

## Characterisation of dystrophin during development of human skeletal muscle

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### Summary

Dystrophin, the  $427 \times 10^3 M_r$  product of the Duchenne muscular dystrophy (DMD) gene, was studied in human foetal skeletal muscle from 9 to 26 weeks of gestation. Dystrophin could be detected from at least 9 weeks of gestation at the sarcolemmal membrane of most myotubes, though there was differential staining with antibodies raised to various regions of the protein. Dystrophin immunostaining increased and became more uniform with age and by 26 weeks of gestation there was intense sarcolemmal staining of all myotubes. On a Western blot, a doublet of smaller relative molecular mass than that seen in adult tissue was detected in all

foetuses studied. There was a gradual increase in abundance of the upper band from 9 to 26 weeks, and the lower band, although present in low amounts in young foetuses, increased significantly between 20 and 26 weeks of gestation. These data indicate that there are several specific isoforms of dystrophin present in developing skeletal muscle, though the role of these is unknown.

Key words: dystrophin, foetal development, skeletal muscle.

### Introduction

Duchenne muscular dystrophy (DMD), the most common and severe form of the muscular dystrophies, is characterised by progressive muscle weakness resulting in premature death, usually before the age of 20 years. Becker muscular dystrophy (BMD) is a less severe form of the disease, but results from a defect in the same gene. The gene has been identified (Koenig et al. 1987) and localises to the short arm of the X-chromosome (Xp21), encompassing at least 2.3Mb of DNA. A 14kb mRNA encodes a  $427 \times 10^3 M_r$  protein, dystrophin (Hoffman et al. 1987). Dystrophin can be detected in all types of muscle and in brain, the levels in brain being 1/10th to 1/100th that of muscle (Chamberlain et al. 1988). Antibodies have been raised to various regions of the protein using either synthetic peptides or fusion protein constructs, and are used to study dystrophin. On a Western blot, dystrophin can be detected in normal muscle samples or muscle from patients with muscular dystrophies other than DMD or BMD as a doublet or triplet of approx.  $400 \times 10^3 M_r$  (Hoffman et al. 1987; Patel et al. 1988). Immunocytochemistry shows that it is uniformly localised to the sarcolemmal membrane (Arahata et al. 1988; Bonilla et al. 1988; Zubrzycka-Gaarn et al. 1988) and ultrastructural studies have localised it to the cytoplasmic surface

(Watkins et al. 1988; Cullen et al. 1990). Using both techniques, dystrophin has been shown to be generally absent from muscle of DMD patients and abnormal in BMD (altered relative molecular mass or reduced amount).

The functional role of dystrophin is, as yet, unclear. The predicted structure reveals a rod-shaped protein composed of several domains: an N-terminal region with homology to  $\alpha$ -actinin; a region of spectrin-like repeats; a cysteine-rich region; and a variable C terminus (Koenig et al. 1988). The homology with spectrin suggests it may have a cytoskeletal role, although recent reports indicate dystrophin may be associated with a membrane-bound glycoprotein (Campbell and Kahl, 1989; Ohlendieck et al. 1991) suggesting that it may be important in protein anchorage.

RNase protection and PCR studies have demonstrated the presence of several isoforms of dystrophin mRNA generated by alternative splicing. This has been shown for both the 5' end, where splicing generates muscle- and brain-specific proteins (Nudel et al. 1989), and the 3' end, which is likely to result in multiple isoforms (Feener et al. 1989). Additionally, alternative splicing has been demonstrated during foetal development in the mouse (Geng et al. 1991).

There have been few published studies on develop-

mental control of dystrophin expression. In cultured cells, dystrophin mRNA is expressed only in fused myotubes, not in myoblasts (Oronzi-Scott et al. 1988), suggesting that it is regulated in a similar way to other muscle-specific proteins. There has been one report on dystrophin localisation in normal foetal skeletal muscle (Wessels et al. 1991), but the study was not extensive and used only the original antibodies raised by Drs E.P. Hoffman and L.M. Kunkel, (Hoffman et al. 1987). Dystrophin was detected at 8 and 11 weeks of gestation in the sarcoplasm, but only at those regions near the myotendinous junctions. By 17 weeks, there was sarcolemmal staining of most fibres.

In this study, dystrophin is characterised in developing skeletal muscle, using both Western blot analysis and immunocytochemistry with a panel of 5 antibodies raised to different regions of dystrophin.

## Materials and methods

### Muscle samples

Foetuses were aborted either by suction (up to 13 weeks of gestation) or prostaglandin termination. The ages and muscle studied is summarised in Table 1. The muscle was processed as soon as possible after the operation (samples were placed on ice immediately after they were obtained and frozen in liquid nitrogen within 4 hours), but prostaglandin termination of older foetuses involved a delivery time of up to 20 hours. Samples were also taken by needle biopsy from the quadriceps of a 41 week still birth, 5 normal volunteers (aged 22 to 44 years), and 6 patients with non-Xp21 neuromuscular disorders (aged 9 months to 10 years). All tissues were used after ethical approval.

### Antibodies

A panel of 5 antibodies to different regions of dystrophin were used: 60kD, the polyclonal antibody raised against a fusion protein containing a  $60 \times 10^3 M_r$  peptide derived from a region towards the N terminus of dystrophin (Hoffman et al. 1987); H12 and P6 polyclonal antibodies raised in our laboratory against fusion proteins derived from regions towards the C terminus of the spectrin-like repeats (corresponding to amino acids 2604-3024 and 2814-3028 respectively, Sherratt et al. unpublished data); dys1 and dys2 monoclonal antibodies were gifts from Dr L. Nicholson (Newcastle-upon-Tyne). They have been previously characterised (Nicholson et al. 1989a,b) and are commercially available. Dys1 was raised to an epitope in the mid-rod region and dys2 to a synthetic peptide corresponding to the C-terminal 17 amino acids of dystrophin. Sections were also

stained for  $\beta$ -spectrin using a monoclonal antibody to erythrocyte- $\beta$ -spectrin (56A), shown to cross react with the muscle isoform (Appleyard et al. 1984). This was a gift of Drs D. Shotton and M. Newton (Oxford).

### Western blot analysis

This was performed as previously described (Clerk et al. 1991). Briefly, frozen muscle was solubilized in 1% SDS containing protease inhibitors (1 mM iodoacetamide, 1 mM

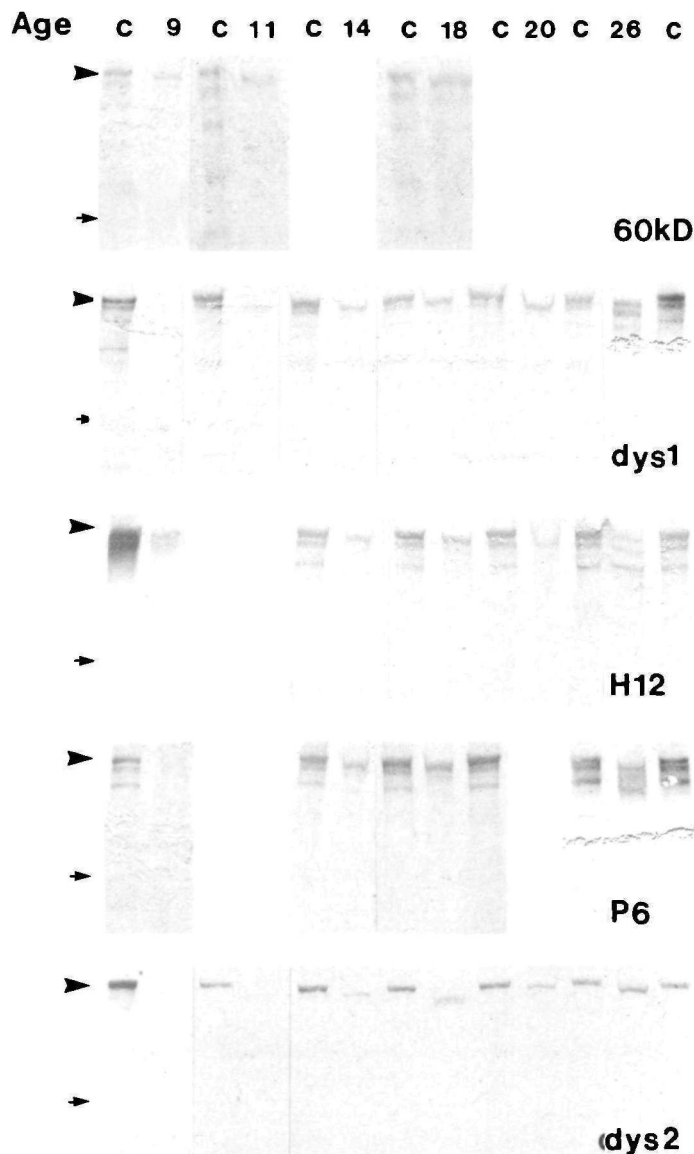


Fig. 1. Dystrophin immunoblots of foetal tissue. [Blemishes in some areas of these blots result from fire and water damage of the negatives. The originals were destroyed]. A smaller relative molecular mass protein than that seen in the adult is detected in all foetal tissue studied. At 9 weeks dystrophin is detectable only with 60kD and H12, but by 14 weeks the protein is seen with all antibodies. Levels of dystrophin increase with gestational age. The doublet detectable in adult tissues with all antibodies except dys2 is not seen until after 20 weeks. C=normal adult control muscle (50  $\mu$ g). Numbers indicate age in weeks of foetal samples (100  $\mu$ g). Arrowheads indicate dystrophin. Arrows indicate  $200 \times 10^3$  relative molecular mass marker.

Table 1. Foetuses studied

Age (weeks)	No. of foetuses	Muscle studied
9	3	Leg
11	1	Thigh
12	2	Thigh
13	1	Thigh
14	2	Thigh
18	1	Quadriceps
20	2	Quadriceps
26	1	Quadriceps
41	1	Quadriceps

benzethonium chloride, 0.5 mM phenylmethylsulphonylfluoride, 0.05 mM pepstatin A) and the protein concentration estimated with the BioRad protein assay. 100  $\mu$ g of foetal muscle or 50  $\mu$ g of control adult muscle was boiled with an equal volume of Laemmli buffer (2 minutes). The proteins were resolved on a 4-20% linear gradient gel and blotted onto nitrocellulose. Blots were incubated with dystrophin antibodies (60kD 1/1000; H12 and P6 1/3000; dys1 and dys2 1/50), followed by a biotinylated secondary antibody (1/400). Protein bands were detected with a streptavidin-peroxidase complex (Dakopatts ABC kit) and visualised with diaminobenzidine containing 0.005% (v/v) hydrogen peroxide.

#### Immunocytochemistry

Muscle was orientated transversely on cork disks, frozen in isopentane cooled in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . 5  $\mu$ m sections were incubated for 30 minutes with primary antibody (60kD antibody and P6 1/800; H12 1/1200; dys1 and dys2 were used undiluted; 56A 1/2), followed by a biotinylated secondary antibody (1/200). Proteins were visualized with a streptavidin-Texas Red conjugate. Control sections, incubated without primary antibody, were negative except for autofluorescence.

## Results

#### Western blot analysis

Normal adult dystrophin was seen as a doublet or triplet of approx.  $400 \times 10^3 M_r$  using all antibodies except dys2 which detected a single band corresponding to the upper band seen with the other antibodies. In human foetal muscle, all dystrophin antibodies detected a

smaller protein than in adult muscle, which persisted until at least 26 weeks of gestation (Fig. 1). We have not yet been able to ascertain when the adult form of the protein is first produced, but by 9 months of age normal relative molecular mass dystrophin is seen (data not shown).

At 9 weeks of gestation, dystrophin was only detectable with 60kD and H12, but by 14 weeks it was seen with all dystrophin antibodies. The levels were low and appeared to increase over the subsequent few weeks. It was apparent, however, that the different isoforms represented by the doublet/triplet seen in adult muscle, were not expressed equally in foetal tissue: although the lower band(s) could be detected in the younger foetuses, the levels were much reduced relative to the upper band. It was not until 26 weeks of gestation that the adult pattern could be clearly seen.

#### Immunocytochemistry

Where possible, sections were stained with the 5 dystrophin antibodies and an antibody to  $\beta$ -spectrin, and serial areas compared to study differential expression of different regions of dystrophin.

In adult muscle, all antibodies gave uniform staining of the sarcolemmal membrane with little internal staining of muscle fibres (Fig. 2).

#### 9 weeks

At 9 weeks of gestation, most myotubes were positive for  $\beta$ -spectrin (Fig. 3A). Distinct populations of myotubes differed in intensity of immunostaining with

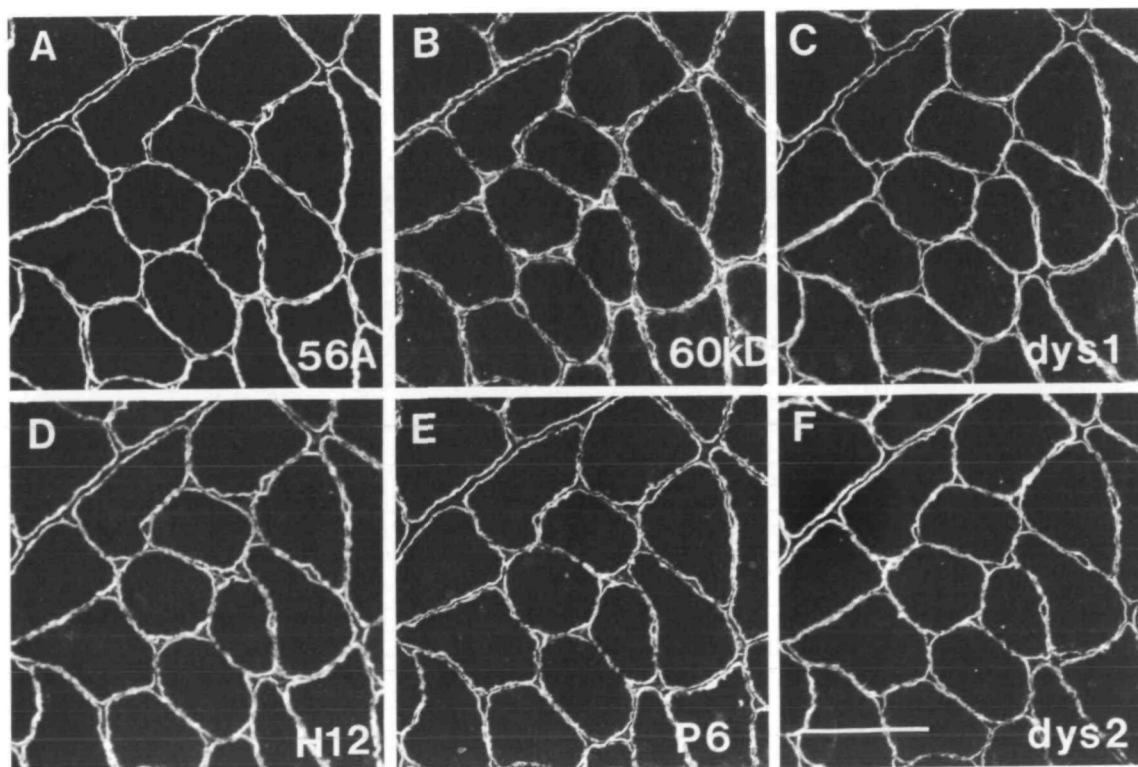
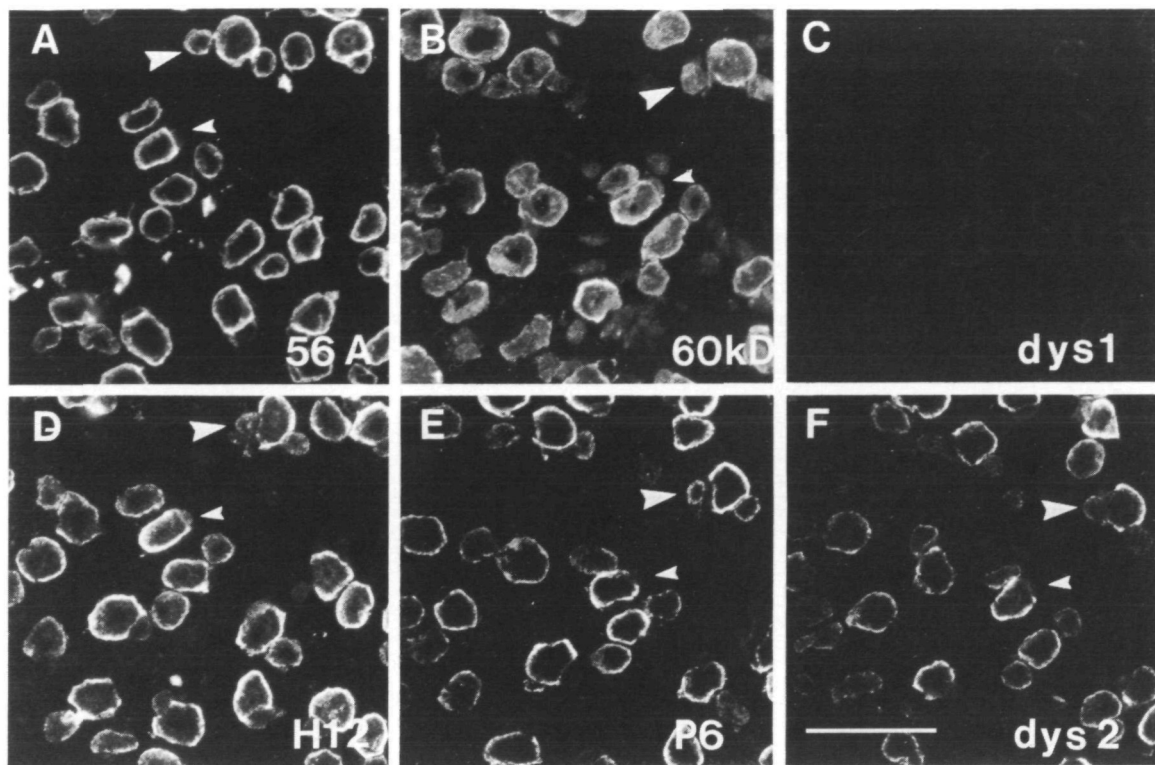


Fig. 2. Immunocytochemical staining of normal adult skeletal muscle shows uniform sarcolemmal staining with antibodies to  $\beta$ -spectrin (A) and all dystrophin antibodies (B to F). A, 56A; B, 60kD; C, dys1; D, H12; E, P6; F, dys2. Bar=100  $\mu$ m.



**Fig. 3.** Differential immunostaining of different classes of myotubes in 9 week foetal muscle. Larger myotubes are uniformly stained with all antibodies except dys1. Smaller myotubes are less brightly stained with P6 and dys2 (large arrowheads), and the smallest myotubes are negative with all antibodies (small arrowheads). Dys1 (C) shows little immunoreactivity. A, 56A; B, 60kD; C, dys1; D, H12; E, P6; F, dys2. Bar=50  $\mu$ m.

dystrophin antibodies (Fig. 3B-D): the largest myotubes were positive with all antibodies except dys1; a second population of smaller myotubes were also positive, but were more weakly stained with P6 and dys2; the very smallest were negative with all antibodies including  $\beta$ -spectrin. Additionally, 60kD gave more internal staining. Dys1 gave very little immunostaining at this stage (Fig. 3C).

#### 11-14 weeks

The largest myotubes gave similar staining as at 9 weeks (Fig. 4), though there was now some sarcolemmal staining with dys1 (Fig. 4C). Smaller myotubes were evenly stained with most antibodies, but showed some variability with P6 (Fig. 4E) and were also weakly stained with dys1. The smallest myotubes were positively stained with 60kD, H12 and P6, but not with dys1 and dys2 (Fig. 4).

#### 20-41 weeks

All dystrophin antibodies gave more even sarcolemmal staining of all myotubes by 20 weeks of gestation (Fig. 5). Levels of staining with dys1 were more comparable with those of the other antibodies, though there was still some variability with weaker staining of the smaller myotubes. Some of the smallest myotubes were very weakly stained with dys1 and dys2.

By 26 weeks, there was even sarcolemmal staining of

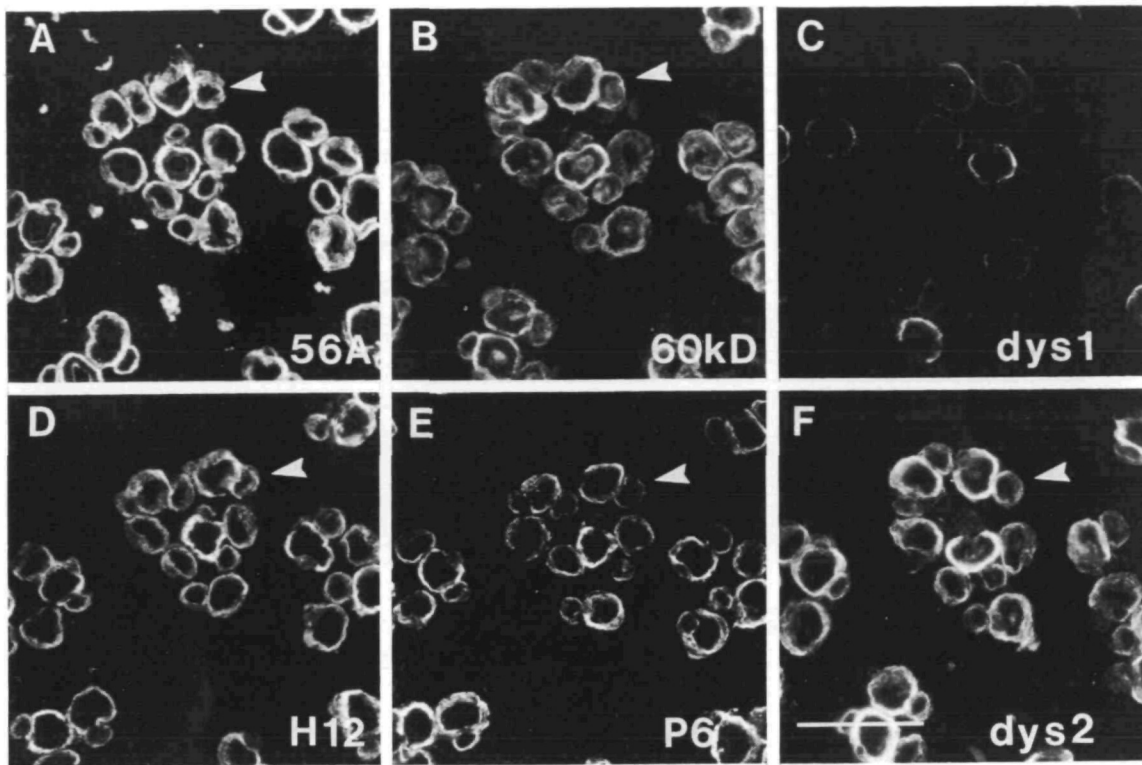
all fibres seen with all antibodies, comparable with the staining seen shortly after birth (Fig. 6).

#### Perinuclear staining

In the younger foetuses dystrophin immunostaining was seen around central nuclei (Fig. 7). This was more apparent with 60kD, but was also seen with H12. The perinuclear staining was seen in foetuses up to 14 weeks of gestation, but could not be detected in older foetuses or in fibres with peripheral nuclei.

#### Discussion

In contrast to previous published work (Wessels et al. 1991), our studies have shown that dystrophin was localised to the sarcolemma of myotubes in foetal muscle from at least 9 weeks of gestation. In the previous study, however, tissue was fixed in formalin, which may affect preservation of the epitope. A contributing factor to the discrepancy may also be the antibody used (60kD) which detects more internal staining of muscle fibres than other antibodies, so that sarcolemmal staining appears less obvious. This internal staining could be due to cross-reactivity with other proteins or may be due to fragments of partially synthesised or degraded dystrophin. The level of internal staining declines with age and is considerably lower in adult muscle.



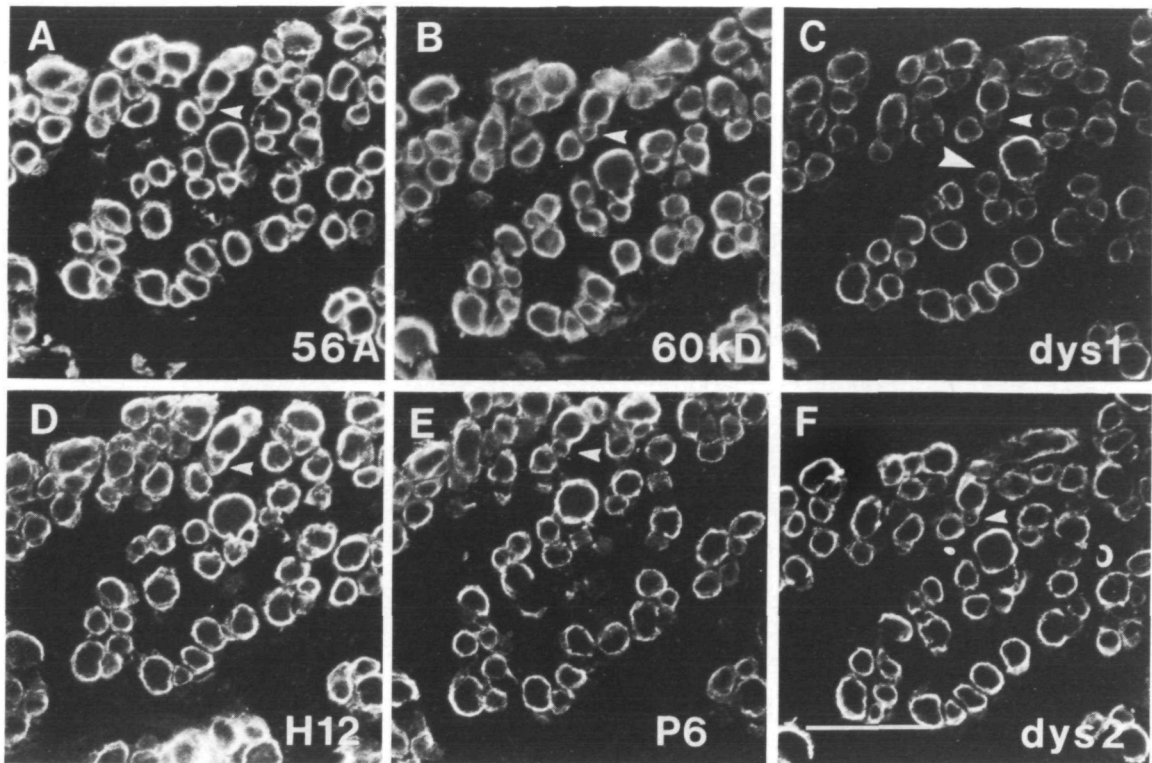
**Fig. 4.** Immunocytochemical staining of skeletal muscle from a 14 week foetus shows smaller myotubes are still weakly stained with P6 and dys2 (large arrowheads), though there is now sarcolemmal staining of some of the smallest myotubes with antibodies to  $\beta$ -spectrin, 60kD and H12 (small arrowheads). Dys1 shows weak staining only of the larger myotubes. A, 56A; B, 60kD; C, dys1; D, H12; E, P6; F, dys2. Bar=50  $\mu$ m.

Although all antibodies except dys1 showed sarcolemmal staining from 9 weeks, the differential staining of myotubes seen with dys2 and P6, suggests that there is more than one isoform of dystrophin expressed in foetal muscle. That the weaker stained myotubes were generally the smaller ones, implies developmental regulation. The significant increase in dys1 immunostaining between 9 and 20 weeks further suggests the presence of several isoforms, particularly since it also shows differential staining of myotubes in younger foetuses. The possibility of autolytic changes in aborted muscle cannot be completely eliminated, particularly in the older prostaglandin terminations, but none of the samples studied showed any signs of protein degradation as studied by Western blotting and it is unlikely that there should be such specific changes of particular dystrophin epitopes in the younger foetuses.

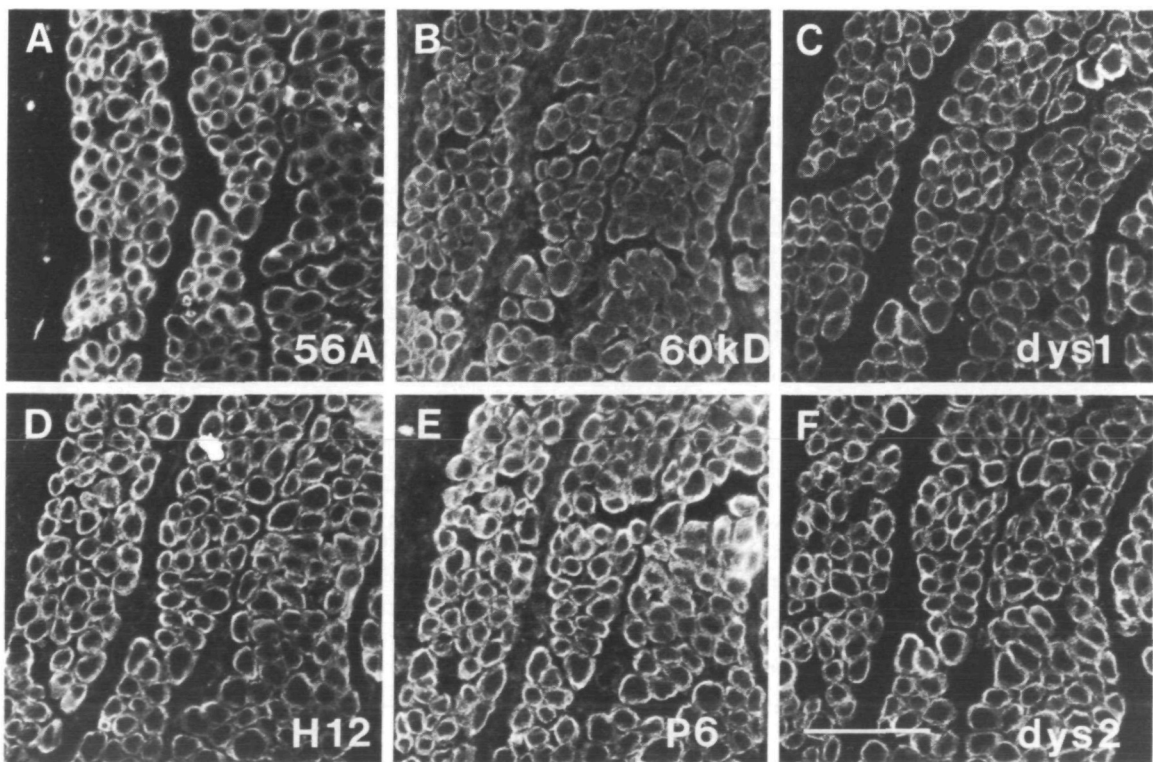
Western blot analysis provides additional evidence of developmental regulation of dystrophin isoforms. In all foetal tissue studied, a lower relative molecular mass protein was detected than in adult tissue. This persisted until at least 26 weeks of gestation, but is known to have switched to the adult form by 9 months of age (data not shown). The smaller foetal protein is unlikely to result from sample degradation, which results in the appearance of multiple smaller bands between  $200$  and  $400 \times 10^3 M_r$ , (unpublished observations and Nicholson et al. 1989a). At 9 weeks of gestation, low levels of dystrophin were detected with 60kD and H12, but other

antibodies detected no protein until 11-14 weeks. The overall levels appeared to increase throughout the gestational period, particularly since the smaller fibre size in younger foetuses results in a greater sarcolemma:sarcoplasm ratio with a correspondingly higher level of sarcolemmal proteins. However, this may be due in part to the presence of less muscle protein in samples from younger foetuses since it was not always possible to dissect the muscle cleanly from other tissues. The relative intensity of staining of the lower band(s) of the triplet/doublet increased significantly between 20 and 26 weeks, correlating with the appearance of normal dystrophin immunostaining, (i.e. uniform sarcolemmal staining with all dystrophin antibodies), and provides further evidence for developmental regulation of dystrophin isoforms.

Different foetal isoforms could be produced either by alternative splicing or by post-translational processing. A distinct embryonic transcript has been detected in mouse foetal muscle (Geng et al. 1991), supporting the former hypothesis. It may be argued that dystrophin antibodies may cross-react with the product of the dystrophin related gene, (DMDL, Khurana et al. 1989; Love et al. 1989, 1991), which could explain both the smaller protein in foetal tissues and the variability in immunostaining given by different antibodies. Although there is significant homology (approx. 80%) of the predicted C terminus, the entire gene has not been sequenced and there is as yet no evidence for



**Fig. 5.** Differential immunostaining seen with dys1 and dys2 antibodies in 20 week foetal muscle. 56A, 60kD, H12 and P6 give uniform sarcolemmal staining of all myotubes. There is still variability with weaker staining of smaller myotubes with dys1 (large arrowhead) and little staining of the smallest myotubes with dys1 and dys2 (small arrowheads). A, 56A; B, 60kD; C, dys1; D, H12; E, P6; F, dys2. Bar=50  $\mu$ m.



**Fig. 6.** Uniform immunostaining of the sarcolemma of all fibres seen in skeletal muscle from a 41 week still birth. A, 56A; B, 60kD; C, dys1; D, H12; E, P6; F, dys2. Bar=50  $\mu$ m.

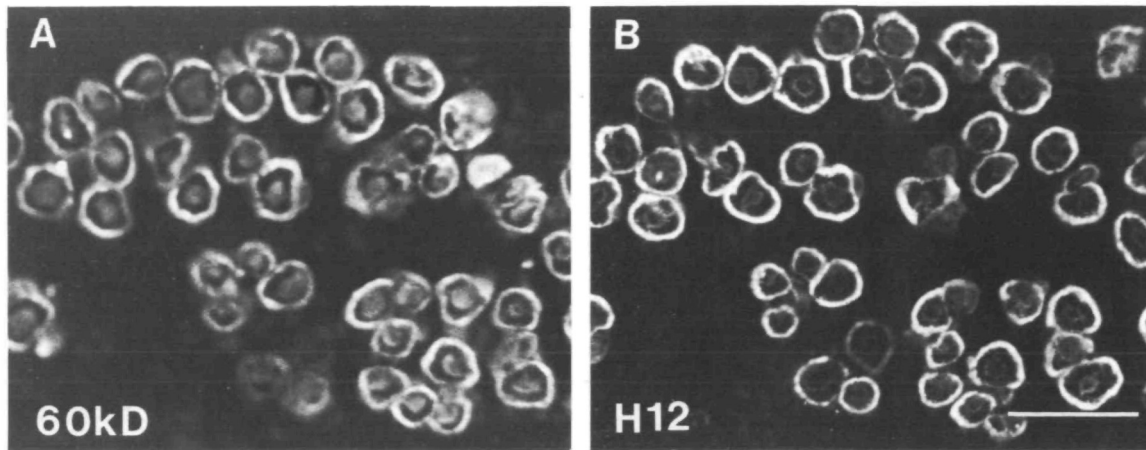


Fig. 7. Perinuclear staining of central nuclei in 9 week foetal muscle with 60kD (A) and H12 (B). Bar=50  $\mu$ m.

cross-reactivity with the dystrophin antibodies used in our study.

The perinuclear staining of central nuclei seen in young foetuses could be dystrophin, particularly since it is seen with more than one antibody. However, considering the homology between dystrophin and spectrin family of proteins, it may be due to cross-reactivity with another protein such as spectrin, (shown to be associated with the nuclear membrane, Vendrell et al. 1990; Bachs et al. 1990), particularly since perinuclear staining is seen in DMD foetuses (unpublished data). The disappearance of perinuclear staining correlated with the migration of nuclei to the periphery, but it is unclear whether the staining was abolished or could no longer be seen with the nucleus so closely associated with the membrane. This is under further study.

Our results suggest there are multiple dystrophin isoforms present during human foetal development. The relevance of these can only be speculated, particularly since the function is unknown.

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