Dystrophin Expression During Skeletal Myogenesis

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Abstract

The factors that regulate dystrophin accumulation, and its association with other proteins in culture, are largely unknown. In this study I have examined the effects of chemical agents on dystrophin accumulation in culture, as well as determined whether dystrophin in culture has associations with glycoproteins in a similar fashion to that found in normal muscle tissue. My results show that the onset of detectable dystrophin accumulation occurs shortly after myoblast fusion has taken place, and increases rapidly thereafter as a percentage of total cell protein. The effects of depolarizing concentrations of potassium ion, resulting in the inhibition of myotube contraction, are negligible, consistent with the fact that dystrophin is a cytoskeletal protein. I have found that 5 bromo-2'-deoxyuridine, known to affect terminal differentiation in myoblasts, markedly inhibits dystrophin accumulation in culture. Finally, I have found that dystrophin in cultured cells associates with Wheat Germ Agglutinin-binding proteins, as reported for adult skeletal muscle tissue, implying that the expression of these glycoproteins either precedes or occurs coordinately with dystrophin expression in culture.

Résumé

Les facteurs régissant l'accumulation de dystrophine, ainsi que son association avec d'autres protéines en milieu de culture sont en grande partie inconnus. Dans cette étude, j'ai examiné les effets d'agents chimiques sur l'accumulation de dystrophine en milieu de culture, et j'ai tenté de déterminer si la dystrophine en culture s'associe avec les glycoprotéines de la même façon que dans les tissus musculaires normaux. Mes résultats indiquent que l'on peut déceler une accumulation de dystrophine immédiatement après que la fusion du myoblaste a eu lieu, et qu'elle augmente ensuite rapidement en proportion avec le pourcentage du nombre total de cellules de protéine. Les effets de la dépolarisation des concentrations des ions potassiques qui entraîne l'inhibition de la contraction du myotube sont négligeables, ce qui me paraît une conséquence logique du fait que la dystrophine est une protéine cytosquelettique. Les résultats de mes recherches indiquent que la 5 Bromo-2'-Deoxyuridine, dont on a constaté qu'elle affecte la différenciation terminale des myoblastes, élimine complètement l'accumulation de dystrophine en milieu de culture. En définitive, j'ai constaté que la dystrophine dans les cellules en milieu de culture s'associe effectivement avec les protéines qui se lient à l'agglutinine du germe de blé, tel qu'on l'a constaté dans les tissus du muscle squelettique adulte, ce qui semble indiquer que l'expression de ces glycoprotéines soit précède l'expression de la dystrophine en milieu de culture, soit se passe de façon congruente.

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List of Abbreviations

aa	Amino Acid(s)
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CEE	Chick embryo extract
ddH_2O	Double distilled water
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DRP	Dystrophin related protein
FBS, FCS	Fetal Calf, Fetal Bovine Serum
g	Acceleration of gravity
HS	Horse serum
KLH	Keyhole Limpet Hemocyanin
MEM	Minimal Essential Medium
mg	Milligram
ml	Millilitre
mm	Millimetre
NaCl	Sodium Chloride
PBS	Phosphate buffered saline
rpm	Revolutions per minute
SDS	Socium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

TC Dish	Tissue Culture dish (plate)
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
TTX	Tetrodotoxin
WGA	Wheat Germ Agglutinin

Chapter One

Introduction

I: Background

Duchenne Muscular Dystrophy (DMD) is one of the most common of human X-linked diseases, and is almost always fatal. Duchenne muscular dystrophy affects approximately 1/3500 males. Affected individuals usually do not live beyond thirty years of age (Koenig *et al*, 1988). The clinical manifestations of Duchenne dystrophy are detectable as early as 1-2 years of age (Kedes, 1985), commencing with motor coordination difficulties and muscle weakness. This weakness is progressive, affecting the extremities more quickly than the chest or abdominal muscles. Often concomitant with this deterioration is a moderate level of mental disability. By age 12, the patient is usually limited to the wheelchair, and death generally occurs by the late teens due to respiratory failure, or complications leading to infections that result in pneumonia (Kedes, 1985). The muscle weakness itself is the result of segmental necrosis of the myofibres, and the near-complete inability to regenerate the deteriorating fibres (Hurko *et al*, 1989). Long before the disease is clinically evident, there is already a pattern of degeneration and regeneration of muscle throughout the body of the patient, and soluble muscle-specific enzymes can be found in the patient's serum. These markers for Duchenne Muscular Dystrophy are found even in female carriers and thus are an aid in understanding the genetics of the disorder, and were invaluable in mapping the gene and the determination of its locus.

Molecular biologists believed that the gene that is altered to cause muscular dystrophy is very large, based on the mutation rate. The cDNA of the DMD gene

has been cloned (Koenig *et al*, 1987). The DMD gene is located on the X-chromosome, at the p21 region (Kedes, 1985). It encodes a product, called *dystrophin*, approximately 427 kilodaltons in weight, estimated by Hoffman to constitute 0.002% of total muscle protein (Hoffman *et al*, 1987). Although generally more abundant in striated muscle, dystrophin has also been found in smooth muscle such as the stomach, and even in brain (Hoffman *et al*, 1987). Dystrophin is composed of 3685 amino acids, separated into 4 domains (Koenig *et al*, 1988).

The carboxy terminal is believed to be involved in membrane attachment, and is the site for extensive alternative mRNA splicing, leading to different isoforms some of which have different C-terminal ends. The C-terminal region is not homologous to other known proteins, whereas the N-terminus and second internal domains show significant sequence homology to α-actinin. The N-terminus of the dystrophin protein is followed by the first internal region which consists of 25 triple-helical segments, which show some similarity to the repeat domains found within spectrin (Koenig *et al*, 1988).

The dystrophin cDNA cloned by Kunkel *et al* (1987), was found to consist of over 60 exons, and to be highly conserved in mammals. Generally the only region of considerable variability has been the carboxy terminus. The dystrophin cDNA is 14 kb in length, while the gene itself is approximately 2000 kb in length (Kunkel *et al*, 1987). Average exon length is 200 bp while introns are around 35 kb in length (Kunkel *et al*, 1987). The dystrophin gene comprises approximately 1/1000 of the total human genome. The sheer size of the gene is sufficient to explain to some

degree the incidence of DMD, since the rate of incidence of mutation in the region of the x-chromosome where the dystrophin gene is located is not unusual (Kunkel *et al*, 1987).

Analysis of the dystrophin amino acid sequence showed that dystrophin is hydrophilic, with 31 percent of its amino acids being charged, and evenly distributed allowing no extensive region that is hydrophobic (Koenig *et al*, 1988), suggesting no segment of the protein is likely to be a membrane-spanning region, and that any association of dystrophin with the myofibre plasma membrane is through an intermediary such as a transmembrane glycoprotein. No evidence exists for a signal peptide.

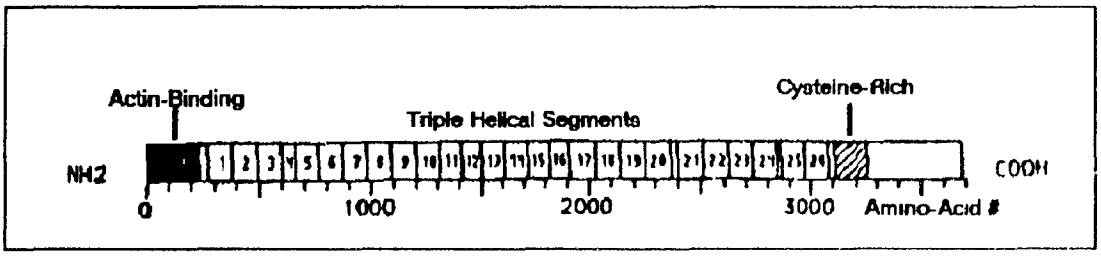

Figure 1: Dystrophin Protein Domains

-Figure adapted from Koenig *et al* (1988)

During screening for enzyme activity mutants in the mouse, Bulfield *et al* (1984) discovered a mouse mutant in C57BL/10 mice that was X linked and produced muscle lesions very similar to human Duchenne Muscular Dystrophy. Furthermore, the genetics of the disease were also as expected for an X-linked disease. Female heterozygotes were carriers with little or no sign of muscle deterioration, similar to normal females or males, while homozygous females and male heterozygotes showed

atrophy of muscle fibres, fibre degeneration, and the muscles showed signs of regeneration in progress. Hence, the disorder in mice was a non-lethal mutation, unlike in humans.

The similarity of the disease in mice and humans, and the fact that both disorders were the result of the absence or truncation of the dystrophin protein gave rise to a valuable animal model for the disease. Sequence studies showed that mouse dystrophin cDNA was over 90% identical at both the amino acid and DNA levels to human dystrophin. This provided researchers in the field with a unique system for use as controls in research involving human dystrophin. The cause of the mouse dystrophy was found by Ryder-Cook *et al* (1988) to be the result of a point mutation resulting in a truncated dystrophin protein.

II: Does dystrophin accumulate in culture?

The size of the dystrophin protein raised other questions. Part of the normal development of cells is the turnover of proteins. Dystrophin, being such a large protein, raised questions as to its susceptibility *in vitro*, since the protein might by its size be much more susceptible to degradation by cellular enzymes. This brought into doubt whether the dystrophin protein would even accumulate substantially in culture and to what extent. The possibility of a dependence upon myoblast fusion had not yet been explored.

Given the necessity of cellular contraction (Bandman *et al*, 1982 and Crisona *et al*, 1983) for the normal accumulation of certain proteins, such as myosin (Crisona

et al, 1983), it became a valid question whether dystrophin would be subject to the same restrictions.

III: What influences the accumulation of dystrophin in culture?

(i) Protein turnover and contractile activity in myotubes

The accumulation of basal lamina in myotube culture is heavily dependent upon myotubes being spontaneously active (Sanes *et al*, 1983). In their study, Sanes and Lawrence found that the addition of tetrodotoxin (TTX) eliminated all contractile activity in the cultured myotubes. TTX has little effect upon protein production in the cells, hence the cells' inability to accumulate basal lamina was found to be dependent not upon a reduced overall protein production, but an alteration of the turnover or a shunting to the production of other proteins, leaving overall protein levels the same (Sanes *et al*, 1983). A comparison was done with Lidocaine and again upon arresting the spontaneous contractions of cultured myotubes, the accumulation of basal lamina was drastically altered, without affecting overall protein levels in the cells.

Bandman, Crisona and Strohman (Bandman *et al*, 1982 and Crisona *et al*, *1983*) confirmed the results by Sanes and went a few steps further. Crisona and Strohman found that the effects of TTX on rat muscle fibres were completely reversible. They also studied the kinetics of the synthesis and accumulation of different protein classes in these cells to determine more specifically which types of proteins were affected by contraction-inhibition.

Their results showed that the kinetics of extramyofibrillar protein accumulation and synthesis were unaffected by the TTX treatment, myofibrillar proteins were significantly reduced for the duration of the treatment, but returned to normal after the removal of TTX from the culture medium (Crisona *et al*, 1983). Among the myofibrillar proteins assayed were troponin-C, myosin fast light chains 1 and 2, α-actin, and tropomyosin. Filament proteins studied included vimentin, α,β-desmin, and β,α-actin. Importantly, of the contractile proteins affected, not all were affected to the same extent nor in the same manner While some were drastically reduced in synthesis, others were more affected in their levels of accumulation. In some cases, the decreased accumulation was not the result of decreased synthesis, but resulted from increased degradation

Further work by Strohman and Bandman (Bandman *et al*, 1982) demonstrated the effects of depolarizing levels of potassium ion on contraction in culture. While cells in mild concentrations of potassium would continue to spontaneously contract, high concentrations of potassium would completely and reversibly inhibit all contraction Again, myosin accumulation was shown to be markedly reduced (Bandman *et al*, 1982) Total protein accumulation in the potassium treated cells was not altered significantly, nor was differentiation affected, as the cells were able to fuse normally and would contract within a day of the removal of the high potassium medium The primary cause of decreased myosin accumulation was found to be the increase in myosin turnover in high potassium cultures (Bandman *et al*, 1982). The

determination of whether dystrophin accumulation is altered due to contraction-inhibition was then considered a valuable endeavour.

(ii) Factors controlling myoblast terminal differentiation

Dystrophin was originally thought to be entirely muscle-specific. Although more recent findings have shown it to be present in other tissues as well, notably brain, the developmental regulation of dystrophin induction in the myoblast is generally unclear. Research into muscle-specific gene activation has shown a single gene can be responsible for myogenic determination. One such gene is known as *MyoD*. MyoD can convert C3H10T1/2 mouse embryo fibroblast clonal cells to myoblasts (Davis *et al*, 1990). The method of myogenic induction by MyoD is the binding of MyoD to cell DNA at the promotor and/or enhancer regions of muscle-specific genes, thereby inducing a cascade of events leading toward the alteration of cell phenotype, and an alteration in their terminal differentiation, preventing cell fusion.

Dystrophin is known to have a MyoD binding site, known as an E Box. The binding of MyoD (or transcription factor heterodimers having MyoD as a component) to the E Box normally activates the promoter and allows transcription to occur. Recent work by Gilgenkrantz *et al* (1992) showed that direct action by MyoD on the dystrophin regulatory elements does not trigger transcription. This does not preclude the possibility that dystrophin could be subject to regulation in its turnover by MyoD controlled elements. Dystrophin is also known to have other regulatory elements, such

as a CCArGG box, which seems to be involved in determining muscle-specific transcription (Gilgenkrantz *et al* 1992). Dystrophin is differentially regulated, with different promoters for muscle and non-muscle tissue (Gilgenkrantz *et al* 1992).

One of the most well-known and effective chemical agents known to alter the terminal differentiation of myoblasts by eliminating the binding of MyoD is 5-bromo 2'-deoxyuridine (BrdU) (Cates *et al*, 1978 and Tapscott *et al*, 1989). BrdU is a mutagen, a thymidine analogue that causes a keto to enol isomerization changing guanidine - cytosine base pairs for adenosine - thymidine. BrdU affects differentiation, but not proliferation of the treated cells. Changes are reversible, and involve normal DNA replication mechanisms. BrdU is believed to work by altering promoters and enhancers on cell DNA, thereby selectively affecting the expression of myogenic regulatory proteins such as MyoD. Overexpression of MyoD in BrdU-treated myoblasts overrides the effects of BrdU on the expression of muscle specific genes such as myosin heavy chain, suggesting that BrdU has an indirect effect on the promoters for these genes and affects their expression secondary to effects on MyoD (Tapscott *et al*, 1989)

The effects of agents that alter terminal differentiation could also be felt by other genes, including dystrophin. Hence, a study of the effects of BrdU on dystrophin levels in cell cultures could reveal whether dystrophin falls into the same category of proteins that are within the MyoD regulatory cascade.

Dystrophin is believed to be a membrane cystoskeletal protein, based upon its sequence. Given the lack of membrane-spanning domains within the protein, it is

logical that it must associate with other proteins that are capable of anchoring to the membrane, or are themselves embedded within it. Since it is believed that the stability of this glycoprotein complex is dependent upon dystrophin associating with the complex, it is not unreasonable to consider the effects of glycoprotein association as important in the expression of dystrophin

IV: Does dystrophin associate with its glycoprotein complex in cultured cells as reported for tissue?

Campbell *et al* (1990) showed that dystrophin is associated to an oligomeric complex of glycoproteins that allow dystrophin attachment to the plasma membrane. This glycoprotein complex consists of cytoskeletal, transmembrane, and extracellular components. A 43K and a 156K glycoprotein have already had their amino acid sequences deduced from cDNA (Ibraghimov-Beskrovnaya *et al*, 1992) Dystrophin appears to associate with a glycoprotein complex consisting of 25K, 35K, 43K, 50K, 59K and 156K proteins at a 1:1 stoichiometry Dystrophin appears to be in direct contact only with the 35K, 43K, and 59K glycoproteins (Ibraghimov Beskrovnaya *et al*, 1992). Use of gentle detergents such as digitonin allows the extraction of the complex intact, allowing the study of the association of dystrophin to its anchor complex in greater detail. It is unknown whether dystrophin associates with glycoproteins in culture, especially at early stages of myogenesis If dystrophin does associate with glycoproteins *in vitro*, then this implies that the expression of the glycoproteins either precedes dystrophin expression, or is occuring concomitantly.

A comparison of the levels of dystrophin accumulation in culture versus the levels of dystrophin in human or animal tissue would also be informative. While *in vitro* studies have considerable usefulness in the study of developing animal systems, hormonal control, immune system interference, and the actions of local cellular environment as well as the environment surrounding the organism as a whole cannot always be assessed in this system. A knowledge of the level of dystrophin expression in culture however provides a yardstick against which results obtained *in vivo* can be measured. The *in vitro* system has the important advantage that the factors controlling gene expression can be manipultated and studied more readily than *in vivo*.

V: Summary of research attempted

In this work I addressed several questions:

i) How is dystrophin expressed *in vitro*? Does it have the same abundance as in adult muscle tissue? Does it start expression early in cell development or is it dependent upon other factors, such as fusion or the presence of other proteins?

ii) What are the regulatory factors that alter the expression of dystrophin from normal levels in culture? Are chemical agents, such as BrdU, which prevent differentiation, or depolarizing agents, such as potassium, that prevent spontaneous cell contraction, capable of modifying the accumulation of dystrophin *in vitro*?

iii) What association occurs with different proteins in culture? Can dystrophin be found associated with membrane glycoproteins as reported, or does early expression of dystrophin occur without coordinate expression of its receptors?

Materials & Methods

Materials:

1) Tissue culture materials:

Growth medium (Ham's F 12), Dulbecco's Modified Eagle Medium (DMEM) chick embryo extract, trypsin (DIFCO 1/250), horse serum, were all obtained from GIBCO, Grand Island, New York, USA.

Supplemented growth medium additives: BSA, dexamethasone, fetuin, and bovine insulin were all obtained from Sigma, St Louis, Mo, USA

Gentamycin sulphate was obtained from Sigma.

Tissue culture dishes, including 100mm and 60 mm dishes were obtained from Nunc (sold by GIBCO)

Gelatin was obtained from BDH, Toronto, Ontario

Potassium chloride was obtained from Fisher Scientific, Lachine, Quebec

5 BromoDeoxyUridine was obtained from Boehringer Mannheim, Laval, Quebec.

IEC Clinical Centrifuge was purchased from International Equipment Company, Needham, Mass, USA

2) Antigen preparation materials:

Freund's Complete and Incomplete Adjuvants were obtained from GIBCO; glutaraldehyde was obtained from JBS Supplies, Pointe Claire, Quebec.

Spectraphor #4 dialysis membranes were obtained from Spectrum Medical Industries, Los Angeles, CA, USA.

Peptides used for injection were obtained from Multiple Peptide Systems, San Diego, CA, USA

Keyhole Limpet Hemocyanin (KLH) was purchased from Sigma.

The sonicator for tissue and immunogen preparation was purchased from Fisher Scientific, Model 150.

3) Affinity purification

Affigel-15 Sepharose beads, 15x0.7cm glass columns, were purchased from Bio Rad, Richmond, CA, USA.

Citrate, sodium chloride, sodium phosphate, and glycine were all purchased from Sigma.

ImmunoPure Ag/Ab Gentle Elution Buffer was purchased from Pierce, Rockford, IL, USA.

4) Protease inhibitors:

Aprotinin, Leupeptin, N-Ethyl-Maleimide, and Pepstatin-A were all obtained from Boehringer Mannheim, Laval, Quebec.

5) Protein assays:

Sodium hydroxide was purchased from Fisher Scientific

BCA Protein Assay reagents were purchased from Pierce.

Model U-1100 UV/Vis Spectrophotometer was purchased from Hitachi.

6) WGA chromatography.

Wheat germ agglutinin-sepharose (WGA-Sepharose) was obtained from Sigma.

Biotinylated wheat germ agglutinin was purchased from Vector Labs, Burlingame, CA, USA.

7) SDS-PAGE & Western blotting, slot blotting including stains

Tris base was purchased from Sigma.

Sodium dodecyl sulphate (SDS) was purchased from Polysciences Incorporated, Warrington, PA, USA.

Bis-acrylamide, acrylamide, ammonium persulphate, and TEMED were all obtained from BioRad.

Blotting and nitrocellulose paper were purchased from Scheicher & Schuell, Keene, NH, USA.

Biotinylated secondary anti-rabbit antibody was purchased from Vector Laboratories.

NBT/BCIP, alkaline-phosphatase conjugated secondary antibody were purchased from Bio/Can Scientific, Mississauga, Ontario, Canada.

8) Image analysis:

Jandel Video Analysis software was purchased from Jandel Scientific, Corte Madera, CA, USA, and Microsoft Excel 4.0 was purchased from Microsoft Corporation, Redmond, WA, USA. Image analysis was performed on a 486/33 IMB-PC clone running MS-DOS 5.0 and Windows 3.1 operating environments

9) Frozen sections:

Potassium biphosphate, sodium biphosphate, diaminobenzidine were obtained from Sigma.

Streptavidin peroxidase was purchased from Bio/Can Scientific.

Biotinylated anti-rabbit antibody was purchased from Vector Laboratories.

10) Stains:

Bromophenol blue and coomassie brilliant blue were purchased from Sigma.

Eosin and trypan blue were obtained from GIBCO.

11) Miscellaneous chemicals:

All other chemicals were purchased from Sigma or Fisher Scientific, depending on price and availability.

Methods:

Peptide-Conjugate preparation:

The method used was modified from Walter *et al* (1980). 5 mg of the selected peptide and 5 mg of KLH (or BSA for use in slot blot assays) were mixed together in 0.8 ml of 0.1 M sodium phosphate solution (pH 7.5) in an eppendorf tube. 0.2 ml of 50% glutaraldehyde was added dropwise and the mixture agitated for 30 minutes at room temperature until the solution turned orange-yellow. The tube was spun at 1000xg for 3 minutes to spin down any precipitate and the supernatant decanted.

The supernatant was placed in a rinsed dialysis membrane (spectraphor #4 MW exclusion limit 12000-14000), and the dialysis bag was then placed in 0.1 M sodium phosphate solution, (pH 7.5) overnight at 4°C.

After dialysis, the solution inside the membrane was removed and aliquoted in 1 ml quantities and frozen at -70°C until needed.

Rabbit injections, bleeds & serum storage:

Methods followed were taken from Goding (1983). Antigen (peptide-KLH conjugate) was thawed and the 1 ml aliquot was mixed with an equal volume of Freund's Complete Adjuvant for the first injection. Subsequent injections were performed using Freund's Incomplete Adjuvant. The adjuvant-conjugate mixture was sonicated for two minutes on ice to prevent excessive heating.

Rabbits were injected subcutaneously in the dorsal region twice monthly using a 1 ml syringe and 22 gauge needle.

Bleeds were started after the third injection and were carried out on weeks that the rabbits were not injected. Bleeds were performed by puncture an artery on the surface of the ear, and collected into 50 ml centrifuge tubes

Blood was allowed to clot overnight at 4°C and then the serum was gently poured out, with as little clot disruption as possible The serum was then vacuum filtered to remove red blood cells, aliquoted into 1 5 ml eppendorf tubes and stored at -70°C.

Slot blots:

Slot blots were performed using a Bio-Rad SF Microfiltration Apparatus The slot blot was selected as the most convenient method should there be a desire to perform densitometric analysis due to the uniformity of distribution of sample in each chamber.

Serial dilution was used to prepare samples of peptide conjugates for loading Upon adsorption by vacuum filtration onto the nitrocellulose membrane, the nitrocellulose was dried overnight and then all unreacted sites on the nitrocellulose were blocked by skim milk and incubated with primary antibody for two hours at room temperature The blot was then rinsed three times for five minutes each with TBST (100 mM Tris HCl pH 7.5, 0.9% NaCl, 0.1% Tween 20) (L.M. Kunkel, personal communication) and secondary biotinylated anti-rabbit antibody was then added at a dilution of 1/400 for one hour.

The blot was then rinsed again three times for five minutes each with TBST and ABC alkaline phosphatase reagent was added and allowed to incubate for one hour, then rinsed as before and NBT/BCIP reagents were added until the stain was no longer appearing to darken. The nitrocellulose was allowed to dry between blotting paper and photographed

SDS-PAGE sample preparation:

i) Tissue samples: Tissue (approx 30 mg) from skeletal muscle of animal or human origin material was minced with a razor blade and then put into a glass test tube with 200 μl SDS sample buffer (75mM tris-HCl pH 6.8, 15% SDS, 20% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue) and polytroned at full speed for 30 seconds for complete homogenization (C. Guerin, personal communication). The samples were then removed and placed in an eppendorf tube and boiled for five minutes, then frozen at -70°C until needed Before use, the samples were thawed, boiled for 2 minutes and sonicated for 30 seconds to disrupt protein aggregates that form during freezing. Before use protein was quantitated using the BCA protein assay as described below

ii) Culture samples. Cultured cells were harvested as described below. The cells were then placed in an equal volume of SDS sample buffer and boiled for 5 minutes, then frozen until needed at -70°C When about to be used, the samples were thawed, boiled for 2 minutes and sonicated for 30 seconds to disrupt protein aggregates that form during freezing. Before use a protein assay was performed to quantitate protein for loading in gels.

Harvesting of cultured cells:

Cultured cells were rinsed twice in the plate with cold (4°C) PBS to remove serum and growth medium, and aspirated until all liquid was removed. Approximately 0.5 ml of cold PBS was then added to the dish. A rubber policeman was gently run over the surface of the dish several times, swirling the PBS periodically to collect all the scraped cells. The PBS was then taken by transfer pipette and put into a 15 ml conical centrifuge tube and spun in a cold (4°C) IEC tabletop centrifuge at 1000 rpm for four minutes. The PBS was then removed with a pasteur pipette, carefully avoiding the pellet.

The pellet was then resuspended in about 200 μl of PBS, and decanted into a cold homogenizer. The suspension was then homogenized with 20 strokes, all the while keeping the whole apparatus covered to the neck with ice to prevent degradation of proteins. The homogenized cells were then aliquoted and frozen at -70°C until needed.

Protein assays:

Protein assays of samples were performed using the bicinchoninic acid (BCA) reagent (Pierce) for spectrophotometric determination of protein concentration.

A standard BSA solution of 2 mg/ml was serially diluted and used for a reference curve. Tissue and culture samples dissolved in SDS were then acetone precipitated by the addition of 10 volumes of acetone to the sample and agitation, followed by evaporation of the acetone and water overnight.

Samples were then digested with 0.1 N NaOH for one hour at room temperature and then the BCA test kit was used as per instructions.

SDS-PAGE & Western blotting:

Methods described here are taken from Zubrzycka-Gaarn *et al* (1991). Tissue or culture homogenates of known concentration were prepared according to the procedure above. A 4.5% polyacrylamide gel was made on the Bio-Rad mini-gel system.

The gel was transferred in the standard tris-glycine transfer buffer at 100 Volts for 3 hours with "Bio-Ice" cooling units changed at 30 minute intervals.

The blot was dried for more than 16 hours between filter paper and incubated in 10% non-fat dry milk in TBST buffer for 45 minutes at 37°C with mild agitation in a petri dish or a modified square tissue culture flask.

The skim milk was removed and the blot rinsed briefly with TBST. The polyclonal antiserum was added in TBST without skim milk and the blot incubated at room temperature on a rocker for 2-3 hours. The blot was washed (3 changes of TBST during 15-20 minutes) and rinsed 30 seconds with the skim milk and 30 seconds with TBST. The secondary anti-rabbit antibody was added to 10 ml of TBST.

Secondary antibody consisted of two sorts:

i) Alkaline Phosphatase conjugated;

ii) Biotinylated.

The biotinylated secondary antibody (goat anti-rabbit) was added at a 1:400 dilution in TBST to the blot and the incubation was continued for one hour at room temperature with gentle rocking. During this time, the ABC alkaline phosphatase reagent (Pierce) was prepared according to the instructions on the package in TBST. The blot was washed as above.

The ABC alkaline phosphatase reagent was added and the incubation continued for one hour at room temperature with gentle rocking. The blot was washed as above, except for 30 minutes and the skim milk rinse was omitted.

The alkaline phosphatase conjugated antibody was added at dilutions of 1/5000 to 1/7500 for one hour, then the blot was rinsed as above with TBST.

Colour development was achieved with the NBT/BCIP reagent in 15 ml of its reaction buffer. Only a few seconds after adding the reagent the dystrophin band appeared over a light background and the reaction was stopped immediately by rinsing with distilled water.

Staining of gels (as opposed to blots described above) was performed using coomassie brilliant blue (0.5% coomassie dye w/v, 25% isopropanol, 10% acetic acid) for 2 to 3 hours and then destained for another 2 to 4 hours using destain (10% isopropanol, 10% acetic acid) until bands were visible clearly over the background

Primary rat muscle cell cultures.

3-day old rat pups were and decapitated with large scissors, then placed on ice. The pups were rinsed briefly in ddH_2O, then 70% ethanol, and then from each pup the calf and thigh muscles were removed and put in a small volume of DMEM in an uncoated tissue culture dish.

Once all the pups have had their leg muscles removed, the excised tissue was minced to a fine pulp in the TC dish, and then decanted into the trypsinization flask Approximately 10 mls of 0.3% trypsin in ddH_2O was added, and the flask put in the

incubator and set to stir slowly. Once the solution became cloudy the flask was removed and the solution carefully decanted into a 15 ml conical tube. Care was taken to avoid large solid tissue particles from leaving the trypsinization flask, since this is still not fully digested. The flask was refilled to the 10 ml mark and replaced in the incubator to continue digestion until again cloudy.

The 15 ml conical tube was filled with an equal volume of DMEM + 10% horse serum and then spun in the centrifuge for 3-4 minutes at 1000xg (setting 4 on the IEC). The supernatant was removed and discarded, and the pellet resuspended 3-5 ml of DMEM + 10% horse serum to inactivate any residual trypsin, and left at room temperature in the hood. These steps were repeated until only fine stringy lumps of connective tissue were left in the trypsinization flask.

The contents of the conical tubes were pooled and then poured into a syringe (60 cc or 12 cc depending on the volume), then passed through a 75 μm Swinney Nitex filter, followed by a 10 μm filter.

An aliquot was taken for cell counting, mixed with trypan blue or eosin stain and counted under the microscope. Cells were plated in 0.5% gelatin-coated TC dishes at 3-5x10^6 cells per 100mm dish. They were given 10 ml of DMEM +10% horse serum + 0.5% chick embryo extract + 50 μg/ml gentamycin sulphate, and placed in a 95% humidity, 5% CO_2 incubator at 37 °C.

The medium was changed every 3-4 days, until harvesting.

Contraction-Inhibited cultures:

Primary rat muscle cell cultures were prepared as described above. Cells were placed in normal medium (DMEM + 10% horse serum + 0.5% chick embryo extract until fusion had taken place, (approximately 3 days) then half the plates were switched to medium containing 12 mM potassium bicarbonate and 19.6 mM sodium bicarbonate. Cells without high potassium concentration were also given fresh medium so that both high and low potassium-containing cultures would have to condition the medium at the same time-point in differentiation. Medium was changed every 3-4 days, continuing to put high potassium for one set of plates, and low potassium for the other set. Cultures were harvested after 9-11 days, when the low potassium plates showed maximum contractile activity.

BrdU cultures:

Primary rat muscle cell cultures were prepared as described above, and placed in normal medium (DMEM + 10% horse serum + 0.5% chick embryo extract. After fusion (approximately 3 days), medium was changed. One half of the plates had normal medium, the other half had normal medium containing 6.5 μM BrdU (Cates *et al*, 1978). Medium was changed as before, every 3-4 days, with one set of plates continuing to receive 6.5 μM BrdU in their medium, and the other half of the plates received normal medium. After 7-11 days, the cells were harvested as described above.

Human cell cultures:

FACS-sorted human cells from tissue were prepared by C. Guerin as described by Champaneria *et al* (1989) and frozen at -70°C. Cells were thawed and plated at $3x10^6$ per 100mm plate in supplemented growth medium (Ham's F-12 + 15% fetal bovine serum, 0.5 mg/ml BSA, 0.39 μg/ml dexamethasone, 10 ng/ml human epidermal growth factor, 0.5 mg/ml fetuin, and 0.18 mg/ml insulin. Cells were allowed to grow to confluence (approximately 5 days) in growth medium.

Once confluent, cells were switched to fusion medium (DMEM + 2% horse serum). Cells were allowed to fuse for another 8 days and then were harvested as described above.

Antiserum affinity purification:

The procedure used was a modification of that followed by Richardson *et al* (1985). 1.0 ml of Affigel-15 affinity support matrix from Bio Rad was placed into an eppendorf tube and spun for 2 minutes in an eppendorf tabletop centrifuge at 15000 rpm. The supernatant was removed and the Affigel-15 was rinsed with 0.5 ml of citrate/NaCl Buffer (0.1 M/1.0 M, pH 6.9) and spun in the eppendorf centrifuge (repeated three times) to remove all traces of isopropyl alcohol (a storage agent).

The Affigel-15 was then mixed until a suspension was formed, then 1.0 ml of BSA-C-terminal DMD peptide conjugate was added. The two were mixed for one hour at room temperature to allow coupling to progress to completion.

The conjugated BSA-C-terminal-Affigel-15 was then resuspended and diluted for ease of transfer in citrate-NaCl buffer and pipetted into a 15 cm long, 7 mm internal bore glass column. This makes for a column of about 1 ml bed volume. Any unreacted sites on the Affigel-15 were blocked by the addition of glycine (1.0 M, pH 8.0) for one hour at room temperature. The glycine was rinsed then with citrate buffer.

1 ml of serum was thawed and then run into the column. The column was allowed to sit overnight at 4°C and for one hour at room temperature to allow the antibody to bind to the free antigenic sites on the Affigel conjugate. The column was washed with 15 ml of citrate buffer, and three 5 ml fractions were taken. Then 5 ml of the Immunopure Gentle Ag/Ab (Pierce) elution buffer was used to elute the IgG. Five 1 ml fractions were taken, and stored at 4°C. The column was regenerated with 5 ml of citrate buffer (pH 2.5, 0.1M) and then rinsed with citrate buffer (pH 6.9, 0.1M), filled with PBS containing 0.05% sodium azide (as preservative), and stored at 4°C.

Wheat Germ Agglutinin extractions:

Human cells were cultured as described above. Harvesting was done somewhat differently from what was previously described on page 19. Cultures were harvested in cold PBS (4°C) with protease inhibitors (Pepstatin A 1.0 μM, Leupeptin 1.1 μM, N Ethyl Maleimide 1.0 μM; Aprotinin 0.0768 μM). Cells were homogenized in a fritted glass homogenizer. Harvesting and homogenization were done on ice at all times.

Cell homogenates were spun in a Ty 65 (Beckman) rotor in an L8 55 (Beckman) ultracentrifuge at 100 000g (42 500 RPM) for one hour at 4°C. The pellet was

solubilized in Buffer A (Buffer A = NaCl 0.50 M; Tris-HCl pH 7.4 50 mM; NP40 1.0%; & protease inhibitors as above), and spun in an eppendorf tube centrifuge at 4°C. The supernatant was kept (the pellet was discarded, as it consists primarily of nuclei).

The supernatant was run into the WGA-Sepharose column at 4°C and allowed to incubate for 2 hours at 4°C. Elution was done with 1% SDS in Buffer B. (Buffer B= Tris HCl pH 7.4 50mM, NP40 0.1%, protease inhibitors as above).

The elutate was mixed with an equal volume of sample buffer and used for SDS-PAGE

Frozen sections:

Tissue was obtained from biopsies performed by either Dr. George Karpati (in the case of human skeletal muscle tissue) or by Mr. Steven Prescott (in the case of mouse tissue). Hereafter the handling of tissue samples is the same.

4 μm sections were sliced using a cryostat and placed on microscope cover slips. The samples were then immersed in acetone for 2 minutes and allowed to air dry. The sections were rehydrated by incubation in PBS for 5 minutes, then excess buffer was removed and the coverslips were place in a humidified chamber

Antisera were applied to the sections, and incubated for 30 minutes at room temperature. The sections were rinsed twice in PBS for 5 minutes each and streptavidin-biotinylated peroxidase was then added and allowed to incubate for another 30 minutes

The rinse was repeated again twice with PBS for 5 minutes each and the sections reacted with 0.5 mg/ml diamino benzidine solution for 5 minutes at room temperature.

Upon reaction the sections were rinsed with distilled water 5 times and dehydrated in a 95%, 95%, 100%, 100%, ethanol series, cleared in xylol and mounted.

Results

Section I: Characterization of the antibody to dystrophin

Initially, it was decided to attempt to raise antisera to synthetic peptides, based on the human dystrophin amino acid sequence (Koenig *et al*, 1987 and 1988). The known sequence was used to select three different regions that were expected to be immunogenic, based on criteria outlined for the selection of suitable epitopes and peptides for injection (Lerner, 1984) The peptides were from areas that were hydrophilic, and considered likley to be exposed sites on the dystrophin protein.The three peptides were: i)GLTGQKLPKFKGSTR-COOH (amino acids 56-70); ii) RQSTRNYPQVNVINFTTS-COOH (aa 145-162); iii)GTGKLSEDEETEVQEQ-COOH (aa 410-423) The 3 selected peptides were conjugated to KLH using glutaraldehyde, and injected into rabbits (Goding, 1983), first with 1 mg peptide-conjugate in complete Freund's adjuvant, followed with biweekly injections of 1 mg in incomplete adjuvant and then the rabbits were bled. The antisera were assayed on slot blots against peptides conjugated to bovine serum albumin (figure 2A, 2B, & 2C) BSA was used to avoid any possible reaction by the antiserum to KLH, which was used as a carrier for the immunogen when it was injected in rabbits.

As can be seen from figure 2, antiserum 1 reacts with its immunogen, but cross-reacts rather strongly with peptides 2 and 3. Antiserum 2 reacts with its own immunogen, as well as peptides 1 & 3. These cross-reactions are likely due to non-specific reactions Antiserum 3 reacted well against its immunogen and cross-reacted

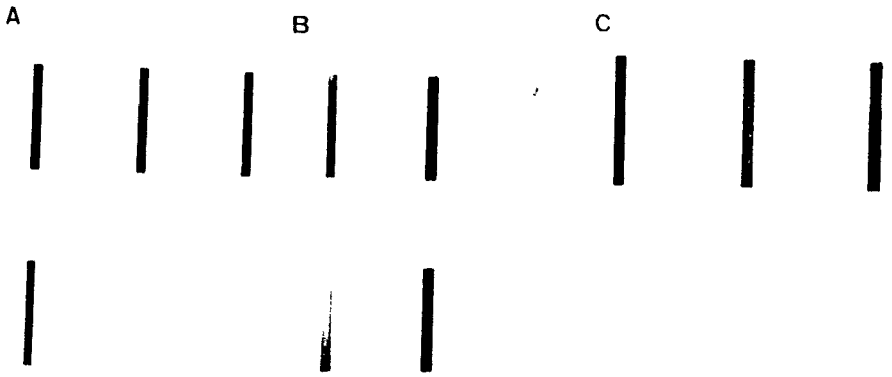

Figure 2A; 2B, 2C: Slot blots of Immunogens tested with their own antisera.

The Peptide-BSA conjugate was prepared as described in Materials & Methods. The blot was probed with the antisera at 1/200 dilutions against varying concentrations of peptide-BSA conjugate. The bound antiserum was then reacted with anti-rabbit biotinylated antibody (Dimension labs) at 1/400 concentration and stained with NBT/BCIP (Bio/Can).

A: The three lanes show the reaction of antiserum to peptide 1 against peptide BSA conjugates to peptides 1,2 and 3 respectively. The upper slot is with antigen concentration at 0.15μg/ml, the lower is at 0 015μg/ml

B: As in A above, except the antiserum to peptide 2 is tested against peptide BSA conjugates 1, 2 and 3.

C: As in A & B above, except the antiserum to peptide 3 is tested against peptide-BSA conjugates 1, 2 and 3.

with peptide 1, but was not as reactive with peptide 2. There was no appreciable activity against BSA alone by any of the three antisera (not shown), so all immunoreactivity can be safely considered specifically directed against the peptide portion of the BSA-peptide conjugates.

These results show that indeed there was an immunoreaction occurring to the injected peptide conjugates, successfully generating antibodies. The next step was to determine whether any of the antisera were immunoreactive against the dystrophin protein (figure 3).

The anti-peptide antisera were used to probe samples of normal and dystrophic (dystrophin-deficient) human tissues, provided by Dr. George Karpati at the MNI. The tissues were homogenized in a glass homogenizer and a polytron, boiled in SDS, and sonicated to ensure complete solubilization. The sample proteins were separated electrophoretically, then transferred by Western blotting to nitrocellulose. A polyclonal antiserum against a 60 kd dystrophin fusion protein (Hoffman *et al*, 1987) was used as a control antiserum for comparison. As can be seen in figure 3, the internal region peptide antisera do not produce any reaction against dystrophin in the normal human tissue, with multiple bands appearing in both dystrophic and non-dystrophic tissues. The dystrophic tissues were intended as controls. By contrast the antiserum of Hoffman *et al* (1987) did elicit a reaction against dystrophin, showing clearly as a band in the high molecular weight region in the normal tissue, and absent in the dystrophic tissue.

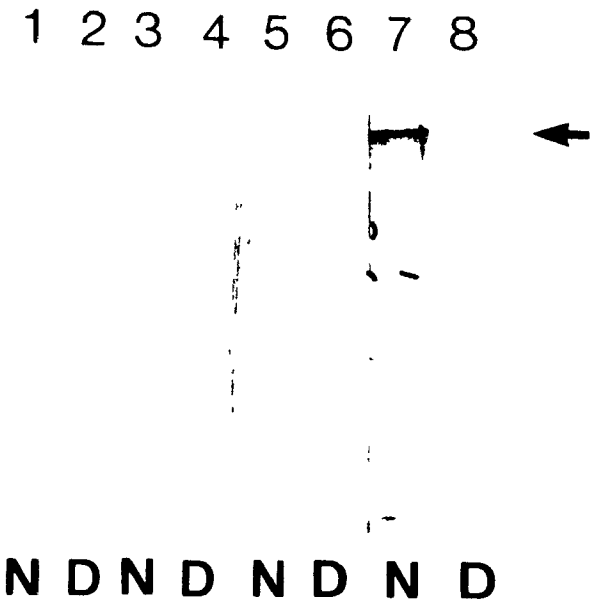

Figure 3: Western Blot of Antisera on Human Tissue

The blot was probed with the antisera at a dilution of 1/400, reacted with biotinylated anti-rabbit antibody, stained with NBT/BCIP and dried

Lanes 1-2 were probed with antiserum 1, lanes 3-4 were probed with antiserum 2, lanes 5-6 were probed with antiserum 3, lanes 7-8 were probed with anti-dystrophin fusion protein antiserum (Hoffman *et al*, 1987) Arro indicates dystrophin band

N = Normal human tissue, 10 μg loading.

D = Dystrophic human tissue, 10 μg loading.

These initial attempts to raise antibodies in rabbit using 3 internal peptides from the DMD gene product were unsuccessful. While the antisera raised could reliably detect their individual immunogens, and to some degree cross-react with each other, there was no evidence that they could detect the intact dystrophin protein. Clearly the antisera did have antibodies to their respective peptides, since they did react to their immunogens, but against the whole protein they were not useful.

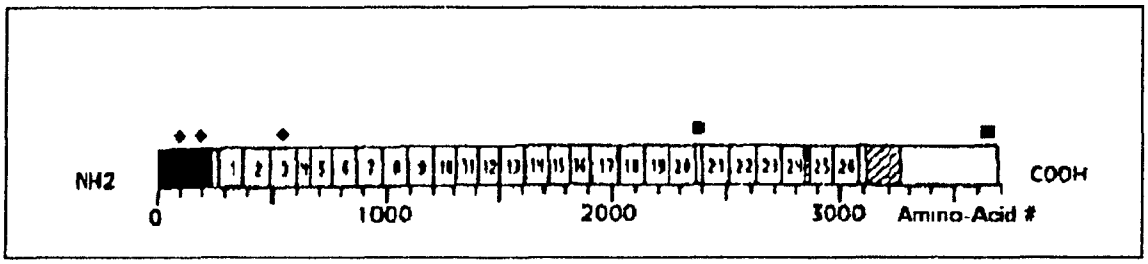

Figure 4· Peptide Selections on the Dystrophin Protein

Diamond: Internal Domain
Circle: Hinge Region
Square: C-terminal

I then decided to attempt to raise antibodies to another hydrophilic region of dystrophin, called the hinge region, as well as to the Dystrophin Related Protein (DRP) (Khuranat *et al*, 1990 and Love *et al*, 1989). DRP is an autosomal homolog to dystrophin, found in very low but nevertheless detectable concentrations in normal muscle. DRP has over 90% homology with human dystrophin.

Two internal sequences (CSMWPEHYDPSQ-COOH and CDPDASGPQFHQAA-COOH) referred to in the following text as DRP1 and DRP2, of the DRP protein were chosen for use as peptides based on their lack of homology

to dystrophin, and injected into rabbits upon conjugation with KLH using glutaraldehyde. The antisera showed no reactivity against DRP on Western blots (figure 5). Anti-DRP antiserum (Karpati *et al*, 1992) did however show a faint band in the DRP region (C Guerin, unpublished data)

When the blot in figure 5, already treated and stained using the DRP1 antiserum and alkaline phosphatase reagent, was reprobed with C-terminal anti dystrophin antiserum (see p 35 for more information about this antiserum), the results were quite different (figure 5)

The appearance of bands in the dystrophin region in figure 5 of the normal tissue lanes indicates that there is dystrophin present, and that high molecular weight proteins were undegraded. Although the concentration of DRP in normal skeletal tissue in mice is low, it is nevertheless normally detectable Yet the anti DRP1 and DRP2 antibodies do not detect DRP on the same Western blot, which C Guerin's DRP3 does. This indicates that the anti-DRP1 and DRP2 antisera are not immunoreactive, and the reasons for this could be simply that the regions chosen for the peptides were not immunogenic enough. Other possibilities are that the region selected has no exposed epitopes, such as was the case with the three unsuccessful anti-dystrophin antisera earlier.

Finally, a proline-rich region of dystrophin, considered to be a hinge region, was selected (Sequence: CKLEMPSSMLE-COOH, aa 2458-2469) Peptides were synthesized and rabbits immunized, but as with the previous immunogen, there was no immune response that was detectable against whole dystrophin on Western blots.

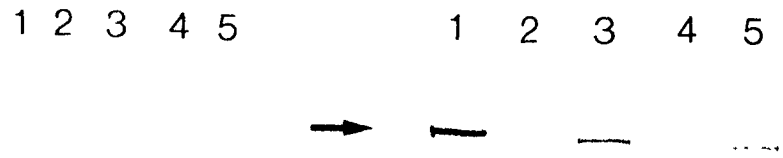

Figure 5: DRP antiserum and reprobing with C-terminal anti-dystrophin antiserum on normal and dystrophic mouse & human tissue.

Human skeletal muscle tissue from patients was taken in biopsies and homogenised, as well as tissue from *cmx* and *mdx* mouse skeletal muscle. The tissues were separately homogenized, boiled in SDS sample buffer, sonicated, and subjected to SDS-PAGE, with 10μg loading per lane The gel was then transferred to nitrocellulose by Western blotting, then the blot was blocked with skim milk and probed with the antisera, reacted with biotinylated anti-rabbit antibody, stained with NBT/BCIP and dried. Arrow indicates dystrophin band.

Probing was done with the anti-DRP antisera, at 1/400 dilution and neither showed any bands(left) and with anti-C-Terminal antiserum at 1/200 dilution (right).

Lane 1 = Human
Lane 2 = MDX
Lane 3 = CMX
Lane 4 = MDX
Lane 5 = CMX

From this it was decided to try using a different region of the DMD gene product. A region composed of the last 17 amino acids of dystrophin was selected. (Sequence: SSRGRNTPGKPMREDTM-COOH, aa 3668-3685) This sequence had previously been shown to be immunogenic by Zubrzycka-Gaarn *et al* (1991) Two rabbits were injected with the C-terminal dystrophin peptide, conjugated to KLH in the same manner as the previous peptides The resulting antiserum was compared on Western blots to the anti-fusion protein antiserum of Hoffman *et al* (1987) and found to elicit the same staining pattern.

The C-terminal antiserum was then tested on mouse tissue, since mouse dystrophin has significant homology to human, rat, and chick dystrophin (Koenig *et al*, 1987 and Hoffman *et al*, 1988), and there is a mouse model of Duchenne Muscular Dystrophy. Normal mice, with intact dystrophin, *cmx*, show a dystrophin band when stained with the C-terminal antibody, while dystrophic mice, *mdx*, do not show any band in the dystrophin region at all This result is consistent with that given by the fusion protein antiserum (figure 6)

In order to be certain that the C-terminal antiserum was truly specific against the DMD gene product, a peptide preincubation experiment was performed (figure 7). Preincubation of the antiserum with the C-terminal peptide should result in binding of the antibody to the peptide immunogen This should then saturate the binding sites on the antibody so that it is unable to bind to dystrophin on the blot

Figure 7 shows that the C-terminal antibody does not bind any proteins transferred to nitrocellulose when preincubated with the C-terminal peptide As a

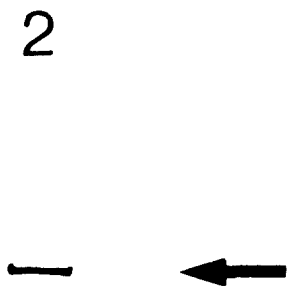

Figure 6: Staining of mouse tissue with the dystrophin C-terminal antibody

Skeletal muscle tissue from both normal and dystrophic mice was homogenised, polytroned, boiled in SDS sample buffer, sonicated and subjected to SDS-PAGE. The gel was then transferred to nitrocellulose by Western blotting, and the blot was blocked with skim milk and probed with the C-terminal antiserum at a dilution of 1/400, reacted with biotinylated anti-rabbit antibody, stained with NBT/BCIP and dried. Arrow indicates dystrophin band.

Lane 1 is from *mdx mice*, 10 μg loading.

Lane 2 is from *cmx* mice, 10μg loading

1 2 3 4 5

Figure 7: Peptide preincubation experiment

Human tissue was transferred to nitrocellulose using Western blotting. The blot was probed with preincubated C-terminal antiserum at a dilution of 1/400, reacted with biotinylated anti-rabbit antibody at a dilution of 1/1000, stained with NBT/BCIP and dried.

C-terminal antiserum was incubated first with peptide for 2 hours and then with the blot for an additional 3 hours. The peptides used were either the immunogen itself or the second peptide from the internal region of the DMD gene product. Controls were the preimmune serum from the c-terminal injected rabbit and the c terminal antiserum without any peptide at all added. Arrow indicates dystrophin band.

Lane 1: Preimmune antiserum

Lane 2: C-terminal antiserum on *mdx* mouse tissue (control)

Lane 3: C-terminal antiserum

Lane 4: C-terminal antiserum preincubated with 20 μg/ml C-terminal peptide

Lane 5: C-terminal antiserum preincubated with 20 μg/ml internal region 2 peptide.

control, another dystrophin peptide from the second internal region (sequence: RQSTRNYPQVNVINFTTS-COOH) was preincubated with the antiserum, and it is clear that this peptide does not prevent antibody binding to dystrophin on nitrocellulose

The negative control, the preimmune serum, does not show any bands in the dystrophin region, while the C-terminal antiserum does bind clearly and specifically to dystrophin when it is not pre-incubated with peptide.

There is a limit to the amount of antigen (dystrophin, in this case) that an antibody can detect. A test was performed to determine the limits of antigen detectability; ie how little antigen was needed to be detectable on Western blots. The results of the blot in figure 8 indicated that the ideal quantity of protein loading in gels required for detection was about 6 μg of total muscle homogenate. Quantities smaller than 6 μg were detectable on Western blots, but fell off rapidly to invisibility below 1.5μg (figure 8). Any increase in loading would not show a proportionate increase in staining of the nitrocellulose, and would increase background due to non-specific interactions of the adhered proteins with the antiserum.

Figure 9 shows graphically the results shown in figure 8. Around 9-10 μg of sample loaded into the gel and transferred to nitrocellulose, the curve reaches a point where large increases in loading will not help in detection of dystrophin. Similarly, below 1 μg, there is little to see on Western blots, regardless of the concentration of antibody.

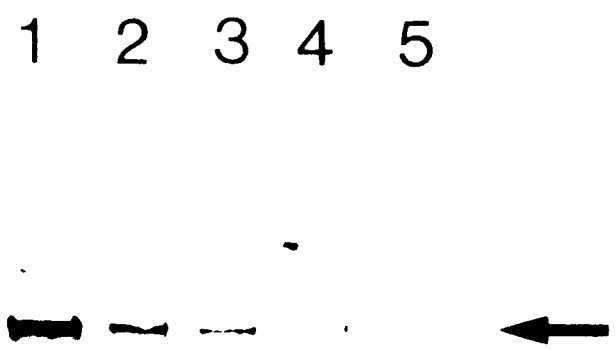

Figure 8: Limits of antigen detection using C-terminal antiserum

Human tissue was transferred by Western blotting and probed with the C terminal antiserum at a dilution of 1/400, reacted with biotinylated anti-rabbit antibody, stained with NBT/BCIP and dried Arrow indicates dystrophin band

Lane 1: 12 μg loading
Lane 2: 6 μg
Lane 3: 3 μg
Lane 4: 1.5 μg
Lane 5: 0.75 μg

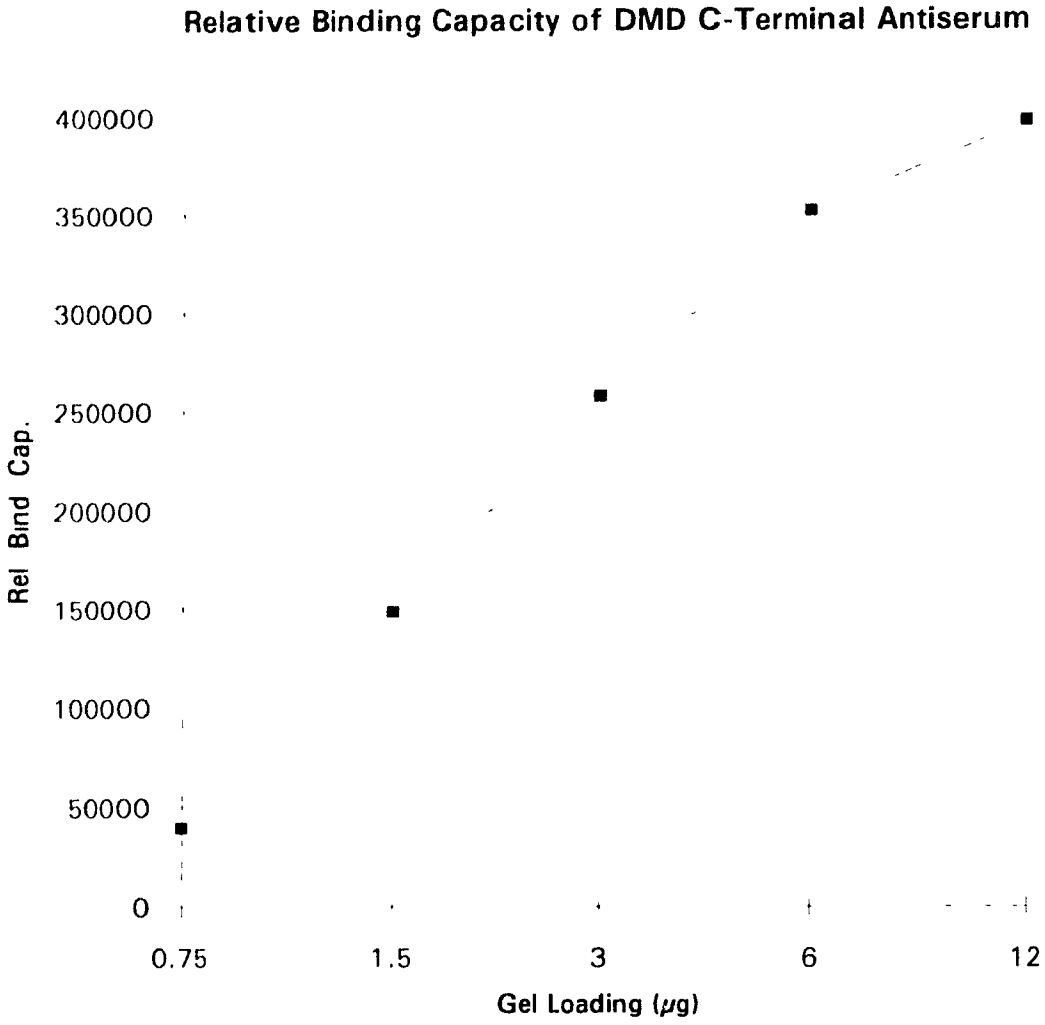

Figure 9 The relative binding capacity of dystrophin to the anti-dystrophin antibody on Western blots was studied by using muscle tissue from patients was taken in biopsies and subjecting it to Western blotting The Westerns were then analyzed in a Jandel Video Analysis system and the results quantitated on computer. Relative binding capacity is the sum of pixel intensities for a band on the nitrocellulose, indicating the darkness of the stain

The limits of dilution were the next stage in the characterization of the antibody. Serial dilutions were used to determine the lowest concentration of antiserum that could be used effectively (figure 10)

It is clear that the antiserum could be used to 1/8000 dilutions When 1/10000 dilution was attempted (not shown), the band was quite faint and such a low dilution was unreliable in circumstances where the loading of protein might be less than the ideal amount, 10μg of protein per lane At very low dilutions (eg: 1/50) detection of dystrophin in tissue homogenates by the antibody was actually impaired This may have been due to coating of the blot with serum proteins and/or lipids As will be seen later, this anomaly was eliminated upon affinity purification of the antiserum

Although useful for immunoblotting, the unpurified C terminal antiserum was inefficient at detecting dystrophin on frozen sections due to high background staining For this reason, and because of the need for affinity purified anti dystrophin antiserum for frozen section analysis of inhibition of myosatellite cell proliferation studies (chapter 2), I carried out an affinity purification of the crude antiserum

Affinity purification was performed using a glass column filled with Affi gel 15 sepharose (Bio Rad) coupled to BSA-DMD-C-terminal peptide conjugate Elution was performed using Gentle Ag/Ab Elution Buffer (Pierce) and separate fractions were collected. Fractions were then assayed for specificity on mouse and human skeletal muscle blots, using both normal and dystrophic tissue for comparison (see page 43 for further information about the affinity purification results)

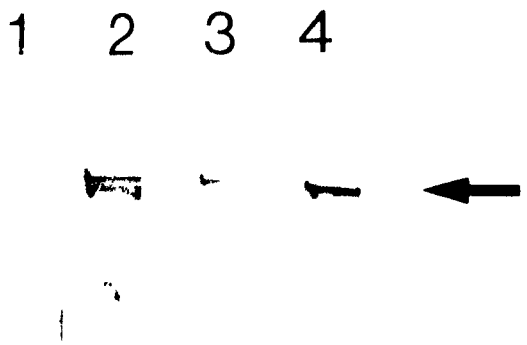

Figure 10: Serial dilution of C-terminal antiserum

Human skeletal muscle tissue from patients was taken in biopsies and homogenised, polytroned, boiled in SDS sample buffer, sonicated, and subjected to SDS PAGE, with 10μl loading per lane. The gel was then transferred to nitrocellulose by Western blotting, then the blot was blocked with skim milk and probed with the C-terminal antiserum at a series of dilutions, reacted with biotinylated anti-rabbit antibody, stained with NBT/BCIP and dried. Arrow indicates dystrophin band.

Lane 1. 1/50 Dilution
Lane 2: 1/1000
Lane 3 1/5000
Lane 4. 1/8000

Further studies of the DMD C-terminal antiserum were then performed on frozen sections of human muscle biopsies from both normal and dystrophic patients (figure 11 a-f). It was found that the Hoffman *et al* (1987) anti dystrophin fusion protein antiserum was inadequate for use in frozen sections (data not shown). Visible in figures 11a, c, and e as a fine line around each fibre, dystrophin is clearly absent in dystrophic tissues. Figures 11b, d, and f show no distinct staining around each fibre. The effects of the purification are visible in figure 11a, and c. Figure 11a is mouse tissue stained with affinity purified antiserum, while 11c was stained using unpurified C-terminal antiserum. The clarity and reduced background achieved is noticeable.

The effects of the purification were apparent in Western blots as well, removing all non-specific interactions and generally resulting in much cleaner blots with lower background (figure 12). This meant that any immunostaining would be an indication of the presence of dystrophin and no other protein. The importance of affinity purified antisera can be seen later, where the antiserum I purified was used for the study of dystrophic mice and myosatellite cell proliferation (chapter 2).

The fractions of purified antibody were collected and tested for the concentration of pure immunoglobulin. The most concentrated fraction was 140 micrograms per millilitre. This as calculated upon measurement of optical density using a constant of 13.5 OD for a 1% solution of IgG (Fasman, 1988). This is about average for an affinity purification (Goding, 1983). Based on the relative abundance

Figure 11a: Normal mouse tissue frozen section probed with affinity purified antiserum.

Figure 11b: Dystrophic mouse tissue frozen section probed with affinity purified antiserum.

4 μm cryostat sections of mouse skeletal muscle were fixed in acetone. Primary antibody (affinity purified C-terminal) was applied at a dilution of 1/40, then biotinylated anti-rabbit secondary antibody. The section was then stained with streptavidin peroxidase and diaminobenzidine and mounted.

Figure 11c: Normal mouse tissue frozen section probed with unpurified C-terminal antiserum.

Figure 11d: Dystrophic mouse tissue frozen section probed with unpurified C terminal antiserum.

4 μm cryostat sections of mouse skeletal muscle were fixed in acetone. Primary antibody (DMD C-terminal) was applied undiluted, then biotinylated anti rabbit secondary antibody The section was then stained with streptavidin peroxidase and diaminobenzidine and mounted.

Figure 11e: Normal human tissue probed with affinity purified antiserum.

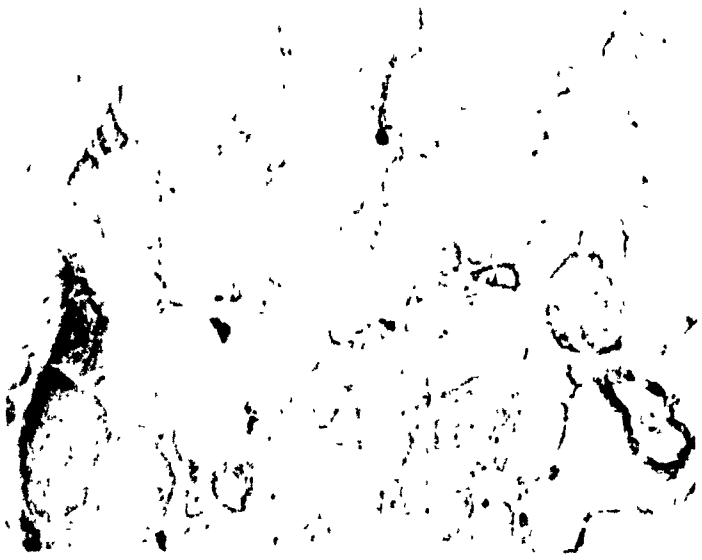

Figure 11f: Dystrophic human tissue probed with affinity purified antiserum.

4 μm cryostat sections from human child or adult skeletal muscle were fixed in acetone. Primary antibody (affinity purified C-terminal) was applied at a dilution of 1/40, then biotinylated anti-rabbit secondary antibody The section was then stained with streptavidin peroxidase and diaminobenzidine and mounted.

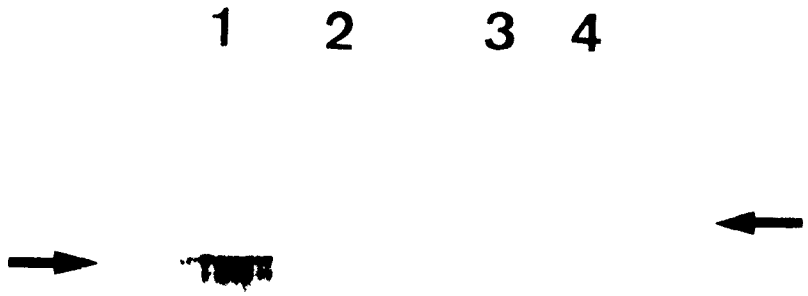

Figure 12: Differences between crude and affinity purified antisera.

Human skeletal muscle tissue from patients was taken in biopsies and homogenised, polytroned, boiled in SDS sample buffer, sonicated, and subjected to SDS-PAGE, with 10μg loading per lane. The gel was then transferred to nitrocellulose by Western blotting, then the blot was blocked with skim milk and probed with the antisera, reacted with biotinylated anti-rabbit antibody, stained with NBT/BCIP and dried. Arrow indicates dystrophin band

Lane 1: Unaffinity purified C-terminal antiserum 1/400, normal tissue
Lane 2: Unaffinity purified C-terminal antiserum 1/400, dystrophic tissue.
Lane 3: Affinity Purified C-terminal antiserum 1/2000, dystrophic tissue.
Lane 4: Affinity Purified C-terminal antiserum 1/2000, normal tissue.

of dystrophin of 0.002% of total muscle protein (Hoffman *et al*, 1987), and knowing that of the 0.75μg of tissue homogenate loaded in a polyacrylamide gel lane, one can estimate the amounts of antigen present. Furthermore, at dilutions of 1/2000 or greater, and a concentration of 140μg/ml, give a better indication of the sensitivity of the antiserum, indicating that the antibody has a high affinity for its epitopes.

Dystrophin is a remarkably conserved protein in nature. It is found in all mammals studied so far, as well as avian species (Hoffman *et al*, 1988). Humans, mice, chickens, and rats all have extensive regions of homology (Koenig *et al*, 1987 and Hoffman *et al*, 1988). The C-terminal dystrophin antibody was specific for human dystrophin, but was immunoreactive on Western blots against a variety of human and non-human tissues (figure 13).

It is clear that the Hoffman *et al* (1987) DMD 60 kd fusion protein antiserum is unable to detect many non-human dystrophins, whereas the DMD C-terminal antiserum shows clear bands in all three tissue types (figure 13 a&b). These results are summarized in figure 14, showing the relative abundance of dystrophin which the C-terminal antiserum can detect in different tissues.

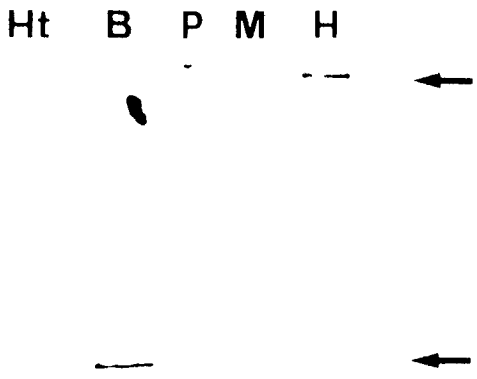

Figure 13: Species and tissue specificity of the C-terminal anti-dystrophin antiserum

Human skeletal muscle tissue from patients was taken in biopsies and homogenised, as well as tissue from chicken brain, heart, and pectoral muscle, as well as mouse skeletal muscle The tissues were separately polytroned, boiled in SDS sample buffer, sonicated, and subjected to SDS-PAGE, with 10μg loading per lane The gel was then transferred to nitrocellulose by Western blotting, blocked with skim milk and probed with the affinity-purified C-terminal antiserum at 1/2000 dilution, reacted with biotinylated anti-rabbit antibody, stained with NBT/BCIP and dried Arrow indicates dystrophin band.

Lane 1=Chick heart
Lane 2=Chick brain
Lane 3=Chick pectoral
Lane 4= Mouse skeletal
Lane 5= Human skeletal

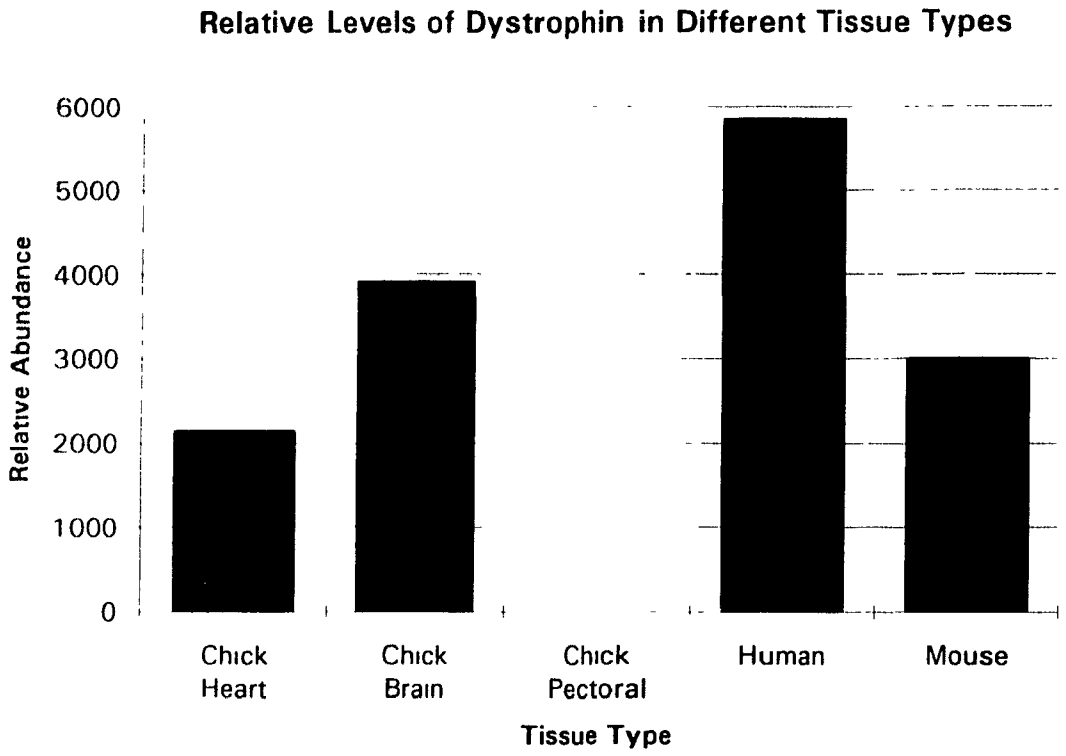

Figure 14· Graphical representation of the results from figure 13. Clearly the C-terminal antiserum used in these assays, based on a human sequence for dystrophin, is best able to detect human dystrophin More curious, the chick brain shows up so strongly, even more so than the mouse homolog, indicating a remarkable similarity at the c-terminal region of chick brain dystrophin to human skeletal dystrophin. Chick pectoral muscle is totally undetectable. Abundance is based on the sum of pixel intensities for a given band on a Western blot.

Section II: A Developmental study of dystrophin production in cultured cells

Several aspects of muscle cell culture growth were examined. First, the time course of dystrophin expression during normal development of rat skeletal muscle was examined. Subsequently, the effects on dystrophin accumulation by various external influences were examined, such as myotube contraction and the effects of BrdU.

Initially chick muscle cultures were chosen for in vitro studies due to their high and sustained rate of spontaneous contraction, however, when homogenates of chick skeletal muscle were assayed for dystrophin, none was detectable. Assumed to be an artifact of culture preparation, chick tissue was homogenized in the same manner as human and mouse tissue had been. Dystrophin is detectable in chick brain, consistent with reports that dystrophin is found in mammalian brain (Nudel *et al*, 1989 and Chelly *et al*, 1988). Heart has, as can be expected for muscle, a clear band indicating the presence of dystrophin, while pectoral muscle, logically an abundant source of dystrophin since it is a skeletal muscle, has no band at all.

This result prompted further study. The subsequent repetition of Western blots using the same and new, fresh samples all yielded the same result. Sequence analysis shows that the C-terminus of chick muscle dystrophin varied from human muscle dystrophin by only three amino acids over the length of the peptide selected. Nevertheless, this apparently affected the antigenic site sufficiently to prevent antibody binding. Differently spliced forms of dystrophin exist, some of which lack the sequence (Lemaire *et al*, 1988) against which the C-terminal antiserum used in my

studies is directed. It is possible that the clone selected for the study of chick dystrophin by Lemaire *et al* (1988) could perhaps have been one that is less abundant than the one detected on Western blots in these experiments. This is unlikely, as ususally the most abundant of clones are statistically likely to be selected, but it is nevertheless a possibility.

The failure of chick muscle culture to provide a system for measuring dystrophin production *in vitro* necessitated a switch to rat muscle cell cultures. Muscle from rat pups was cultured over varying periods of time and harvested to assay dystrophin accumulation and content using the affinity purified C-terminal antibody. Rat cell lines were not considered an alternative to primary cell cultures because cell lines show different levels of terminal differentiation from primary cells and some are known to be dystrophin deficient as previously described (Nudel *et al*, 1988). Chick cells were even more suitable due to their earlier maturation and even more vigorous contractions, but a suitable antibody had yet to be developed.

Early in development, after 1-2 days, the rat cells became confluent. As they matured, they became elongated, mononucleate cells with a characteristic spindle shape (figure 15)

Cell fusion started at 3 days, and after 4 days, fusion was extensive and cells could be seen to have central nuclei, clustered together as seen in figure 16. By day 6, contractions began to spontaneously occur. The fused cells at this point were

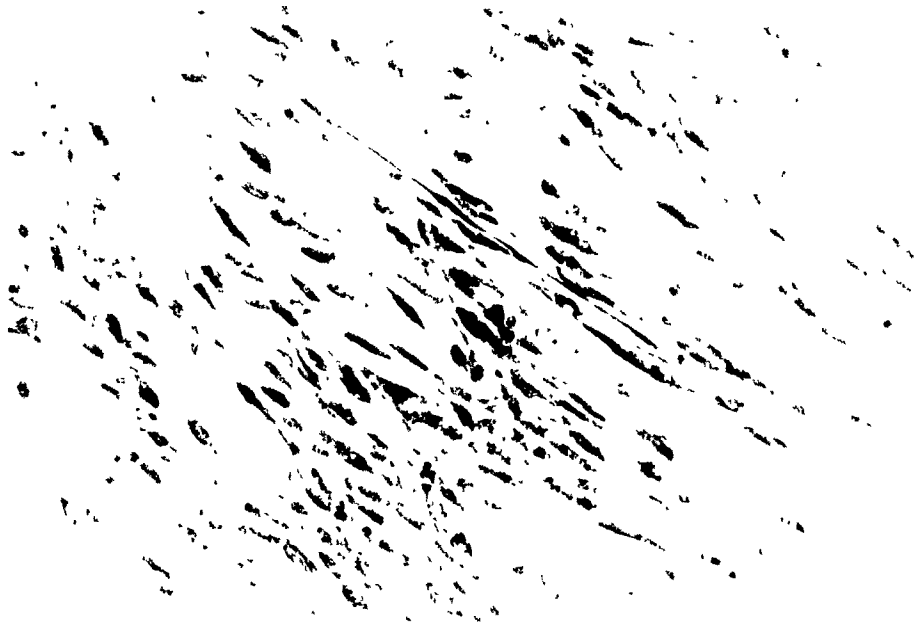

Figure 15: Unfused rat muscle cells
Rat cell primary culture after 3 days, (DMEM + 10% Horse serum, 0.5% Chick Embryo Extract.)

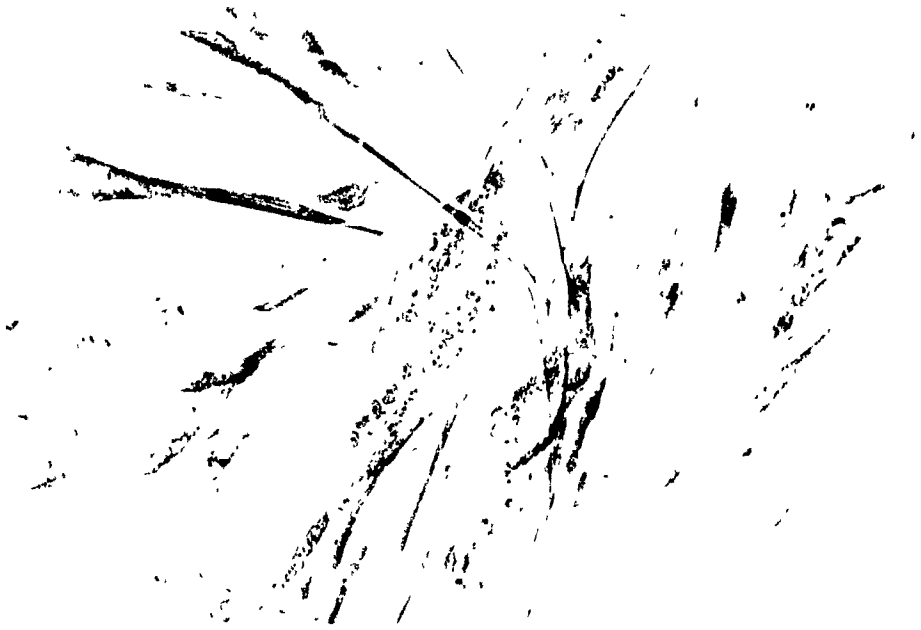

Figure 16: Late fused rat muscle cells
The same culture as in photo 1 after 11 days, in the same medium

myotubes, forming vast interconnected networks of fibres running all over the plate and locally contracting in unison

The fused cells were then assayed over a period of 4 days for their levels of dystrophin From initial plating up to day three, rat myoblasts do not show any amounts of dystrophin production that would be detectable on Western blots using the affinity purified C' terminal antiserum Starting on day 4, there is a measurable level of dystrophin in rat culture that barely increased by day 5, remaining at steady state until day 6, when the levels rose rapidly and continued to increase steadily at day 7 (figure 17)

The increase of dystrophin over the course of natural myofiber development would in itself not be unusual if one considers that the cells themselves should also be growing As a response to this consideration, the measurement of the cultures' total protein content was performed, measuring the total protein content of the plates. As total protein content was assayed over the course of the 4 day period, it was found to have already attained a relatively steady state, with only a slow, gradual increase in overall protein content This was then compared to the rate of dystrophin increase to yield figure 18

From figure 18 it can be seen that dystrophin shows a dramatic increase in production around day 5-6, while the overall amount of cell matter (total protein content) in the plates remains relatively constant. This is indicative that amount of dystrophin shown is truly changing, and is not merely an increase in the levels of dystrophin because there is more total protein content.

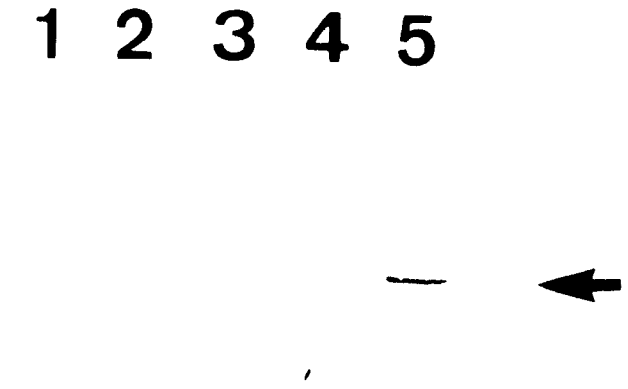

Figure 17: Dystrophin production in rat muscle cell culture

Rat cells from hind legs muscles were cultured in DMEM + 10% horse serum & 0.5% chick embryo extract. They were harvested at days 4, 5, 6 and 7, then homogenized, boiled in SDS sample buffer, and then subjected to SDS PAGE and Western blotting. Human tissue sample was used as a control. The blot was blocked with skim milk and probed with the C terminal affinity purified antiserum at 1/2000 dilution, reacted with alkaline-phosphatase conjugated secondary anti-rabbit antibody, stained with NBT/BCIP and dried. Loadings in each lane was 10 μg. Arrow indicates dystrophin band.

Lane 1 = Day 4 rat cells
Lane 2 = Day 5 rat cells
Lane 3 = Day 6 rat cells
Lane 4 = Day 7 rat cells
Lane 5 = Human tissue

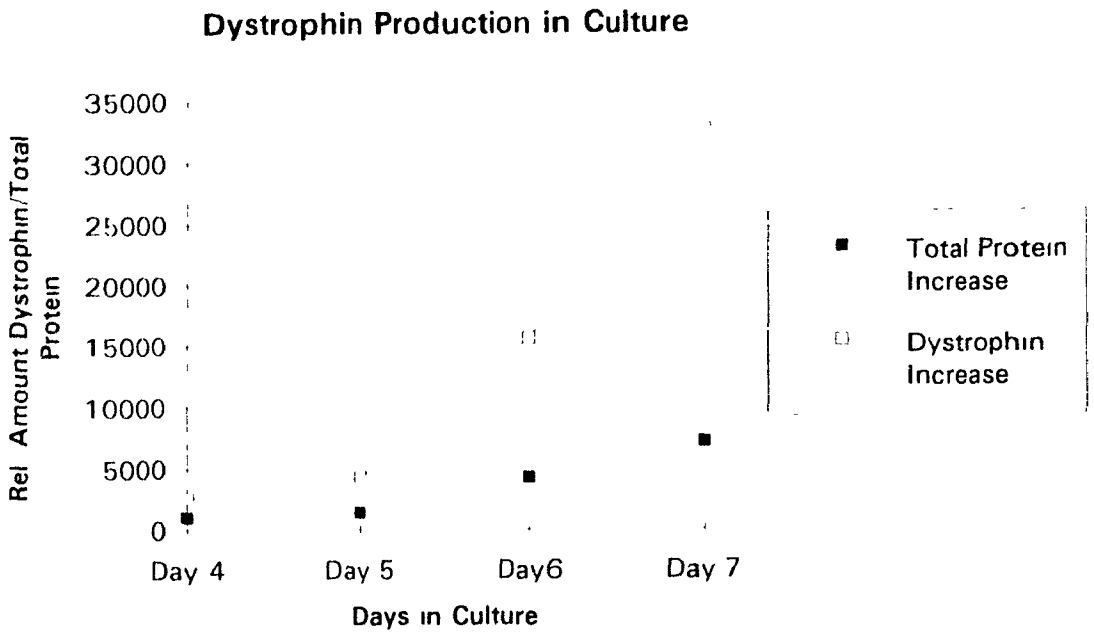

Figure 18: Total protein content was quantitated by measuring average total protein in plates of cultured cells at different time points. The amount of dystrophin in culture was quantitated by taking aliquots of culture homogenate and performing SDS-PAGE and Western blotting as described before, then quantitating on the Image Analysis System. The scale is arbitrary and not absolute. Each curve is independent of the other, and is merely a means of representing the level of protein or dystrophin relative to the starting amount at day 4. The scale is the pixel intensity summation.

Section III: Contractile activity and the effects of bromodeoxyuridine on dystrophin accumulation

There is evidence that contraction of muscle cells in culture after fusion affects the production and turnover of certain proteins. Rat cells that were allowed to fuse began to spontaneously contract (Bandman *et al*, 1982 and Crisona *et al*, 1983). They were so vigorous in their contractions that eventually the entire cell layer would pull itself off the tissue culture plate. Due to the dramatic effects of contraction-inhibition on certain cell proteins, it was decided to investigate what effects might be visible on dystrophin production.

I found that the inhibition of contraction by the addition of high concentrations of potassium into the culture medium was not successful in reproducibly altering the production of dystrophin. Although slight variations occurred, they were not reproducible and showed the same level of variations in dystrophin content as the non potassium-treated cells (figure 19).

The potassium treatment had no conclusive effect on dystrophin accumulation, but it was considered possible that the potassium treatment was not having the desired effect for unknown reasons. Thus it was decided to determine the effect of high potassium concentration upon myosin heavy chain, which is known to be affected by contraction-inhibition (Bandman *et al*, 1982). The coomassie staining of electrophoresed cultured myoblasts showed a definite effect on myosin accumulation (figure 20), demonstrating clearly that growth of cells in high potassium

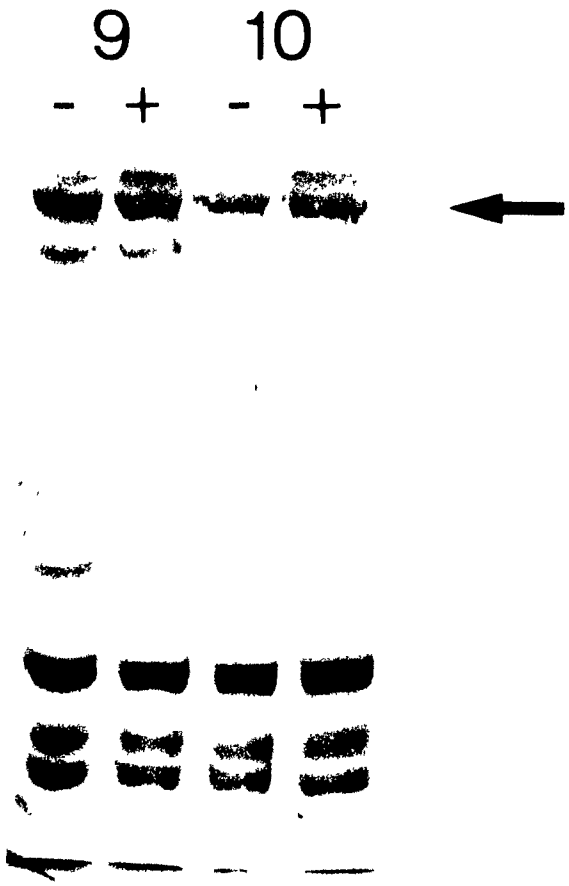

Figure 19: The levels of dystrophin production in potassium-treated and non-treated cultures of different age

Rat cells were cultured in DMEM, horse serum and chick embryo extract. One series of cultures was normal, the other had 12 mM potassium added. Plates were harvested after a number of days in pairs, one normal, the other high potassium. The above blot is a representative one showing two days Harvested cultures were homogenized, boiled in SDS sample buffer, and then subjected to SDS-PAGE and Western blotting. The blot was blocked with skim milk and probed with the C-terminal affinity purified antiserum at 1/2000 dilution, reacted with alkaline-phosphatase conjugated secondary anti-rabbit antibody, stained with NBT/BCIP and dried Loadings in each lane was 10 μg. Arrow indicates dystrophin band.

Pairs of Lanes are as follows day 9, day 10; the first in each pair is without potassium, the second is potassium treated.

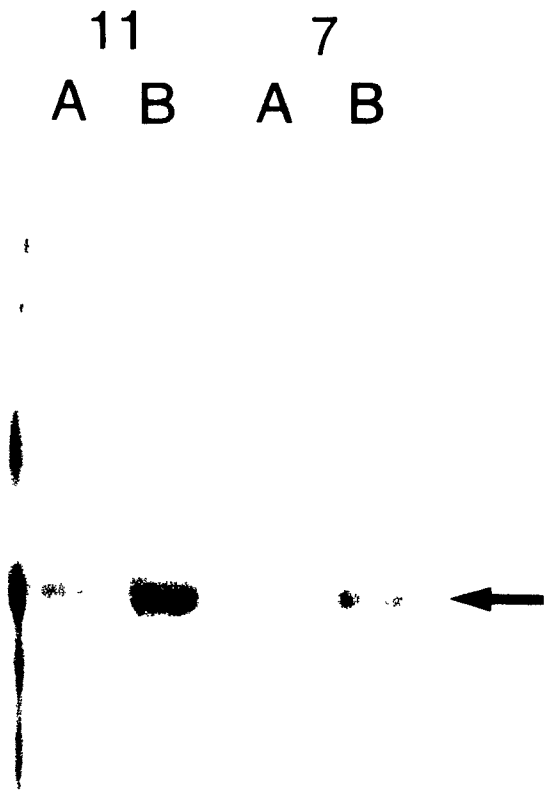

Figure 20: Coomassie staining of SDS-PAGE of potassium-treated and non-treated cultures

Rat cells were cultured in DMEM, horse serum and chick embryo extract One series of cultures was normal, the other had 12 mM potassium added Plates were harvested after a number of days in pairs, one normal, the other high potassium The above gel is a representative one showing two days Harvested cultures were homogenized, boiled in SDS sample buffer, and then subjected to SDS PAGE, then stained with coomassie brilliant blue dye Arrow indicated myosin heavy chain band

Pairs of lanes are as follows: Day 11,7. "A" in each pair is with potassium, while "B" was not potassium treated. Loadings in each lane were 10 μg

concentrations did have the expected effect. The gel shows degradation products, which were visible in newer cultures as well as later ones.

BrdU is known to have pleiotropic effects in muscle cells, mediated via its strong effect on muscle regulatory genes such as MyoD (Cates *et al*, 1978 and Tapscott *et al*, 1989). The effects upon dystrophin production would hence be interesting to discover, giving an insight as to whether dystrophin might fall into the MyoD cascade of events that affects terminal differentiation.

Rat muscle cells were cultured both in the presence and absence of BrdU (Cates *et al*, 1978). The effects were markedly different from the rather poor effects of contraction-inhibition. It is clearly visible that dystrophin production is almost entirely eliminated in BrdU treated cultures (figure 21). Note that only the normal, untreated cells show any dystrophin band, and that the intensity of staining for dystrophin increases with culture age, as seen previously (figure 17).

Myosin is one protein known to be affected by BrdU treatment (Tapscott *et al*, 1989), and as can be seen in figure 22, there appears to be a significant effect upon myosin accumulation

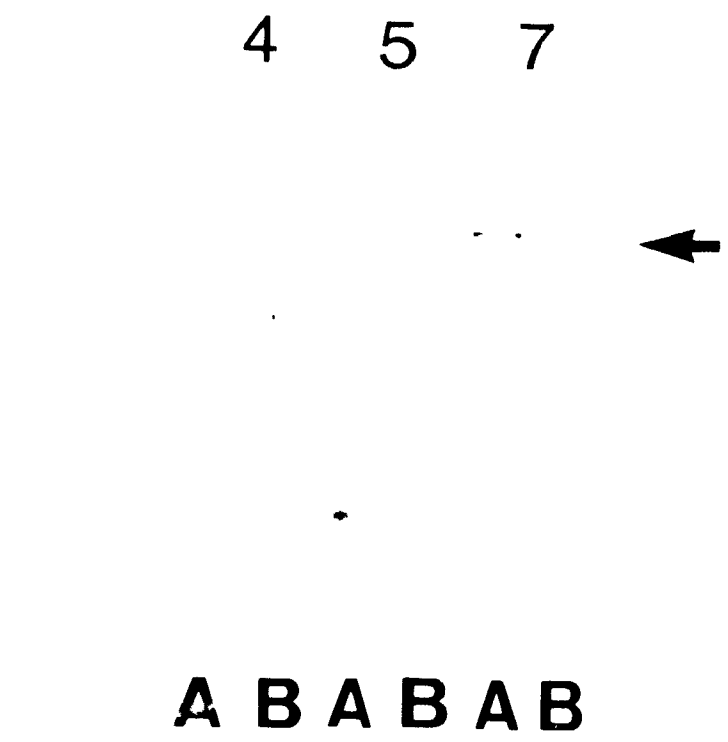

Figure 21: Western blot of BrdU-treated rat muscle cultures

Rat cells were cultured in DMEM, horse serum and chick embryo extract One series of cultures was normal, the other had 6.5 μM BrdU (30) in the medium after day 3. Plates were harvested after a number of days in pairs, one normal, the other BrdU. The above blot is a representative one showing three days Harvested cultures were homogenized, boiled in SDS sample buffer, and then subjected to SDS PAGE and western blotting The blot was blocked with skim milk and probed with the C-terminal affinity purified antiserum at 1/2 x00 dilution, reacted with alkaline phosphatase conjugated secondary anti-rabbit antibody, stained with NBT/BCIP and dried. Loadings in each lane was 10 μg Arrow indicates dystrophin band

Pairs of lanes are as follows: Day 4, 5, 7

"A" in each pair is normal, "B" is BrdU treated

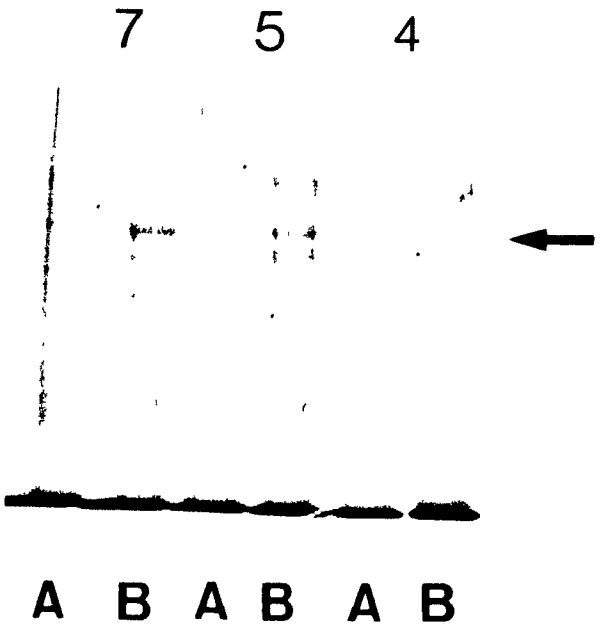

Figure 22: Myosin staining in gels of cultured cells

Rat cells were cultured in DMEM, horse serum and chick embryo extract. One series of cultures was normal, the other had 6.5 μM BrdU (30) in the medium after day 3. Plates were harvested after a number of days in pairs, one normal, the other BrdU. The above blot is a representative one showing three days. Harvested cultures were homogenized, boiled in SDS sample buffer, and then subjected to SDS-PAGE and stained with coomassie brilliant blue dye. Loadings in each lane was 10 μg. Arrow indicates myosin heavy chain band.

Pairs of lanes are as follows. Day 7, 5, 4

"A" in each pair is BrdU-treated, "B" is normal.

Section IV: Wheat-Germ Agglutinin binding studies

Wheat Germ Agglutinin (WGA) is known to bind to sialated glycoproteins with low affinity or N-acetyl-glucosamine residues with high affinity (Beeley, 1985). Since it is has been shown (Yoshida *et al*, 1980 and Campbell *et al*, 1989 and Ervasti *et al*, 1991 and Ibraghimov-Beskrovnaya *et al*, 1992) that in adult skeletal muscle dystrophin is associated with WGA-binding membrane glycoproteins, I chose to determine if dystrophin colocalizes with wheat germ agglutinin binding proteins on Western blots of homogenates from differentiating cultured cells

In adult skeletal muscle, all dystrophin is found to be bound to WGA binding proteins (glycoproteins) (Campbell *et al*, 1989 and Ervasti *et al*, 1991 and Ibraghimov-Beskrovnaya *et al*, 1992) It is unknown what the associations of dystrophin in culture might be. I found that in culture dystrophin did indeed show association to WGA-binding glycoproteins, although not all the dystrophin appeared to bind.

Homogenised rat skeletal muscle cultures were incubated with WGA-sepharose and then rinsed repeatedly and eluted At each step an aliquot was taken and tested for the presence of dystrophin and WGA binding glycoproteins

Since the samples were adsorbed in non-ionic detergents under non denaturing conditions, WGA should have adsorbed glycoproteins and anything bound to the glycoproteins Upon rinsing to remove non-adsorbed material, followed by elution with SDS, it was found that while there was a significant fraction of dystrophin that

was associated with WGA (figure 23, lane E), a large part of the dystrophin in cultured cells was found to be in the unbound pool (figure 23 Lanes R_1-R_3) This could imply that a only fraction of dystrophin is complexed to WGA-binding glycoproteins, or that insufficient lectin was used to quantitatively adsorb WGA binding glycoproteins from the extract

Incubation times of cell homogenate with WGA-sepharose were initially overnight, but excessive degradation was found to be a problem, therefore times between 30 minutes to four hours were tested. Two hours was chosen as sufficient to allow glycoprotein binding to WGA-sepharose, while being short enough to prevent extreme degradation of the homogenate Degradation was observed with these experiments despite the use of protease inhibitors.

To be certain that the dystrophin was indeed associated with WGA, a staining of the same samples was performed using biotinylated WGA. The staining pattern shows that a fraction of WGA binding proteins were retained through three rinses and were eluted with dystrophin upon treatment of the rinsed WGA-sepharose with SDS (figure 24)

Although no WGA-binding proteins were released until elution by SDS (figure 24) after the first rinse, dystrophin showed a slow leaching effect throughout the rinses and was then released in even greater quantity upon elution. This suggests that dystrophin may have low-affinity non-specific interactions with the WGA-sepharose. Fraction R_4 contains dystrophin and the virtual absence of WGA-binding glycoproteins

suggest a "free" dystrophin pool may exist in the cultured cells. Further work is required to establish this interesting possibility.

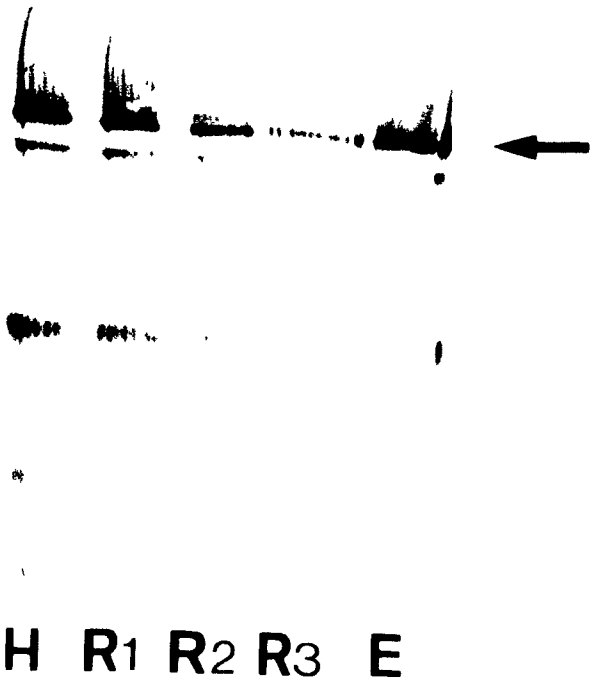

Figure 23: Dystrophin staining of Western blot after WGA adsorption and elution

Human cells were grown in supplemented F-12 medium for 5 days, switched to fusion medium (DMEM, 2% horse serum) for 8 days, then harvested and homogenized in PBS with protease inhibitors, and mixed with WGA-sepharose. After incubation for 2 hours, the suspension was spun in centrifuge at 15 000 RPM for 2 minutes and supernatant removed (H) Pellets were then rinsed three times, with a spin between each rinse and the supernatant was kept (R1, R2, R3). Finally the pellet was incubated for 5 minutes with SDS-sample buffer to elute all bound glycoproteins.

Samples were then subjected to SDS-PAGE and Western blotting. The blot was blocked with skim milk and probed with the C-terminal affinity purified antiserum at 1/2000 dilution, reacted with alkaline-phosphatase conjugated secondary anti rabbit antibody, stained with NBT/BCIP and dried. Loadings in each lane was 10 μg. Arrow indicates dystrophin band

H=Cell Homogenate & WGA

R1, R2, R3= first, second and third rinses

E=Eluted Dystrophin that came off with WGA.

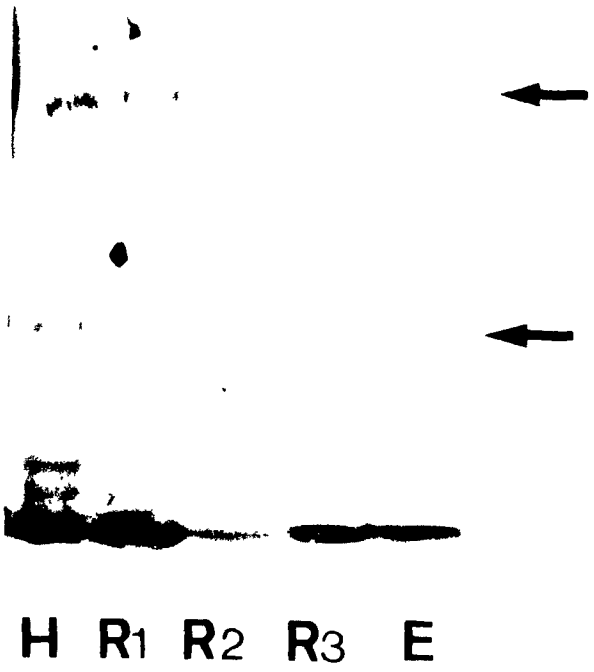

Figure 24: WGA staining of Western blot after adsorption and elution

Human cells were grown in supplemented F-12 medium for 5 days, switched to fusion medium (DMEM, 2% horse serum) for 8 days, then harvested and homogenized in PBS with protease inhibitors, and mixed with WGA Sepharose. After incubation for 2 hours, the suspension was spun in centrifuge at 15 000 RPM for 2 minutes and supernatant removed (H). Pellets were then rinsed three times, with a spin between each rinse and the supernatant was kept (R1, R2, R3). Finally the pellet was incubated for 5 minutes with SDS-sample buffer to elute all bound glycoproteins.

Samples were then subjected to SDS-PAGE and Western blotting. The blot was blocked with skim milk and probed with biotinylated WGA at 1/5000 dilution, stained with NBT/BCIP and dried. Loadings in each lane was 10 μg. Arrows indicate WGA-binding proteins that are virtually absent in Rinse 3.

H=Cell Homogenate
R1, R2, R3= first, second and third rinses
E=Eluted Dystrophin that came off with WGA

Section V Collaborative study of dystrophin expression in MDX mice

The antiserum that I prepared and affinity purified based on the dystrophin C-terminal peptide was used in a series of experiments in collaboration with Dr. Karpati of the Montreal Neurological Institute, Neuromuscular Research Laboratory (chapter 2).

Discussion

I: Overview

Several questions were addressed in this study, all of which were critically dependent upon the development of a specific means of detecting dystrophin. Analyses were performed initially in tissue to establish that native dystrophin was indeed being detected, and then in culture, which is the area of interest in these studies. While tissue localization of dystrophin had been performed in the past (Hoffman *et al*, 1988 and Zubrzycka-Gaarn *et al*, 1988 and Yoshida *et al*, 1990 and Campbell *et al*, 1989 and Ervasti *et al*, 1991 and Ibraghimov-Beskrovnaya *et al*, 1992 and Duncan, 1989 and Nudel *et al*, 1989 and Champaneria *et al*, 1989 and Wessels *et al*, 1991), there have as yet been only limited studies of dystrophin expression and localization in cultured cells (Park-Matsumoto *et al*, 1991 and Nudel *et al*, 1988). Hence, it was necessary to develop an antibody that would have the required specificity to detect dystrophin in both tissue and culture, and preferably across a wide range of species. Secondly, a study of the developmental expression of dystrophin in culture was performed to allow one to address any questions of external influences on development. Third, what factors were involved in controlling the expression of dystrophin in cultured cells; specifically, the effects of BrdU, a known inhibitor of muscle cell differentiation, as well as the effects of the inhibition of myotube contraction, accepted as an important factor in skeletal muscle myotube development, were studied. Finally, the association of dystrophin with sarcolemmal glycoproteins in

adult muscle is now well established (Yoshida *et al*, 1990 and Campbell *et al*, 1989 and Ervasti *et al*, 1991 and Ibraghimov-Beskrovnaya *et al*, 1992). Is dystrophin associated with glycoproteins at an early stage of development such as in tissue culture?

Answers to such questions would give a background to the developmental expression of dystrophin *in vitro*, and may enable one to understand part of the process of accumulation of dystrophin into the muscle fiber and its association with glycoproteins of the plasma membrane.

II: Use of synthetic peptides to raise antisera

The development and characterization of the anti-dystrophin antibody proved to be time-consuming and rather frustrating in that of several synthetic peptides used as immunogens, only one yielded a useful antiserum. While many great strides have been taken toward understanding of immunology and the development of antisera to specific protein products and epitopes (Lerner, 1984), immunological procedures are often still a mystery and more art than science. The results seem to bear this out.

Initial attempts to raise synthetic peptides based on the DMD sequence were unsuccessful. While it is clear that an immune response was triggered by the injections, as seen in slot blots against the immunogens themselves, it is equally clear that dystrophin was not detected by the antisera against all three initial DMD peptides, the hinge region peptide or the Dystrophin Related Protein (DRP).

The reason for the lack of immunospecificity against dystrophin by the antisera could be the result of several different factors: First and most likely is that the immunoglobulins produced from the KLH-conjugated peptides are unable to recognize the native conformation of the segment of the DMD gene product they were targeted against. The peptide segment injected would likely not have the precise folding that would exist in the intact protein, since the segment is relatively short and not subject to the full conformational restraints of the intact molecule. Secondly, it is possible that the lack of immunostaining on Western blots could be a result of insufficient affinity of the antiserum for its antigen. Thirdly, it is possible that post translational modifications such as phosphorylation might alter the antigenic site on dystrophin so as to prevent antibody attachment.

When the culture homogenates or tissue homogenates are prepared for SDS PAGE, they are boiled with SDS to prevent denaturation, and ensure complete solubilization of all proteins in the sample. Most importantly, SDS treated samples will cause the unfolding and elongation of the entire protein, creating an evenly distributed negative charge that is wholly dependent on protein molecular weight. The greater the size of molecule, the more negative charge is accumulated. The charge/mass ratio is constant and equal for all molecules, thus migration into an acrylamide gel is wholly dependent upon molecular weight, smaller molecules migrate faster than large ones for a given applied electric field. Once the samples are drawn into the gel, the proteins are transferred to nitrocellulose via Western blotting. In the course of this process, the SDS is removed from the samples, allowing the native

conformation of the proteins to be restored as closely as possible to their original state.

The peptides conjugated to KLH and injected in rabbits represent sequences that were chosen for their amino acid moieties, their uniqueness for certain species or their conservation in different species, and were not selected due to any knowledge about the local environment other than care taken to select relatively hydrophilic sequences. Hence, it is possible that the peptide sequences selected for injection may be part of internal regions of the dystrophin gene product and therefore may not be exposed to immunoglobulin binding.

The apparent cross-reactivity seen between antisera to unrelated peptides (figure 2) is very likely the result of non-specific interactions, due either to hydrophobic attraction or aggregation. The regions are too dissimilar to expect a specific immunoreaction to occur, since the sequences were selected with the intent of making them immunospecific against different regions of the dystrophin gene product. Although the antisera did not show any reactivity against BSA alone, it is possible that BSA coupled to a peptide could be sufficient to allow the non-specific binding to occur.

Contrary to the results obtained with the internal DMD peptides, the peptide-based C terminal anti-human dystrophin antiserum was, as expected from previous work by Zubrzycka-Gaarn *et al* (1988), generated a high-titre antiserum that detected dystrophin across a wide variety of species and tissue types. The peptide was injected into 4 different rabbits, all of which gave a positive response, although some yielded

a higher titre antiserum than others. This is indicative of the critical choice of peptide necessary for successful antiserum generation.

The titre of the antiserum was sufficient that very low concentrations, as low as 1/8000 could be used with Western blots. At lower dilutions (1/50) the crude antiserum showed a reduced binding capacity to samples on nitrocellulose. While unusual, this finding could be explained by the presence of serum proteins or lipids in the crude antiserum that inhibited the action of the antibodies, most likely through non-specific interactions and protein aggregation.

III: Characterization of antisera

The C-terminal antiserum was first tested using the mouse *mdx* muscular dystrophy model. The results showed, as expected, that no dystrophin could be detected in immunoblots of skeletal muscle of dystrophic mice. Similar results were obtained when using other sources of anti-dystrophin antibody, such as the Kunkel 60 kd fusion protein-based antiserum. Human tissue showed a clean dystrophin band, while dystrophic human tissue was lacking reactivity with the antiserum. Similar results were obtained using frozen sections, although there was a higher background, eliminated later by affinity purification of the C-terminal antiserum.

Subsequently, the antiserum was tested against human samples with antiserum preincubated with the C-terminal peptide itself. The peptide, if the antibody is specifically targeted against it, should bind with high affinity to the immunoglobulin molecule and prevent antibody attachment to the intact protein on the nitrocellulose.

Control peptides for this competition experiment consisted of peptides that were based on the dystrophin protein, but were not used as the immunogen for the generation of the C-terminal antibody

The peptide preincubation experiment showed clearly that the antiserum was specifically targeted against the C-terminal peptide The control peptide was no hindrance to antibody binding to antigen The antiserum also showed no reactivity against BSA or KLH alone, so the antibodies generated were highly specific against the DMD gene product

IV: Affinity purification of the C-terminal antiserum

Antiserum affinity purification is a vital process for the generation of monospecific antisera Crude antisera showed bands in regions aside from the known 400 kda dystrophin protein band, and it is only through the use of rigorous positive and negative controls that the specificity the antiserum can be determined Affinity purification allows one to select specifically only those antibodies directed against the synthetic peptide injected and therefore against the specific protein targetted.

The affinity purification introduced changes in the antiserum Following affinity purification, one could be assured that any band on a Western blot was indicative of the presence of dystrophin alone Faint bands could now be assessed for relative levels of dystrophin, and bands well below the normal molecular weight of dystrophin became significant as possible breakdown products Later studies of the time course of degradation of dystrophin (data not shown) indicated that the low

molecular weight bands were indeed dystrophin degradation products. Frozen section staining also showed clearly the staining of the sarcolemma using the affinity purified antiserum, whereas dystrophic tissue had no staining at all, demonstrating that all antibodies were specifically targeted against dystrophin.

After affinity purification, the most concentrated fraction of eluted antiserum had a final immunoglobulin concentration of 140 μg/ml. Hoffman *et al* (1987) estimated that the relative abundance of dystrophin in normal human skeletal muscle was approximately 0.002% of total muscle protein. I found the limit of detection in my assay to be 0.75 μg of total protein. Of the 0.75 μg loaded into a SDS PAGE gel and transferred to nitrocellulose, a maximum of 15 picograms of protein could have been dystrophin, assuming 100% of the sample was transferred, which is known to not occur in real conditions. Given that the 140 μg/ml antiserum is further diluted to 1/2000 or more, one can estimate that 70 nanograms of antibody can detect 15 picograms of antigen, a high degree of sensitivity indeed.

The high degree of conservation of dystrophin in nature accounts for the ability of the antiserum directed against the human dystrophin C terminal sequence to detect rat, mouse or even chicken dystrophin. The antiserum was able to detect dystrophin in human brain, where it is known that dystrophin can be found (Nudel *et al*, 1989 and Chelly *et al*, 1988), as well as chicken brain, but surprisingly not in chicken skeletal muscle.

The failure of the C-terminal DMD antiserum to detect skeletal muscle dystrophin in chick tissues is difficult to explain. Studies of the C terminal human

DMD sequence used for the generation of antisera shows that it differs from the chick only in three amino acids, all of which are non-consecutive. One possible explanation has already been addressed (see pp 51-52). Spliced forms of dystrophin lacking the C-terminal sequence have been detected in mammalian muscles but are of low abundance (Feener *et al*, 1989) Other possibilities are that the epitopes required for detection may have been spliced out during mRNA processing, or that dystrophin is phosphorylated at the antibody binding site, the dystrophin C-terminal does have serine residues that potentially could act as phosphorylation sites.

V. Dystrophin expression in culture

Dystrophin expression in rat cell culture was found to be initially nonexistent, with the onset of dystrophin accumulation occurring around day 4. The dramatic increase in dystrophin accumulation over the next three days cannot be accounted for by an increase in culture total protein content, as dystrophin accumulation quickly outstrips the increase in culture protein One could speculate that the accumulation of dystrophin is dependent upon differentiation, perhaps the presence of a receptor that is only fully expressed in adult tissue

It is known from previous studies (Zubrzycka-Gaarn, 1988 and Yoshida *et al*, 1990 and Campbell *et al*, 1989 and Ervasti *et al*, 1991 and Ibraghimov-Beskrovnaya *et al*, 1992 and Duncan, 1989 and Zubrzycka-Gaarn, 1991) that dystrophin in adult skeletal muscle associates with a glycoprotein complex in the sarcolemma This anchor to the sarcolemma accounts for 100% of the dystrophin in muscle, with no

dystrophin free in the cytosol (Ervasti *et al*, 1990). Although in cultured cells not all dystrophin appeared to be associated with WGA-binding glycoproteins, more work would be required to elucidate whether dystrophin exists in two pools, one bound to WGA-binding glycoproteins, and one unbound.

From the Western blots, it became clear that while WGA-binding proteins were retarded in a WGA-sepharose column, dystrophin continued to be released during rinses, in gradually diminishing amounts. WGA ceased to be released after two rinses, and then was released upon elution, along with a large amount of dystrophin. These results indicate that not all dystrophin is associated with WGA binding glycoproteins in cultured myotubes, contrasting with the results reported for adult tissue. This suggests that further development is required for complete attachment of dystrophin to the sarcolemma, as there is apparently a surplus of dystrophin for its receptor. These results, more importantly, indicate that the WGA-binding glycoproteins are present in culture either before dystrophin is found in culture or they are coordinately expressed.

VI: Regulation of the accumulation of dystrophin in culture

Contraction-inhibition did not reproducibly alter the levels of dystrophin in culture. This is consistent with the fact that dystrophin is considered a cytoskeletal protein. Crisona *et al* (1983) found that cytoskeletal proteins as opposed to contractile proteins were unaffected by contraction inhibition. Dystrophin is a very large protein, however, and is thus more likely to be susceptible to degradation, so variations that

were seen, given their unpredictability, are likely to be the result of degradation, particularly in light of the steady diminution of myosin under the same conditions when the cultures were contractilely inhibited (Crisona *et al*, 1983)

BrdU is a known altering agent of differentiation in muscle cells. I found that it eliminated detectable dystrophin production in cultured cells. Myosin was also decreased, consistent with the fact that it is known to be BrdU-sensitive (Tapscott *et al*, 1989) From these results alone it is impossible to know whether the effect of BrdU on dystrophin is due to repression of muscle regulatory genes such as MyoD, or whether the effect of BrdU is direct and acts upon the DMD locus itself It is known that MyoD is not solely responsible for the regulation of dystrophin expression; the dystrophin promoter has a CCArGG box (Gilgenkrantz *et al*, 1992) The E box in the dystrophin promotor is unable to be transactivated by MyoD (Gilgenkrantz *et al*, 1992) However, it is still possible the MyoD does have an effect upon dystrophin accumulation in culture, as it may be required for the activation of another gene whose product is required for the stabilization of dystrophin Further work would be required to study this, including attempting overexpression of MyoD as done by Tapscott *et al* (1989) to determine if the effects of BrdU on dystrophin accumulation can be overcome by transfection of cells with MyoD coupled to a BrdU insensitive promoter

In conclusion, the present study demonstrates that dystrophin accumulation is regulated in terminally differentiated skeletal muscle cultures in a manner similar to many other muscle-specific genes in that it increases coincident with the onset of

myoblast alignment and fusion. Accumulation is eliminated by BrdU but is unaffected by contractile activity. Finally, as in adult muscle, dystrophin in cultured myotubes was found in association with WGA-binding glycoproteins indicating this association takes place very early in development of muscle fibres.

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Chapter Two

Preface to Chapter 2

As heterozygous female *mdx* mice age, the pattern of dystrophin positive and negative muscle fibres alters such that the number of dystrophin negative fibres decreases with time. The initial pattern of dystrophin positive and negative fibres is a result of random X chromosome inactivation in accordance with Lyon's hypothesis. The mechanism by which dystrophin negative segments are gradually changed to dystrophin positive is unknown, but two hypotheses were proposed.

First, spontaneous fusion of dystrophin competent cells into the myofibre as it regenerates or grows; second, that dystrophin gene expression in the segments that are positive has a gradual "diffusion" effect that spreads dystrophin throughout the muscle fibre. The study conducted here attempted to demonstrate whether the first hypothesis was correct.

For the purpose of data analysis a reliable and sensitive detection method was required for the assay of dystrophin competence in frozen muscle sections. My role was the preparation of the antibody used in the assay, and its affinity purification.

The results of the study showed that elimination of myosatellite cell proliferation did not prevent the gradual increase in dystrophin positive fibre segments in the mice. This clearly demonstrated that the satellite cells were not involved in this process.

Inhibition of Myosatellite Cell Proliferation by Gamma Irradiation Does Not Prevent the Age-related Increase of the Number of Dystrophin-positive Fibers in Soleus Muscles of mdx Female Heterozygote Mice

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In skeletal muscles of young mdx female heterozygote mice, there is a mosaic of dystrophin positive and dystrophin negative fiber segments. In older animals there is a marked decline in the number of dystrophin negative fiber segments. This phenomenon might be due to a fusion of dystrophin competent satellite cells into the originally dystrophin negative fiber segments during growth. To study this possibility, soleus muscles of 10 day old mdx female heterozygotes were gamma irradiated (2000 rads) to inhibit subsequent myosatellite cell proliferation and fusion. In the irradiated soleus muscles of animals at 60 days, the relative amount of dystrophin measured by quantitative immunoblots was not significantly different from that of the contralateral nonirradiated muscles. The prevalence of dystrophin negative fibers in the 60 day old irradiated soleus was not higher than in the nonirradiated contralateral muscles, implying that dystrophin competent satellite cell fusion was not a significant factor in the observed conversion. A longitudinal expansion of the cytoplasmic domain of the original dystrophin competent myonuclei during growth could explain the observed conversion phenomenon. *(Am J Pathol 1991, 138:1497–1502)*

In the highly inbred mdx strain of mice, skeletal and cardiac muscle fibers lack dystrophin because of a point mutation in the dystrophin gene on the X chromosome.[1] Dystrophin deficiency leads, in some groups of muscle fibers, starting at the age of 15 days, to degeneration, very efficient and regenerated fibers are relatively resistant to recurrent necroses.

Previous studies showed that on cross sections of skeletal muscles of very young heterozygote female mdx mice there was a mosaic of dystrophin positive [dys(+)] and dystrophin negative [dys(−)] muscle fibers,[2] as expected according to random X chromosome inactivation in female cells, during early embryogenesis.[3] A more striking finding was a marked gradual diminution of the percentage of dys(+) fibers, between the ages of 10 and 60 days. Two explanations were suggested to explain this phenomenon.[2] 1) A postnatal fusion of dystrophin competent satellite cells into the dys(−) fiber segments, during normal growth and 2) an overexpansion of the cytoplasmic domain of dystrophin competent myonuclei in the original dys(+) fiber segments.

We have designed experiments to determine if the fusion of dystrophin competent myosatellite cells into the growing dys(−) muscle fibers had a role in the conversion process. We have inhibited proliferation of satellite cells (and thus fusion) by gamma irradiation delivered to the hind segments of 10 day old mdx female heterozy-

[illegible]

[illegible] already demonstrated the ability of [illegible] to inhibit myoblast cell [illegible]

Materials and Methods

Irradiation Procedure

[illegible] the night [illegible] 10 day old female heterozygote [illegible] 2000 rads generated by a cobalt 60 [illegible] (Theratron 780, Atomic Energy of Can[illegible] irradiation was delivered in 13 minutes. Five [illegible] killed at age 35 days and 4 at 60 days, and [illegible] both sides, removed. Muscles were also [illegible] 10 day old nonirradiated heterozygote [illegible] to provide reference data.

Microscopic Study of Muscles

Light Microscopy

The irradiated and nonirradiated muscles were snap frozen in isopentane chilled with liquid nitrogen and 4 μ thick transverse cryostat sections were prepared from the midpoint of each muscle. The sections were immunostained for dystrophin using a rabbit polyclonal antibody raised against a synthesized polypeptide corresponding to the last 17 amino acids at the carboxy terminal of dystrophin (1:40 dilution).[4] The immunoperoxidase sections were counterstained with hematoxylin and eosin for determination of the ratio of transverse fiber area and the number of myonuclei in each fiber on a given section (nuclear cytoplasmic ratio). Six micron serial sections of all muscles were reacted for the activity of myofibrillar adenosine triphosphatase (ATPase) after pH 4.2 incubation that clearly distinguishes fiber types in the rodent normal soleus muscles.[5] On these preparations, the fiber type ratio and the fiber type specific muscle fiber cross sectional area were measured.

Morphometry

Photographic prints of the dystrophin and ATPase stained sections representing the entire cross section of the midpoint of soleus of 24 animals were analyzed with a MOP/3 image analysis system (Carl Zeiss Co., Munich FRG). The following parameters were measured: 1) The degree of dystrophin staining of the fibers (expressed as the percentage of dys(+), dys(−) and dystrophin intermediate [dys(IM)] fiber segments), 2) the overall and fiber type specific mean cross sectional area of muscle fibers, 3) the cytoplasmic nuclear ratio (the cross-sectional fiber area in square microns divided by the number of nuclei in that section), and 4) the numerical ratio of type 1 type 2 fibers.

A fiber was scored dys(+) if the sarcolemmal staining was similar to control fibers in normal mouse soleus. Dys(−) fibers were totally void of any immunostaining. In dys(IM) fibers, sarcolemmal staining was clearly discernable but weaker than normal.

Dystrophin Quantitation by Immunoblotting

Frozen muscle tissue was finely minced and homogenized in approximately 20 volumes of 15% sodium dodecyl sulfate (SDS), 20% glycerol, 75 mmol/l (nanomolar) TRIS HCl, pH 6.8, 5% mercaptoethanol, 0.001% bromophenol blue, using a polytron homogenizer (at one half of maximal speed) for 30 seconds. The homogenate was immediately placed in boiling water for 5 minutes and then spun at 10,000*g* for 5 minutes. Aliquots of 5 μl in triplicate were taken for protein determination. These aliquots were precipitated with 20 volumes of acetone to remove SDS, spun at 10,000*g* for 5 minutes, and the pellets were air dried and redissolved in 0.1 N NaOH for 1 hour at room temperature and protein was determined by the method of Lowry et al.

Samples of homogenates were loaded on 5.5% SDS polyacrylamide mini-gels.[6]

After electrophoresis the gels were cut between the myosin heavy-chain and the dystrophin bands using prestained molecular weight markers (Rainbow, Amersham Corp., Boston, MA) as reference points. The lower portion (myosin) was stained with Coomassie blue and the upper part (dystrophin) was transferred to nitrocellulose by the method of Towbin et al[7] with cooling using the BioRad mini transfer cell (BioRad, Mississauga, Ontario, Canada). The blots were rinsed with water and air dried for 16 hours.

The blots were rehydrated with water, incubated at 37°C for 45 minutes in 10% skim milk in TBST (50 mmol/l [millimolar] TRIS HCl, pH 7.5, 0.9% NaCl, 0.1% Tween-20). The blots were rinsed with TBST and incubated for 2 hours with polyclonal antibodies against a synthetic peptide corresponding to the last 17 amino acid residues of the C-terminus of dystrophin. Anti-dystrophin antibodies were raised as previously described.[4] The antiserum was was used at 1:500 dilution in TBST without skim milk. An avidin-biotin complex alkaline phosphatase detection system (Pierce, Rockford, IL) was used with biotinylated anti-rabbit gamma G immunoglobulin (IgG) as a secondary antibody (Dimension Labs, Mississauga, Ontario, Canada). Myosin and dystrophin bands were quantitated as pixel number × density using a computerized-video image analyzer system (CAVA, Jandel Scientific, Mont-

real Quebec) A linear relationship between the amount of homogenate loaded and the pixel number × density for myosin and dystrophin was demonstrated over the range of 2 to 12 mg homogenate protein (data not shown)

Statistical analysis was performed by the analysis of variance (fiber dystrophin distribution and mean cross sectional area) and Student's *t* test (nuclear cytoplasmic ratio and fiber type ratios)

Results

Approximately 5000 muscle fibers in the 24 muscles were assessed

{OUTL}

I Dystrophin immunostaining of muscle fibers

The percentage distribution of dys(+) dys(−) and dys(IM) fiber per muscle is shown in Figure 1 and illustrated in Figure 2 Table 1 shows the significance values of these parameters to be at the level of P 0 05

1 Dys(+) fibers

a) In the nonirradiated muscles there was significant increase in the percentage of dys(+) fibers between 10 and 35 days whereas no significant change occurred after 35 days

b) In the irradiated muscles there was significant increase in the percentage of dys(+) fibers between 10 and 35 days and between 35 and 60 days

c) There was no significant difference in the percentage of dys(+) fibers at 35 days between irradiated and nonirradiated soleus At 60 days the percentage of dys(+) fibers was significantly higher in the irradiated muscles as compared with the nonirradiated muscles

2 Dys(−) fibers

a) In the nonirradiated muscles there was significant decrease in the percentage of dys(−) fibers between 10 and 35 days whereas no significant change occurred after that age

b) In the irradiated muscles the percentage of dys(−) fibers decreased but not significantly between 10 and 35 days and between 35 and 60 days The decrease was however significant between 10 and 60 days

c) There was no significant difference in the percentage of dys(−) fibers at 35 and 60 days between irradiated and nonirradiated muscles

3 Dys(IM) fibers

a) In the nonirradiated muscles there was a significant decrease in the percentage of dys(IM) fibers between 10 and 35 days whereas no significant change occurred after this age

b) In the irradiated muscles there was a significant decrease in the percentage of dys(IM) fibers between 10 and 35 days and to a lesser extent but still significantly between 35 and 60 days

c) There was no significant difference in the percentage of dys(IM) fibers at 35 days between irradiated and nonirradiated muscles At 60 days the percentage of dys(IM) fibers was significantly lower in the irradiated muscles

II Mean cross sectional area (Table 2)

The overall mean cross sectional area was larger at 35 days in the nonirradiated muscles than in the irradiated muscles but the difference was not significant At 60 days this difference became significant There was a significant difference in the overall mean cross sectional area in the nonirradiated muscles but not in the irradiated ones between 35 and 60 days

The fiber type specific mean cross sectional areas (μ^2) in the heterozygote mice were 10 day type 1 2884 ± 24 type 2 2891 ± 19 60 day irradiated type 1 7866 ± 89 type 2 9709 ± 120 60 day nonirradiated type 1 13133 ± 190 type 2 14488 ± 167

There also was no significant difference between 35 and 60 days in the mean cross sectional area in soleus of normal mice (8911 ± 74 versus 8956 ± 75) and soleus of mdx mice (9561 ± 207 versus 11617 ± 166)

III The Cytoplasmic Nuclear Ratio (Table 2)

The cytoplasmic nuclear ratio was larger in the nonirradiated muscles than in the irradiated ones but the difference was not significant at 35 or 60 days In fact there was no change in this ratio between 35 and 60 days in the nonirradiated or in the irradiated muscles

IV Mean fiber type ratio

The mean ratio of type 1 type 2 fibers was 65 35 in the

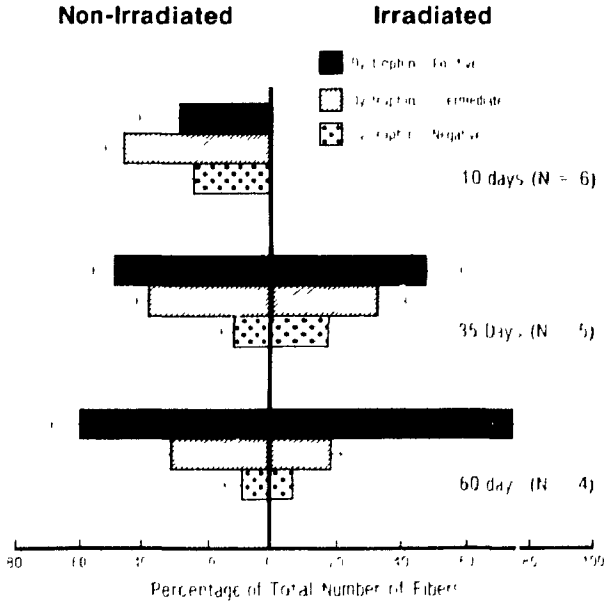

Figure 1 *Prevalence of dystrophin positive intermediate and dystrophin negative fibers (mean ± 1 SD) in intact and irradiated muscles of mdx female heterozygote mice at 10 35 and 60 days*

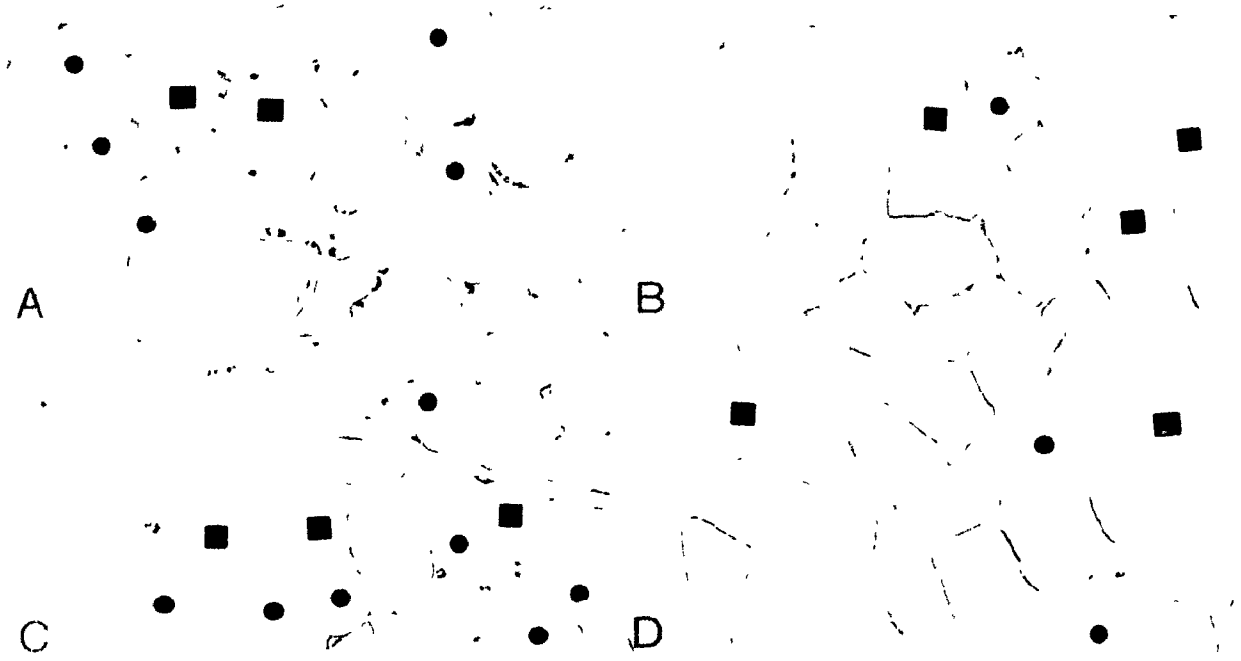

Figure 2. Transverse sections of heterozygote mdx soleus muscles showing dystrophin immunostaining. A, 35 days nonirradiated. B, 60 days nonirradiated. C, 35 days irradiated. D, 60 days irradiated. Dys(−) fibers are marked by dots. Dys(IM) fibers are marked by spaces. Much fewer dys(−) fibers are present in the 60 day old soleus than in the 35 day old, after but there is no significant difference between irradiated and nonirradiated muscles.

10 day heterozygotes, 67.33 in the irradiated 60 day old heterozygotes, and 66.34 in the 60 day old nonirradiated heterozygotes. There was no statistical significance between these values.

V. Relative dystrophin content by quantitative immunoblots (Figure 3)

The 10 day old intact heterozygote muscle contained approximately 24.5% of the 10 day old normal mouse muscle, confirming our earlier data.[3] By 60 days in the heterozygote muscle the dystrophin content has doubled, and irradiation had no significant effect on this change.

Discussion

The age related diminution of dys(−) muscle fiber segments in mdx heterozygotes is striking.[3,4] A similar phenomenon has been demonstrated in dystrophin-deficient cross breeds of golden retriever dogs.[10] We also have demonstrated in our previous[3] and present study that there is a significant relative increase of dystrophin content in heterozygote mdx muscles between 10 and 60 days of age. The present experiments were designed to investigate the possible mechanisms that underlie this phenomenon. The results of the present studies suggest that in older mdx heterozygote females the dys(−) and dys(IM) fiber segments have probably converted to dys(+) segments, and inhibition of satellite cell proliferation by gamma irradiation not only did not prevent this transformation, but actually seemed to enhance it. There was a continued significant increase in the number of dys(+) fibers in the irradiated muscle between 35 and 60 days of age, and a corresponding decrease of the dys(−) and dys(IM) fibers. In quantitative terms, how-

Table 1 *Significance Values for Figure 1*

Group of animals	Positive fibers	Intermediate fibers	Negative fibers
Nonirr. 10 days vs. nonirr. 35 days	S ↑	S ↓	S ↓
Nonirr. 35 days vs. nonirr. 60 days	NS	NS	NS
Nonirr. 10 days vs. nonirr. 60 days	S ↑	S ↓	S ↓
Nonirr. 10 days vs. irr. 35 days	S ↑	S ↓	NS
Nonirr. 10 days vs. irr. 60 days	S ↑	S ↓	S ↓
Irr. 35 days vs. irr. 60 day	S ↑	S ↓	NS
Nonirr. 35 days vs. irr. 35 days	NS	NS	NS
Nonirr. 60 days vs. irr. 60 days	S *	S ↓	NS

[illegible] NS, not significant at the [illegible] level of 0.05; * greater [illegible]

Table 2 *Mean Cross-sectional Area of Muscle Fibers and Cytoplasmic Domain Size [illegible] Soleus Muscles of Heterozygote mdx Mice at 35 and 60 Days of Age*

	[illegible]		60 days [illegible]	
	Mean cross-sectional area (μ[illegible] ± SD)	Cytoplasmic domain size (μ[illegible] per nucleus number ± SD)	Mean cross-sectional area (μ[illegible] ± SD)	Cytoplasmic domain size (μ[illegible] per nucleus number ± SD)
Nonirradiated	[illegible]27 ± 15[illegible]	[illegible]	[illegible]	[illegible]
Irradiated*	528 ± [illegible]48	[illegible]68 [illegible]	[illegible]	[illegible]

Irradiated [illegible]
* indicates significant [illegible] between [illegible]
* indicates significant difference between [illegible]

ever the dystrophin content (relative to a myosin heavy chain reference base) in the 60 day old irradiated muscles was not different from the 60 day old nonirradiated ones.

We have previously suggested that a spontaneous fusion of dystrophin-competent satellite cells into the dys(−) fiber segments in mdx female heterozygotes could bring about such a change.[7] Spontaneous satellite cell fusion into growing muscle fibers is known to take place during normal growth and development.[11] Because myosatellite cells can migrate beneath the basal lamina of muscle fibers,[11,12] some dystrophin competent myonuclei added to the previously dys(−) fibers segments could be expected to convert such segments to dys(+) or at least dys(IM). Such a mechanisms could be suppressed by gamma or X irradiation, which is known to cause an inhibition of myosatellite cell proliferation.[13,14] Previous studies have shown that this amount of irradiation causes an effective inhibition of satellite cell proliferation in mdx mice.[9,16] If the fusion of dystrophin competent myosatellite cells into dys(−) fiber segments were responsible for the conversion of dys(−) to dys(+) fiber segments, the suppression of satellite cell proliferation could be expected to reduce this conversion. Such an effect was not observed. In the irradiated muscles, the percentage of dys(+) fibers at 60 days was even higher than in the nonirradiated muscles, although the relative amount of dystrophin by quantitative immunoblot was about the same. Because the employed radiation dosage does not affect the normal expression of the dystrophin gene,[4,9] the putative dystrophin competent satellite cell fusion into dys(−) fiber segments cannot explain the conversion phenomenon. An alternate explanation is an expansion of the cytoplasmic domain of dystrophin expression of the originally dystrophin competent myonuclei.

In the muscles of young mdx heterozygote females, dys(+) and dys(−) muscle fiber segments are interspersed. It is possible that a longitudinal expansion of the cytoplasmic domain of dystrophin expression could increase the length of dys(+) fiber segments, resulting in a higher percentage of dys(+) or dys(IM) fibers on a given cross section of the muscle. Such putative expansion of the dystrophin domain by the originally dystrophin competent myonuclei[3] could be enhanced by the presence of unoccupied intramembranous dystrophin binding proteins in the dys(−) fiber segments. An integral membrane glycoprotein has been identified as a

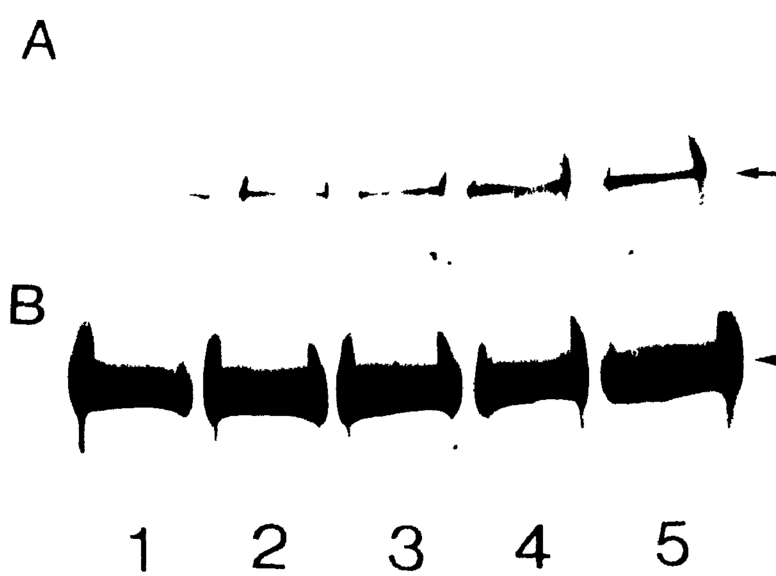

Figure 3 *Mouse skeletal muscle was solubilized and electrophoresed as described in Experimental Procedures. Samples corresponding to 6 μg of muscle homogenate protein were subjected to SDS-PAGE.* A *shows the upper portion of the gel after transfer to nitrocellulose and incubation with antibody to dystrophin.* B *shows myosin heavy chain in a lower portion of the same gel stained with Coomassie blue. Lane 1: 10 day heterozygote; lane 2: 60 day heterozygote irradiated; lane 3: 60 day heterozygote nonirradiated; lane 4: 10 day normal control mouse; lane 5: 60 day normal control mouse. The dystrophin content was normalized to the myosin content in the 60 day old normal control muscle as 100 (reference point); the corresponding values in each lane were: lane 1, 27; lane 2, 54; lane 3, 56; lane 4, 111. The dystrophin normalization was achieved by first calculating the dystrophin to myosin pixel number × density ratio for each sample and expressing each of these ratios as a percentage of the same ratio obtained for the 60 day old normal control muscle.*

[illegible] the dystrophin anchoring molecule. The higher percentage of dys(+) fibers in the irradiated soleus of older animals, as compared with the nonirradiated ones, may be explained by the smaller overall caliber of muscle fibers in irradiated muscles of 60-day-old animals, so that there is a smaller surface area over which the same amount of dystrophin is distributed.

The results of these studies might be relevant to Duchenne muscular dystrophy (DMD). In DMD muscle fibers lack dystrophin. Injection of normal myoblasts derived from a normal muscle of a donor into DMD muscles (myoblast transfer) may result in scattered dys(+) fiber segments as a result of the fusion of the normal myoblasts into the dystrophic muscle. The dys(+) fiber segments eventually may undergo the same longitudinal expansion because of an increase of the cytoplasmic domain of dystrophin, as we hypothesize for the dys(+) fiber segment expansion of mdx female heterozygotes.

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