and breeding.

**Protein analysis.** Total muscle extracts were prepared by homogenization in 1 ml extraction buffer (75 mM Tris, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol, 5%  $\beta$ -mercaptoethanol) then heated to 95 °C for 5 min. Usually 50  $\mu$ g of total protein (quantified using Biorad DC protein assay kit) was loaded onto 6% polyacrylamide gels and transferred to nitrocellulose. Utrophin transgene expression was detected using a 1/200 dilution of mouse anti-utrophin monoclonal antibody (MANCHO7; ref. 24) and visualized using anti-mouse IgG-POD and chemiluminescence (Boehringer). For sectioning, skeletal muscle samples were removed and immersed in OCT compound (BDH) and frozen in liquid-nitrogen-cooled isopentane. The diaphragm was removed, cut in half, then rolled longitudinally and sandwiched between ox liver to facilitate orientation and easier sectioning. The sandwich was then frozen. Immunostaining of unfixed 8  $\mu$ m cryosections was performed by blocking the sections in 10% heat-inactivated fetal calf serum in 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS), then the primary antibody diluted in TBS added and incubated for 1 h at room temperature. The slides were washed four times in TBS for 5 min each, then incubated for a further hour at room temperature with conjugated second antibody diluted in TBS. Finally, the slides were washed as before, mounted with VectaShield (Vector) and photographed using a Leica DMRBE microscope and photomicrograph system.

**Antibodies used for immunofluorescence.** Antibodies were used at the following dilutions: polyclonal rabbit against utrophin (G3, 1/25), dystrophin (P6 (ref. 25), 1/400),  $\beta$ 1-syntrophin (syn35, 1/50)  $\alpha$ -sarcoglycan<sup>26</sup> (1/5); goat polyclonal against  $\alpha/\beta$ -dystroglycan (FP-B<sup>27</sup>, 1/10); FITC conjugated secondary antibody to goat (Sigma) and Cy3 conjugated secondary antibody to rabbit (Jackson) were diluted 1/50 and 1/200, respectively.

**Creatine kinase assay.** Serum CK levels from mice 4–5 weeks old generated from four F<sub>3</sub> litters resultant from a male transgenic mouse crossed with female *mdx* were assayed. The tail tips were cut off and DNA prepared for Southern blotting to establish the transgenic status of each mouse. Blood was collected simultaneously, allowed to clot, and serum was removed. Serum CK levels were measured using the Boehringer NAC-CK kit and 5  $\mu$ l of serum. The rate was averaged over 4 min and calculated as U l<sup>-1</sup>.

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