# Localization of the DMDL Gene-encoded Dystrophin-related Protein Using a Panel of Nineteen Monoclonal Antibodies: Presence at Neuromuscular Junctions, in the Sarcolemma of Dystrophic Skeletal Muscle, in Vascular and Other Smooth Muscles, and in Proliferating Brain Cell Lines

Nguyen thi Man,* J. M. Ellis,* D. R. Love,‡ K. E. Davies,‡ K. C. Gatter,§ G. Dickson,‖ and G. E. Morris*

*Research Division, N. E. Wales Institute, Deeside, Clwyd, CH5 4BR, Great Britain; ‡Molecular Genetics Group, Institute of Molecular Medicine; and §Department of Pathology, John Radcliffe Hospital, Oxford, OX3 9DU, Great Britain; and ‖Department of Experimental Pathology, Guy's Hospital Medical School, London, SE1 9RT, Great Britain

*Abstract.* mAbs have been raised against different epitopes on the protein product of the DMDL gene, which is an autosomal homologue of the X-linked DMD gene for dystrophin. These antibodies provide direct evidence that DMDL protein is localized near acetylcholine receptors at neuromuscular junctions in normal and *mdx* mouse intercostal muscle. The primary location in tissues other than skeletal muscle is smooth muscle, especially in the vascular system, which may account for the wide tissue distribution previously demonstrated by Western blotting. The DMDL protein was undetectable in the nonjunctional sarcolemma of normal human muscle, but was observed in nonjunctional sarcolemma of Duchenne muscular dystrophy patients, where dystrophin itself is absent or greatly reduced. The expression of DMDL protein is not restricted to smooth and skeletal muscle, however, since relatively large amounts are present in transformed brain cell lines of both glial and Schwann cell origin. This contrasts with the low levels of DMDL protein in adult brain tissue.

An autosomal homologue (DMDL) of the dystrophin gene is found on chromosome 6 in man (10) and close to the *dystrophia muscularis* (dy) locus on chromosome 10 in the mouse (2). Unlike dystrophin, which is expressed predominantly in muscle tissues and localized at the sarcolemma (5), the DMDL protein is found in varying amounts in most tissues (7, 11) and its subcellular location has not yet been defined. The homologue belongs to a family of related proteins, which include dystrophin, spectrin and actinin (9), as well as possible protein products of alternative COOH-terminal transcripts of the dystrophin gene (1). Antibody cross-reactions can occur within the family, complicating the interpretation of immunolocalization results (13). This problem has been largely avoided in the case of dystrophin by taking advantage of null mutations in the dystrophin gene in Duchenne muscular dystrophy patients and the *mdx* mouse. Without a specific mutation which eliminates the DMDL gene product, the use of a number of different epitope-specific mAbs for immunostaining offers the best approach to tissue and subcellular localization of authentic DMDL protein.

The preparation of 19 dystrophin-specific mAbs using, as immunogen, a pEX2 bacterial fusion protein (lacZ-108kD) containing nearly half of the central helical sequence of dystrophin has already been described (12). We now report the preparation of 19 new mAbs against a pEX2 fusion protein (lacZ-Bfm3) containing the last 329 amino-acids of the DMDL protein (10, 11) and their use in the localization of DMDL protein in normal and dystrophic muscle and in non-muscle tissues.

## Materials and Methods

### mAb Production

The expression plasmid was produced by cloning a 1.05-kb fragment of Bfm3 cDNA (10, 11) into the SmaI site of pEX2. The fragment starts at nucleotide 510 in the sequence (10) and the plasmid was introduced into *E. coli* POP2136. Induction by incubation at 42°C and purification of the 155-kD β-galactosidase fusion protein by extraction of inclusion bodies with 2% SDS and gel filtration on UltragelAcA34 (LKB Instruments, Inc., Gaithersburg, MD) were performed as described previously (12). mAbs were produced by immunization of Balb/c mice and fusion of spleen cells with Sp2/0 myeloma cells as described previously (12). Hybridoma culture supernatants were screened initially by ELISA using microtiter plates coated with fusion protein or with β-galactosidase alone and were further selected for binding to a 400-kD protein on Western blots of human lung extracts. All hybridoma cell lines were subcloned twice by limiting dilution. The immunoglobulin subtype of each antibody was determined with an isotyping kit (Serotec, Oxford, UK).

### Rat Cell Lines

The C6 rat glial tumour cell line (ATCC No. CCL107) and the SWA transformed rat Schwann cell line (18) were grown in culture dishes in DME with 10% FCS and, for SWA cells, 0.1 mM $ZnCl_2$.

### Western Blotting

Freshly dissected mouse or frozen human tissues were weighed, dropped into 4 ml/g of boiling SDS extraction buffer (10% SDS, 10% EDTA, 10% glycerol, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.7), homogenized with a Silverson blender, boiled for 2 min, and centrifuged at 100,000 *g* for 20 min. This procedure produces extracts containing approximately the same concentration of total protein for all tissues studied. Cultured cells were harvested in sample buffer containing 2% SDS and 5% 2-mercaptoethanol, boiled, and centrifuged. Extracts (10 μl) were loaded onto 4–12.5% gradient gels with a 4% stacking gel, together with 5 μl of prestained molecular mass markers (Sigma Chemical Co., St. Louis, MO; 180, 116, 84, 58, 48.5, 36.5, and 26.6 kD; 1 mg/ml each). After electrophoresis, proteins were transferred electrophoretically (BioRad Transblot; Bio-Rad Laboratories, Cambridge, MA) to nitrocellulose sheets (Schleicher and Schull, BA85) at 100 mA overnight in 25 mM Tris, 192 mM glycine, 0.003% SDS. The SDS concentration is critical and must be adjusted to the minimum required for efficient transfer of very high $M_r$ proteins.

Blots were blocked in 3% skimmed milk powder in incubation buffer (0.05% Triton X-100 in PBS 25 mM sodium phosphate, pH 7.2, 0.9% NaCl). After two 5-min washes in PBS, blots were incubated with mAb culture supernatant (1/100 dilution in incubation buffer/1% horse serum/1% FCS/0.3% BSA) for 1 h at 20°C. After three 5-min washes with PBS, blots were incubated with biotinylated anti-mouse Ig and a peroxidase-avidin detection reagent (Vectastain ABC kit), according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). After four 5-min washes with PBS, substrate was added (0.4 mg/ml diaminobenzidine (Sigma Chemical Co.) in 25 mM phosphate-citrate buffer, pH 5.0, with 0.012% $H_2O_2$).

To screen a single sample with a large number of antibodies (up to 28), extracts were subjected to SDS-PAGE as a strip (250 μl), and a "miniblotter" apparatus (Immunetics, Cambridge, MA) was used to apply the mAbs as vertical lanes across all the protein bands on the blot.

### Immunohistochemistry

Frozen sections (7–10 μm) of human and mouse tissues were allowed to attach to untreated glass slides and either stored at −80°C or fixed with acetone and air-dried. After incubation for 30 min at 20°C with mAb culture supernatants, diluted 1+3 with PBS, and three 5-min washes with PBS, sections were incubated with second antibody for 30 min at 20°C. The second antibody (DAKOpatts Ltd; 1/40 dilution in PBS containing 1% horse serum/1% FCS/0.3% BSA) was anti-mouse Ig labeled with fluorescein or peroxidase. For double labeling, fluorescein-labeled α-bungarotoxin (Sigma Chemical Co.; 10 μg/ml) and a rhodamine-labeled anti-mouse Ig were used simultaneously. After three 5-min washes in PBS, slides for immunofluorescence were mounted in 70% glycerol in PBS and peroxidase slides were incubated with diaminobenzidine substrate. Immunofluorescence slides were examined with a Leitz (Leica) epifluorescence photomicroscope with appropriate filters and photographed for a fixed time (60 s) using Kodak Tri-X Pan film.

*Table I. Characterization of 19 mAbs against the COOH-terminal Region of the Chromosome 6 Homologue of Human Dystrophin*

| Antibody | Subtype | Dys* | Lung‡ Blot | NMJ§ | Group‖ | DMD¶ membrane |
|---|---|---|---|---|---|---|
| Homologue-specific | | | | | | |
| MANCHO1 | G1 | − | ++ | ND | 4 | + |
| MANCHO2 | G1 | − | + | ND | 4 | ND |
| MANCHO3 | G1 | − | ++ | + | 4 | ND |
| MANCHO4 | G1 | − | + | w | 4 | − |
| MANCHO5 | G1 | − | + | ND | 4 | ND |
| MANCHO6 | G1 | − | + | + | 4 | ND |
| MANCHO7 | G2a | − | + | + | 5 | + |
| MANCHO8 | G2a | − | ++ | + | 5 | + |
| MANCHO9 | G2a | − | w | w | 5 | + |
| MANCHO10 | G2a | − | + | + | 2 | + |
| Cross-reactive with dystrophin | | | | | | |
| MANCHO11 | G1 | + | ++ | + | 3 | ND |
| MANCHO12 | G1 | + | + | + | 3 | + |
| MANCHO13 | G1 | + | w | + | 3 | ND |
| MANCHO14 | G1 | + | ++ | + | 3 | ND |
| MANCHO15 | G1 | + | + | ND | 6 | + |
| MANCHO16 | G3 | + | + | − | 6 | ND |
| MANCHO17 | G2a | + | ++ | + | 6 | + |
| MANCHO18 | G1 | + | ++ | − | 7 | − |
| MANCHO19 | G1 | + | + | + | 1 | ND |

* Only MANCHO11-19 cross-reacted with the corresponding region of dystrophin in a fusion protein on ELISA plates and they also gave typical dystrophin membrane staining (Dys) on frozen human muscle sections.
‡ All antibodies reacted with a 400K homologue band on Western blots of human lung and *mdx* mouse lung, though with different intensities.
§ Antibodies were tested for neuromuscular junction staining as in Fig. 2.
‖ Antibodies were placed in seven different epitope mapping groups from their binding to different subfragments of the DMDL fusion protein (manuscript in preparation; see refs 8, 12, 13).
¶ Antibodies were tested for staining of Duchenne sarcolemma as in Fig. 3. w, weak.

## Results

19 mAbs raised against the DMDL protein were screened for specificity by Western blotting and immunohistochemistry on muscle sections (Table I). Nine of the antibodies (MANCHO11–19) were found to cross-react with dystrophin and this is consistent with our earlier observation that polyclonal mouse antisera raised against the same lacZ-Bfm3 fusion protein cross-react with dystrophin (11). On Western blots, the 10 DMDL-specific mAbs (MANCHO1-10) bind to a 400K protein in both normal and *mdx* muscle and, in characteristically larger amounts, in both normal and *mdx* lung (Fig. 1); our previous results with polyclonal antisera have shown that *mdx* lung expresses more DMDL protein than *mdx* muscle (11). Dystrophin, in contrast, is found in large amounts in normal muscle and in smaller amounts in normal lung, but is absent from *mdx* tissues (Fig. 1). Both dystrophin-specific and cross-reactive antibodies show characteristic sarcolemmal immunostaining of normal human and mouse frozen muscle sections, but DMDL-specific antibodies do not stain normal muscle sarcolemma (Figs. 2 and 3). These results show the specificity of the mAbs for their respective antigens in both SDS-denatured and native states.

Fardeau et al. (4) reported material cross-reacting with antidystrophin antibodies at neuromuscular junctions in *mdx* mouse muscle. We therefore examined sections of short intercostal muscles which are relatively rich in neuromuscular junctions. We used double labeling with a rhodamine-labeled anti-mouse Ig antibody in the presence of fluorescein-labeled α-bungarotoxin to locate both DMDL protein and acetylcholine receptors in the same transverse sections of intercostal muscle. Fig. 2 shows that DMDL-specific mAb binding is localized near acetylcholine receptors at neuromuscular junctions on the membranes of both normal and *mdx* muscle fibers. In normal mouse muscle, the dystrophin-specific antibody, MANDYS1, binds to the sarcolemma with more intense labeling at the neuromuscular junctions,

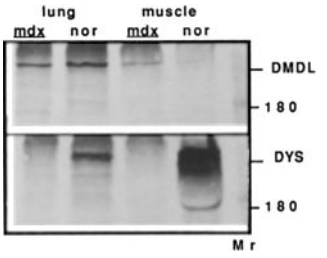


*Figure 1.* Western blots of DMDL protein and dystrophin (*DYS*) in normal and *mdx* mouse lung and muscle extracts. The blots were incubated with MANCHO7 antibody for DMDL protein and MANDYS1 for dystrophin. We have optimized this method for detection of the low levels of DMDL protein in muscle and, consequently, the dystrophin band in normal muscle appears overloaded and distorted. The smaller band at ~180 kD is a degradation product of dystrophin. MANCHO7 is among the weaker of the DMDL-specific monoclonals on Western blots of SDS-denatured proteins (Table I and Fig. 5), but is shown because it reacts well with native DMDL protein on frozen sections and was used in Figs. 2–4 for this reason.

but shows no binding to *mdx* muscle (since dystrophin is absent in *mdx*). Other mAbs that recognize five additional DMDL epitopes also showed neuromuscular junction staining in *mdx* muscle (Table I). Two mAbs (MANCHO16 and 18) failed to stain neuromuscular junctions because they require denatured DMDL antigen and so bind on Western blots but not frozen sections.

Fig. 3 shows that in human thigh muscle (where neuromuscular junctions are uncommon) DMDL-specific mAbs show little or no detectable staining of the sarcolemma of normal fibers, but they do show significant membrane staining on a large proportion of fibers from Duchenne patients. The situation is the reverse of that seen with antidystrophin antibodies, which stain normal but not Duchenne muscle (Fig. 3). Although the extent and intensity of staining varied between fibers, at least 50–80% of Duchenne fibers showed some membrane staining with anti-DMDL antibodies, compared with the very much smaller proportion of dystrophin-positive fibers (1–3%) frequently reported in Duchenne patients. This result has been obtained with antibodies in five epitope groups tested on three different Duchenne patients. Sarcolemmal staining of DMDL protein is also detectable in *mdx* muscle (Fig. 2), even though background staining is higher on mouse than on human sections. One antibody, MANCHO4, which was negative on Duchenne membranes, was also weak on neuromuscular junctions and is probably too weak to detect low antigen levels (Table I). It is not uncommon for mAbs to show a strong preference for either native or denatured antigen.

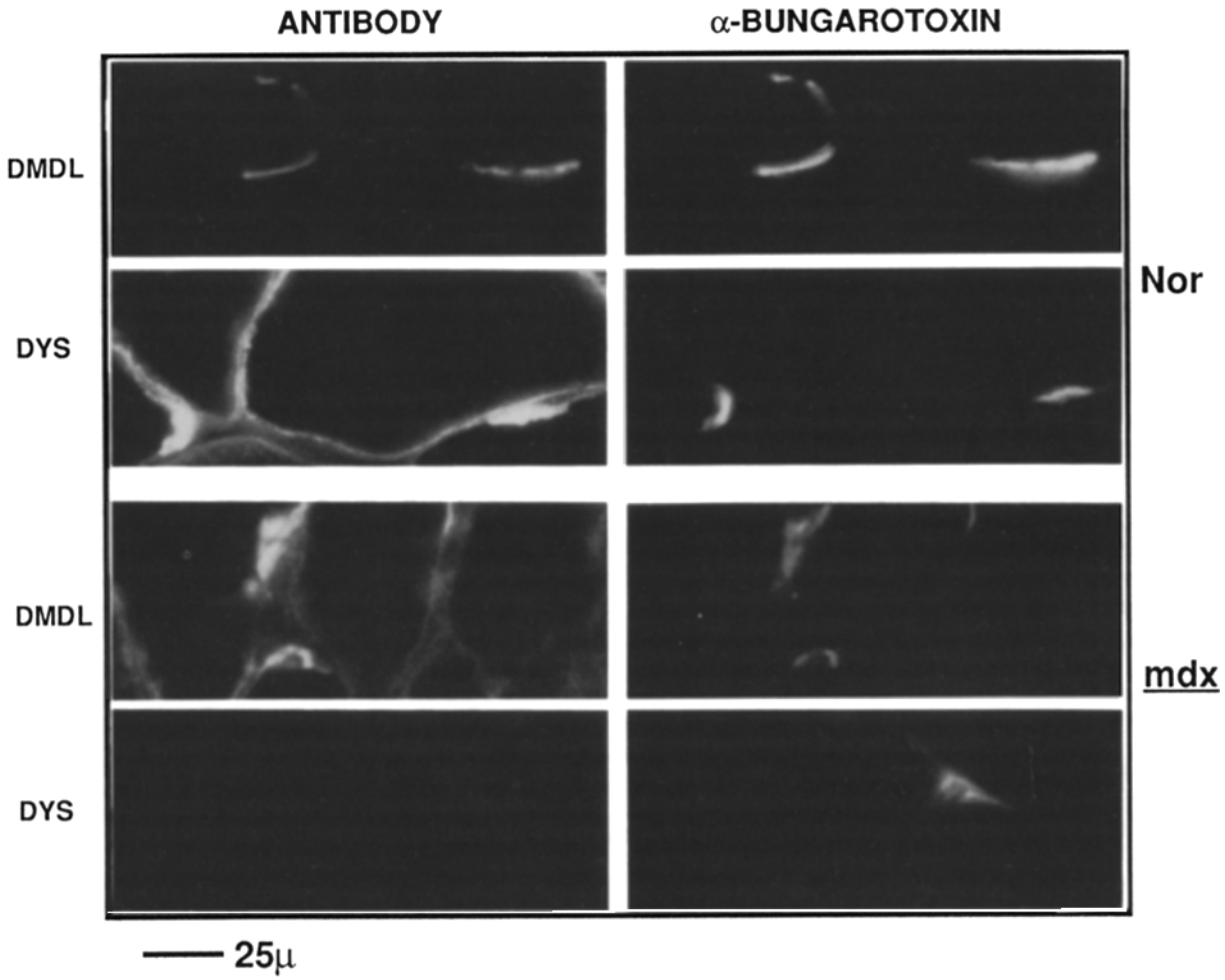


*Figure 2.* Immunolocalization of DMDL protein and dystrophin (DYS) with acetylcholine receptors in normal and *mdx* mouse muscle sections by double labeling. MANCHO7 antibody for DMDL and MANDYS1 for dystrophin were used, followed by a mixture of rhodamine-labeled rabbit anti-(mouse Ig) and fluorescein-labeled α-bungarotoxin.

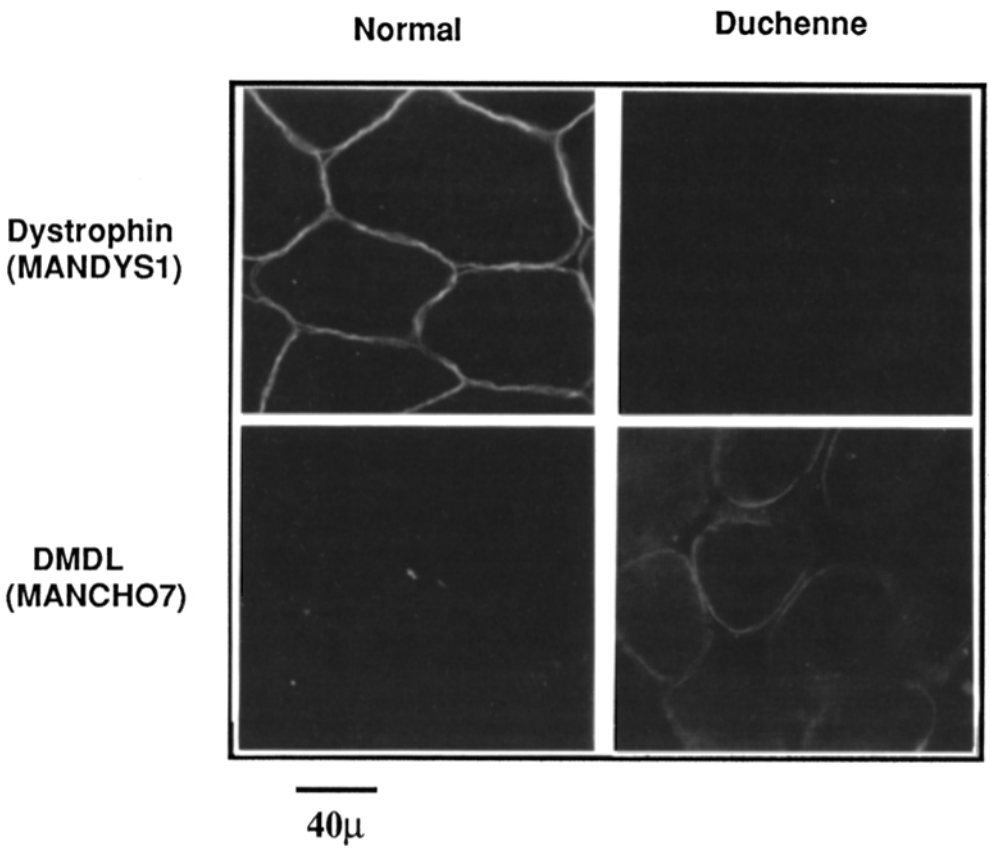


*Figure 3.* Immunolocalization of DMDL protein and dystrophin in normal and Duchenne human muscle sections. Antibodies and conditions as in Fig. 2, except that a fluorescein-labeled anti-(mouse Ig) was used. Duchenne biopsies were taken from boys of 4–6 yr for the purpose of confirmation of clinical diagnosis and dystrophin was undetectable using both rod and COOH-terminal antidystrophin antibodies.

Fig. 4 shows the localization of DMDL protein in human uterus sections using the homologue-specific antibody, MANCHO7, and illustrates both the intense staining of vascular smooth muscle in blood vessels and the rather less intense staining of bands of myometrial smooth muscle throughout the surrounding tissue (Fig. 4 *a*). At lower magnification (Fig. 4 *c*), it is clear that each of these sites makes a significant contribution to the total DMDL protein content of this tissue. We have observed similar binding of DMDL-specific antibodies to blood vessels in other tissues, including lung, heart, liver, brain, and testis (results not shown).

Fig. 5 *a* shows that a 400K protein comigrating with dystrophin can be detected by all anti-DMDL protein antibodies tested on Western blots of extracts of proliferating tumor cell lines of both glial and Schwann cell origin. Variation in intensity of the 400K band probably reflects differences in antibody affinity for SDS-denatured DMDL protein, since it correlates well with intensity of staining of the human lung 400-kD protein by the same antibodies (Table I), notably the weak reaction of MANCHO9 in lane 12. These differences make it difficult to obtain an absolute value for the ratio of DMDL protein to dystrophin. It is clear, however, that the ratio of DMDL protein to dystrophin is much higher in the cell lines than in adult brain tissue, when Western blots of the same samples are compared using dystrophin-specific MANDYS1 and DMDL-specific MANCHO1 (Fig. 5 *a*, lanes *2* and *9*, and Fig. 5 *b*). The subcellular localization of DMDL protein in these cultured cells has not yet been determined, because of technical problems arising from their small size and rounded morphology.

## *Discussion*

We have shown that several different mAbs against the 400-kD autosomal homologue of dystrophin localize this protein to the neuromuscular junction and to smooth muscle. The latter observation explains our recent demonstration that lung has high levels of DMDL protein, relative to other tissues, and that high levels of DMDL transcripts are found in placenta (11). It remains to be seen whether all highly vascular tissues contain high levels of the homologue, but its presence in tissue culture cells (Fig. 5) shows that it is not merely a smooth muscle isoform of dystrophin. Nevertheless, the uniform staining of myometrial smooth muscle cells in uterus (Fig. 4) contrasts strongly with the absence of staining in skeletal muscle fibers (Fig. 3), except at neuromuscular junctions. Both DMDL protein and dystrophin itself are present at neuromuscular junctions (Fig. 2). Pons et al. (14) have described neuromuscular junction staining in *mdx* and Duchenne skeletal muscle by antibodies raised against the COOH-terminal region of dystrophin and have suggested that cross-reaction with a dystrophin-like protein is responsible. Our neuromuscular junction staining using several mAbs raised against DMDL sequences provides direct evidence that the antigen involved is an authentic product of the DMDL gene, as opposed to indirect evidence obtained using cross-reactive antidystrophin antibodies.

The DMDL-specific mAbs do not stain nonjunctional membranes of normal human muscle, but they do stain Duchenne membranes, though rather weakly. Similar results have been obtained by Tanaka et al. (16, 17). They raised a

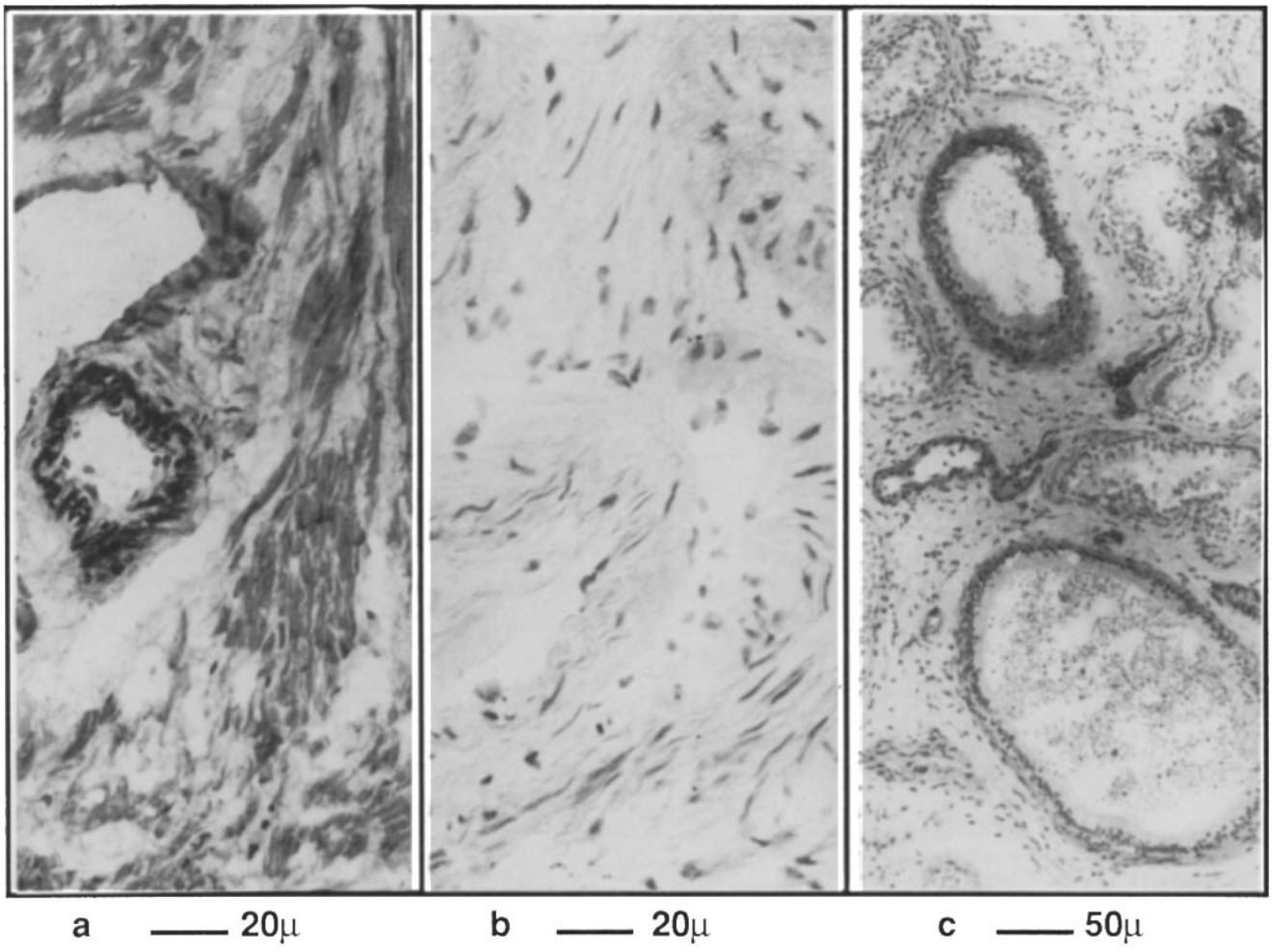


*Figure 4.* Immunolocalization of DMDL protein in acetone-fixed sections of human uterus. In *a* and *c*, the mAb MANCHO7 was used as in Fig. 2, except that the detection system was a peroxidase-labeled anti-(mouse Ig) with DAB substrate. The control section (*b*) was incubated with culture medium without antibody. Sections were counterstained with haemotoxylin to reveal nuclei. Binding to a large blood vessel is visible in the center left of *a*, while areas of myometrial smooth muscle staining occupy much of the rest of this frame. The overall extent of myometrial staining can be seen in the lower power micrograph (*c*).

polyclonal antiserum against a 51 amino-acid synthetic peptide near the COOH terminus of the DMDL homologue and observed weak staining of normal muscle membranes, but much more intense staining of Duchenne and *mdx* mouse membranes. These authors point out that, although the DMDL protein is likely to be responsible, the possibility of cross-reaction by other proteins that share an epitope with DMDL protein cannot be ruled out. Antibodies (monoclonal or polyclonal) may cross-react with epitope-sharing proteins on immunostained sections, even though they do not cross-react on Western blots, and vice versa. A polyclonal antiserum raised against dystrophin, for example, has been shown to stain both dystrophin and actinin in frozen sections, though dystrophin was difficult to detect on Western blots (6). Without an available mutation resulting in absence of the DMDL homologue (like the *mdx* and Duchenne mutations for dystrophin), it is impossible to entirely rule out cross-reactions with proteins other than DMDL and dystrophin. mAbs against different epitopes on the DMDL protein, however, do resolve this problem to a considerable extent and the fact that a number of different mAbs bind to the neuromuscular junction, vascular smooth muscle, the Duchenne membrane, and transformed brain cell lines makes it more likely that they are binding to authentic DMDL antigen at all four locations. These data are a considerable advance on our earlier studies (11) and those of Khurana et al. (7), in which potentially cross-reactive polyclonal antisera against large fragments of recombinant DMDL protein were used for Western blotting.

There is evidence to suggest that there is more DMDL mRNA and protein in fetal tissues than in adult tissues (7, 11) and several proteins associated with the neuromuscular junction, including the acetylcholine receptor, have a more general membrane distribution on immature, denervated, or regenerating fibers (15). Regenerating fibers might explain the presence of DMDL-positive membranes in Duchenne muscle (Fig. 3). The high levels of DMDL protein in proliferating brain cell tumor lines, relative to adult brain tissue (Fig. 5), are also consistent with expression in predifferentiation cells and tissues. Fetal tissues, however, may also be more highly vasculated than adult tissues so that blood vessels could make a significant contribution to fetal DMDL

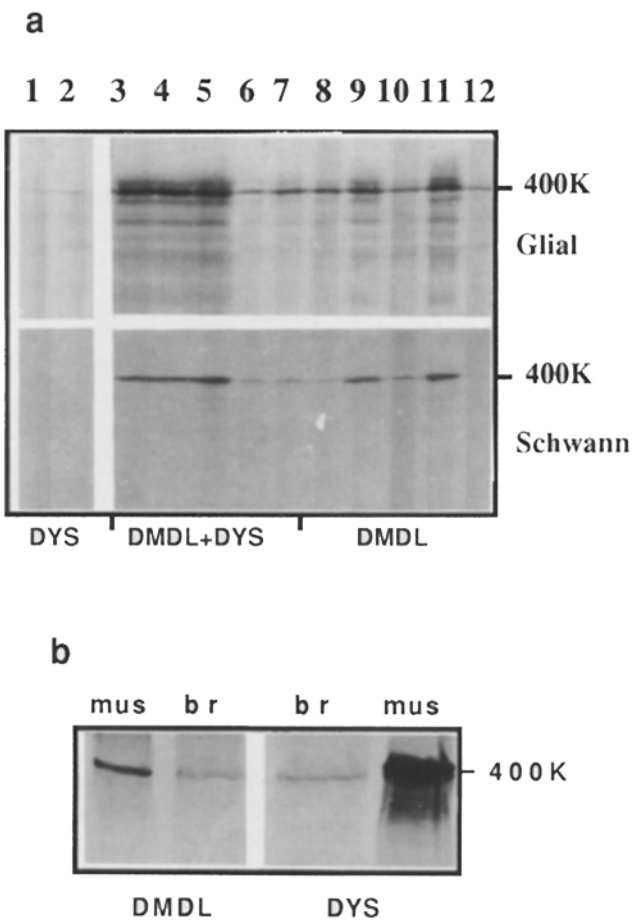


*Figure 5.* Presence of DMDL protein and dystrophin (*DYS*) in extracts of (*a*) rat glioma and Schwannoma cultured tumor cells and (*b*) adult mouse brain and muscle. (*a*) Extracts were subjected to SDS-PAGE as a strip and the Western blot was developed with 12 different mAbs applied in the "miniblotter" multiple lane blotting apparatus. Two dystrophin-specific antibodies (lanes *1* and *2*; MANDYS16 and MANDYS1 [12]), five DMDL-specific antibodies (lanes *8–12*; MANCHO 7, 1, 2, 3, and 9), and five antibodies that bind both DMDL and dystrophin (lanes *3–7*; MANCHO 17, 15, 11, 18, and 19) were used. The intensity differences between glioma and Schwannoma blots may be accounted for by different total protein concentrations in the two extracts. (*b*) Identical amounts of the same extracts of Balb/c mouse brain and muscle were used for the two blots, which were developed with MANCHO1 (lane *9* in *a*) for DMDL and MANDYS1 (lane *2* in *a*) for dystrophin (*DYS*). As in Fig. 1, when conditions are used that reveal the low levels of both DMDL and dystrophin in adult brain, the dystrophin band in adult muscle appears overloaded and distorted.

protein and mRNA levels in some tissues. In dystrophic muscle, the homologue may be occupying some of the membrane sites left vacant by the absence of dystrophin. This raises the intriguing question of whether the homologue can perform any dystrophin-like functions at the muscle membrane. The immunohistochemical analysis of the developmental expression of the DMDL protein and identification of proteins with which it interacts should provide some insight into its function.

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